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FUNDAMENTAL INVESTIGATION AND APPLIED STUDIES ON NON-*SACCHAROMYCES* YEASTS IN NON-ALCOHOLIC AND LOW ALCOHOL BEER BREWING

Thesis presented by

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BSc, MSc Brewing and Beverage Technology

for the degree of

Doctor of Philosophy – PhD in Food Science and Technology

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Declaration

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Date: 11/12/2019

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Abbreviations

°P	Degree Plato
°C	Degree Celsius
AA	Amino acid
AACC	American Association for Clinical Chemistry
ABV	Alcohol by volume
ADF	Apparent degree of fermentation
ADH	Alcohol dehydrogenase
AFB	Alcohol-free beer
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSG	Brewers' spent grain
BLAST	Basic Local Alignment Search Tool
DLG	Deutsche Landwirtschafts-Gesellschaft e.V.
DNA	Deoxyribonucleic acid
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDTA	Ethylenediaminetetraacetic acid
EFFCA	European Food and Feed Cultures Association
EFSA	European Food Safety Authority
FAN	Free amino nitrogen
FAA	Free amino acids
FID	Flame ionization detector
GC	Gas chromatography
GNPD	Global New Products Database
GRAS	Generally recognized as safe
GRYC	Genome Resources for Yeast Chromosomes
HDMS	Hexamethyldisilazane
HPLC	High performance liquid chromatography
IBU	International bitterness unit
IDF	International Dairy Federation
LAB	Low alcohol beer
LDH	Lactate dehydrogenase
LOD	Limit of detection
MEBAK	Mitteleuropäische Brautechnische Analysenkommision

MENA	Middle East and North Africa
MIC	Minimal inhibitory concentration
NAB	Non-alcoholic beer
NABLAB	Non-alcoholic beer and low alcohol beer
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NCBI	National Center for Biotechnology Information
OD	Optical density
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDC	Pyruvate decarboxylase
PI	Prediction interval
pKa	Acid dissociation constant
POF	Phenolic off-flavor
QPS	Qualified presumption of safety
RID	Refractive index detector
rRNA	Ribosomal ribonucleic acid
RSP	Retail Sale Price
TCA	Tricarboxylic acid
UV/DAD	Ultra violet-diode array detector
WGS	Whole genome sequencing
YM	Yeast-mold
YPD	Yeast extract peptone dextrose

Abstract

Non-alcoholic and low alcohol beer (NABLAB) is enjoying growing popularity owing to consumer lifestyle changes, improved production methods and stricter legislation. Among the biological methods for their production, particularly research into non-*Saccharomyces* yeasts has gained momentum in recent years in order to produce NABLAB with novel flavor characteristics in an easy-to-apply manner. In a proof-of-concept study, five selected non-*Saccharomyces* species isolated from kombucha showed to perform just as well in laboratory-scale trials in wort as commercially applied species *Saccharomycodes ludwigii*. In a subsequent study, species of the *Cyberlindnera* genus were found to produce a pleasant, fruity flavor in wort. Fermentation parameters were optimized by means of response surface methodology (RSM) and the resulting non-alcoholic beer (NAB; 0.36% ABV) produced with *Cyberlindnera subsufficiens* on pilot-scale (60 L) had a significantly more fruity and significantly less wort-like aroma compared to two commercial NABs. Regarding low alcohol beer (LAB), the yeast species *Lachancea fermentati* was introduced to create LAB by harnessing the species' uncommon ability to produce significant amounts of lactic acid (LA) during alcoholic fermentation. Compared to a *Saccharomyces cerevisiae* brewers' yeast, *L. fermentati* produced less ethanol (–15%) while producing 1.3 g/L lactic acid, giving the beer a sour taste. In a follow-up study, four *L. fermentati* isolated from individual kombucha cultures were investigated in detail. The strains genotypes and phenotypes where shown to be diverse, correlating with the strains' geographical origin. LA production was optimized via RSM, where low pitching rate, high fermentation temperature, and a high initial glucose concentration resulted in the highest LA concentrations (max. 1.6 g/L). LAB (1.26 %ABV) produced with *L. fermentati* by stopped fermentation showed to have a balanced ratio of acidity from lactic acid to residual wort sweetness. In conclusion, the results of this thesis give prospect to future studies with non-*Saccharomyces* yeasts and strengthen their position as a serious and applicable alternative to established methods in NABLAB brewing.

Chapter 1:

Introduction

1.1 Introduction

Beer brewing has been a human activity ever since the beginning of urbanization and civilization in the Neolithic period. First evidence was recovered from ancient Egypt [1] and it has since grown into a global phenomenon. In the past years, the global annual beer production amounted to approximately 194 billion liters [2], which is about 80 times the volume of the Great Pyramid of Giza [3,4], a development that certainly would have made the ancient Egyptian brewers very proud. However, overall beer production volumes have been stagnating over the past years. Notwithstanding the stagnation, the non-alcoholic and low alcohol beer (NABLAB) sector of the beer market has enjoyed strong growth which is forecast to continue [5]. Emerging lifestyle trends, stricter legislation, and improved production methods have led to a growing interest in NABLAB by consumers, the beer industry, and researchers around the world. Research on NABLAB production in recent years focused on improved physical dealcoholization techniques [6–8], novel biological production methods using non-conventional yeast strains [5,9–11], and combinations thereof [12]. While the principle behind dealcoholization techniques is the gentle removal of ethanol from standard-strength or low alcohol beer (LAB), biological methods are based on limited alcohol formation in the first place. Of the biological methods, especially research into non-*Saccharomyces* yeasts for non-alcoholic beer (NAB) production is on the rise. The principle is to apply yeast species which are incapable of utilizing the most abundant wort sugars maltose and maltotriose and would thus naturally cease fermentation at low ethanol values. Consequently, those sugars remain in the finished product, creating the often-criticized sweet taste of this type of NAB. A wort-like flavor is another criticized off-flavor owed to the insufficient reduction of wort aldehydes. Dealcoholized beer, on the other hand, is criticized for its bitter and sour taste, and poor flavor, caused by the simultaneous removal of important flavor compounds along with ethanol [13].

From an economical point of view, the application of non-conventional yeasts in NAB brewing does not require special equipment, compared to the substantial investment that is required for physical dealcoholization systems. This gives opportunity for small and middle-sized brewing companies to expand their product portfolio into the NAB sector with little investment in order to satisfy growing consumer demands and produce innovative NAB with novel flavor characteristics. However, 0.0% ABV (more precisely,

< 0.05% ABV) NAB cannot be achieved with biological methods but requires dealcoholization. Therefore, biological methods for NAB production aim for an ethanol concentration below 0.5% ABV.

Ultimately, NAB must have a good flavor and taste to overcome the moderate consumer acceptance owed to the previously described taste deficits. This is where non-*Saccharomyces* yeasts can come into play. They are known for their important flavor contribution in all sorts of alcoholic beverages such as wine, fruit wine, tequila, mezcal, and cachaça. Formerly regarded as spoilage yeasts, they are now deployed purposefully to enhance the composition and aroma profile of those beverages. In winemaking, for example, non-*Saccharomyces* species are already applied as a means to improve wine aroma complexity [14,15]. In brewing, non-*Saccharomyces* species are found, for example, in Belgian style Lambic and Geuze beers, and many spontaneously fermented cereal-based, alcoholic drinks around the world [16]. But the use of non-*Saccharomyces* yeasts is not only limited to alcoholic beverages. Low-alcoholic and non-alcoholic fermented beverages such as kefir and kombucha, which are produced by symbiotic cultures of bacteria and yeasts (SCOBY), are on the rise [17,18]. Those SCOBYs are alive with non-*Saccharomyces* species [19], waiting to be isolated, and their special metabolic traits harnessed, to create innovative NABLABs with novel flavor characteristics.

In this thesis, the main objective was to investigate the suitability of selected non-*Saccharomyces* species to produce NAB or LAB on laboratory-/ and pilot-scale. The yeasts' special metabolic traits (e.g., high ester production, lactic acid production) were harnessed to improve the flavor profile of the NABLABs produced and to create NABLABs with novel flavor characteristics.

A study with selected non-*Saccharomyces* strains isolated from kombucha served as a proof of concept to investigate the suitability of non-*Saccharomyces* yeasts in NAB brewing compared to a commercially applied *Saccharomycodes ludwigii* strain (**Chapter 3**). The strains were characterized for important brewing characteristics and screened in wort, followed by a sensorial comparison.

Strains from the *Cyberlindera* genus were investigated to produce a fruity NAB with reduced wort-like off-flavor (**Chapter 4**). Known for their high ester production, five different *Cyberlindera* species from various sources were characterized and screened in wort. The best performing strain, *Cyberlindnera subsufficiens* C6.1, was investigated further,

and the fermentation parameters were optimized for an enhanced fruity aroma. A NAB (< 0.5% ABV) was produced on pilot-scale and compared to two commercial NABs in a sensory evaluation.

Some species of the *Lachancea* genus have the for yeasts uncommon ability to produce significant amounts of lactic acid during alcoholic fermentation. *Lachancea fermentati* strain KBI 12.1, isolated from kombucha, was investigated to produce a LAB, and its significant lactic acid production was introduced as a potential means to counteract residual wort sweetness, and to produce LAB with novel flavor characteristics (**Chapter 5**).

In a follow-up study, whole genome analysis of four *Lachancea fermentati* strains isolated from individual kombucha cultures was applied in an attempt to link the strains' genotypes to their phenotypes in wort fermentations (**Chapter 6**). Crucial parameters for lactic acid production by *Lachancea fermentati* were identified and optimized for a maximal lactic acid production. Finally, a LAB (< 1.3% ABV) was produced on pilot-scale.

Figure 1.1–1 gives an overview over the structure of this thesis and Table 1.1–1 summarizes the chapters/publications, including their objectives, methods, and main findings.

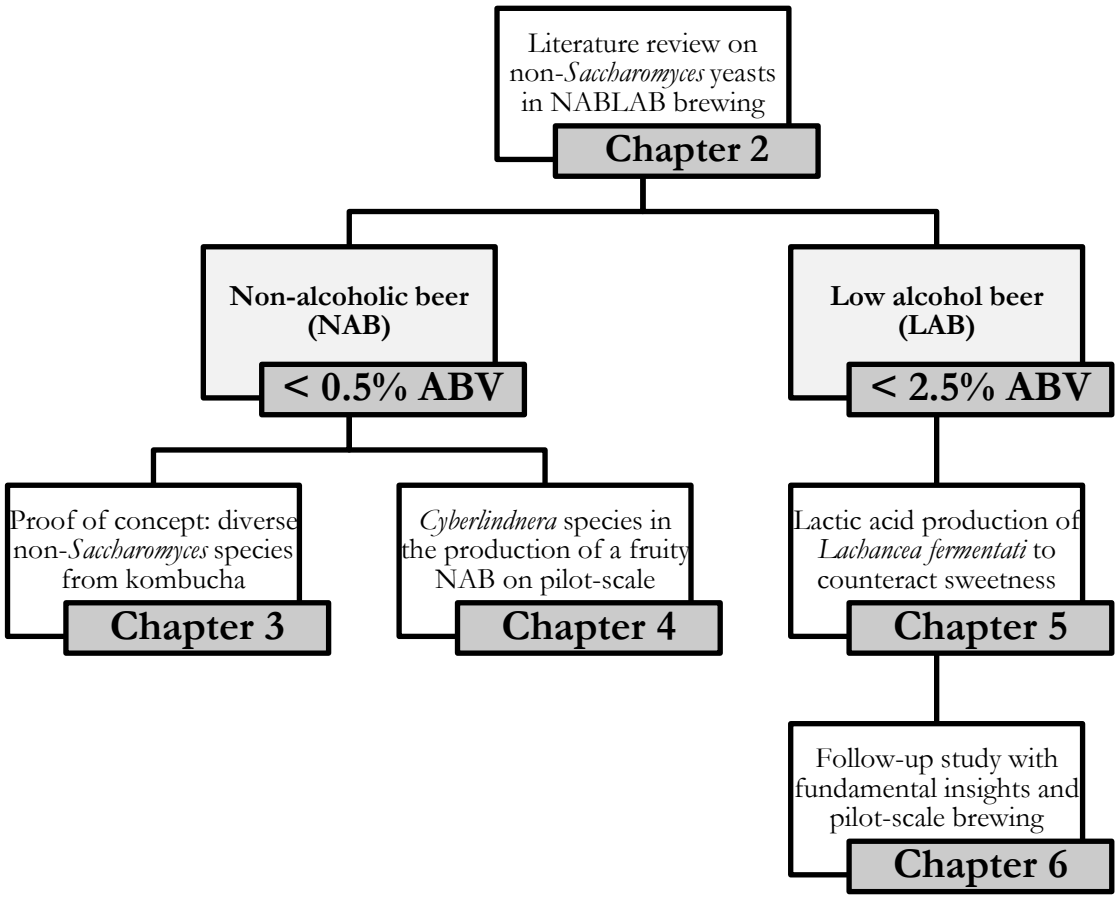


Figure 1.1–1 Structural overview over the thesis.

Table 1.1–1 Overview over publications including objective, methods, and main findings.

Chapter 2	Chapter 3	Chapter 4	Chapter 5	Chapter 6
Literature review – Chance and Challenge: Non-*Saccharomyces* yeasts in non-alcoholic and low alcohol beer brewing	Application of non-*Saccharomyces* yeasts isolated from kombucha in the production of alcohol-free beer	Screening and application of *Cyberlindnera* yeasts to produce a fruity, non-alcoholic beer	Investigation into the application of *Lachancea fermentati* strain KBI 12.1 in low alcohol beer brewing	*Lachancea fermentati* strains isolated from kombucha: fundamental insights, and practical application in low alcohol beer brewing
Objective				
To gain an overview over the NABLAB market, elucidate the consumers' perspective and summarize the existing studies on non-*Saccharomyces* yeasts for NABLAB brewing	Proof of concept: Are non-*Saccharomyces* species suitable for producing NAB and can they compete with commercially applied NAB strain *Saccharomycodes ludwigii*?	To produce a fruity NAB with *Cyberlindnera*, masking the often criticized worty off-flavor in NAB produced by limited fermentation.	To investigate the suitability of *Lachancea fermentati* to produce a low alcohol beer, and to introduce the idea of harnessing its lactic acid production to counteract residual sweetness.	To gain fundamental insights into the lactic acid production by *Lachancea fermentati* strains in wort, and optimize lactic acid production to create a low alcohol beer on pilot-scale.
Methods				
Market overview; combining sensory and consumer studies on NABLAB; combining literature from the past; critical comparison of outcome of different studies	Sugar utilization; hop resistance test; flocculation assay; phenolic off-flavor test; propagation performance; trial fermentation; analyses of sugars, amino acids and volatile compounds; sensory; PCA	Identification via PCR; sugar utilization; stress factor tests (hop, alcohol, pH); scanning electron microscopy (SEM); wort fermentations; optimization via response surface methodology (RSM); pilot-scale (60 L) brewing trial; sensory	Sugar utilization; hop resistance test; flocculation assay; phenolic off-flavor test; propagation performance; trial fermentation; analyses of sugars, amino acids and volatile compounds; sensory	Whole genome sequencing; antifungal susceptibility; sugar utilization; SEM; flocculation; stress factor tests; fermentation trial; sugars, acids, volatiles analysis; RSM; pilot-scale (60 L) brewing trial; sensory
Main findings				
The NABLAB sector is a strongly growing market sector. NAB lacks consumer acceptance due to poor flavor and taste. *Saccharomycodes ludwigii* is extensively studied but lacks acceptable flavour. Few studies on other non-*Saccharomyces* species exist, but topic is gaining momentum.	The investigated species were just as suitable in terms of yeast handling (e.g. propagation performance) and NAB production on laboratory scale as the commercially applied strain of *Saccharomycodes ludwigii*. However, all NABs exhibited a worty off-flavor.	The investigated *Cyberlindnera* species produced fruity aroma in wort while producing little alcohol. Low temperature and low pitching rate enhanced the fruity character. A fruity NAB was produced which was significantly fruitier and significantly less worty than two commercial NABs.	The investigated *Lachancea fermentati* strain produced significant amounts of lactic acid during alcoholic fermentation. Combined with missing maltotriose utilization, a LAB was produced that was characterized by a fruity aroma and sour taste.	Lactic acid production was enhanced by a low pitching rate, high fermentation temperature, and a high amount of glucose present in the wort. The pilot-scale LAB (< 1.3% ABV) exhibited a balanced sweetness-acidity ratio but exhibited high diacetyl values.

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Chapter 2:

Literature review – Chance and Challenge: Non-*Saccharomyces* yeasts in non-alcoholic and low alcohol beer brewing

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2.1 Abstract

The non-alcoholic and low alcohol beer (NABLAB) market has enjoyed significant growth in the past years and is forecasted to keep growing. However, NABLAB has organoleptic issues and lacks acceptance from many consumers. While dealcoholization methods focus on gentle and the most selective ways possible to remove ethanol from normal strength beers so as not to compromise the taste, biological methods focus on the limited production of ethanol during fermentation. In particular, investigations into the application of yeasts from the non-*Saccharomyces* sector have gained momentum in the recent years, which can show great potential to introduce new flavors to NABLAB without the necessity of any special equipment. This paper gives comprehensive insight into the NABLAB market. Consumer studies with NABLAB give recommendations for marketers and product developers. Finally, the use of non-*Saccharomyces* yeasts in the production of NABLAB is discussed in detail. Research into the use of non-*Saccharomyces* yeasts for the production of NABLAB demonstrates promising results. However, for most species, the research is still in the early stages and requires further investigation into flavor characteristics and the practicality of up-scaling. Nonetheless, the application of non-*Saccharomyces* species could introduce new, non-conventional flavors into NABLAB brewing in an easy to apply manner.

2.2 Introduction

Non-alcoholic and low alcohol beer (NABLAB) is experiencing growing popularity in a society that is more conscious about health and well-being and a beer industry that is observing a slowing down of the overall market growth and is seeking to extend their product portfolio to benefit from a growing NABLAB market and to satisfy consumer demands [1].

Different names exist for non-alcoholic beer (hereafter NAB), such as 'alcohol-free beer', 'near beer', 'small beer', 'dealcoholized beer', which all generally define a beer ethanol content somewhere in the range 0.00–0.50% alcohol by volume (ABV). In this review, NAB is defined as beers ≤ 0.5% ABV. Low alcohol beer (hereafter LAB), also 'low-alcoholic beer', 'lower alcohol beer', 'low-point beer', 'alcohol-reduced beer' and sometimes referred to as 'light beer' has different definitions concerning the alcohol limit depending on the legislation of individual countries [2–4]. This review follows the definition for beer with an ethanol content between 0.6–3.5% ABV.

Researchers are investigating improvements in dealcoholization processes and innovations in fermentation practices to produce NABLAB, which enables the consumer to enjoy a beer with all the benefits of health promoting beer ingredients (i.e. B vitamins, minerals, phenolic substances) without the downside of excessive intake of alcohol [5–7]. However, NABLAB faces organoleptic issues due to process practices that leave the taste compromised, which is reflected in modest consumer acceptance [8]. While dealcoholization focuses on removal of ethanol from a standard strength beer, biological methods focus on limited formation of ethanol. On the biological side, especially research on the use of non-*Saccharomyces* yeasts, has gained momentum.

This article gives an insight into the NABLAB market and factors influencing its growth dynamics and continues with a short review of recent consumer studies linked to the consumption of NABLAB and the marketing thereof. Finally, the main body of this paper focuses on a comprehensive review of the use of non-*Saccharomyces* yeasts for the production of NABLAB.

2.3 NABLAB market insights

The enhanced performance of the NABLAB sector in existing and emerging markets can be attributed to new policies, demographics, lifestyle trends and improved production methods. The world-wide NABLAB market experienced total volume growth of 20% from 2011 to 2016 and is forecast to grow another 24% until 2021 [9]. The non-alcoholic beer segment (NAB, ≤ 0.5% ABV), grew in total volume by 21% from 31.9 to 38.7 Mio. hl and in total value RSP (Retail Sale Price) by 38% from 7.1 to 9.9 billion Euros in the 5-year period 2012 to 2017 (Figure 2.3–1). The Middle East and Africa and Western Europe regions represent the biggest markets in terms of volume and value (Figure 2.3–1). However, the largest growth could be observed for the Latin American region with increases of 168% and 296%, respectively [10]. All regional markets exhibited growth over the past years, except for the North American market which showed stagnation and even a decrease by 1% in volume (Figure 2.3–1).

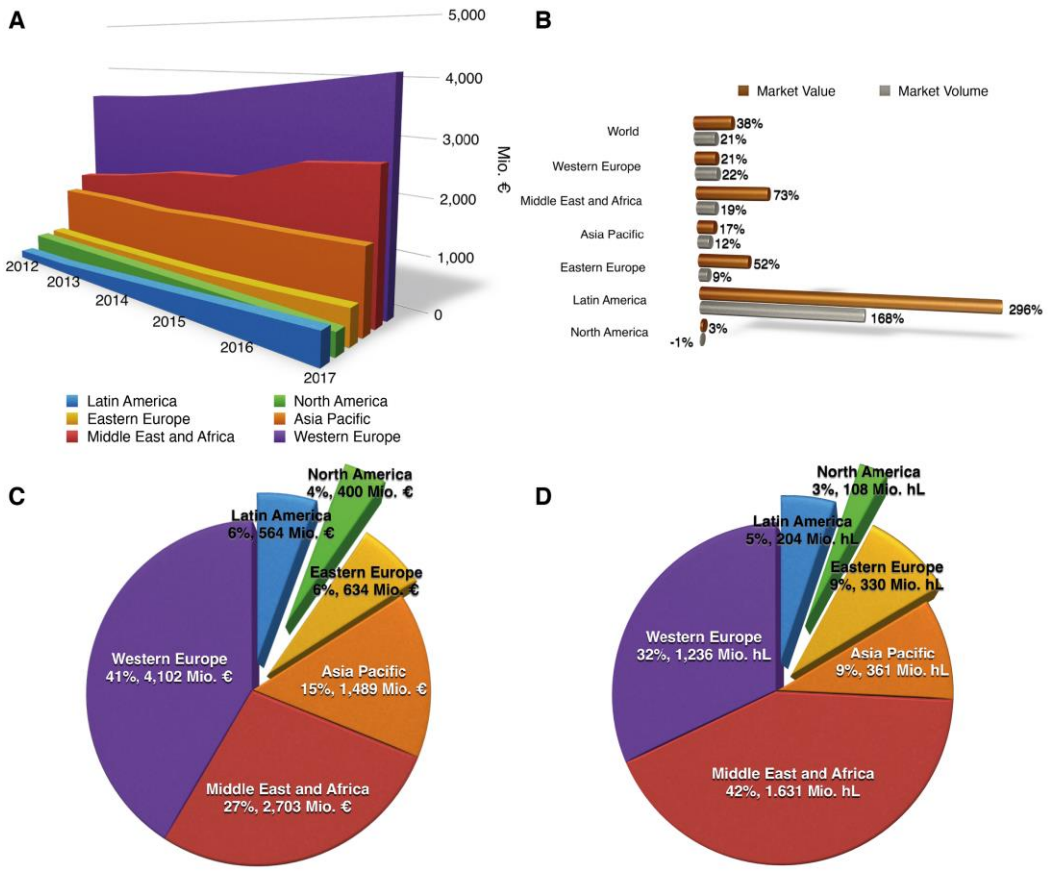


Figure 2.3–1 Insights into Non-Alcoholic Beer (NAB, ≤ 0.5% ABV) market. (A) Regional development in market value (Research Sales Price RSP, fixed 2017 exchange rates). (B) Percentage increase in market value and market size in the 5-year period from 2012 to 2017. (C) Regional NAB market share in 2017 in value (RSP, fixed 2017 exchange rates). (D) Regional NAB market share in 2017 in volume. [10]

The Western European NAB market was with 41% the biggest region in 2017 in terms of market value. In particular, the German NABLAB market is one of the biggest in the world. In 2016, it accounted for 41% of total volume in the NABLAB market in the Western Europe region (followed by Spain with 38%), taking up 14% of the world-wide NABLAB market (Figure 2.3–2). According to the German Brewers' Association (DBB), Germany's non-alcoholic beer was taking over 6% share of the country's total beer market in 2017, including over 400 different brands of non-alcoholic beer [11]. In a study in 2013, Mintel [12] found that 50–65% of European consumers would drink lower alcohol beer if the taste was comparable to the taste of standard beers. However, despite the omnipresent taste challenge of NAB, they appear to be enjoying a reasonably good taste reputation in Germany. As opposed to other European countries like France and Spain, where about 50% of beer consumers expect lower-alcohol beers not to taste as good as standard beers, in Germany this number is only at 28% [13]. In 2017, about one-quarter (23%) of German adults reported drinking NAB, with key motivators being health and well-being [14].

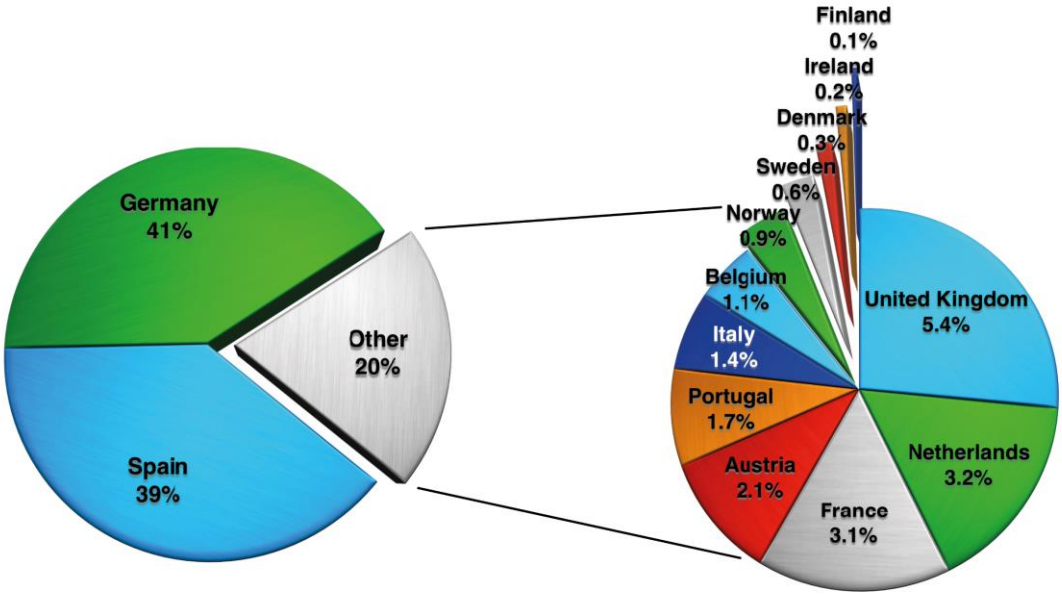


Figure 2.3–2 NABLAB market share in volume of individual countries in the Western European region [9].

Growth of NAB has been particularly strong in the Middle East and North Africa (MENA) region for the past five years (Figure 2.3–1). This region now accounts for 27% of total NAB market value. According to Mintel's GNPD (Global New Products Database), in 2016, every third new beer launched in the region was non-alcoholic

(< 0.5% ABV) [15]. NAB enjoys high popularity in MENA mostly on religious grounds since Muslims are forbidden to drink alcohol. However, prominent Saudi and Egyptian clerics have issued *fatwas* (rulings on points of Islamic law given by a recognized authority) declaring it permissible for Muslims to drink zero-alcohol beers and the Saudi ruling names the key issue to be whether one could become intoxicated consuming a large amount of the drink, making it permissible to consume NAB [16]. By contrast, in Muslim-dominated Malaysia, the country's Department of Islamic Development (Jakim), has so far refused to grant halal-certifications to any NAB, even if it is confirmed that they contain no traces of alcohol [15]. With 40% of the population being Millennials, they are a large target group in the MENA region [17]. Beer, even if non-alcoholic, is a statement of a globalized lifestyle for MENA Millennials who are increasingly embracing modern values. They prefer Western brands and engage more and more with social media and the English language as the Arab Youth Surveys from the past years have shown [18–21]. NAB allows Muslim Millennials to imitate Western lifestyles without compromising their religious beliefs. However, some NAB brands are positioning themselves as adult soft drinks rather than zero strength beers to avoid putting off more conservative consumers and governments. While the focus of NAB innovation in the past had increasingly been focused on fruit-flavored variants, some brands are now tapping into the field of increased functionality such as added minerals and vitamins in order to satisfy rising health trends, migrating from Europe and North America into the MENA region [15].

North America holds a special position in the NAB market because – as opposed to all other regions – it did not experience growth over the past five years. Indeed, nearly every second new beer released into the US market (88% total market volume of North America region) in 2015 had a high ABV of 6.6% or more, compared to only one in 50 with a low ABV of 0–3.5% [22]. The reason for the high number of high ABV beer launches is believed to be due to the influence of the craft beer trend. A high ABV is a way for craft brewers to distance their beers from milder mainstream lager beers and has dominated retail releases in the past decade, with their beers pushing the limits of traditionally acceptable ABV (4–5%) products [23]. However, data collected by GlobalData showed that young Americans consider alcohol in a more negative light than older generations with 54% of 25–34-year-old Americans stating that they are actively trying to reduce alcohol consumption compared to 28% of Americans overall and 22% of global consumers overall [24]. Combined with the fact that non-alcoholic craft breweries have started to emerge (i.e. Nirvana Brewery, London, UK and WellBeing Brewing Company,

Missouri, USA) and that many other craft breweries have added NAB and LAB to their product portfolio, it could mean that the growth in the North American NABLAB market is yet to come [25].

A growing factor in enforcing the brewers' focus on NAB is the introduction of novel government legislation. More countries are introducing stricter legislation concerning driving under the influence of alcohol or the sales ban on alcoholic products. After the passage of a zero-tolerance drunk driving law in Colombia in December 2013, brewers have increased non-alcoholic beer launches. Although still being a small segment of the overall category, non-alcoholic beer releases increased from 6% of Colombia's beer launches in 2014 to 16% in 2015 [26]. Another example for the influence of new legislation could be seen in Indonesia where the ban on beer sales in Indonesian mini-marts in 2015 stimulated the NAB market. The Indonesian government banned sales of alcoholic beverages with an ABV between 1–5% from mini-marts, small shops and kiosks – a channel which previously accounted for an estimated 60% of all beer sales in Indonesia [27] – which led to the escalation of NAB innovation meaning that a third of all new beer launches in Indonesia in 2016 have been non-alcoholic compared to just one in 25 in 2014 [28].

2.4 Consumer studies related to NABLAB

Taste is an omnipresent factor, when dealing with NABLAB. The taste preference for standard strength and higher strength beers as opposed to NABLAB becomes evident when looking at the beer rating website *ratebeer.com* and the ratings of the best rated beers of different ethanol categories (Figure 2.4–1). The taste deficits of low alcohol beer have been reviewed by Blanco et al. [8]. For dealcoholized beer, it mainly manifests in a bitter and sour taste, while NABLAB produced by limited fermentation are often characterized by a worty off-flavor and sweet taste.

Besides taste problems, there may be marketing, or labeling problem. Should the taste of NAB copy its alcoholic counterpart or stand as a beverage on its own? This chapter reviews recent consumer studies related to the taste, expectations and the liking and emotions of NABLAB as well as labeling and marketing issues.

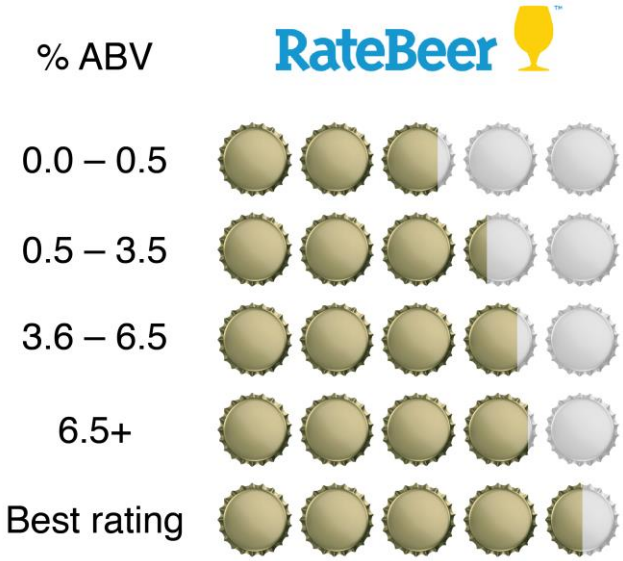


Figure 2.4–1 Average rating of beers with different ABV (alcohol by volume) values on ratebeer.com. (ratings n ≥ 100; average rating of best 10 beers listed; category for 0.0–3.5% ABV: "Low Alcohol", category for 3.6–6.5+% ABV: "Lagers", category for Best rating: "All Styles".) [29].

In a study dating from 2014, Lachenmeier et al. [30] showed that consumers are unable to discriminate alcoholic strength of high spirits but are well able to discriminate between non-alcoholic beer (0.5% ABV) and standard strength beer (5% ABV). There is a lack of research on minimal detectable differences in alcoholic strength in beer, but for white wine, King and Heymann [31] found that consumers were unable to detect differences in alcoholic strength of 1% ABV.

Missbach et al. [32] investigated the flavor life cycle of beers with varying alcohol contents to study the temporal flavor dominance during consumption with a trained and experienced panel. The tested attributes were worty, fruity, bitter, astringency and malty. The study included three different brands with their regular strength lager beer (4.9–5.4% ABV), alcohol-reduced beer (3.0–3.5% ABV) and NAB (< 0.5% ABV). The study found that the undesirable worty off-flavor was most pronounced in NAB, but only prior to swallowing. After swallowing, malty flavors and the hop bitterness were dominant. Therefore, the authors recommend consumers to swallow alcohol-free beer faster and focus on the flavor characteristics of the post-swallowing phase with malty flavor and bitterness from the hops. The findings might also be interesting for brewers (i.e. experimenting with hops and malts) and marketers (i.e. focus on consumption from the bottle instead of from the glass).

In a study by Schmelzle et al. [33], a sensory descriptive analysis was conducted on twelve NAB (< 0.5% ABV), five of which produced by physical dealcoholization and seven produced by limited ethanol formation and hybrid methods. The trained panel (n = 21) identified 21 attributes concerning the taste, smell and mouthfeel of the NAB. The attributes were used to assess the intensity in the individual NAB and a principal component analysis (PCA) was conducted to illustrate sensory similarities and differences. The collected data indicated that the dealcoholized NABs were perceived to have a sour and bitter taste, boiled cabbage-like aroma and a mouth coating texture. The NABs produced with limited fermentation (and hybrid methods) were perceived as sweet, with a malty and honey-like aroma or with a hop aroma. Sugar analysis revealed, that the NABs produced by limited fermentation had residual sugar concentrations above 24 g/L, while dealcoholized NABs had sugar concentrations less than or equal to 9 g/L. The authors showed a clear correlation existed between the perceived sweet taste and the amount of residual sugars, classifying the NABs into two groups according to their taste and production methods. In an acceptance study, nine of the twelve beers were selected to represent the different sensory groups and evaluated in a consumer test (n = 116). It was shown that the consumers preferred sweet and slightly fruity NABs. Malty and honey-like odors, which were found in some NABs produced by limited fermentation, were not particularly favored – neither was the bitter and sour taste from the NABs which were produced by dealcoholization. Although most participants stated that the taste of NAB should not differ from regular strength beer, their acceptance rating did not differ significantly from the participants who disagreed with that statement. Therefore, the authors pose the question whether NABs should be developed in line with normal strength beers, or should they be regarded as a product category on its own, stressing that the preferred sensory attributes (sweet and slightly fruity) were underrepresented among the tested NABs [33].

Silva et al. [34] explored functional and emotional associations that consumers (n = 56) have with NAB consumption, compared to regular strength beer and wine. It was found that the conceptualization of NAB was mostly functional, while beer and wine were also rich in emotional content. NAB was mostly seen as a substitute for beer and soft drinks and a healthier alternative. Amongst the emotional responses were: responsible (positive), conscious and safe (neutral), and disappointed (negative). It was reported that NAB consumers appear to be divided into two groups in terms of their motivation for NAB consumption. In one group. the flavor was the main motivation for consumption which

is comparable to the findings of Chrysochou [35] in a study of Icelandic light beer consumers. For the other group, the primary reason for NAB consumption was to avoid alcohol. The authors state that some participants described NAB as a fake beverage, comparable to plastic flowers, reflecting the high level of comparison of NAB to regular beer, which can leave the consumer with unfulfilled expectations. The authors conclude, that to prevent or minimize disappointment by the consumers, NAB should be treated as a beverage in its own right and that direct conceptual comparisons with beer, especially regarding the flavor, should be avoided [34].

Jaeger et al. [36] reported similar findings in emotional associations with the consumption of NABLAB. The authors tested nine commercially available beers with ABVs between 2.5 and 7.0% in a consumer tasting procedure (n = 128) and recorded – amongst other assessments – their emotional responses. The beer with the lowest alcohol content (2.5% ABV) among the nine beers in the study, was most strongly associated with the emotional associations "secure/at ease" (13%). Conversely, the beer with the highest alcohol content (7.0% ABV) had the weakest association with this emotion (2%). The authors suggest tentatively, that the alcohol content underpinned this difference and hold out the prospect for future research with a stronger focus on low alcohol- and alcohol-free beers [36].

In another study, Silva et al. [37] investigated the expectations, liking and emotional responses related to the consumption of regular beer (5.0% ABV) and NAB (0.0% ABV) in connection with different labeling. In 4 sessions in a bar setting, consumers (n = 155) were given a glass of regular beer or NAB under two different conditions, labeled either correctly or incorrectly (BEER or NON-ALCOHOLIC BEER) with respect to the actual content of the glass. When NAB was labeled as "BEER"; liking significantly increased and emotional responses slightly changed in a more positive direction with participants feeling more fulfilled. Without name manipulation, the consumers' expectations of drinking a NAB were more positive than the actual experience in terms of liking and emotional responses, again resulting in unfulfilled expectations as already reported in the study from 2016 [34]. Conversely, expected liking of the standard beer correctly labeled as "BEER" was equal to the actual liking, meaning that in this case expectations were fulfilled. When the standard beer was labeled as "NAB", the emotional response of six positive emotions decreased [37]. The results show that product labeling is a powerful tool for creating specific sensory expectations that can influence the consumer, leaving his/her expectations fulfilled or unfulfilled.

Vasiljevic et al. [38] analyzed marketing messages in text and image for the sale of low/er (low: < 1.2% ABV; lower: 1.2–2.8% ABV) and regular strength beer (> 2.8% ABV) on the websites of the four main UK retailers, in order to evaluate whether they were marketed as substitutes for standard strength beers or as additional products. It was found that the low/er strength equivalents were more often marketed in association with outdoor events or for sports and fitness occasions. Compared to regular strength beer, they were presented as suitable for consumption on a wider range of occasions, suggesting they may be marketed to replace soft drinks rather than regular strength beer. Therefore, the authors raise the question to which extent low/er alcohol beer would contribute to a public health strategy to reduce alcohol consumption. Furthermore, compared with regular strength beer, low/er strength equivalents were more frequently marketed with images or text with explicit reference to health benefits, suggesting that the industry and retailers may be targeting the health conscious "Millennials" who now form a large portion of the drinks market [39]. Analysis of the marketing messages concerning low/er and regular strength wine products painted a similar picture [38].

Concerning gender targeted marketing, Porretta et al. [40] found during a consumer study in Italy from 2008, that male participants believed that NAB should be targeted directly at them and should not be marketed in a way that appeals to females. Conversely, the female participants found that NAB should move towards a more gender-neutral positioning.

2.5 NABLAB by special yeasts

The production of NABLAB can generally be divided into two main categories: physical methods and biological methods [41] (Figure 2.5–1). While physical methods are based on the dealcoholization of a finished beer, biological methods are based on limited ethanol production by the yeast during fermentation processes. The physical methods for the dealcoholization of beer and other beverages have recently been reviewed and discussed in detail in two comprehensive reviews by Müller et al. [2] and Mangindaan et al. [42] with all their advantages and disadvantages and will not be discussed further in this review.

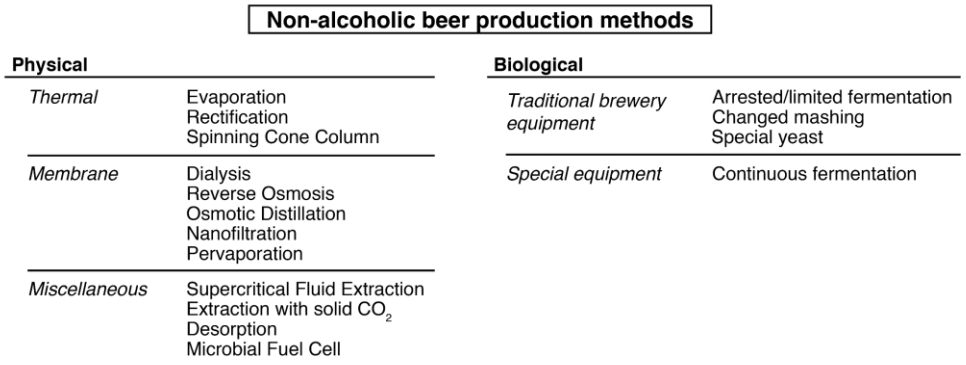


Figure 2.5–1 Non-alcoholic beer production methods. Adapted from Brányik et al. [41] and extended [2,42].

The biological methods can further be divided into methods that require special equipment and methods that can be used with standard brewing equipment (Figure 2.5–1). While NABLAB production by continuous fermentation requires investment in special equipment, changed mashing, arrested/limited fermentation and the use of special yeast can be performed with standard brewery equipment. Information about changed mashing [43], arrested or limited fermentation, and continuous fermentation in the production of NABLAB, has not experienced major advances in terms of new research papers and is available in the comprehensive review of NABLAB production methods by Brányik et al. from 2012 [41].

Unlike the other biological methods, research for the use of special yeasts has gained momentum in recent years. The application of so-called non-conventional or non-*Saccharomyces* yeasts with limited abilities to ferment wort sugars for the production of low-alcohol and non-alcoholic beers in single culture fermentation is not a new approach. The non-*Saccharomyces* species *Saccharomycodes ludwigii* has been applied commercially for this purpose for many years and is the most popular species with regards to the number of studies conducted in the past [44]. A summary of studies conducted with *Saccharomyces ludwigii* for the production of NABLAB is shown in Table 2.5–1. However, the use of non-*Saccharomyces* yeasts for the purpose of brewing NAB other than *S. ludwigii* can potentially present a whole new set of different flavors. In winemaking, non-*Saccharomyces* are already applied as a means to improve wine aroma complexity [45,46]. Changing the yeast culture is also one of the easiest modifications for breweries to make since it does not require investments in additional brewing equipment which makes it accessible for

breweries of all sizes. Species that have been investigated in a wort substrate include: *Candida shehatae, Candida zemplinina, Cyberlindnera mrakii* (former *Williopsis saturnus* var. *mrakii*), *Cyberlindnera fabianii, Torulaspora delbrueckii, Hanseniaspora valbyensis, Hanseniaspora vineae, Mrakia gelida, Pichia kluyveri, Pichia kudriavzevii, Zygosaccharomyces bailii, Zygosaccharomyces kombuchaensis and Zygosaccharomyces rouxii*, which are discussed in this review. An overview about these studies is shown in Table 2.5–2. Some of the yeast species discussed in this paper have also been reviewed by other authors. Michel et al. [44] discusses non-*Saccharomyces* yeasts as pure starter cultures for beer fermentation with focus on the production of secondary metabolites. Capece et al. [47] presented the wide choice of available conventional and non-conventional yeasts for brewing, with an emphasis on new biotechnological approaches to target the characteristics of beer and to produce different or completely new beer styles. A review by Varela et al. [48] covered the impact of non-*Saccharomyces* yeasts on the volatile composition and sensory profile of beer, wine, spirits and other fermented beverages. Gibson et al. [49] highlighted "modern approaches in brewing yeast design and development" such as hybridization. The approach to use non-*Saccharomyces* yeasts for the production of NABLAB is strongly dependent on the substrate and its sugar composition, therefore, only studies with wort substrates are included in this review. Furthermore, this review is limited to applications where the outcome were NABLABs due to the yeasts' limited ability to ferment wort sugars with a focus on beers produced below 0.5% and 1.2% ABV.

2.5.1 *Saccharomycodes ludwigii*

Saccharomycodes ludwigii has been investigated thoroughly in the past and has been applied as an example of a commercial NAB starter strain in comparison to other non-*Saccharomyces* strains employed in more recent studies. The yeast species, mentioned in a patent by Glaubitz and Haehn [50] in 1929, was used to produce a beer with low alcohol content and high concentration of residual unfermented maltose. This was discussed again in a 1990 patent by Huige et al. [51].

Narziss et al. [52] investigated the use of *S. ludwigii* to brew NAB (< 0.5% ABV) in comparison to the use of brewers' yeast with an arrested fermentation in a 11.5 °P wort. The strain produced 0.68% ABV ethanol and the authors suggested the use of a wort with 7.5 °P to stay below 0.5% ABV. In comparison to the NAB produced with a brewers' yeast strain through arrested fermentation, the *S. ludwigii* fermented beer contained higher

ester and higher alcohol concentrations. Also, diacetyl production was increased and identified by a sensory tasting panel. The authors stated a positive influence of biological wort acidification during the process leading to a slight suppression of the worty off-flavor and diacetyl off-flavor, but the NABs were all criticized by the panel for their worty taste.

Liu et al. [53] fermented a 8.1 °P wort with *S. ludwigii* at 12 °C. Ethanol reached 0.47% ABV with the low production of esters (1.9 mg/L) and higher alcohols (39 mg/L). The NAB was reported to exhibit a weak aroma and sweet taste.

In a more fundamental approach, Sohrabvandi et al. [54] investigated the *S. ludwigii* strain DSM 3447 for its performance in synthetic media containing different fermentable sugars. It was reported that the fastest growth occurred in the presence of fructose, followed by glucose and sucrose. In the media containing maltose as the sole fermentable sugar no growth was observed. Mohammadi et al. [55] investigated the same *S. ludwigii* strain DSM 3447 immobilized on brewers' spent grain (BSG) and found that the immobilized strain was able to consume maltose, presumably due to reduced intracellular pH values and increased enzymatic activity. It was reported that the strain produced 1.7% ABV ethanol (7 °C) and 2.7% ABV ethanol (12 °C) in 6.5 °P wort. Mortazavian et al. [56] fermented a 6 °P wort for 48 hours at different temperatures (4, 12 and 24 °C) and with two different pitching rates (10^6 and 4×10^6 cells/mL) of the same *S. ludwigii* strain DSM 3447 under anaerobic conditions or with periodic aeration (every 12 h). Ethanol levels ranged from 0.15 to 1.20% ABV and the beers were reported to have a low acceptance rate during sensory evaluation. This was due to sweet and immature flavors in the samples fermented at cooler temperatures (4 and 12 °C) and lactic sour flavors for the sample fermented at 24 °C.

Meier-Dörnberg et al. [57] used *S. ludwigii* strain TUM SL 17 to ferment a 12.8 °P and 7 °P wort at 15 °C or 20 °C to produce an alcohol-free wheat beer. Ethanol concentrations ranged between 1.00–1.16% ABV (12.8 °P) and 0.50–0.62% ABV (7 °P). The alcohol-free wheat beer (7 °P, 15 °C; 0.5% ABV) exhibited increased concentrations of higher alcohols compared to the average of 20 commercial alcohol-free wheat beers. However, the typical wheat beer aroma compounds ethyl acetate, isoamyl acetate and 4-vinylguaiacol were missing.

De Francesco et al. [58] screened six *S. ludwigii* strains, mostly isolated from grape must, for their applicability to produce a low alcohol beer. Small scale fermentations of 50 mL 12 °P wort were performed at 20 °C under aerobic conditions. Ethanol concentrations ranged from 0.51 to 1.36% ABV, while ester concentrations ranged from 1–15 mg/L and higher alcohols from 43–77 mg/L. Diacetyl values were reported to be below the threshold of 0.1 mg/L [59] and it was concluded that the strain with the lowest ethanol production would be a potential yeast especially for the production of NABLAB.

Since *S. ludwigii* is already applied in commercial NAB brewing [44,60], it was recently used by different authors as a control strain to compare the performance of different non-*Saccharomyces* yeasts [60–62]. Saerens et al. [60] found *S. ludwigii* inferior to a *Pichia kluyveri* strain in laboratory scale fermentations in order to produce NAB. The *S. ludwigii* strain employed was reported to produce similar amounts of higher alcohols, lower ester concentrations, and high decanoic acid concentrations that could potentially lead to a rancid, cheesy off-flavor in the beer. The ethanol concentration was 0.3% ABV with a 7 °P malt extract following 5 days of fermentation at 20 °C.

De Francesco et al. [61] compared the *S. ludwigii* strain TUM SL 17 (alternative name WSL 17) to a *Mrakia gelida* yeast strain during the fermentations of 12 °P wort. After 10 days fermentation at 23 °C, the *S. ludwigii* strain produced 1.23% ABV ethanol and following additional re-fermentation (bottle conditioning) with addition of 5 g/L glucose, the ethanol concentration rose to 1.32% ABV. Ester values ranged from 9–15 mg/L with higher alcohol levels of approximately 43 mg/L. The beers produced with *S. ludwigii* were described as cereal-like and malty.

The same *S. ludwigii* strain TUM SL 17 was used by Bellut et al. [62] in comparison to five different non-*Saccharomyces* strains. The *S. ludwigii* strain produced 0.5% ABV alcohol with a 6.6 °P wort after three days fermentation at 25 °C. Ester production was reported to be very low (0.8 mg/L) as well as low higher alcohol production (21 mg/L) and diacetyl production, which was below the flavor threshold. During a tasting, the NAB was described exhibiting a sweet taste, and worty, bread-like flavors.

During a combination of physical and biological methods, Jiang et al. [63] used a *S. ludwigii* strain to ferment a 12.2 °P wort produced with both barley and wheat malt followed by vacuum distillation to remove the ethanol. Blending with small quantities of regular beer was used to develop a beer with a normal aroma. The beer was produced on a 2000 L

scale with the fermentation temperature at 18 °C and a pitching rate of 15×10^6 cells/mL. After vacuum distillation, re-dilution and the addition of 9% ABV regular beer with 4.5% ABV ethanol, the final ethanol concentration was < 0.5% ABV and the concentration of flavor substances was reported to be similar to a commercial alcohol-free beer.

Table 2.5–1 *Saccharomycodes ludwigii* strains in wort substrates

Saccharomycodes ludwigii **Strain designation**	**Wort gravity** **°P**	**Scale** **L**	**Ethanol content** **% ABV**	**Fermentation conditions** **Time (d) / Temperature (°C) / Pitching rate (×10⁶ cells/mL)**	**Secondary metabolites ΣEsters (mg/L)/ ΣHigher alcohols (mg/L)**	**Sensory**	**Reference**
6 DPVPG[1] strains	12.0	0.05	0.51–1.36	10 / 20 / NA	1.21–14.92 / 43.31–76.62	NA	[58]
WSL 17 (=TUM SL 17[2])	12.0	25	1.23–1.32	10 / 23 / 0.4	9.3–14.9 / 42.2–43.4	Cereal, malty	[61]
NA	8.1	2	0.47	NA / 12 / NA	1.88 / 39.10	Weak aroma, sweet	[53]
#303[3]	12.2	2000	< 0.5	NA / 18 / 15	7.95 / 8.70	NA	[63]
DSM 3447[4]	6.5	0.2	1.7 2.7	10 / 7 / NA 7 / 12 / NA	NA	NA	[55]
TUM SL 17[2]	12.8 7.0	ca. 2	0.99–1.16 0.50–0.62	6 / 15&20 / 8	NA 0.75 / 22.94	NA	[57]
TUM SL 17[2]	6.6	1.5	0.50	3 / 25 / 8	0.80 / 21.05	Worty, honey, bread-like, sweet	[62]
NA	11.5	NA	0.68	5 / 20 / NA	1.88 / 31.80	Worty taste, diacetyl	[52]
DSM 3447[5]	6.0	NA	0.15–1.2	2 / 4 / 10 (periodic aeration) 2 / 24 / 40 (anaerobic)	NA	Low acceptance, lactic acid sourness; sweet and immature flavor	[56]
NA	7.0 E	0.5	0.3	5 / 20 / 1	NA	NA	[60]

NA not available; E wort from wort extract; [1] Industrial Yeast Collection (DBVPG), University of Perugia, Italy; [2] Research Center Weihenstephan for Brewing and Food Quality, Freising, Germany; [3] Doemens Academy, Germany; [4] immobilized on brewers' spent grain (BSG); [5] Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany

2.5.2 *Candida spp.*

Estela-Escalante et al. [64] investigated the application of a ***Candida zemplinina*** strain (Y.01670), isolated from overripe grapes, for craft beer production. Trial fermentations were carried out at 350 mL laboratory scale at 18 °C for 8 days in different wort extracts with and without adjuncts: malt wort, malt wort plus glucose syrup, malt wort plus glucose

syrup and yeast extract, and malt wort plus apple juice. All worts were adjusted to an extract concentration of 12 °P and a pH of 4.8. When malt wort alone was used, the *C. zemplinina* strain produced only 1.5% ABV ethanol, owing to the yeast's inability to consume maltose. The addition of glucose syrup to the wort led to a final ethanol concentration of 1.7% ABV. Additional supplementation with yeast extract led to increased numbers of viable cells but did not influence the final ethanol content significantly. When apple juice was used as an adjunct, the production of ethanol increased in correlation with the reducing sugar consumption and final beers had an ethanol content of about 4.1% ABV. Unfortunately, no sensory study was conducted. During a second study with the same strain, the use of additional adjuncts was investigated [65]. Under the same fermentation conditions (350 mL, 18 °C, 8 days), the fermented substrates were 12 °P wort extract, wort extract plus glucose syrup (1:1; 6 °P wort plus 6 °P glucose syrup DE45), wort extract plus grape juice (1:1) and wort extract plus high fructose syrup (1:1). In pure wort, the yeast produced 1.67% ABV ethanol. When glucose syrup was added, final ethanol content was only insignificantly higher with 1.85% ABV, due to the high content of di- and oligosaccharides in the glucose syrup which the yeast is not able to ferment. The addition of high fructose syrup or apple juice, in which both have a high content of monosaccharides, resulted in final ethanol concentrations of 4.69% ABV and 4.46% ABV, respectively. The authors concluded, that *C. zemplinina* strain Y.01670 would be suitable to produce a variety of beers when brewing with adjuncts (addition of monosaccharides). Conversely, wort without the use of adjuncts led to a low alcohol beer. No sensory study was conducted.

In a patent by Li et al. [66], a ***Candida shehatae*** strain is used to produce an alcohol-free beer from wort. In 300 mL laboratory scale fermentations, *Candida shehatae* strain CICC 1766 was used to ferment a 9 °P wort at 14 °C with an approximate 3% inoculum. The final beer had an ethanol content of 0.47% ABV and a diacetyl concentration below 0.05 mg/L. The NAB was reported to contain a high ester content and lack the sweet and worty off-taste, that is typical of many NABs produced by limited fermentation. A 200 L trial led to a final ethanol content of 0.37% ABV, a diacetyl concentration below 0.05 mg/L and a reportedly similar flavor to normal beer.

2.5.3 *Cyberlindnera spp.*

Cyberlindnera yeasts have previously been reported to produce high concentrations of acetate esters, in particular isoamyl, ethyl and 2-phenylethyl acetate [67,68]. During a study by Van Rijswijck et al. [69] on the "performance of non-conventional yeasts in co-culture with brewers' yeast for steering ethanol and aroma production", 49 wild yeast isolates belonging to the species *S. cerevisiae* (16 isolates), ***Cyberlindnera fabianii*** (9 isolates) and *Pichia kudriavzevii* (24 isolates), were screened in a 12 °P wort from barley wort extract in 100 mL laboratory scale as single culture fermentations. After 7 days incubation at 20 °C, ethanol and volatile organic compounds concentration were analyzed. Due to total yeast uptake of glucose but only very limited consumption of maltose and no maltotriose utilization, ethanol levels of the worts fermented with *C. fabianii* only reached 0.6% ABV. The relative abundance of volatile esters to volatile alcohols were found to have an approximate 40:60 (esters : alcohols) ratio as opposed to a 15:85 ratio for the *Saccharomyces cerevisiae* isolates.

Cyberlindnera mrakii (formerly *Williopsis saturnus* var. *mrakii*) strain NCYC 500 was investigated by Liu et al. [70] to evaluate its potential to produce a fruity beer. A wort containing wort extract, barley malts and glucose was produced, and hops added during the boil. The final wort had an extract content of 13.8 °P and contained about 2.3% (w/v) glucose (through the addition of glucose), 0.3% (w/v) fructose, 0.3% (w/v) sucrose and 5% (w/v) maltose. Fermentation was conducted in 400 mL laboratory scale at 21 °C for 14 days. The final beer had an ethanol content of 1.7% ABV due to the yeasts' inability to consume substantial amounts of sugars other than glucose. Conversely, the *Saccharomyces cerevisiae* brewers' yeast (Safale US-05), fermented the wort to a final ethanol content of 6.9% ABV, depleting all the sugars. The concentrations of ethyl and isoamyl acetate detected in the beer fermented with *C. mrakii* were significantly higher than those detected in the beer fermented with brewers' yeast, despite its limited fermentation capabilities. In particular, isoamyl acetate levels in a beer fermented with *C. mrakii* were approximately 20 times higher than in those fermented with Safale US-05. Thus, the authors suggest that the use of *Cyberlindnera spp.* to ferment wort would result in a beer with a distinct fruity, banana-like aroma. However, the authors raise concern that the higher production of ethyl acetate by the NCYC 500 strain could lead to a solvent-like off-flavor in beer. It was concluded that the high ester production in combination with

the strain's low fermentative ability would make *Cyberlindnera mrakii* very suitable to produce extra-fruity, low-alcohol beer.

2.5.4 *Pichia spp.*

In a study by Saerens et al. [60], two ***Pichia kluyveri*** strains (PK-KR1, PK-KR2) were investigated to produce NAB on the 1000 L scale. The all malt wort (62% barley, 38% wheat) with 8.3 °P was inoculated with 5×10^6 cells/mL, and hop extract was used for bitterness. The fermentation was carried out at 20 °C for three weeks. The beer produced with PK-KR1 reached an alcohol concentration of 0.1% ABV, while the beer produced with PK-KR2 had an alcohol percentage of 0.2% ABV. A low alcohol beer produced with PK-KR1 that contained 0.7% ABV by the end of fermentation was also produced with the same wort on a 1500 L scale not only with hop extract but with the addition of different hops during hot (boiling) and cold (fermentation) phase. It was reported that by the end of the fermentation, all the wort glucose had been consumed. Esters and higher alcohols were analyzed and compared to three commercial beers (Carlsberg pilsner, Heineken lager, Stella premium lager) that contained alcohol volumes between 4.6–5.2% ABV and three commercial NABs with 0.0% ABV. It has to be stated that the commercial NABs are unfermented NABs, neither dealcoholized full-strength beers nor NAB produced by limited fermentation [71,72]. Compared to the commercial beers, the NAB produced with *Pichia kluyveri* had similar levels of the flavor compounds isoamyl alcohol, ethyl hexanoate and ethyl octanoate. Isoamyl acetate was absent in the commercial NAB but present in the NAB produced with yeast strains PK-KR1 and PK-KR2 in double or higher than the amount in commercial beers, despite the limited fermentation. The authors reported the flavor profile of esters and higher alcohols to be closer to that of the commercial beers with 4.6–5.2% ABV alcohol than the flavor profile of any of the commercial NAB measured. Taste assessment by a tasting panel of brewers and beer consumers revealed a very beer-like flavor of the *Pichia kluyveri* NAB and a preference over the commercial NAB. Diacetyl production by *P. kluyveri* PK-KR1 was studied in a laboratory brewing trial compared to a beer produced with a *Saccharomyces cerevisiae* brewers' yeast strain. It was found that *P. kluyveri* produced much less diacetyl compared to the *Saccharomyces cerevisiae* brewers' yeast strain. The low alcohol *Picha kluyveri* beer proved to have a similar flavor profile to the *Pichia kluyveri* NAB. Therefore, Saerens and Swiegers [60] suggest that *P. kluyveri* is a yeast that is ideally suited to produce alcohol-free and low-alcohol beers. In a direct comparison to a *Saccharomycodes ludwigii* in 1.6 L lab

scale fermentations in a 7 °P hopped wort from pilsner malt extract, it was concluded that *P. kluyveri* was better suited to produce a NAB owing to lower alcohol production (0.1% ABV as opposed to 0.3% ABV), a higher production of wanted ester compounds (especially isoamyl acetate and phenylethyl acetate) and lower production of unwanted acids (especially octanoic acid and decanoic acid) [60].

In the above-mentioned study by Van Rijswijck et al. [69], the 24 ***Pichia kudriavzevii*** strains, screened in a 12 °P wort from barley wort extract, exhibited final ethanol concentrations of 0.5–0.8% ABV, due to the very limited consumption of maltose. The relative abundance of volatile esters to volatile alcohols was 50:50 (esters : alcohols), slightly higher esters than with *Cyberlindnera fabianii* (40:60).

2.5.5 *Torulaspora delbrueckii*

Michel et al. [73] investigated ten *Torulaspora delbrueckii* strains for their application in brewing. From a total of 10 strains, 9 strains exhibited low alcohol production ability due to their inability to utilize maltose. In 2 L trial fermentations in 12 °P wort from barley malt extract at 27 °C, the final beers exhibited an ethanol content of 0.83–0.94% ABV. Additionally, the strains were investigated for phenolic off-flavor (POF) production and sensitivity to hop compounds, specifically iso-α-acid concentration. None of the investigated yeast strains showed any positive POF behavior. The presence of 90 mg/L iso-α-acids in wort resulted in a slightly longer lag phase and lower slope for the log phase as compared to an unhopped wort. Diacetyl concentrations were between 0.1 and 0.3 mg/L. Concentration of secondary metabolites was low but sensory analysis with a trained panel revealed the beers to have a honey and pear-like character and two of them had an additional citrus fruit-like character.

Canonico et al. [74,75] investigated the use of *Torulaspora delbrueckii* strains in mixed culture fermentations with *Saccharomyces cerevisiae* brewers' yeast for bioflavoring and to reduce the alcohol content. In a pre-screening of 28 *T. delbrueckii* strains, 20 exhibited no maltose utilization. One maltose positive strain was selected for further investigation in mixed culture fermentations. However, single culture fermentations were also conducted in 12.7 °P and 12.3 °P all barley malt worts, respectively. In single culture fermentations, ethanol contents of only 2.66% and 2.62% ABV were achieved due to only partial maltose utilization. Despite the fact that the strain was able to utilize maltose, the real attenuation was poor at only 37%. Ester and higher alcohol concentrations in the final beers were

lower compared to the ones brewed with brewers' yeast. In accordance with Michel et al. [73] the beers brewed with single culture *Torulaspora delbrueckii* were characterized as fruity and citric in sensory trials. Additionally, Canonico et al. [75] reported the beers were full-bodied.

Tataridis et al. [76] fermented 100% malt worts (12.2 °P, pH 5.3) in 100 L scale at 20 °C. Two *T. delbrueckii* strains were used as well as one reference ale strain (*S. cerevisiae*). While the *S. cerevisiae* reached a final apparent attenuation of 79%, one *T. delbrueckii* strain showed 63% apparent attenuation, while the other only reached 36% apparent attenuation. Fermentation with the strain with low attenuation was also reported to have been progressing very slowly and the final ethanol content was only 2.34% ABV. Concentration of esters was also lower but the authors state that it had an equally pleasant yet slightly less intense flavor. Twelve panelists judged the beers and described the *T. delbrueckii* fermented beers, in accordance with the findings of Canonico et al. [74,75], as highly estery and fruity as well as full bodied.

In a study by Bellut et al. [62], several pre-screened non-*Saccharomyces* yeasts were applied in NAB brewing and compared to a commercially applied NAB strain (*Saccharomycodes ludwigii*) and a *Saccharomyces cerevisiae* brewers' yeast strain. The study included one *Torulaspora delbrueckii* strain. During characterization of the yeasts, it was found that the *Torulaspora delbrueckii* strain was only able to ferment the wort sugars glucose, fructose and sucrose (no maltose or maltotriose). In accordance with Michel et al. [73], the *T. delbrueckii* strain was found to be suitable for brewing applications. It did not develop a POF flavour and was able to grow in highly hopped worts containing up to 100 mg/L iso-α-acids. A 1.5 L fermentation trial was carried out in a 6.6 °P all barley malt wort at 25 °C with a pitching rate of 8×10^6 cells/mL. The NAB reached a final ethanol content of 0.50% ABV. It was reported that the strain consumed only a small amount of free amino nitrogen (FAN) and amino acids (AA). Ester levels were very low with 0.8 mg/L and the concentration of higher alcohols was also reported to be low at 18 mg/L. Diacetyl levels were reported to be below the threshold of 0.1 mg/L [59]. In contrast to the findings of other studies [73–75], the NAB produced with *T. delbrueckii* exhibited a low fruity character and was described as "wort-like" and "bread-like". However, an experienced expert taste panel was unable to discriminate the NAB produced with *T. delbrueckii* from the NAB produced with the commercially applied NAB strain *S. ludwigii* TUM SL 17.

2.5.6 *Zygosaccharomyces spp.*

Sohrabvandi et al. [77] investigated the successive application of two *Z. rouxii* strains (DSM 2531, DSM 2535) following a primary fermentation with *S. cerevisiae*. After 48 hours fermentation with *S. cerevisiae* at 12 and 24 °C, respectively, the yeast cells were inactivated (85 °C, 15 min) and the wort inoculated with *Z. rouxii*. It was then fermented for another 48 hours at 12 and 24 °C, respectively, with periodic aeration while monitoring the pH decrease, wort gravity and alcohol content. At end of fermentation, acetaldehyde, diacetyl and 2,3-pentandione were determined. Ethanol levels in the young beers after 96 hours fermentation with single culture *S. cerevisiae* fermentations reached 2.75% (12 °C) and 1.91% ABV (24 °C). Conversely, the inoculation with *Z. rouxii* after 48 hours led to a significant decrease in ethanol between 0.78–1.29% ABV with the resulting beers exhibiting alcohol levels between 0.36–0.40% ABV. The authors explained the ethanol reduction as follows: During primary fermentation, the *S. cerevisiae* consumed the wort monosaccharides (glucose and fructose), making them unavailable for *Z. rouxii* which is not able to consume maltose, the most abundant sugar in wort. Together with periodic aeration and the yeasts' ability to consume ethanol under aerobic conditions this led to a decrease in ethanol content. A sensory evaluation with a trained panel showed a higher acceptance for the fermentations with *Z. rouxii* at 24 °C, presumably due to the lower acetaldehyde content in the final beer owing to a fermentation temperature above the boiling point of acetaldehyde (20.2 °C). However, general acceptance was also significantly higher for the single strain culture *S. cerevisiae* fermentations compared to the mixed strain fermentations, owing to extended fermentations along with a more extensive aroma production.

Mohammadi et al. [55] studied the ethanol production of the *Z. rouxii* strain DSM 2531 after its immobilization on brewer's spent grain (BSG). Unlike in the study by Sohrabvandi et al. [77], this strain exhibited strong maltose utilization, fermenting the 6.5 °P all barley malt wort used in the study, to a final ethanol content of 2.0% ABV after 9 days at 7 °C, and 3.3% ABV after 7 days at 12 °C. The authors state the data indicated that immobilization affected the metabolic activity of the yeast strain, enabling it to consume maltose which led to higher ethanol concentrations than in other reported studies where *Z. rouxii* strains were unable to consume maltose [54,77].

Mortazavian et al. [56] investigated two *Z. rouxii* strains (DSM 70531, DSM 70535) for their ethanol production in 6 °P wort. Worts were fermented for 48 hours at 4, 12, and

24 °C with pitching rates of 10^7 and 4×10^7 cells/mL under anaerobic conditions and periodic aeration (every 12 hours). Ethanol values ranged from 0.04% (4 °C, 10^7 cells/mL, periodic aeration) to 0.40% (24 °C, 4×10^7 cells/mL, anaerobic). During sensory evaluation, the NABs were reported to show low acceptance.

De Francesco et al. [58] investigated five ***Zygosaccharomyces rouxii*** strains for their suitability to produce low-alcohol beers (< 1.2% ABV) from 12 °P all barley malt wort. Small fermentation tests were carried out in the 50 mL scale at 20 °C under aerobic conditions. Only one strain produced low alcohol with 0.93% ABV for the final beer. The other strains produced between 1.46% and 3.32% ABV. The differing ethanol contents were explained by their partial inability to ferment maltose. The low alcohol strain exhibited relatively high ester production with 34 mg/L and higher alcohols production of 92 mg/L. The low alcohol strain exhibited the highest diacetyl production amongst the strains studied with 0.85 mg/L diacetyl. However, all strains exhibited diacetyl levels above the flavor threshold of 0.1 mg/L. Unfortunately, no sensory analysis was conducted.

Two *Zygosaccharomyces* strains, ***Zygosaccharomyces bailii*** and ***Z. kombuchaensis***, were also included in the study by Bellut et al. [62]. The strains fermented the 6.6 °P wort to final ethanol concentrations of 0.42% and 0.48% ABV respectively, after 4 days fermentation at 25 °C. Like the *T. delbrueckii* strain, the strains showed no signs of hop sensitivity or production of POF. Ester production was again very low with 1 mg/L and higher alcohol production with 23 and 22 mg/L, respectively. Diacetyl values of the samples fermented with *Z. kombuchaensis* were with 0.15 mg/L above the flavor threshold, mirroring the descriptive part of the sensory where a diacetyl character was described for the *Z. kombuchaensis* sample together with the attributes wort-like and honey-like, while Z. *bailii* was described as being wort-like, honey-like, grassy, fruity and white wine-like. Again, the sensory panel was unable to discriminate the *Zygosaccharomyces* NAB from the NAB produced with the commercial NAB strain (*Saccharomycodes ludwigii*). The NAB produced with *Z. bailii* was perceived as less sweet in comparison to the other NABs produced during the study, but without statistical significance [62].

2.5.7 Other non-*Saccharomyces* species

Two *Hanseniaspora* strains, ***Hanseniaspora valbyensis*** and ***H. vineae***, were included in the study by Bellut et al. [62]. During characterization of the yeasts, it was found that the

Hanseniaspora spp. were only able to ferment the wort sugars glucose and fructose (no sucrose, maltose nor maltotriose utilization), and again showed no signs of sensitivity towards iso-α-acids concentrations of up to 100 mg/L and also no sign of producing POF. In the 1.5 L fermentation trial with 6.6 °P wort at 25 °C and a pitching rate of 8×10^6 cells/mL, the NAB reached final ethanol contents of 0.35% and 0.34% ABV, respectively. As with the *T. delbrueckii* and *Zygosaccharomyces* strains, it was reported that these strains consumed only small amounts of FAN and AA. Ester levels were low with a concentration of 0.9 mg/L and 6.0 mg/L, respectively. Levels of higher alcohols were also reported as low at 20–23 mg/L. In the sensory analysis with the expert panel, the NAB produced with *Hanseniaspora spp.* could again not be discriminated from the NAB produced with the commercially employed *S. ludwigii* strain. However, a substantial wort-like character was described for all the NABs. *H. valbyensis* produced 0.2 mg/L diacetyl, above the threshold value of 0.1 mg/L [59], mirroring the descriptive part of the sensory where *H. valbyensis* was described to have a diacetyl character, while *H. vineae* was given the attributes of "black tea" and "caramel-like".

De Francesco et al. [61] investigated the use of the psychrophilic yeast ***Mrakia gelida*** to produce a low alcohol beer. The species had previously been mentioned in connection with brewing by Thomas-Hall et al. [78] who reported the use of one *Mrakia* strain, isolated from soil in Antarctica, to brew a beer using a home brewing kit. De Francesco et al. used the *M. gelida* strain to ferment a 12 °P all barley malt wort at 10 °C. Fermentation came to a halt after 22 days with a final ethanol content of 1.16% ABV. The strain was shown to deplete fructose, glucose and sucrose but only very small amounts of maltose, hence the low alcohol production. Re-fermentation in bottles (bottle-conditioning) for 15 days at 10 °C after the addition of 5 g/L glucose led to a final ethanol content of 1.40% ABV. Fermentation performance and the low alcohol beers produced were compared to the commercial *Saccharomycodes ludwigii* yeast strain WSL 17. The beers fermented and re-fermented with *S. ludwigii* reached final ethanol contents of 1.23 and 1.32% ABV, respectively, showing a similar sugar utilization pattern. Diacetyl production was low with 5–8 μg/L. The sum of higher alcohols was lower for the *M. gelida* fermented samples with about 26 mg/L compared to about 43 mg/L for the *S. ludwigii* fermented samples. Although the ester content of beers produced with *M. gelida* was lower than the *S. ludwigii* counterparts (3.5 versus 15 mg/L), the beers produced with *M. gelida* were evaluated to be significantly fruitier determined during a sensory analysis. The panelists gave the beer fruity descriptors like apricot, grape and litchi, while only apricot was found

in the *S. ludwigii* sample. The authors reported low evaluation of sweetness (2.8–3.0 on a scale of 9) despite the low degree of fermentation (18–22%), hence the high amount of residual extract (9.3–9.8 °P). The *M. gelida* fermented samples additionally demonstrated to have a higher value for body with 5.5 compared to 2.0 for the *S. ludwigii* sample. The authors conclude *M. gelida* to be a good and candidate to be used for brewing [61]. Concerning yeast safety, the authors mention its inability to grow at human body temperature and that no abnormalities have been observed in rats that were fed with beer produced using *Mrakia* strains [61,78].

2.5.8 Different approaches

Apart from the use of non-*Saccharomyces* yeasts, other approaches include the use of yeast mutants [79] or more invasive methods such as gene knock-out [80,81]. Strejc et al. [79] investigated the performance of two spontaneous *Saccharomyces pastorianus* mutants resistant to 5,5,5-trifluoro-DL-leucin. The resistance to 5,5,5-trifluoro-DL-leucin is associated with an overproduction of the flavor active secondary metabolites isoamyl alcohol and isoamyl acetate. Elevated ester and higher alcohol levels were indeed observed in the alcohol-free beers (diluted to 0.5% ABV) produced with the mutant strains. Sensory analysis confirmed a fruitier (banana) taste compared to the NAB produced with the parental strain. Navrátil et al. [80] and Selecký et al. [81] investigated the use of *Saccharomyces cerevisiae* strains deficient in tricarboxylic acid (TCA) cycle enzyme activities. The strains with enzyme deficiencies produced less ethanol and the finished beers had considerably higher amounts of residual sugars. Some samples, fermented with enzyme deficient strains, showed over five times increased levels of organic acids [80].

Table 2.5–2 Non-*Saccharomyces* strains in wort substrates.

Yeast species	Strain designation	Wort gravity °P	Scale L	Ethanol content % ABV	Fermentation conditions Time (d) / Temperature (°C) / Pitching rate (×10⁶ cells/mL)	Secondary metabolites ΣEsters (mg/L) / ΣHigher alcohols (mg/L)	Sensory	Reference
Candida shehatae	CICC[1] 1766	9 9	0.3 200	0.47 0.37	NA / 14 / NA NA / 14 / NA	NA	NA	[66]
Candida zemplinina	Y.01670[2]	12 E	0.35	Ca 1.5	8 / 18 / NA	NA	NA	[64]
Candida zemplinina	Y.01670[2]	12 E	0.35	1.67	8 / 18 / NA	NA	NA	[65]
Cyberlindnera fabianii	9 strains[3]	12 E	0.1	0.6	7 / 20 / NA	40:60 ratio	NA	[69]
Cyberlindnera mrakii	NCYC[4] 500	13.80 E[12]	0.4	1.7	14 / 21 / 0.1	NA	NA	[70]
Hanseniaspora valbyensis	[5]	6.6	1.5	0.35	2 / 25 / 8	0.90 / 23.30	Wort-like, honey-like, cereal-like, diacetyl	[62]
Hanseniaspora vineae	[5]	6.6	1.5	0.34	2 / 25 / 8	6.00 / 20.2	Wort-like, honey-like, black tea, caramel	[62]
Mrakia gelida	DBVPG[6] 5952	12	25	1.16 1.40	22 / 10 / 0.4 15 d bottle-conditioned	0.6 / 25.5 3.5 / 27.7	Fruity (apricot, grape, litchi) , malty, hoppy,	[61]
Pichia kluyveri LAB	PK-KR1[7]	8.3	1500	0.7	21 / 21 / 5	2.9 / 1.8	NA	[60]
Pichia kluyveri NAB	PK-KR1[7] PK-KR2[7]	8.3	1000	0.1 0.2	21 / 20 / 5	2.5 / 2.0 5.4 / 2.0	Preference over commercial unfermented NAB	[60]
Pichia kudriavzevii	24 strains[3]	12 E	0.1	0.5–0.8	7 / 20 / NA	50:50 ratio	NA	[69]

Table 2 continued Non-*Saccharomyces* strains in wort substrates

Yeast species	Strain designation	Wort gravity °P	Scale L	Ethanol content % ABV	Fermentation conditions Time (d) / Temperature (°C) / Pitching rate (×10⁶ cells/mL)	Secondary metabolites ΣEsters (mg/L) / ΣHigher alcohols (mg/L)	Sensory	Reference
Torulaspora delbrueckii	DiSVA[8] 254	12.3	0.5	2.62	NA / 19 / 5	4.56 / 61.99	NA	[75]
Torulaspora delbrueckii	[5]	6.6	1.5	0.50	3 / 25 / 8	0.77 / 18.1	Wort-like, honey-like,	[62]
Torulaspora delbrueckii	9 strains from various depositors	12 E	2	0.83–0.94	NA / 27 / 15	2.23–5.96 / 20.53–36.79	Honey, pear-like, citrusy	[73]
Torulaspora delbrueckii	[9]	12.2	100	2.34	9 / 20 / NA	NA	Estery/fruity, high in body	[76]
Zygosaccharomyces bailii	[5]	6.6	1.5	0.42	4 / 25 / 8	1.00 / 23.1	Wort-like, honey-like, grassy, fruity, white wine	[62]
Zygosaccharomyces kombuchaensis	[5]	6.6	1.5	0.48	4 / 25 / 8	1.00 / 22.0	Wort-like, honey-like, diacetyl	[62]
Zygosaccharomyces rouxii	5 DBVPG[6] strains	12	0.05	0.93–3.32	10 / 20 / NA	2.16–71.15 / 61.80–196.77	NA	[58]
Zygosaccharomyces rouxii	DSM[10] 2531[11]	6.5	0.2	2.0 3.3	10 / 7 / NA 7 / 12 / NA	NA	NA	[55]
Zygosaccharomyces rouxii	DSM[10] 2531 DSM[10] 2535	6	NA	0.36–0.40	4 / 12,24 / 10[13]	NA	NC	[77]
Zygosaccharomyces rouxii	DSM[10] 70535, DSM[10] 70531	6	NA	0.04–0.40	2 / 4,12,24 / 10,40 anaerobic or periodic aeration	NA	Low acceptance	[56]

NA not available; E wort from malt extract; NC not comparable; [1] China Industrial Culture Collection; [2] National Collection of Agricultural and Industrial Microorganisms, Hungary; [3] isolated from fermented masau fruit [89]; [4] National Collection of Yeast Cultures, Norwich, UK; [5] isolated from kombucha; [5] Industrial Yeast Collection, DBVPG (University of Perugia, Italy); [7] National Measurement Institute, South Melbourne, Australia; [8] Yeast Collection of the Department of Life and Environmental Sciences (DiSVA), Polytechnic University of Marche, Italy; [9] isolated from wine; [10] Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; [11] immobilized on brewer's spent grain (BSG); [12] plus addition of glucose; [13] secondary fermentation after *S. cerevisiae* (48h)

2.6 Conclusion and holistic future perspective

The market figures and consumer demands are demonstrating that the future of NABLAB is current. In light of growing market and consumer trends, the large brewing companies are dedicated to extending their product portfolio with regards to non-alcoholic and low alcohol beer. However, the challenge of an inferior taste in comparison to normal strength beers has yet to be met.

The number of recent papers and their results give the production of NABLAB by non-*Saccharomyces* yeasts its justified existence beside physical dealcoholization methods. Also, given the reported, predominantly poor sensorial evaluation of NABLAB produced with *Saccharomycodes ludwigii*, which is already applied in NABLAB brewing, investigations into new non-*Saccharomyces* species are justified (Table 2.5–1). However, research into these species is mostly still at the stage of screenings and lab-scale fermentations, except for *Pichia kluyveri* which made it ready for commercialization. Furthermore, many studies are lacking sensory analysis of the end product even though the taste is an important factor.

Sensorial analysis (where available) with non-*Saccharomyces* fermented end products, often revealed fruity notes (i.e. apricot, litchi, pear, pear, citrus fruit) which are usually not common flavors in beer. However, the slight separation from beer-like flavors and towards a more fruity flavor, to stand as a category on its own, might even be beneficial in terms of consumer acceptance [33,34]. The application of non-*Saccharomyces* yeasts for NABLAB brewing could be used as a chance to tap into unconventional, atypical flavors while physical dealcoholization focuses on the most selective way possible to remove ethanol and leave the initial flavor profile intact. Indeed, a differentiation between dealcoholized NABLAB and NABLAB produced with biological methods already seems to be reasonable due to the substantial differences in residual extract (mostly maltose) and the consequentially reported sweet taste for biologically produced NABLAB [33].

NABLAB production by the use of non-*Saccharomyces* yeasts can also be seen as an opportunity for small and craft breweries, since changing the yeast is an easy modification compared to the substantial investment into equipment necessary for dealcoholization [2]. However, sterile and careful handling of the yeast is very important to avoid contamination and pasteurization becomes essential due to the residual sugars in the finished product [57].

The use of non-*Saccharomyces* yeasts is a substrate-dependent process since the amount of fermentable sugars in the wort (mostly monosaccharides and sucrose) defines the final ethanol content of the finished NABLAB. When brewing with adjuncts, it has to be considered which type of sugar is introduced into the wort and whether or not the applied yeast is able to ferment. Changed mashing procedures have also been considered as a way to alter the wort sugar composition and could potentially be applied in combination with non-conventional yeasts [43]. Another factor that has to be taken into consideration, is that dilution of the wort to lower original extract values also dilutes the FAN content which is required by the yeast [82]. However, emerging results have indicated that non-*Saccharomyces* yeasts are not as demanding as brewers' yeasts with regard to FAN (and AA) availability and consumption, presumably due to the less extensive fermentation [62,64].

Finally, another factor that has to be considered with seldom applied species is their safety with regards to consumption. However, even though without QPS ('Qualified Presumption of Safety', European Food Safety Authority) or GRAS ('Generally Recognized As Safe', American Food and Drug Administration) status, most species that are discussed in this review are on diverse lists of microorganisms that are applied in food production such as the "Inventory of Microorganisms with a documented history of use in food" [83] or its extended version [84], which is an important factor in food safety regulations [85,86]. More information about this topic can be found in diverse reviews and other sources [84,87,88].

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Chapter 3:

Application of non-*Saccharomyces* yeasts isolated from kombucha in the production of alcohol-free beer

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3.1 Abstract

Alcohol-free beer (AFB) is no longer just a niche product in the beer market. For brewers, this product category offers economic benefits in the form of a growing market and often a lower tax burden and enables brewers to extend their product portfolio and promote responsible drinking. Non-*Saccharomyces* yeasts are known for their flavor-enhancing properties in food fermentations, and their prevailing inability to ferment maltose and maltotriose sets a natural fermentation limit and can introduce a promising approach in the production of AFB (≤ 0.5% ABV). Five strains isolated from kombucha, *Hanseniaspora valbyensis*, *Hanseniaspora vineae*, *Torulaspora delbrueckii*, *Zygosaccharomyces bailii* and *Zygosaccharomyces kombuchaensis* were compared to a commercially applied AFB strain *Saccharomycodes ludwigii* and a *Saccharomyces cerevisiae* brewer's yeast. The strains were characterized for their sugar utilization, phenolic off-flavors, hop sensitivity and flocculation. Trial fermentations were analyzed for extract reduction, ethanol formation, pH drop, and final beers were analyzed for amino acids utilization and fermentation by-products. The performance of non-*Saccharomyces* strains and the commercial AFB strain were comparable during fermentation and production of fermentation by-products. An experienced sensory panel could not discriminate between the non-*Saccharomyces* AFB and the one produced with the commercial AFB strain, therefore indicating their suitability in AFB brewing.

3.2 Introduction

In many countries nowadays, alcohol-free beer (AFB) is no longer just a niche product in the beer market. For brewers, this product category offers economic benefits in the form of a steadily growing market and often a lower tax burden. At the same time, consumer preference for low-alcohol and alcohol-free beer is increasing due to greater interest in health, concern about weight, and considering the encouragement of responsible drinking, especially when driving. Furthermore, consumers benefit from the health effects of alcohol-free beers, which lie in the healthy beer components (antioxidants, soluble fiber, vitamins and minerals), lower energy intake and absence of negative aspects of alcohol consumption [1].

The terminology of alcohol-free beer and the corresponding alcohol limits are not uniform. The classifications of alcohol-free beers are defined in the statutory regulations of the individual countries. In many European countries such as Germany, Switzerland, Austria, Finland and Portugal, the term "alcohol-free" describes a maximum alcohol limit of 0.5% alcohol by volume (ABV). In Denmark and in the Netherlands the term "alcohol-free" may be applied to beers with < 0.1% ABV [2]. In the UK, the term "alcohol-free" can be applied to beer with < 0.05% ABV and the term "de-alcoholised" when the alcohol content is < 0.5% ABV [3]. In the USA and China, the limit of < 0.5% ABV is described by the term "non-alcoholic". Other countries like Spain or France are more tolerant towards the term "alcohol-free" with limits of 1.0% and 1.2% ABV, respectively [2].

The strategies to produce alcohol-free beers can be divided into two main groups: physical and biological processes. The physical processes, divided into thermal and membrane-based methods, are based on the removal of alcohol from regular beer and require considerable investments into special equipment [4]. In the case of thermal processes, the beer is heated to evaporate the ethanol, whereby also volatile aroma components are partly or completely evaporated. During membrane-based processes, ethanol (as well as aroma components) is removed mainly by its molecular size. Both cases can lead to less aromatic beers with reduced body and a significant acidity [2]. The most widespread biological approaches are based on limited ethanol formation by the yeast during the beer fermentation. Limited fermentation is usually performed in traditional brewery equipment and hence does not require additional investment. However, the beers are often perceived as sweet because of the interruption of the fermentation; fermentable sugars are not or only partly metabolized by the yeast, and the aromatic secondary metabolites are formed

only in small quantities or have not yet been generated due to the short fermentation time. In the field of limited fermentation different approaches are being pursued to improve the taste impression, which include the reduction of worty taste caused by Strecker aldehydes [5,6], the use of immobilized yeasts [7], and the use of alternative yeast strains or yeast mutants [8]. The use of non-*Saccharomyces* yeasts (other than *Saccharomycodes ludwigii*) for the production of AFB has not been studied to a great extent, though changing the yeast is an easy adjustment for breweries to make. By using yeast strains which are unable to ferment the most abundant wort sugars maltose and maltotriose, a natural fermentation limit is set. It is unnecessary to stop the fermentation by cooling or yeast separation, since the fermentation will naturally come to a halt by the depletion of the fermentable sugars. However, the challenge is to discover non-*Saccharomyces* yeasts, that are able to produce flavors that can mask the wort-like off-flavors created by residual wort sugars and aldehydes [5,6].

There are few published studies on the application of non-*Saccharomyces* yeasts in the production of alcohol-free beer [9]. Mostly known as spoilage yeasts for beer or other beverages, they can form a range of flavors which could potentially benefit the alcohol-free beer [10–12]. In a recent patent application, Saerens and Swiegers [13] used *Pichia kluyveri* to produce a low-alcohol or alcohol-free beer with a flavor profile very close to a beer of at least 4% ABV. Another patent by Li et al. [14] suggests the use of *Candida shehatae* to produce an alcohol-free beer. Sohrabvandi et al. [15] investigated the use of *Zygosaccharomyces rouxii* in a successive application after *Saccharomyces cerevisiae* in order to produce an alcohol-free beer. A significant alcohol reduction could be shown; however, the taste was compromised. De Francesco et al. [3] investigated strains of *Z. rouxii* and *Saccharomycodes ludwigii* for the production of low-alcohol beers. In contrast to the results from Sohrabvandi et al. [15], *Z. rouxii* strains were found unsuitable to produce low alcohol beer due to the production of a high concentration of ethanol, however, *S. ludwigii* was identified as a yeast species with great potential for the production of low-alcohol and alcohol-free beer.

3.3 Materials and Methods

3.3.1 Materials

All reagents used in this study were at least analytical grade from Sigma-Aldrich (St Louis MO, USA) unless stated otherwise. Malt extract used for the flocculation test, hop resistance test and propagation was supplied by Muntons (Spraymalt Light, Muntons plc, Suffolk, UK). Pilsner malt for wort production was sourced from Weyermann® (Malzfabrik Weyermann, Bamberg, Germany).

3.3.2 Yeast strains

The yeast strains investigated in this study were isolated from kombucha. DNA of the isolates was extracted using an extraction kit (Yeast DNA Extraction Kit, Thermo Fisher Scientific, Waltham MA, USA). To amplify the D1/D2 domain of the 26S rRNA gene the primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') were used. PCR was performed using the temperature protocol: 95 °C / 2 min; 30 cycles of 95 °C / 30 s, 56 °C / 15 s; 72 °C / 60 s; 72 °C / 5 min.

Stocks were kept in glycerol at –80 °C. Table 3.3–1 lists the yeast strains that were used in this study.

Table 3.3–1 Yeast strain designation, species and origin of yeast strains used in this study.

Strain designation	Species	Origin
KBI 5.4	*Zygosaccharomyces kombuchaensis*	UCC Culture Collection (Kombucha, Australia)
KBI 7.1	*Hanseniaspora vineae*	UCC Culture Collection (Kombucha, USA)
KBI 22.1	*Hanseniaspora valbyensis*	UCC Culture Collection (Kombucha, Australia)
KBI 22.2	*Torulaspora delbrueckii*	UCC Culture Collection (Kombucha, Australia)
KBI 25.2	*Zygosaccharomyces bailii*	UCC Culture Collection (Kombucha, USA)
TUM SL 17	*Saccharomycodes ludwigii*	FZW BLQ[1], Weihenstephan, Germany
WLP001	*Saccharomyces cerevisiae*	California Ale Yeast®, Whitelabs, San Diego CA, USA
TUM 68[2]	*Saccharomyces cerevisiae*	FZW BLQ[1], Weihenstephan, Germany

[1] Research Centre Weihenstephan for Brewing and Food Quality, Technische Universität München
[2] only used as positive control for POF test.

Strains were grown on PDA agar plates for 72 h at 25 °C and stored in a sterile environment at 2–4 °C. During this study, strains were subcultured at intervals of 2 weeks. The strains were chosen from a collection of 64 isolated strains by their performance in a pre-screening in wort (data not shown).

3.3.3 Flocculation test

Flocculation of the yeast strains was evaluated using a slightly modified Helm's assay [18,19]. Essentially, all cells were washed in EDTA and the sedimentation period was extended to 10 min to allow slowly flocculating strains to show their potential. Fermentation wort was 75 g spray-dried malt extract (Spraymalt Light, Muntons plc, Suffolk, UK) in 1000 mL brewing water with 30 IBU (30 mg/mL iso-α-acids; from 30% stock solution; Barth-Haas Group, Nürnberg, Germany). Cultures recovered from fermentation were washed with 5 mM EDTA (pH 7) to break the cell aggregates. Flocculation was assayed by first washing the yeast pellets with 3.7 mM CaSO4 solution and resuspending them in flocculation solution containing 3.7 mM CaSO4, 6.8 g/L sodium acetate and 4.05 g/L acetic acid (pH 4.5). Yeast cells in control tubes were resuspended in 5 mM EDTA (pH 7) without undergoing the flocculation step with CaSO4. After a sedimentation period of 10 min, samples were taken from just below the meniscus and dispersed in 5 mM EDTA. The absorbance at 600 nm was measured (Helios Gamma Spectrophotometer, Thermo Fisher Scientific, Waltham MA, USA), and percentage of flocculation was determined from the difference in absorbance between control and flocculation tubes.

3.3.4 Sugar utilization

Substrate utilization tests YT MicroPlate™ (Biolog Inc., Hayward CA, USA) were used to analyze the biochemical spectrum of the yeast isolates. The yeast strains were cultured on Sabouraud agar for 72 h at 25 °C. Individual colonies were taken from the surface using sterile inoculation loops and suspended in 20 mL of sterile water. Colonies were gradually added to increase the turbidity until 46±1%. From this yeast solution, 100 µL were added to each of the 96 wells of the YT MicroPlate™. After incubation at 25 °C for 72 h, the YT MicroPlate™ was read with the Microplate reader (Multiskan FC, Thermo Fischer Scientific) at a wavelength of 590 nm. Results are shown as "+" for a significant

increase in optical density (OD) compared to the OD of the water control and a "–" for showing no difference. The substrate utilization test was carried out in duplicate.

3.3.5 Hop resistance

Three 100 ml flasks containing sterile filtered wort (75 g Muntons Spraymalt Light in 1000 mL brewing water) were adjusted to 0, 50 and 100 mg/L iso-α-acids respectively by using an aliquot of a stock solution of 3% iso-α-acids in 96% (v/v) ethanol (Barth-Haas Group, Nürnberg, Germany). The pure grown yeast cells were added to a total cell count of 10^5 cells/mL. Optical density (OD_{600}) was measured every 40 min at 25 °C without shaking over a time period of 96 h (Multiskan FC, Thermo Scientific, Waltham, Massachusetts, USA).

3.3.6 Phenolic off-flavor test

The phenolic off-flavor (POF) test was conducted according to Meier-Dörnberg et al. [20]. Yeast strains were spread on yeasts and mold agar plate (YM-agar) containing one of the following precursors: ferulic acid, cinnamic acid or coumaric acid. After three days of incubation at 25 °C, plates were evaluated by sniffing to detect any of the following aromas: ferulic acid becomes 4-vinylguaiacol (clove-like), cinnamic acid becomes 4-vinylstyrene (Styrofoam-like) and coumaric acid becomes 4-vinylphenol (medicinal-like). TUM 68 (Research Center Weihenstephan for Brewing and Food Quality, Freising-Weihenstephan, Germany) was used as a positive control.

3.3.7 Propagation

Propagation wort was prepared by dissolving 75 g spray-dried malt (Muntons Spraymalt light, Muntons plc, Suffolk, UK) and 30 g glucose (Gem Pack Foods Ltd., Dublin, Ireland) in 1000 mL brewing water, followed by sterilization (15 min, 121 °C). Investigated pure yeast strains were inoculated into a 140 ml of sterile propagation wort. The flask was covered with sterile cotton and placed in an incubator with orbital shaker (ES-80 shaker-incubator, Grant Instruments (Cambridge) Ltd, Shepreth, UK) and incubated for 48 h at an orbital agitation of 170 rpm and 25 °C. Viability was measured by staining with Löffler's methylene blue solution (MEBAK 10.11.3.3) and cells were counted with a Hemocytometer (Blaubrand, Thoma pattern).

3.3.8 Wort production

Wort for fermentation trials was produced on a 60 L pilot-scale brewing plant comprising of a combined mash-boiling vessel, a lauter tun and a whirlpool tank. Weyermann® Pilsner Malt was milled with a two-roller mill fitted with a 0.8 mm gap size between the rollers. Seven kg of malt were mashed in with 40 L of brewing water. The following mashing regime was employed: 40 min at 50 °C, 20 min at 62 °C, 20 min at 72 °C and 5 min at 78 °C for mashing off. The heating rate was 1 °C/min between the temperature rests. The mash was pumped in the lauter tun and lautering was performed using three sparging steps of 5 L each. Collected wort was boiled for 45 min. 25 g Magnum hop pellets (10.5% iso-α-acids) were added at the start of the boil for a calculated IBU content of 10.4. Hot trub precipitates and hop residue were removed by means of the whirlpool with a rest of 20 min. Wort was pumped back to the boiling vessel, corrected to a specific gravity of 6.6 °P extract by the addition of brewing water, and heated to 100 °C before filling into sterile 5 L containers, which were kept for short-term storage at 2 °C.

3.3.9 Fermentation

Fermentation trials were carried out in 2-litre sterile Duran glass bottles (Lennox Laboratory Supplies Ltd, Dublin, Ireland), equipped with an air lock to control CO_2 under sterile conditions. Bottles were filled with 1600 mL wort. Respective fermentation temperature was 25 °C, a temperature that suits most non-*Saccharomyces* species [21]. Fermentation was performed until no change in extract could be measured for 24 h. Yeast cells for pitching were washed by centrifugation at 5000 *g* for 5 min and resuspension in sterile water. Supernatant was discarded to ensure no carryover of sugars from the propagation wort into the fermentation wort and yeast cells were resuspended in sterile water. The pitching volume was 30 mL with a pitching rate of 8×10^6 CFU/mL at a viability of at least 96% for all fermentations.

3.3.10 Analyses of the produced beers

50 mL samples of each fermentation were withdrawn every day. Cell count was performed using the Hemocytometer (Blaubrand, Thoma pattern). Yeast was separated by centrifugation at 5000 *g* for 5 min and specific gravity and ethanol content of the supernatant were measured using a density meter DMA 4500M with Alcolyzer Beer ME

(Anton-Paar GmbH, Graz, Austria). The pH value was determined using a digital pH meter (Mettler Toledo LLC, Columbus OH, USA).

Analyses of the final beers were performed by the following methods. Sugars and ethanol were determined by high performance liquid chromatography HPLC Agilent 1260 Infinity (Agilent Technologies, Santa Clara CA, USA) equipped a refractive index detector (RID) and a Sugar-Pak I 10 μm, 6.5 mm × 300 mm column (Waters, Milford MA, USA) with 0.1 mM Ca-EDTA as mobile phase and a flow rate of 0.2 mL/min. Differentiation of maltose and sucrose was achieved with a Nova-Pak 4 μm, 4.6 mm × 250 mm column (Waters, Milford MA, USA) with acetonitrile/water 75:25 (v/v) as mobile phase and a flow rate of 1.2 mL/min.

Free vicinal diketones were quantified by a Clarus 500 gas chromatograph (Perkin-Elmer, Waltham MA, USA) with a headspace unit and Elite-5 60 m × 0.25 mm, 0.5 μm column using a 2,3-hexanedione internal standard. The final concentrations of fermentation by-products (e.g. acetaldehyde, ethyl acetate, n-propanol, i-butanol, isoamyl acetate, amyl alcohols) were quantified using a gas chromatograph with a headspace unit and INNOWAX cross-linked polyethylene-glycol 60 m × 0.32 mm 0.5 μm column (Perkin-Elmer, Waltham MA, USA). The amino acid content was quantified using the HPLC MEBAK 2.6.4.1 method. Free amino nitrogen (FAN) was measured using a ninhydrin-based dying method where absorbance is measured at 570 nm against glycine (MEBAK 2.6.4.1). Free vicinal diketones, fermentation by-products and amino acids were quantified in duplicate.

3.3.11 Sensory evaluation

All beer samples were tasted and judged by a sensory panel of 11 panelists with long-standing experience in the sensory analysis of beer. "Fruity", "floral" and "wort-like" were chosen as attributes for the smell. "Acidic/sour" and "sweet" were chosen as attributes for the taste and the panelists were additionally asked to evaluate the "body". Panelists were asked to evaluate the attributes in its intensity on a scale from 0, nothing, to 10, extremely. Before the evaluation of the intensity, a descriptive sensory was performed where the panelists were asked to record the flavors they perceived from the samples. Samples were given in dark glasses with a three-digit code.

3.3.12 Statistical analyses

Fermentations and analyses were carried out in triplicate, unless stated otherwise. The data was statistically analyzed using RStudio, Version 1.1.423 with R version 3.4.4 (RStudio Inc, Boston MA, USA; R Core Team, r-project). For the analysis of sensory data and constructing the multidimensional sensory profile, the R package "SensoMineR" was used [22]. One-way ANOVA was used to compare means and Tukey's test with 95% confidence intervals was applied for the pairwise comparison of means. The statistical significance value for both ANOVA and multiple comparison analysis was set at $p = 0.05$. Values are given as means ± standard deviation.

3.4 Results and Discussion

3.4.1 Yeast characterization

When characterizing non-*Saccharomyces* yeasts for their suitability in alcohol-free beer production, several key attributes should be investigated. The first attribute is the ability to utilize the sugars in the wort, as for all-malt beers the average composition of fermentable wort sugars is 12% glucose and fructose (0.8–2.8%), 5% sucrose, 65% maltose and 17.5% maltotriose [23]. For its suitability to produce alcohol-free beers it should not be able to ferment maltose. Considering the sugars that are important for brewing (glucose, fructose, sucrose, maltose, maltotriose), all strains were capable of utilizing glucose and fructose (Table 3.4–1).

Table 3.4–1 Substrate utilization profile by BioLog YT plate test, phenolic off-flavor (POF) performance and flocculation performance of the investigated yeasts.

Attribute	WLP001	TUM SL 17	KBI 22.1	KBI 7.1	KBI 22.2	KBI 25.2	KBI 5.4
Maltose	+	–	–	–	–	–	–
Maltotriose	+	–	–	–	–	–	–
Glucose	+	+	+	+	+	+	+
Fructose[1]	+	+	+	+	+	+	+
Sucrose	+	+	–	–	+	+	+
Melibiose	–	–	–	–	+	–	–
Raffinose	+	+	–	–	+	–	+
Cellobiose	–	+	+	+	–	–	–
POF	–	–	–	–	–	–	–
Flocculation (%)	83 ± 3^{d}	60 ± 7^{c}	11 ± 8^{a}	41 ± 4^{b}	17 ± 0^{a}	45 ± 0^{bc}	44 ± 3^{bc}

[1] by HPLC sugar analysis; fructose was not detected in final beers.
Different superscripts of values within a row indicate a significant difference ($p \leq 0.05$).

All investigated strains except KBI 22.1 and KBI 7.1 (*Hanseniaspora spp.*) were able to utilize sucrose. The inability to utilize sucrose by KBI 22.1 and KBI 7.1 can be traced to the absence of the enzyme invertase, which converts sucrose into glucose and fructose [24]. In kombucha (source of investigated yeasts), where sucrose is the main or only sugar source, the conversion of sucrose by yeast invertase is required for *Acetobacter spp.* to subsequently produce acetic acid [25]. Looking at the main sugars of wort, only the control strain WLP001 was able to utilize maltose and maltotriose. The disability to utilize maltose and maltotriose indicates the absence of a maltose transporter and the enzyme maltase [26,27]. The sugar utilization patterns from the BioLog YT plate test were confirmed by the sugar analysis of the final beers.

The second criterion for a yeast to be applied in brewing is its capability of growing in the presence of hop-derived iso-α-acids. The resistance against iso-a-acids and their induced weak organic acid stress were studied for the *Saccharomyces* species but it has barely been investigated for non-*Saccharomyces* species [28–30]. All investigated strains were able to grow in wort with 0, 50 and 100 IBU (international bitterness units). Figure 3.4–1 shows the exemplary growth of the investigated strains at 50 IBU (due to all strains exhibiting the same behavior at different IBU values, the rest of the data is not shown).

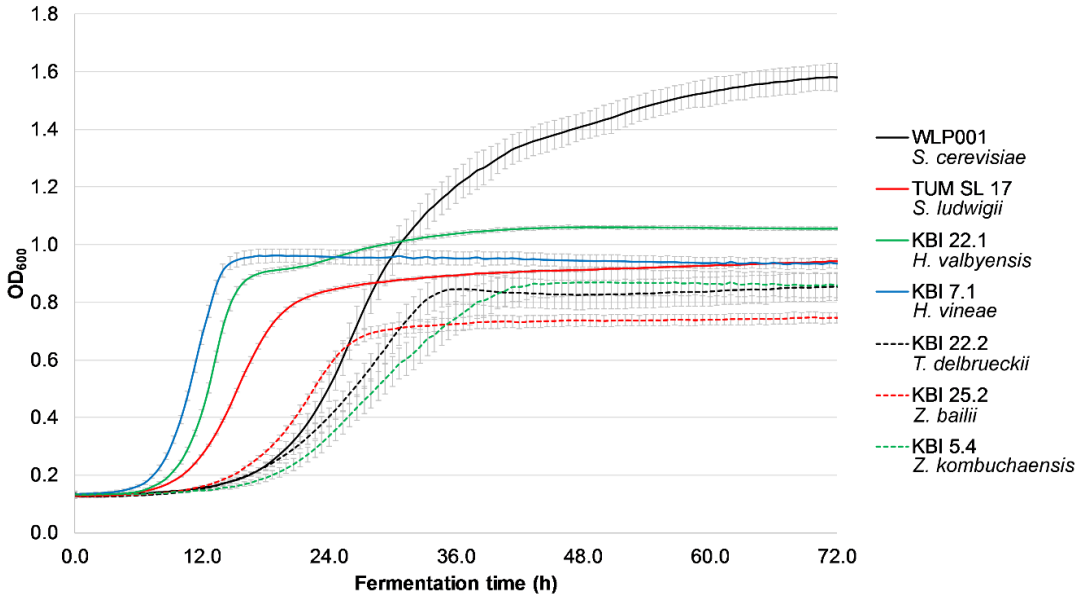


Figure 3.4–1 Growth curves of investigated yeast strains in 7 °P wort with 50 IBU.

KBI 7.1, KBI 22.1 and TUM SL 17 had the shortest lag time with log phases starting between 8 and 13 hours after inoculation, followed by the rest of the investigated strains with log phases between 19 and 23 hours. However, all the investigated yeast strains were able to grow in high iso-α-acid concentrations and are therefore able to ferment even highly hopped worts. The presence of iso-α-acids did not have any influence on the growth of the investigated yeast strains. This is in contrast to a study by Michel et al. [29], where the presence of 90 IBU resulted in a longer log phase as well as a lower slope during log phase compared with 50 and 0 IBU with several *Torulaspora delbrueckii* strains.

None of the investigated yeast strains, except the positive control TUM 68, showed any positive POF behavior on plate when exposed to precursors, suggesting the absence of functional *PAD1* and *FDC1* genes [31] (Table 3.4–1). Those results were consistent with the sensory of the final beers where no panelist detected any phenolic off-flavors. POF

are produced by decarboxylation of ferulic acid, coumaric acid and cinnamic acid, which are present in beer wort. Ferulic acid becomes 4-vinylguaiacol, which is described as having a clove-like flavor [32]. Apart from the wheat beer style this flavor is usually unwanted [33]. Coumaric acid is decarboxylated to 4-vinylphenol, having a solvent-like flavor, and cinnamic acid becomes 4-vinylstyrene, which has a Styrofoam-like flavor [34].

In terms of flocculation, a prerequisite for bulk sedimentation of yeast during brewery fermentation, the control yeast WLP001 performed as most flocculent of all the investigated strains. The method defines flocculation values of 85–100% as "very flocculent", 20–80% as "moderately flocculent" and less than 20% as "non-flocculent" yeasts [18]. By that definition WLP001 was with 83.3% at the very upper scale of moderately flocculent yeasts. KBI 22.1 and KBI 22.2 fell with 11.0% and 17.0%, respectively into the category of non-flocculent yeasts, while the rest qualified as moderately flocculent (Table 3.4–1). The most common mechanism of yeast flocculation is generally accepted to be the lectin-mediated adhesion of adjacent yeast cells to form large cell aggregations [35]. The flocculation characteristics of yeast are strongly strain-dependent and largely defined by which members of the *FLO* genes, which encode for lectin proteins, are functional in each strain. Rossouw et al. [36] showed that for 17 out of 18 investigated, non-*Saccharomyces* strains the flocculation phenotypes were calcium-dependent, thus indicating a *FLO*-dependency much like in *Saccharomyces cerevisiae*.

3.4.2 Fermentation performance

The aim of propagation is to get a high quantity of yeast cells with high viability and vitality. After propagation for 48 h, cell counts ranged from 7.1×10^7 cells/mL for TUM SL 17 to 6.5×10^8 cells/mL for KBI 22.1, as illustrated in Figure 3.4–2.

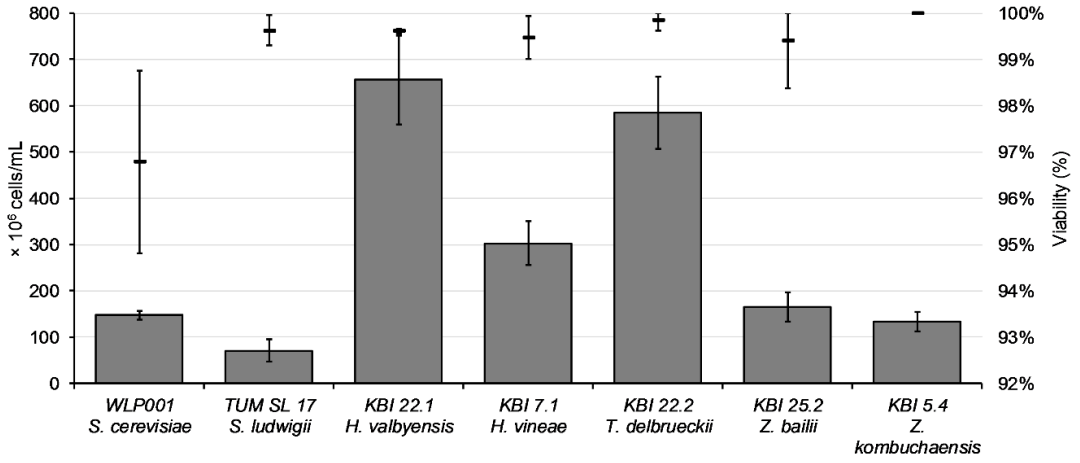


Figure 3.4–2 Cell count (bars) and viability (lines) of investigated strains after propagation for 48 h in 10 °P propagation wort (7% wort extract spiked with 3% glucose).

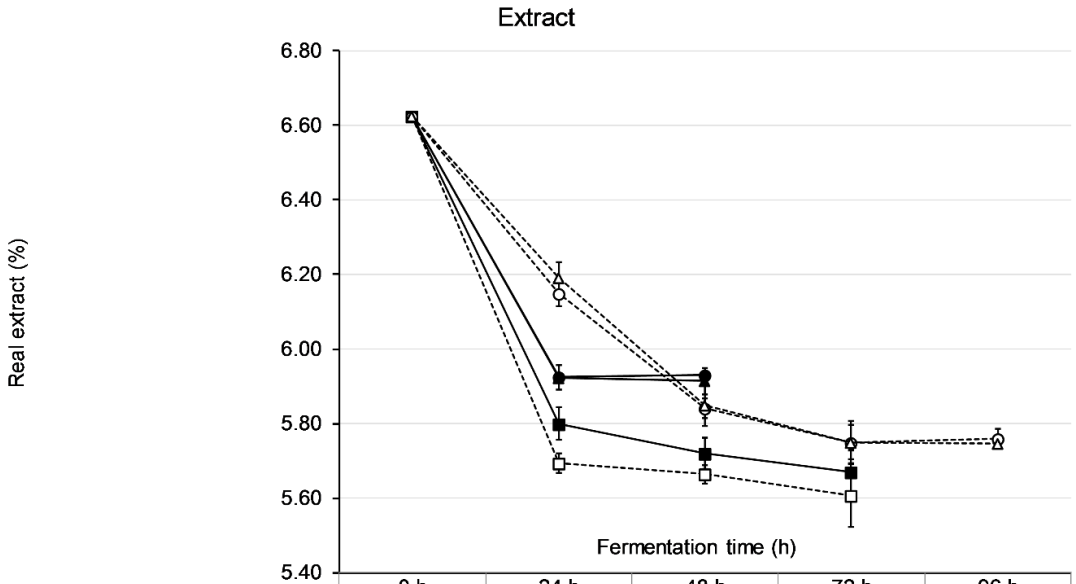
Except for WLP001 with a viability value around 97%, viability values after propagation were over 99%. The composition of the wort used for the fermentation trials is shown in Table 3.4–2.

Table 3.4–2 Wort composition of fermentation wort.

Wort composition	Unit	Value
Extract	°P	6.63 ± 0.01
pH		5.73 ± 0.01
Maltose	g/L	26.60 ± 0.25
Maltotriose	g/L	5.09 ± 0.04
Glucose	g/L	5.46 ± 0.01
Sucrose	g/L	1.70 ± 0.04
Fructose	g/L	1.29 ± 0.02
Total amino acids	mg/100 mL	98.31 ± 0.86
Free amino nitrogen	mg/L	110 ± 5

The wort was fermented until no change in extract was measurable for 24 hours. KBI 22.2 showed the steepest decrease in extract with an extract drop of nearly 1°P extract in the first 24 h followed by TUM SL 17 (0.8°P) and the *Hanseniaspora spp.* KBI 22.1 and KBI 7.1 (0.7°P) (Figure 3.4–3). The *Zygosaccharomyces spp.*, KBI 25.2 and KBI 5.4 followed a lesser decrease in extract with a linear decrease of about 0.45°P per 24 h for the first 48 hours. Consequently, KBI 22.2 reached an ethanol concentration of 0.42% ABV after 24 h, while KBI 25.2 and KBI 5.4 produced only 0.21% ABV and 0.20% ABV, respectively. Fermentation ceased fastest for KBI 22.1 and KBI 7.1 after 24 h when fructose and glucose were depleted while sucrose remained untouched. TUM SL 17 and KBI 22.2 reached their final extract after 48 h of fermentation. KBI 25.2 and KBI 5.4,

demonstrating the slowest metabolism, ceased fermentation after 72 h. WLP001 fermented the wort to a final extract (real) of 2.13 °P after 96 hours (data not shown).



		0 h	24 h	48 h	72 h	96 h
TUM SL 17	*S. ludwigii*	6.63	5.80	5.72	5.67	
KBI 22.1	*H. valbyensis*	6.63	5.93	5.93		
KBI 7.1	*H. vineae*	6.63	5.92	5.91		
KBI 22.2	*T. delbrueckii*	6.63	5.69	5.66	5.61	
KBI 25.2	*Z. bailii*	6.63	6.15	5.84	5.75	5.76
KBI 5.4	*Z. kombuchaensis*	6.63	6.19	5.85	5.75	5.75

Figure 3.4–3 Drop in real extract for the investigated maltose-negative yeast strains.

The pH value dropped during the first 24 hours of fermentation by values ranging from 0.7 for KBI 5.4 to 1.0 for KBI 22.2 with only marginal changes thereafter (data not shown). Due to the high starting pH of the wort of 5.7, the beers, except WLP001, did not reach pH values below 4.5, which are desired in order to serve as one of the microbial hurdles for beer spoiling bacteria to overcome [37]. However, lower pH values can be reached with a lower starting pH of the wort, which can be adjusted i.e. by lactic acid, the use of sour malt or biological acidification. Visual evaluation of the finished beers matched the analyzed flocculation behavior from Table 3.4–1. KBI 22.1 and KBI 22.2 showed the highest turbidity and cells in suspension while TUM SL 17 and WLP001 were the clearest beers with a layer of flocculated yeast at the bottom.

Sugar analysis of the final beers revealed a complete depletion of all fermentable sugars by WLP001. Consistent with the sugar utilization patterns from Table 3.4–1, the other investigated strains showed a complete depletion of monosaccharides. Sucrose was not

fermented by the *Hanseniaspora spp.* KBI 22.1 and KBI 7.1 but was depleted by the other strains as predicted before.

Analyses of the ethanol content of the final beers showed all investigated maltose-negative strains at or below 0.5% ABV. WLP001, the maltose-positive control reached an ethanol content of 2.61% ABV. The maltose-negative control, TUM SL 17, together with KBI 22.2 showed an ethanol content of 0.50% ABV, followed by KBI 5.4 and KBI 25.2 with 0.48% ABV and 0.42% ABV, respectively. The least ethanol content showed KBI 7.1 and KBI 22.1 with 0.34% ABV and 0.35% ABV, respectively. The lower ethanol production by KBI 7.1 and KBI 22.1 was due to the inability to ferment sucrose, which reflected in a higher final gravity of the beers (Table 3.4–3). Corresponding to a lower degree of fermentation, pH values for the alcohol-free beers are higher, ranging between 4.61 (KBI 5.4) and 4.84 (KBI 22.1).

Table 3.4–3 Analysis of final beers after fermentation with investigated yeasts.

	WLP001	**TUM SL 17**	**KBI 22.1**	**KBI 7.1**	**KBI 22.2**	**KBI 25.2**	**KBI 5.4**
	S. cerevisiae	*S. ludwigii*	*H. valbyensis*	*H. vineae*	*T. delbrueckii*	*Z. bailii*	*Z. kombuchaensis*
Ethanol (% ABV)	2.61 ± 0.10^{d}	0.50 ± 0.01^{c}	0.35 ± 0.01^{ab}	0.34 ± 0.02^{a}	0.50 ± 0.01^{c}	0.42 ± 0.07^{abc}	0.48 ± 0.01^{bc}
Final real extract (°P)	2.13 ± 0.02	5.67 ± 0.06	5.93 ± 0.00	5.91 ± 0.04	5.61 ± 0.09	5.76 ± 0.03	5.75 ± 0.01
pH	4.18 ± 0.02^{a}	4.76 ± 0.04^{cd}	4.84 ± 0.02^{e}	4.78 ± 0.03^{de}	4.69 ± 0.02^{c}	4.71 ± 0.02^{cd}	4.61 ± 0.02^{b}
FAN (mg/L)	48 ± 3^{a}	90 ± 6^{b}	91 ± 0^{b}	91 ± 0^{b}	83 ± 0^{b}	83 ± 17^{b}	93 ± 1^{b}

Different superscripts of values within a row indicate a significant difference ($p \leq 0.05$).

3.4.3 Amino acid metabolism

The amino acid (AA) catabolism is very important for the formation of higher alcohols in the final beer. AA are important for the formation of higher alcohols such as propanol, isobutanol and isoamyl alcohol via the Ehrlich pathway [38]. The AA are transaminated to α-keto acids and decarboxylated to form the respective aldehyde, which are further reduced to higher alcohols [10]. AA analysis revealed a substantial AA consumption only by WLP001 with a consumption of 76.4% of AA and depleting six AA namely aspartic and glutamic acid, asparagine, methionine, leucine and isoleucine (Table 3.4–4), owing to its longer fermentation time and higher sugar uptake. WLP001 also formed higher concentrations of higher alcohols (4 times higher) than the other strains, as seen in Table

3.4–5. Adequate levels of amino acids and free amino nitrogen (FAN) in wort are necessary for a "healthy" fermentation [39–41]. Only one depletion of methionine for KBI 22.1 revealed that, for the low-alcohol strains, every AA was available in the wort in sufficient amounts. The high amount of residual amino acids and FAN after fermentation indicated that the diluted wort (6.64°P) used in this study held a sufficient amount of amino acids and free amino nitrogen for a healthy fermentation. Generally, AA consumption was strain dependent with TUM SL 17 and KBI 22.2 being on the higher end with 26.6% and 25.5% of consumption, respectively. KBI 5.4 consumed with 11.2% the lowest amount of AA (Table 3.4–4). KBI 7.1 formed serine, which is shown at a significantly higher value after fermentation in Table 3.4–4.

3.4.4 Volatile compounds

Analysis of the volatile fraction of the beers fermented with the different yeasts showed mostly only small differences in higher alcohols, esters and diacetyl (Table 3.4–5). Regarding higher alcohols, n-propanol, isobutanol and isoamyl alcohol contents were significantly higher for the maltose-positive control WLP001, owing to the extensive fermentation compared to the low-alcohol strains, which showed no significant differences amongst each other for n-propanol and isobutanol. Small, yet significant differences could be found for isoamyl alcohol values with KBI 22.1 exhibiting highest (16.5 mg/L) and KBI 22.2 exhibiting lowest (10.4 mg/L) values amongst the strains. The odor threshold for isoamyl alcohol, which is considered to have a fruity, brandy-like aroma, is reported to lay between 50–70 mg/L [10]. All the investigated low-alcohol yeasts produced a fifth to a third of the odor threshold of isoamyl alcohols. In sum, the low-alcohol strains produced an average of 21 mg/L of higher alcohols compared to 82 mg/L by WLP001. Other major contributors to the aroma of beer are acetate esters [11]. Volatile esters are the product of an enzyme-catalyzed condensation reaction between acyl-CoA – a product of the sugar and lipid metabolism – and a higher alcohol, originating from the nitrogen metabolism [42,43]. Ethyl acetate represents approximately one third of all esters in beers [44]. Sum of acetate ester concentration was low in all the beers ranging from 0.77 mg/L for KBI 22.2 to 6.00 mg/L for KBI 7.1 (Table 3.4–5).

Table 3.4–4 Amino acid analysis of wort and final beers fermented with the investigated strains; values in mg/100 mL.

	Wort	WLP001 *S. cerevisiae*	TUM SL 17 *S. ludwigii*	KBI 22.1 *H. valbyensis*	KBI 7.1 *H. vineae*	KBI 22.2 *T. delbrueckii*	KBI 25.2 *Z. bailii*	KBI 5.4 *Z. kombuchaensis*
Aspartic acid	3.16 ± 0.08^{b}	$< 0.5^{a}$	2.45 ± 0.08^{b}	3.10 ± 0.13^{b}	3.29 ± 0.49^{b}	2.73 ± 0.51^{b}	3.31 ± 0.05^{b}	3.24 ± 0.04^{b}
Glutamic acid	3.13 ± 0.02^{b}	$< 0.5^{a}$	3.91 ± 0.03^{c}	3.21 ± 0.15^{b}	2.91 ± 0.01^{b}	3.04 ± 0.11^{b}	3.26 ± 0.02^{b}	3.14 ± 0.05^{b}
Asparagine	6.36 ± 0.12^{d}	$< 0.5^{a}$	1.04 ± 0.07^{ab}	4.14 ± 0.15^{bcd}	2.63 ± 0.81^{abc}	5.58 ± 0.89^{cd}	5.23 ± 0.05^{cd}	4.14 ± 1.24^{bcd}
Serine	4.34 ± 0.04^{bc}	0.73 ± 0.21^{a}	2.21 ± 0.10^{ab}	2.99 ± 0.06^{abc}	6.00 ± 0.25^{c}	3.91 ± 0.40^{abc}	3.64 ± 0.03^{abc}	5.08 ± 1.53^{bc}
Glutamine	3.12 ± 0.03^{d}	0.59 ± 0.01^{a}	1.23 ± 0.01^{ab}	1.34 ± 0.06^{b}	2.28 ± 0.08^{c}	2.81 ± 0.34^{cd}	2.76 ± 0.00^{cd}	2.64 ± 0.01^{cd}
Histidine	3.37 ± 0.12^{c}	1.51 ± 0.09^{a}	2.23 ± 0.20^{ab}	2.98 ± 0.06^{bc}	3.01 ± 0.10^{bc}	3.18 ± 0.31^{c}	3.05 ± 0.02^{c}	3.26 ± 0.03^{c}
Glycine	2.31 ± 0.05^{b}	1.39 ± 0.11^{a}	2.33 ± 0.12^{b}	2.27 ± 0.04^{b}	2.57 ± 0.26^{b}	2.27 ± 0.01^{b}	2.03 ± 0.03^{b}	2.06 ± 0.05^{b}
Threonine	4.07 ± 0.17^{c}	0.55 ± 0.04^{a}	1.62 ± 0.03^{ab}	2.25 ± 0.06^{b}	3.75 ± 0.20^{c}	3.70 ± 0.54^{c}	3.71 ± 0.01^{c}	3.78 ± 0.09^{c}
Alanine	6.36 ± 0.02^{bc}	4.10 ± 0.42^{a}	7.10 ± 0.10^{c}	6.55 ± 0.23^{bc}	5.82 ± 0.06^{bc}	5.86 ± 0.52^{bc}	5.20 ± 0.01^{ab}	5.21 ± 0.07^{ab}
Arginine	10.70 ± 0.04^{b}	1.07 ± 0.22^{a}	7.99 ± 0.14^{b}	9.73 ± 0.28^{b}	8.16 ± 0.37^{b}	7.31 ± 3.36^{ab}	8.22 ± 0.02^{b}	9.39 ± 0.15^{b}
Tyrosine	5.65 ± 0.02^{b}	1.68 ± 0.14^{a}	5.02 ± 0.01^{b}	5.06 ± 0.17^{b}	5.35 ± 0.16^{b}	5.19 ± 0.49^{b}	4.63 ± 0.04^{b}	5.09 ± 0.09^{b}
Valine	7.59 ± 0.05^{b}	2.86 ± 0.28^{a}	6.09 ± 0.03^{b}	5.90 ± 0.27^{b}	6.42 ± 0.01^{b}	6.69 ± 0.95^{b}	6.50 ± 0.02^{b}	6.57 ± 0.01^{b}
Methionine	1.72 ± 0.02^{c}	$< 0.5^{a}$	0.79 ± 0.06^{abc}	$< 0.5^{a}$	0.93 ± 0.02^{abc}	1.17 ± 0.54^{abc}	1.07 ± 0.05^{abc}	1.28 ± 0.06^{bc}
Isoleucine	5.00 ± 0.04^{b}	$< 0.5^{a}$	3.81 ± 0.03^{b}	3.52 ± 0.16^{b}	4.04 ± 0.00^{b}	4.23 ± 0.82^{b}	4.38 ± 0.03^{b}	4.31 ± 0.08^{b}
Tryptophan	2.71 ± 0.05^{c}	1.62 ± 0.03^{a}	2.47 ± 0.03^{bc}	2.36 ± 0.05^{b}	2.46 ± 0.04^{bc}	2.67 ± 0.10^{c}	2.44 ± 0.04^{bc}	2.67 ± 0.06^{c}
Phenylalanine	7.95 ± 0.09^{b}	1.48 ± 0.08^{a}	6.10 ± 0.00^{b}	4.94 ± 1.27^{b}	6.04 ± 0.00^{b}	7.11 ± 0.93^{b}	5.48 ± 0.06^{b}	6.58 ± 0.07^{b}
Leucine	9.61 ± 0.07^{b}	$< 0.5^{a}$	6.79 ± 0.09^{b}	6.75 ± 0.04^{b}	6.89 ± 0.04^{b}	8.19 ± 1.48^{b}	6.92 ± 0.05^{b}	7.91 ± 0.17^{b}
Lysine	5.77 ± 0.02^{bc}	0.55 ± 0.00^{a}	3.57 ± 0.08^{b}	6.24 ± 0.99^{bc}	5.20 ± 0.02^{bc}	5.00 ± 0.76^{bc}	5.56 ± 0.02^{bc}	5.46 ± 0.06^{bc}
Total AA	98.31 ± 0.86^{f}	23.25 ± 1.60^{a}	72.14 ± 0.21^{b}	80.10 ± 2.69^{cd}	82.98 ± 1.17^{de}	73.24 ± 0.33^{bc}	82.85 ± 0.34^{de}	87.32 ± 0.69^{e}
AA consumption (%)	-	76.4	26.6	18.5	15.6	25.5	15.7	11.2

Different superscripts of values within a row indicate a significant difference ($p \leq 0.05$).

Table 3.4–5 Analysis of fermentation by-products of final beers fermented with investigated yeasts; values in mg/L.

	WLP001	**TUM SL 17**	**KBI 22.1**	**KBI 7.1**	**KBI 22.2**	**KBI 25.2**	**KBI 5.4**
	S. cerevisiae	***S. ludwigii***	***H. valbyensis***	***H. vineae***	***T. delbrueckii***	***Z. bailii***	***Z. kombuchaensis***
n-Propanol	13.7 ± 3.1^{b}	2.6 ± 0.9^{a}	2.1 ± 0.1^{a}	2.2 ± 0.0^{a}	2.9 ± 0.5^{a}	2.7 ± 0.1^{a}	2.1 ± 0.0^{a}
Isobutanol	17.9 ± 1.8^{b}	6.4 ± 0.1^{a}	4.8 ± 0.1^{a}	4.6 ± 0.3^{a}	4.9 ± 0.1^{a}	5.7 ± 0.1^{a}	7.1 ± 0. 5^{a}
Isoamyl alcohols	50.8 ± 3.0^{c}	12.1 ± 0.4^{ab}	16.5 ± 1.1^{b}	13.4 ± 0.1^{ab}	10.4 ± 0.3^{a}	14.8 ± 0.2^{ab}	12.9 ± 0.5^{ab}
Σ Higher alcohols (HA)	82.4 ± 7.9^{b}	21.1 ± 0.4^{a}	23.3 ± 1.1^{a}	20.2 ± 0.4^{a}	18.1 ± 0.1^{a}	23.1 ± 0.0^{a}	22.0 ± 1.0^{a}
Ethyl acetate	4.05 ± 0.21^{b}	0.80 ± 0.01^{a}	0.90 ± 0.05^{a}	6.00 ± 0.14^{c}	0.77 ± 0.02^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}
Isoamyl acetate	0.20 ± 0.00	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Σ Esters (E)	4.25 ± 0.21^{b}	0.80 ± 0.01^{a}	0.90 ± 0.05^{a}	6.00 ± 0.14^{b}	0.77 ± 0.02^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}
Diacetyl, total	0.04 ± 0.01^{a}	0.03 ± 0.00^{a}	0.21 ± 0.03^{b}	0.05 ± 0.01^{a}	0.06 ± 0.01^{a}	0.03 ± 0.00^{a}	0.15 ± 0.04^{b}
Ethyl formate	1.05 ± 0.07	1.01 ± 0.13	0.78 ± 0.06	0.76 ± 0.03	0.90 ± 0.05	0.56 ± 0.03	0.72 ± 0.07
Acetaldehyde	7.8 ± 0.4^{c}	8.5 ± 0.7^{c}	3.3 ± 0.4^{a}	4.1 ± 0.4^{ab}	9.1 ± 0.4^{c}	4.9 ± 1.3^{ab}	6.8 ± 2.6^{bc}

Different superscripts of values within a row indicate a significant difference ($p \leq 0.05$).

Ethyl acetate production by KBI 7.1 was with 6.00 mg/L the highest of all investigated strains and outperformed even the maltose-positive control yeast WLP001 with 4.05 mg/L, which is described by the supplier to have a clean taste and has been reported to produce low concentrations of esters in previous studies [45]. Threshold values for ethyl acetate in beer range from 21–30 mg/L which is usually higher than the amount found in alcohol-free beers [11]. However, synergistic effects of different volatile aroma compounds could contribute to the overall flavor, as suggested by Sterckx et al. [46]. The concentration of isoamyl acetate was below the detection level of 0.1 mg/L in all alcohol-free beers. The concentrations of ethyl formate (light estery, fruity, solvent) were with 1 mg/L and lower far below their individual threshold of 150 mg/L [47]. The concentration of ethyl propionate, ethyl butyrate and ethyl caproate, did not reach higher than the LOD of 0.01 mg/L in either of the beers (data not shown). Diacetyl levels were strain dependent with KBI 22.1 and KBI 5.4 producing values above the flavor threshold in light beers of 0.1 mg/L with 0.21 mg/L and 0.15 mg/L, respectively, while diacetyl production of the other strains stayed below the threshold [48]. Diacetyl is known for its undesired buttery flavor, which usually undergoes reduction during maturation of the beer [49]. Acetaldehyde is the most important aldehyde of beer and is formed in the metabolic pathway leading from carbohydrate to ethanol. Its level varies during fermentation and aging and in beers, it usually lies in the range 2–20 mg/L, while its threshold lies between 10-25 mg/L [44,47]. Acetaldehyde concentrations were below the threshold for all beers produced (Table 3.4–5). The overall flavor of beer depends on the relative contents of all the flavor-active compounds [44]. The presence of different esters can have a synergistic effect on the individual flavors, which means that esters can also have a positive effect on beer flavor, even at amounts below their individual threshold concentrations [50].

3.4.5 Sensory

To evaluate and compare the flavor of the beers, a panel of 11 trained and experienced beer tasters judged the beers by individual description of the aroma, followed by the evaluation of the intensity descriptors “fruity”, “wort-like” and “floral” smell, “sweet” and “acidic/sour” taste, and the body of the beer. Each descriptor was given a value on a scale from 0 (nothing) to 10 (extremely). A spider web graph of the means for the descriptors is shown in Figure 3.4–4.

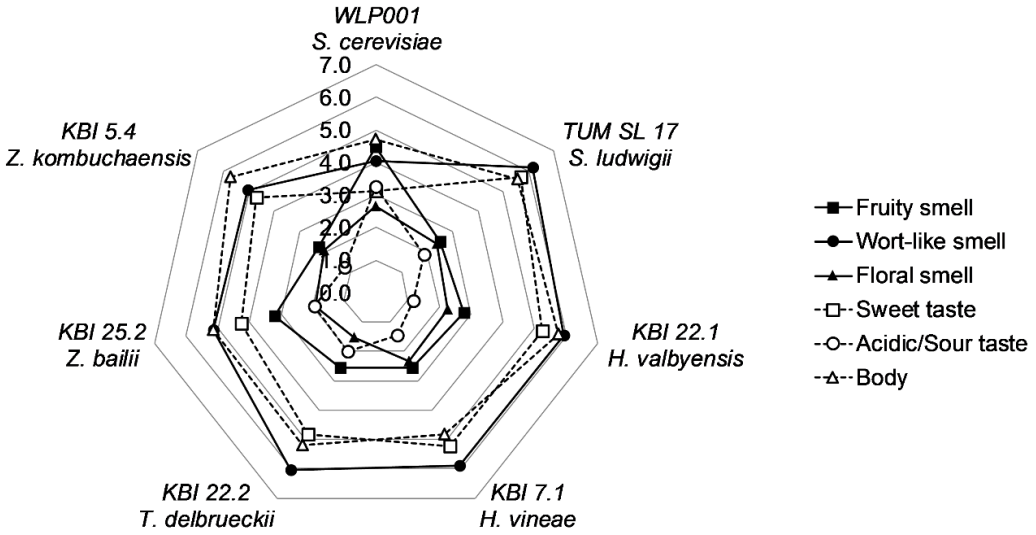


Figure 3.4–4 Spider web of the means of the descriptors from the sensory of the final beers.

WLP001 showed to have a less wort-like and fruitier smell and a less sweet, but more acidic/sour taste, owing to the longer fermentation time and higher extract consumption. The body of the beers was evaluated as being a little lower compared to the alcohol-free beers. Floral smell and acidic/sour taste were generally described to be low in intensity. Overall, the differences between the alcohol-free beers were small. KBI 25.2 was described to have a slightly fruitier smell and lower wort-like smell and sweet taste amongst the alcohol-free beers. However, analysis of variance (ANOVA) revealed no significant difference ($p \leq 0.05$) between the AFB. ANOVA analysis between all beer samples revealed significant differences in acidic/sour taste ($p < 0.001$) and differences in sweet taste ($p < 0.1$) and fruity smell ($p < 0.1$).

To create a multidimensional sensory profile of all beers, a principal component analysis (PCA) was conducted. PCA is a tool used to transform and combine a large amount of data into new components, based on variation and correlation within a data set. As descriptors, wort-like and fruity smell were selected as well as sweet and acidic/sour taste and body. If descriptors do not discriminate the products, they cause distortion in the PCA. Hence the descriptor "flora smell", having a P value for the F-test of the product effect greater than the default value of 0.5, was excluded from the PCA [22]. The Variables

factor map (Figure 3.4–5) presents the observed variables projected into the plane, spanned by the first two principal components. It shows the structural relationship between the variables and helps to name the components. The projection of a variable vector onto the component axis allows to directly read the correlation between the variable and the component.

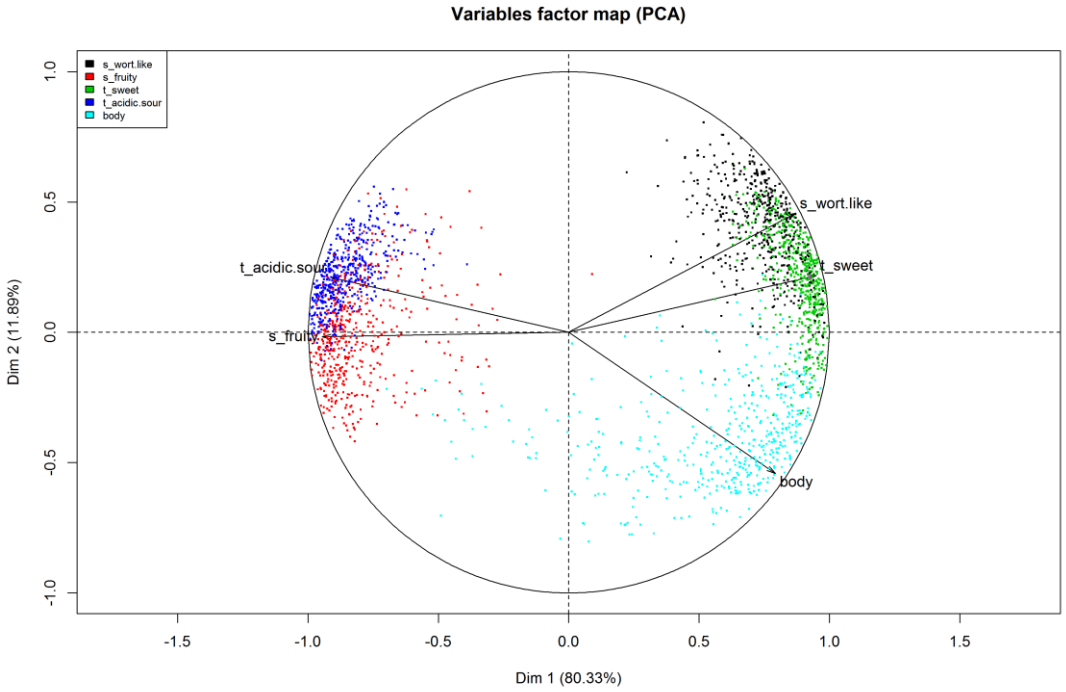


Figure 3.4–5 Variables factor map of the PCA of the sensory of the final beers. Criteria for descriptors to be included in the PCA was a P value of the F-test of below 0.5. For the descriptors, "s_" stands for smell, and "t_" stands for taste.

The variables factor map should be interpreted in terms of angles, either between each variable or between a variable and the component axes. Narrow angles reflect positively linked variables (i.e. sweet taste and wort-like smell). Right angles depict variables that are unrelated to each other (i.e. body and wort-like smell) and obtuse angles represent negative relationships (i.e. wort-like and fruity smell). The first principal component described about 80% of the total variation and showed an almost perfect correlation to the variable fruity smell and a very strong correlation to sweet and acidic/sour taste and wort-like smell. The second principal component explained an additional 12% of the total variation with a correlation to the body of the beers. Combined, the first two principal components explained about 92% of the total variance of the data.

In the PCA graph (Figure 3.4–6), confidence ellipses (α = 0.05) around each beer were added to visualize the uncertainty as for the position of the beer given by the panel. Well separated confidence ellipses indicate a great discriminant power of the panel.

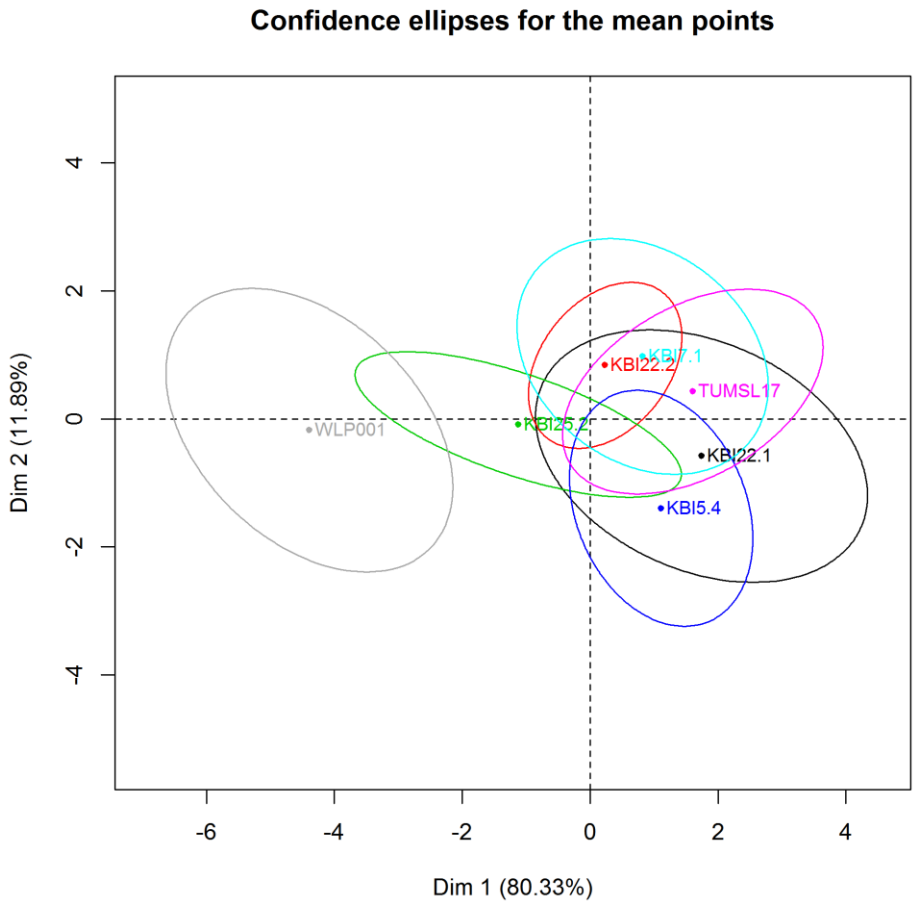


Figure 3.4–6 Mean points with confidence ellipses (α = 0.05) of the PCA from the sensory of the final beers.

As expected, WLP001 could be well discriminated from the other beers in the direction of a fruitier smell, more acidic/sour taste and away from a sweet taste and wort-like smell. The alcohol-free beers were not well discriminated but were all located in the direction of a sweeter taste and wort-like smell. However, a tendency of KBI 25.2 separating from the group of alcohol-free beers in the direction of WLP001 could be observed. The highest means for body by KBI 5.4 and KBI 22.1 also reflected in the PCA. The results of the sensory reflect the marginal differences of the alcohol-free beers between each other from the analyses of secondary metabolites. However, the significantly higher ester content of

the beer fermented with KBI 7.1 did not show in the sensory analyses of the different beers.

In the descriptive part of the sensory, the panelists gave all the alcohol-free beers attributes like: "wort-like", "bread-like" and "honey-like". TUM SL 17 was described using at least one of those attributes, by 90% of the panelists. KBI 22.1 was additionally given a "cereal-like" character and half of the panel detected the diacetyl flavor as expected from the metabolites analysis (Table 3.4–5). KBI 7.1 was also described with attributes like "black tea" and "caramel". KBI 25.2 was given additional attributes like "slightly grassy", "fruity" and "white wine". The elevated diacetyl values for KBI 5.4 were again detected by 50% of the panelists. The problem of wort-like off-flavor in alcohol-free beers is very common. Aldehydes are reported to be the cause, with 3-methylthiopropionaldehyde seemingly being the key compound responsible for the worty off-flavor [51,52]. Wort aldehydes form mainly during mashing and boiling but are also partially formed during fermentation by the yeast. They can originate from oxo-acids via the anabolic process, and from exogenous amino acids via the catabolic pathway [53]. Ethanol plays a significant role in the reduction of the worty character of the beer. As a flavor component, it contributes directly to the flavor of beer, giving rise to a warming character and influencing the partitioning of flavor components between the liquid beer, foam and the headspace above the beer [54]. Additionally, Perpète and Collin [6] reported, that aldehyde retention caused by its solubilization in ethanol leads to a lower perception of the worty taste. In regular beers the retention of aldehydes is 32-39% as opposed to 8-12% retention in alcohol-free beers [4]. It is also known that yeast metabolism reduces wort aldehydes to less flavor active ones [55]. The absence of ethanol, the lack of aldehyde reduction due to shortened fermentation times and the higher level of mono-/ and disaccharides such as maltose intensify undesirable worty flavors [6]. The results of the sensory indicate, that none of the investigated maltose-negative strains were able to mask the worty off-flavors. However, they neither stood out negatively compared to TUM SL 17, which is already commercially applied in the production of alcohol-free beers. KBI 25.2 showed the highest potential of non-*Saccharomyces* yeasts to become a serious alternative in the brewing of alcohol-free beer with an improved sensorial profile.

3.5 Conclusion

This study on the application of five non-*Saccharomyces* yeasts in the production of AFB, gave a comprehensive overview of their suitability and characteristics. After ruling out undesirable traits during characterization, such as POF production and hop sensitivity, the non-*Saccharomyces* yeasts showed excellent performance during propagation, outperforming TUM SL 17 in cell numbers and showing very high viability rates. In fermentation trials the non-*Saccharomyces* yeasts exhibited a comparable performance, and analysis of volatile compounds revealed only marginal differences. The AFB fermented with the commercial AFB yeast (TUM SL 17) could not be discriminated from the alcohol-free beers fermented with the investigated non-*Saccharomyces* yeasts, which indicates the potential of their application in alcohol-free beer brewing. All fermentations were performed at 25 °C to be able to compare the strains. Twenty-five degrees Celsius most likely was not the optimum for each of the yeast strains in terms of fermentation performance or production of secondary metabolites, but it allows an indication of the suitability of the investigated strains in alcohol-free beer production.

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Chapter 4:

Screening and application of *Cyberlindnera* yeasts to produce a fruity, non-alcoholic beer

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4.1 Abstract

Non-alcoholic beer (NAB) is enjoying growing demand and popularity due to consumer lifestyle trends and improved production methods. In recent years in particular, research into the application of non-*Saccharomyces* yeasts to produce NAB via limited fermentation has gained momentum. Non-*Saccharomyces* yeasts are known to produce fruity aromas, owing to a high ester production. This trait could be harnessed to mask the often-criticized wort-like off-flavors of NAB produced via limited fermentation. Six *Cyberlindnera* strains were characterized and screened in wort extract. Four of the six strains produced a pleasant, fruity aroma while exhibiting low ethanol production. The strain *Cyberlindnera subsufficiens* C6.1 was chosen for fermentation optimization via response surface methodology (RSM) and a pilot-scale (60 L) brewing trial with subsequent sensory evaluation. A low fermentation temperature and low pitching rate enhanced the fruitiness and overall acceptance of the NAB. The NAB (0.36% ABV) produced on pilot-scale was significantly more fruity and exhibited a significantly reduced wort-like off-flavor compared to two commercial NABs. This study demonstrated the suitability of *Cyberlindnera subsufficiens* to produce a fruity NAB, which can compete with commercial NABs. The outcome strengthens the position of non-*Saccharomyces* yeasts as a serious and applicable alternative to established methods in NAB brewing.

4.2 Introduction

While the overall market growth of beer is slowing down, non-alcoholic and low alcohol beer (NABLAB) is growing in volume and popularity, owed to stricter legislation, lifestyle trends and improved production methods [1]. The increasing interest has fueled research in NABLAB production methods, especially in recent years, aimed at overcoming taste deficits compared to regular beer and consequently improve consumer acceptance. The two major production methods, physical dealcoholization and limited fermentation, both compromise the taste of the beer. Dealcoholized beer is often criticized for its lack of body and aromatic profile, a consequence of the removal of volatile esters and higher alcohols in conjunction with ethanol. Apart from a sweet taste due to residual sugars, one of the main points of criticism of NAB produced by limited fermentation is its wort-like off-flavor caused by aldehydes present in the wort [2]. In regular beer, ethanol significantly increases aldehyde retention, reducing the perceptibility of the wort-like flavor. However, in NAB produced by limited fermentation, the low ethanol content and higher level of mono- and disaccharides intensify this undesired off-flavor [3].

It is known that esters, which yeast produce as a by-product of alcoholic fermentation, are extremely important for the flavor profile of beer [4,5]. The lack thereof, as well as their overproduction can significantly compromise the flavor. Aside from strain-specific differences, the process parameters such as the fermentation temperature, pitching rate and wort gravity have been shown to have a significant influence on ester formation [4,6]. In non-alcoholic beers, ester concentrations are lower compared to regular beer independent of the production method [7,8]. While the physical dealcoholization removes esters that were previously produced, a limited fermentation adversely affects the production of substantial amounts in the first place.

Non-*Saccharomyces* yeasts are known for their important contribution to the flavor profile of fermented foods and beverages and have therefore been investigated for their targeted application in bioflavoring and, not least, NABLAB brewing [1,9,10]. Species that have been mentioned in the context of NABLAB production, belong to the genera *Cyberlindnera*, *Hanseniaspora*, *Lachancea*, *Mrakia*, *Pichia*, *Torulaspora*, *Saccharomycodes*, *Scheffersomyces* and *Zygosaccharomyces* [1,11–16]. Especially the *Cyberlindnera* species are known for their high ester production, which was shown in studies with *Cyberlindnera saturnus* (formerly *Williopsis saturnus*), *C. mrakii* (formerly *Williopsis saturnus* var. *mrakii*) and *C. subsufficiens* (formerly *Williopsis saturnus* var. *subsufficiens*) [17–20]. Furthermore, it has

been proposed to use yeasts with high production of flavor compounds (i.e. esters, higher alcohols) to mask the wort-like flavor of NAB produced by limited fermentation. However, research in that direction is sparse [21,22]. In addition, such yeasts are capable of reducing aldehydes to their correspondent alcohol, which can also enhance the reduction of the often-criticized wort-like off-flavor [23,24].

In this study, six strains of the genus *Cyberlindnera* were investigated to create a fruity NAB. After identification, the strains were characterized for their substrate utilization, flocculation behavior and stress responses. A screening in diluted wort extract was performed to investigate the strains' potential to produce a pronounced fruity flavor without the production of high concentrations of ethanol. Interspecific differences in sugar consumption and the production of volatile fermentation by-products was investigated by means of high-performance liquid chromatography (HPLC) and gas chromatography (GC). The most promising strain was studied further to determine the optimal fermentation conditions to enhance the fruity flavor, which was performed by means of response surface methodology (RSM). Finally, a non-alcoholic beer was produced on pilot-scale (60 L) and its analytical attributes, aroma, and taste compared to two commercial NABs were examined.

4.3 Materials and Methods

4.3.1 Materials

All reagents used in this study were at least analytical grade from Sigma-Aldrich (St Louis MO, USA) unless stated otherwise. The wort extract applied in this study was spray-dried wort from 100% barley malt (Spraymalt Light, Muntons plc, Suffolk, UK). For the pilot-scale brewing, Pilsner Malt and acidulated malt were sourced from Weyermann (Malzfabrik Weyermann, Bamberg, Germany).

4.3.2 Yeast strain origin and identification

Strain 837A was isolated from a brewery cellar, NT Cyb originates from a dried fermentation starter for rice wine, strain C6.1 originates from a coconut, and L1 from "Lulo", the fruit of *Solanum quitoense*. The type strains CBS 1707 and CBS 5763 originate from soil samples. For identification, the D1/D2 domain of the 26S rRNA gene was

amplified, sequenced and compared to publicly available sequences on the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The DNA of the yeast isolates was extracted using an extraction kit (Yeast DNA Extraction Kit, Thermo Fisher Scientific, Waltham MA, USA). To amplify the D1/D2 domain of the 26S rRNA gene the primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') were used. Polymerase chain reaction (PCR) was performed using the temperature protocol: 95 °C / 2 min; 30 cycles of 95 °C / 30 s, 56 °C / 15 s; 72 °C / 60 s; 72 °C / 5 min. Stock cultures were kept in 50 % (v/v) glycerol at – 80 °C.

4.3.3 Yeast characterization

4.3.3.1 Flocculation assay and phenolic off-flavor (POF) test

The flocculation test was performed using a slightly modified Helm's assay [25,26]. Essentially, all cells were washed in ethylenediaminetetraacetic acid (EDTA) and the sedimentation period was extended to 10 min. Wort was composed of 75 g/L spray-dried malt extract (Spraymalt Light, Muntons plc, Suffolk, UK) adjusted to 15 International Bitterness Units (IBU) (15 mg/mL iso-α-acids; from 30% stock solution; Barth-Haas Group, Nürnberg, Germany).

The phenolic off-flavor test was performed according to Meier-Dörnberg et al. [27]. In short, yeast strains were spread on yeast and mold agar plates (YM-agar) containing only one of the following precursors: either ferulic acid, cinnamic acid or coumaric acid. After three days of incubation at 25 °C, plates were evaluated by a trained panel by sniffing to detect any of the following aromas: clove-like (4-vinylguajacol), Styrofoam-like (4-vinylstyrene) and medicinal-like (4-vinylphenol). *Saccharomyces cerevisiae* LeoBavaricus - TUM 68® (Research Center Weihenstephan for Brewing and Food Quality, Freising-Weihenstephan, Germany) was used as a positive control.

4.3.3.2 Substrate utilization

To analyze substrate utilization by the *Cyberlindnera* strains, the test kit API ID 32C (BioMérieux, Marcy-l'Étoile, France) was used. Preparation of inoculum and inoculation of the strips was performed according to the manufacturers' instructions. Colonies for the inoculum were grown on YPD agar plates for 48 h at 27 °C. After inoculation, API ID 32C strips were incubated for 2 days at 28 °C. The samples were evaluated visually for turbidity in the wells, differentiating positive (+), negative (-), and weak (w) growth.

4.3.3.3 Stress tests

Stress tests were performed via the measurement of yeast growth in a microplate, through the repeated measurement of absorbance over a time period of 96 h (Multiskan FC, Thermo Scientific, Waltham, Massachusetts, USA).

The substrate for the hop sensitivity test was sterile-filtered wort extract (75 g/L Muntons Spraymalt Light) adjusted to 0, 50 and 100 mg/L iso-α-acids (1 mg/L = 1 International Bitterness Unit, IBU), respectively by using an aliquot of a stock solution of 3% iso-α-acids in 96% (v/v) ethanol (Barth-Haas Group, Nürnberg, Germany).

For testing ethanol sensitivity, the sterile-filtered wort extract was adjusted to 0%, 2.5%, 5% and 7.5% ABV with an aliquot of 100% (v/v) ethanol.

For testing pH sensitivity, the sterile-filtered wort extract was adjusted to the following pHs with 2 M HCl: 5.5 (control without addition of HCl), 5.0, 4.0 and 3.0.

For inoculation, strains were grown in sterilized wort extract for 24 h at 25 °C under aerobic conditions. The microtiter plate wells were inoculated with a concentration of 10^5 cells/mL. The wells contained 200 µL of the respective wort substrates. Plates were incubated at 25 °C and absorbance was measured every 30 min at 600 nm without shaking over a time period of 96 h (Multiskan FC, Thermo Scientific, Waltham, Massachusetts, USA). Stress tests were performed in triplicate.

4.3.4 Yeast screening

4.3.4.1 Propagation

Single colonies of the respective strains were taken from yeast extract peptone dextrose (YPD) agar plates after 72 h growth at 25 °C and transferred into a 250 mL sterile Duran glass bottle (Lennox Laboratory Supplies Ltd, Dublin, Ireland) containing 150 mL propagation wort consisting of 75 g/L spray-dried malt (Spraymalt light, Muntons plc, Suffolk, UK) and 30 g/L glucose (Gem Pack Foods Ltd., Dublin, Ireland), sterilized at 121 °C for 15 min. The bottles were covered with sterile cotton and placed in an incubator with orbital shaker (ES-80 shaker-incubator, Grant Instruments (Cambridge) Ltd, Shepreth, UK) and incubated for 24 h at an orbital agitation of 170 rpm at 25 °C (Strain 837A was incubated for 48 h). Cell count was performed using a Thoma Hemocytometer with a depth of 0.1 mm (Blaubrand, Sigma-Aldrich, St. Louis, MO, USA).

4.3.4.2 Fermentation

Fermentation wort was prepared by dissolving 75 g/L spray-dried malt extract (Munton Spraymalt light) in 1 L of brewing water and sterilized at 121 °C for 15 min, followed by filtration through sterile grade 1V Whatman filter (Whatman plc, Maidstone, UK) to remove hot trub formed during sterilization. The analytical attributes of the fermentation wort for the yeast screening trial and RSM trial is shown in Table 4.3–1.

Table 4.3–1 Attributes of screening wort from wort extract.

Attribute	Unit	Value
real Extract	°P	6.97 ± 0.00
pH	-	5.20 ± 0.01
FAN	mg/L	115 ± 1
Maltotriose	g/L	8.12 ± 0.15
Maltose	g/L	32.37 ± 0.57
Sucrose	g/L	0.83 ± 0.04
Glucose	g/L	5.68 ± 0.91
Fructose	g/L	1.45 ± 0.10

Fermentation trials were carried out in 1 L sterile Duran glass bottles, equipped with an air lock. Per yeast strain, triplicate bottles were filled with 400 mL of wort and left untouched throughout the fermentation. Yeast cells for pitching were washed by centrifugation at 900 *g* for 5 min and resuspended in sterile water to ensure no carryover

of sugars from the propagation wort into the fermentation wort. Pitching rate was 3×10^7 cells/mL. Fermentation temperature was 25 °C. Fermentation was performed until no change in extract could be measured for two consecutive days.

4.3.5 Scanning electron microscopy (SEM)

Yeast cultures for scanning electron microscopy (SEM) were prepared following the protocol for cultured microorganisms by Das Murtey and Ramasamy [28]. Single colonies were taken from a YPD agar plate and grown in YPD broth for 24 h at 25 °C. One milliliter of sample was centrifuged at 900 g for 2 min for pellet formation and resuspended in 5% glutaraldehyde solution prepared in 0.1 M phosphate buffer (pH 7.2) for fixation. After 30 min, the sample was centrifuged, the supernatant was discarded, and the pellet was washed twice in 0.1 M phosphate buffer. Consequently, the pellet was resuspended in 1% osmium tetroxide prepared in 0.1 M phosphate buffer. After 1 h, cells were again washed twice in 0.1 M phosphate buffer. The sample was then dehydrated through an ethanol series of 35%, 50%, 75%, 95%, absolute ethanol, and hexamethyldisilazane (HDMS), with 30 min per step (last two ethanol steps twice), centrifuging and discarding the supernatant at each change. Lastly, the second HDMS was discarded and the sample left drying overnight in a desiccator.

The dehydrated yeast sample was mounted onto plain aluminum stubs using carbon double surface adhesive and coated with a 5 nm gold-palladium (80:20) layer using a Gold Sputter Coater (BIO-RAD Polaron Division, SEM coating system, England) and observed under a constant accelerating voltage of 5 kV under a JEOL scanning electron microscope type 5510 (JEOL, Tokyo, Japan).

4.3.6 Response surface methodology (RSM)

To investigate optimal fermentation conditions for C6.1 to produce a fruity, non-alcoholic beer, response surface methodology (RSM) was performed using DesignExpert 9 software (StatEase, Minneapolis MN, USA). A two factorial, face-centered, central composite design with single factorial points and 5 replications of the center point was chosen. The predictor factors were temperature (17, 22, 27 °C), and pitching rate (10, 35, 60×10^6 cells/mL).

Spray-dried malt extract (Spraymalt light, Muntons plc, Suffolk, UK) served as substrate. Wort preparation, propagation and inoculation was carried out as outlined in 4.3.4.1. The wort was the same as in the screening (Table 4.3–1). Fermentation volume was 150 mL in 250 mL Duran glass bottles equipped with an air lock. Fermentation was performed until no change in extract could be measured for two consecutive days. Table 4.3–2 shows the experimental design.

Table 4.3–2 Response surface methodology (RSM) experiment design: Two-factorial, face-centered central composite design with five repetitions of the center point. Factor 1, A: Temperature, Range 17, 22, 27 °C. Factor 2, B: Pitching rate, Range 10, 35, 60×10^6 cells/mL.

Run	Factor 1 A: Temperature (°C)	Factor 2 B: Pitching Rate (×10^6 cells/mL)
1	22	60
2	22	10
3	17	35
4	27	35
5*	22	35
6*	22	35
7	17	60
8*	22	35
9*	22	35
10*	22	35
11	17	10
12	27	10
13	27	60

* Center point

4.3.7 Pilot-scale brewing

4.3.7.1 Wort production

Wort for the pilot brew was produced in a 60 L pilot-scale brewing plant consisting of a combined mash-boiling vessel, a lauter tun and whirlpool (FOODING Nahrungsmitteltechnik GmbH, Stuttgart, Germany). The grain bill comprised of 6.65 kg Weyermann Pilsner Malt and 0.35 kg Weyermann Acidulated Malt (Malzfabrik Weyermann, Bamberg, Germany). Grains were milled with a two-roller mill ("Derby", Engl Maschinen, Schwebheim, Germany) at a 0.8 mm gap size. The crushed malt was mashed-in with 30 L of brewing water at 50 °C. The following mashing regime was employed: 20 min at 50 °C, 20 min at 62 °C, 10 min at 72 °C and mashing out at 78 °C. The mash was pumped into the lauter tun and lautering was performed after a 15 min

lauter rest, employing four sparging steps of 5 L hot brewing water each. Boil volume was 50 L at a gravity of 1.030 (7.0 °P) and total boiling time was 60 min. Thirty minutes into the boil, 15 g of Magnum hop pellets (14% iso-α-acids) were added for a calculated IBU content of 9. After boiling, gravity was readjusted to 1.030 (7.0 °P) with hot brewing water and hot trub precipitates and hop residue were removed in the whirlpool with a rest of 20 min. Clear wort was pumped through a heat exchanger and filled into 60 L cylindroconical fermentation vessels at a temperature of 17 °C.

4.3.7.2 Propagation, fermentation and aftercare

A first propagation step was employed as described in 4.3.4.1. A second propagation step was performed by transferring the small-scale propagated wort into a 5 L carboy filled with 2 L of sterile wort extract at 7 °P and closed with sterile cotton. The second propagation step was conducted for 24 h under constant agitation at ambient temperature (20 ± 2 °C).

Yeast was pitched into the fermenter at a pitching rate of 10^7 cells/mL. Fermentation was carried out in cylindroconical fermentation vessels with a capacity of 60 L, at ambient pressure and at a glycol-controlled fermentation temperature of 17 °C. Samples were withdrawn every day. Fermentation was carried out until no change in extract could be measured for two consecutive days. The beer was then filled into a 50 L keg and carbonated by repeated pressurization with CO_2 to 1 bar at 2 °C. After 5 days, the carbonated beer was filled into 330 mL brown glass bottles with a counter-pressure hand-filler (TOPINCN, Shenzen, China) and capped. Bottles were pasteurized in a pilot retort (APR-95; Surdry, Abadiano, Vizcaya, Spain) with spray water at 65 °C for 10 min resulting in approximately 23 pasteurization units (PU). The successful pasteurization was confirmed by plating the pasteurized NAB on agar plates. Beer bottles were stored at 2 °C in a dark place for further analysis and sensory evaluation.

4.3.8 Sensory evaluation

The sensory evaluation of the samples produced during yeast screening and RSM trial were judged by a panel of 12–15 experienced tasters. Samples were given at ambient temperature (20 °C) with a three-digit code. Each panelist evaluated the samples in an individual cubical at ambient temperature (20 °C). The tasters were asked to desribe the

sample in their own words, followed by the evaluation of the intensity of a fruity smell and an overall acceptance of the smell of the sample on a hedonic scale from 0 ("not fruity"/"dislike extremely") to 5 ("extremely fruity"/"like extremely") according to MEBAK Sensory Analysis 3.2.1 "Simple Descriptive Test" and 3.2.2 "Profile Test", respectively.

The non-alcoholic beer samples (C6.1 pilot scale and commercial samples) were tasted and judged by a sensory panel of ten experienced and certified (DLG International Certificate for Sensory Analysis – beer and beer-based mixed drinks; Deutsche Landwirtschafts-Gesellschaft e.V.) panelists. A "Simple Descriptive Test" and "Profile Test" were performed according to MEBAK Sensory Analysis 3.2.1 and 3.2.2, respectively. Attributes for the aroma were "wort-like", "floral", "fruity", "citrus-like" and "tropical". A taste attribute "sweet taste" was also included. Panelists were asked to evaluate the attributes in its intensity on a line-marking scale from 0, "not perceptible", to 5, "strongly perceptible". Before the evaluation of the intensity, a descriptive sensory was performed, where the panelists were asked to describe the aroma of the samples in their own words. Samples were provided in dark glasses with a three-digit code and evaluated at a temperature of 20 °C in order to evaluate the full flavor profile (following DLG guidelines). The commercial samples NAB A and NAB B were non-alcoholic beers produced by limited fermentation [29] and "dialysis technology" [30], respectively. Each panelist tasted the samples in an individual cubical at ambient temperature (20 °C). The amount of sample tasted was 50 mL per sample.

4.3.9 Wort and beer analyses

4.3.9.1 HPLC analyses

Sugars and ethanol were determined by HPLC Agilent 1260 Infinity (Agilent Technologies, Santa Clara CA, USA) equipped a refractive index detector (RID) and a Sugar-Pak I 10 μm, 6.5 mm × 300 mm column (Waters, Milford MA, USA) with 50 mg/L Ca-EDTA as mobile phase and a flow rate of 0.5 mL/min at 80 °C. Differentiation of maltose and sucrose was achieved with a Nova-Pak 4 μm, 4.6 mm × 250 mm column (Waters, Milford MA, USA) with acetonitrile/water 78:22 (v/v) as mobile phase and a flow rate of 1.0 mL/min. Quantification was achieved by external standards in a calibration range of 0.5 to 30 mM.

4.3.9.2 GC analyses

Free vicinal diketones were quantified by a Clarus 500 gas chromatograph (Perkin-Elmer, Waltham MA, USA) with a headspace unit and Elite-5 60 m × 0.25 mm, 0.5 μm column using a 2,3-hexandione internal standard. Fermentation by products (esters, higher alcohols) were quantified using a Clarus 580 (Perkin-Elmer, Waltham MA, USA) gas chromatograph with a headspace unit and INNOWAX cross-linked polyethylene-glycol 60 m × 0.32 mm 0.5 μm column (Perkin-Elmer, Waltham MA, USA). Vials containing beer samples were equilibrated for 25 min at 60 °C. The samples were injected at 50 °C, rising to 85 °C after one minute by heating at 7 °C/min. A temperature of 85 °C was maintained for one minute and then elevated to 190 °C at a heating rate of 25 °C/min.

4.3.9.3 Other

Glycerol was determined via enzymatic assay kit (glucokinase method), following the recommended procedure (K-GCROLGK, Megazyme, Bray Co. Wicklow, Ireland). The method is based on the use of ADP-glucokinase and an increase in absorbance on conversion of NAD^+ to NADH, and is performed at ambient temperature at a sample volume of 2 mL.

Free amino nitrogen (FAN) was measured using a ninhydrin-based dying method, where absorbance is measured at 570 nm against a glycine standard (ASBC Method Wort-12 A). The method is performed at a total volume of 10 mL. Following the color reaction at 95 °C, the samples are measured at ambient temperature.

Extract (apparent and real) and ethanol (for fermentation monitoring) were analyzed via density meter DMA 4500M with Alcolyzer Beer ME (Anton-Paar GmbH, Graz, Austria) at 20 °C and a sample volume of 30 mL.

The pH was determined using a digital pH meter (Mettler Toledo LLC, Columbus OH, USA).

4.3.10 Statistical analyses

Screening fermentations and analyses were carried out in triplicate. Statistical analysis was performed using RStudio, Version 1.1.463 with R version 3.5.2 (RStudio Inc, Boston MA, USA; R Core Team, r-project). One-way analysis of variance (ANOVA) was used to

compare means and Tukey's post hoc test with 95% confidence intervals was applied for the pairwise comparison of means. When available, values are given as the mean ± standard deviation. Statistical analyses during the RSM trials were performed using the DesignExpert 9 software (StatEase, Minneapolis MN, USA).

4.4 Results and Discussion

4.4.1 Yeast strain characterization

To identify the species of the yeast strains, amplification of the D1/D2 domain via PCR was performed and sequenced. The obtained sequences were compared to publicly available sequences in the NCBI nucleotide database via BLAST. The results of the strain identification are shown in Table 4.4–1.

Table 4.4–1 Yeast strain designation, species and origin of yeast strains used in this study.

Strain designation	Species	Origin	Yeast bank
837A	*Cyberlindnera misumaiensis*	Brewery cellar	FZW BLQ[1], Weihenstephan, Germany
NT Cyb	*Cyberlindnera fabianii*	Dried yeast starter for rice wine	FZW BLQ[1], Weihenstephan, Germany
L1	*Cyberlindnera jadinii*	Fruit of *Solanum quitoense*, "Lulo"	UCC Culture Collection, Cork, Ireland
C6.1	*Cyberlindnera subsufficiens*	Coconut	UCC Culture Collection, Cork, Ireland
CBS 1707[T]	*Cyberlindnera mrakii*	Soil	Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands
CBS 5763[T]	*Cyberlindnera subsufficiens*	Soil	Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands

[1] Research Centre Weihenstephan for Brewing and Food Quality, Technische Universität München
[T] Type strain

The yeast strains were found to belong to the species *Cyberlindnera misumaiensis* (837A), *C. fabianii* (NT Cyb), *C. jadinii* (L1), and *C. subsufficiens* (C6.1). The *Cyberlindnera mrakii* type strain CBS 1707 (former *Williopsis saturnus* var. *mrakii*; synonym NCYC 500) was included in this study as a strain that has previously been investigated for the production of a low alcohol beer with high levels of esters [20]. The *Cyberlindnera subsufficiens* type strain CBS 5763 was included as an example to investigate potential intraspecific differences to C6.1.

4.4.2 API substrate utilization

Before considering non-conventional yeasts for NABLAB brewing, their behavior regarding utilization of important wort sugars like maltose and sucrose should be investigated. An API ID 32C test was performed to investigate the utilization of those sugars and to show general, interspecific differences between the strains. The results of the API test are shown in Table 4.4–2.

Table 4.4–2 Results of the API ID 32C substrate utilization test of the individual strains. Substrates without brewing-relevance which were negative for all strains are not shown. '+' positive, '–' negative, 'w' weak.

Substrate	837A	NT Cyb	L1	C6.1	CBS 1707	CBS 5763
Cycloheximide (Actidione)	+	–	–	–	–	–
D-Cellobiose	+	+	+	+	+	+
D-Galactose	–	–	w	–	–	–
D-Glucose	+	+	+	+	+	+
D-Maltose	–	+	+	–	+[1]	–[1]
D-Mannitol	+	+	w	w	w	w
D-Melibiose	–	–	–	–	–	–
D-Melezitose	–	+	+	–	+	–
D-Raffinose	–	+	+	+	+	+
D-Sorbitol	+	+	w	+	–	–
D-Sucrose	–	+	+	+	–	+
D-Trehalose	–	+	–	–	+	–
D-Xylose	–	+	+	+	+	+
Esculin ferric acid	+	+	+	+	+	+
Glucosamine	–	–	–	w	–	–
Glycerol	+	+	+	+	+	+
Lactic Acid	–	+	+	+	+	+
Levulinic acid	–	w	w	w	w	+
L-Sorbose	–	–	–	–	–	+
Methyl-αD-Glucopyranoside	–	+	–	–	–	–
N-Acetyl-Glucosamine	–	–	w	–	w	–
Palatinose	–	+	+	–	+	–
Potassium Gluconate	w	w	–	+	w	+

[1] Growth "variable" according to Kurtzman et al. [31].

Maltose utilization was positive for NT Cyb, L1 and CBS 1707, in accordance with reported literature, although assimilation of maltose by CBS 1707 is classified as "variable" [31]. Sucrose utilization was positive for four of the six strains and negative for 837A and CBS1707. The results suggest that in brewers' wort, where maltose is the most abundant fermentable sugar, only NT Cyb, L1 and CBS 1707 have the capability to achieve high attenuations. However, the API test investigates substrate utilization under aerobic conditions. Sugar consumption during fermentation, under anaerobic conditions, can differ significantly [31] which is described by the Kluyver effect [32]. Due to the inability of 837A and CBS 1707 to utilize sucrose, lower attenuations in fermentations in wort could be expected.

4.4.3 Stress tests

When considering non-*Saccharomyces* yeast strains for brewing purposes, several brewing-relevant parameters such as flocculation behavior, POF production and stress responses should be investigated [33]. The flocculation behavior can give initial indications regarding yeast handling in terms of potential bottom cropping. POF behavior is important because in most beer styles, POF is not desired. Substances like hop-derived iso-α-acids, ethanol content, or the pH value of the wort can have significant influences on yeast activity, manifesting mainly in a prolonged lag time, and even complete growth inhibition [33–34]. With the investigated yeast strains, iso-α-acid concentrations of up to 100 IBU had no significant effect on the yeast growth (data not shown) which is in accordance with previous reports on seven different non-*Saccharomyces* species [34]. However, Michel et al. [33] reported a minor prolongation in lag time of *Torulaspora delbrueckii* strains in concentrations of up to 90 IBU. The results of the investigated characterization attributes is shown in Table 4.4–3.

Table 4.4–3 Characterization of yeast strains for flocculation behavior, phenolic off-flavor (POF) production and lag time in wort with and without stressor at different concentrations. '—' no growth.

Characterization attributes		**Unit**	**837A**	**NT Cyb**	**L1**	**C6.1**	**CBS 1707**	**CBS 5763**
Flocculation		%	78 ± 3	22 ± 2	35 ± 4	32 ± 1	85 ± 2	51 ± 4
POF		-	negative	negative	negative	negative	negative	negative
Ethanol	0% ABV	h	18	6	9	6	9	9
	2.5% ABV	h	120	12	18	18	12	18
	5% ABV	h	—	24	36	24	48	—
	7.5% ABV	h	—	42	—	—	126	—
pH	5.5	h	18	6	9	6	9	9
	5	h	18	6	9	6	9	9
	4	h	66	6	9	6	9	9
	3	h	—	12	24	18	78	42

CBS 1707 exhibited the strongest flocculation behavior, at 85%, followed by 837A and CBS 5763, at 78 and 51%, respectively. NT Cyb, L1 and C6.1 exhibited very low flocculation of below 35%. All strains were negative for POF behavior. NT Cyb and C6.1 exhibited the fastest growth in wort (without stress factor), overcoming the lag time after only 6 hours, followed by L1 and the CBS strains after 9 hours. Strain 837A exhibited a long lag phase of 18 hours (Figure 4.4–1). Concentrations of 2.5% ABV ethanol in the wort affected the lag time of all investigated strains. 837A was especially susceptible, with a prolonged lag phase of 120 hours. The remainder of the strains showed an extension of

the lag phase of 3 to 12 hours. At 5% ABV, growth was fully inhibited for 837A and CBS 5763 while the other strains again exhibited an extension of the lag phase, of up to a maximum of 48 hours in CBS 1707. Complete growth inhibition was observed for L1 and C6.1 at 7.5% ABV, while the lag phase of NT Cyb and CBS 1707 was prolonged to 42 and 126 hours, respectively. All strains except 837A, which showed a significant extension of the lag phase to 66 hours, remained unaffected by a lower pH of 4. Only at pH 3 were lag times affected, while 837A was fully inhibited. Growth at low pH is important when considering the yeast for sour beer production where the yeast must withstand pH values of below 4 [35]. However, it has been shown that organic acids like lactic acid can have a stronger inhibitory effect on yeasts and other microorganisms than HCl, which is caused by its chemical properties as a weak acid [36]. Inhibition by lactic acid could therefore be more pronounced than the HCl inhibition observed in this study. Figure 4.4–1 shows the growth of the investigated yeast strains in wort without addition of a stressor.

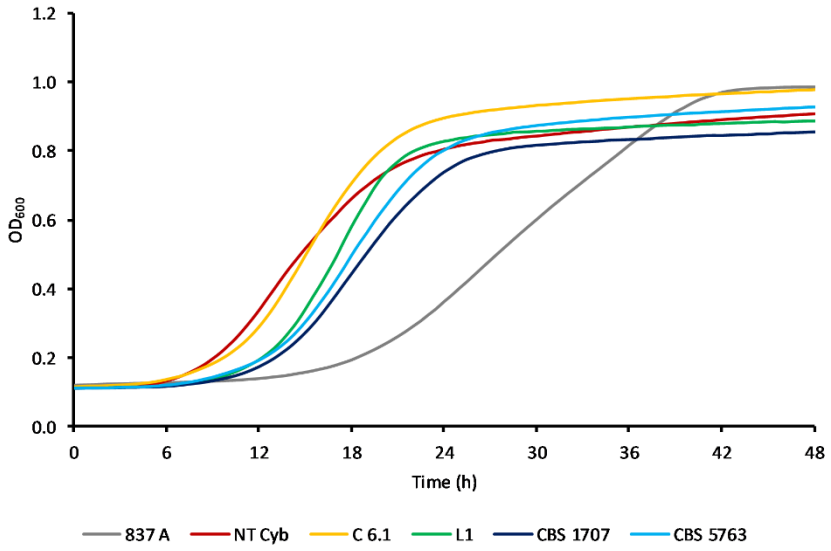


Figure 4.4–1 Growth of yeast strains in 7 °P wort extract at 25 °C without stressor. Growth curves shown are the mean of a triplicate.

4.4.4 Screening

To investigate interspecific differences in the fermentation of wort, fermentation trials were performed in a diluted wort extract of 7 °P. Previous studies have shown that extract contents of around 7 °P will yield ethanol concentrations of around 0.5% ABV, a popular legal limit for NAB [37], in fermentations with maltose-negative yeast strains [1,14,34,38]. After aerobic propagation for 24 hours, NT Cyb exhibited the highest number of cells, at 2×10^9 cells/mL, more than four-fold the amount of cells compared to L1, C6.1, and the

CBS strains with counts between 3.4 and 4.9×10^8 cells/mL (Table 4.4–4). Due to a delayed growth (compare Figure 4.4–1), 837A had to be propagated for 48 hours, reaching a cell count of 6.1×10^8 cells/mL. For the screening in wort, yeast cells were added at a concentration of 3×10^7 cells/mL, after a gentle washing step in water to prevent carry-over of propagation wort sugars. The results from the yeast screening are shown in Table 4.4–4. The fermentations were carried out until no change in extract could be measured for two consecutive days.

Strains 837A and CBS 1707 exhibited the lowest attenuation of only 18 and 17%, respectively, owing to their inability to utilize sucrose (Table 4.4–2), which was confirmed by the lack of sucrose consumption. Liu and Quek [20] also reported the absence of sucrose utilization by CBS 1707. The other strains, which depleted sucrose completely, reached attenuations of 21 to 24%. Consequently, 837A and CBS 1707 also produced, at 0.55 and 0.56% ABV, the lowest amounts of ethanol ($p \leq 0.05$) compared to the remaining strains, where ethanol concentrations ranged from 0.63 to 0.67% ABV. The final pH of the fermented samples ranged from 4.33 (CBS 5763) to 4.51 (NT Cyb). Residual FAN ranged from 78 (CBS 1707) to 88 mg/L (837A). As expected, none of the strains consumed maltotriose. Maltose consumption was also neglectable in all strains, although the species *Cyberlindnera fabianii* (like NT Cyb) has been reported to be able to ferment maltose [31,40]. The observations also underlined that results from the API substrate utilization test (where NT Cyb, L1 and CBS 1707 were positive for maltose) are not necessarily reflected in practice, especially since sugar utilization during respiration and fermentation can differ [31,32,40]. While glucose was depleted by all strains, fructose was only fully depleted by L1. The remaining strains exhibited glucophilic behavior and consumed only 73 to 83% of fructose during fermentation.

Table 4.4–4 Results of the screening of the investigated *Cyberlindnera* strains in wort extract. Different superscripts of values within a row indicate a significant difference ($p \leq 0.05$).

NAB attributes		Unit	837A	NT Cyb	L1	C6.1	CBS 1707	CBS 5763
Propagation	Cell count (24h)[1]	$\times 10^6$ cells/mL	611 ± 34 [1]	2055 ± 21	486 ± 27	445 ± 4	338 ± 25	386 ± 48
Fermented wort	real Extract	°P	6.53 ± 0.03 [b]	6.40 ± 0.04 [ab]	6.45 ± 0.05 [ab]	6.36 ± 0.03 [a]	6.57 ± 0.10 [b]	6.35 ± 0.10 [a]
	Attenuation	%	18 ± 1 [ab]	23 ± 1 [bc]	21 ± 2 [abc]	24 ± 1 [c]	17 ± 3 [a]	24 ± 3 [c]
	Ethanol	% ABV	0.55 ± 0.01 [a]	0.63 ± 0.01 [b]	0.66 ± 0.00 [cd]	0.63 ± 0.00 [bc]	0.54 ± 0.01 [a]	0.67 ± 0.02 [d]
	pH	-	4.41 ± 0.02 [ab]	4.51 ± 0.02 [c]	4.44 ± 0.01 [bc]	4.38 ± 0.03 [b]	4.37 ± 0.06 [ab]	4.33 ± 0.01 [a]
	FAN	mg/L	88 ± 1 [b]	83 ± 2 [ab]	80 ± 1 [a]	81 ± 4 [a]	78 ± 1 [a]	84 ± 5 [ab]
Sugar consumption	Maltotriose	%	3 ± 2	6 ± 1	4 ± 1	5 ± 1	3 ± 1	5 ± 3
	Maltose	%	4 ± 2	4 ± 0	3 ± 1	4 ± 0	4 ± 1	4 ± 4
	Sucrose	%	2 ± 10	100	100	100	2 ± 2	100
	Glucose	%	100	100	100	100	100	100
	Fructose	%	81 ± 1	75 ± 1	100	80 ± 1	73 ± 8	83 ± 2
Fermentation by-products	Glycerol	g/L	0.25 ± 0.05 [ab]	0.23 ± 0.04 [a]	0.36 ± 0.03 [b]	0.30 ± 0.04 [ab]	0.18 ± 0.05 [a]	0.21 ± 0.05 [a]
	Acetaldehyde	mg/L	9.70 ± 2.83 [b]	8.05 ± 1.48 [b]	2.60 ± 0.14 [a]	3.37 ± 0.71 [a]	3.83 ± 0.45 [a]	2.57 ± 0.21 [a]
	Ethyl acetate	mg/L	65.70 ± 14.57 [b]	22.55 ± 2.90 [a]	9.27 ± 3.23 [a]	4.90 ± 0.85 [a]	8.10 ± 0.28 [a]	5.17 ± 0.29 [a]
	Isoamyl acetate	mg/L	0.90 ± 0.14 [ab]	<LOD	0.15 ± 0.07 [a]	1.60 ± 0.62 [b]	1.67 ± 0.12 [b]	1.03 ± 0.23 [ab]
	Ethyl formate	mg/L	0.53 ± 0.04 [a]	0.31 ± 0.06 [a]	0.57 ± 0.09 [a]	0.25 ± 0.03 [a]	2.70 ± 0.57 [c]	1.45 ± 0.07 [b]
	Ethyl propionate	mg/L	0.13 ± 0.04 [a]	0.13 ± 0.01 [a]	<LOD	<LOD	0.16 ± 0.01 [a]	0.17 ± 0.03 [a]
	Isoamyl alcohols	mg/L	11.20 ± 0.14 [a]	16.40 ± 0.57 [b]	23.15 ± 0.92 [c]	11.67 ± 1.74 [a]	11.93 ± 0.93 [a]	10.50 ± 0.14 [a]
	n-Propanol	mg/L	4.03 ± 0.84 [ab]	3.73 ± 0.21 [ab]	4.40 ± 0.62 [b]	3.27 ± 0.15 [ab]	2.93 ± 0.29 [a]	3.33 ± 0.15 [ab]
	Isobutanol	mg/L	7.57 ± 1.24 [ab]	7.70 ± 0.36 [b]	8.27 ± 1.38 [b]	8.03 ± 0.40 [b]	5.33 ± 0.55 [a]	7.20 ± 0.20 [ab]
	Σ Esters	mg/L	67.26 ± 14.79 [b]	22.99 ± 2.87 [a]	9.99 ± 3.31 [a]	6.75 ± 0.61 [a]	12.62 ± 1.48 [a]	7.82 ± 0.30 [a]
	Σ Alcohols	mg/L	22.80 ± 0.14 [a]	27.83 ± 0.64 [b]	35.82 ± 1.48 [c]	22.97 ± 1.97 [a]	20.20 ± 0.17 [a]	21.03 ± 0.21 [a]
Sensory	Aroma	-	Solvent-like, unpleasant	Cabbage-like, unpleasant	Fruity, pleasant	Fruity, pleasant	Fruity, pleasant	Fruity, pleasant

[1] Cell count after 48 h due to delayed growth compared to other strains (compare Figure 4.4–1).

Regarding fermentation by-products, glycerol concentrations were low, ranging from 0.18 to 0.36 g/L. The strains 837A and NT Cyb accumulated significantly higher amounts of acetaldehyde, at 9.7 and 8.1 mg/L respectively, compared to 2.6 to 3.8 mg/L in the remaining samples. The sample fermented with *Cyberlindnera misumaiensis* 837A exhibited extremely high values of ethyl acetate, at 65.7 mg/L, twice the flavor threshold concentration in beer [2,41]. Ethyl acetate is described to have a fruity, estery character but also solvent-like, especially in high concentrations. The remaining strains exhibited ethyl acetate production between 4.9 (C6.1) and 22.6 mg/L (NT Cyb). Isoamyl acetate, which is predominantly described by a fruity, banana-like aroma, has a much lower flavor threshold of only 1.4–1.6 mg/L [2,41]. The strains C6.1 and CBS 1707 produced the highest amounts of isoamyl acetate, at 1.67 and 1.60 mg/L, followed by CBS 5763, 837A and L1, at 1.03, 0.90 and 0.15 mg/L, respectively. NT Cyb did not produce detectable amounts of isoamyl acetate. Concentrations of ethyl formate and ethyl propionate in the fermented samples were low, ranging from undetectable to 2.7 mg/L. Ethyl butyrate and ethyl caproate were not detected in either of the samples (data not shown). The strain L1 produced a significantly higher amount of higher alcohols, at 35.8 mg/L, followed by NT Cyb, at 27.8 mg/L, and the remaining strains at 20–23 mg/L. During sensory evaluation, the high ethyl acetate concentration in the sample fermented with 837A was indeed perceptible and described as an unpleasant, solvent-like aroma. The sample fermented with NT Cyb was described as having an unpleasant cabbage-like aroma. The remaining samples were characterized by a pleasant, fruity aroma.

The unpleasant, solvent-like aroma in the sample fermented with 837A was attributed to the very high ethyl acetate concentration, well above the flavor threshold. However, the cabbage-like aroma, which is generally associated with sulfides or thiol compounds [41], that was detected in the sample fermented with NT Cyb could not be linked to the volatile by-products that were measured. Interestingly, ethyl acetate concentrations in the remaining samples, characterized by a pleasant, fruity aroma were low, at only 2.6–3.8 mg/L. However, C6.1, CBS 1707 and CBS 5763 exhibited higher amounts of isoamyl acetate, a desired ester in beer (particularly ales) [42], when compared to the samples with unpleasant aroma. The concentrations of 1.0–1.6 mg/L are within, the reported flavor threshold in beer of between 0.5–2.0 mg/L [43]. Additionally, it is also well known that synergistic effects between esters occur that can push the concentration of perception below their individual flavor thresholds [42,44,45]. Isoamyl acetate could therefore have been a cause of the fruity aroma in the samples fermented with C6.1, CBS 1707 and CBS

5763. However, the sample fermented with L1, which was also characterized by a fruity aroma, only contained a very low isoamyl acetate concentration of 0.15 mg/L. It is noteworthy, however, that the L1 sample contained a significantly higher amount of isoamyl alcohol, at 23.2 mg/L, which is described to have an alcoholic, fruity and banana-like flavor [2]. The results have confirmed that not a high amount of esters, but rather a balanced profile will lead to a pleasant, fruity aroma [5].

Based on the results from the screening, *Cyberlindnera subsufficiens* C6.1 was chosen for optimization of fermentation conditions by means of response surface methodology, followed by an up-scaled brewing trial at 60 L to create a fruity, non-alcoholic beer (≤ 0.5% ABV). Strains 837A and NT Cyb were eliminated because of their poor flavor characteristics. CBS 1707 was eliminated due to its inability to ferment sucrose, which apart from the lower attenuation, would remain in the wort after fermentation, acting as an additional sweetening agent and potential contamination risk. *Cyberlindnera jadinii* strain L1 was eliminated due to its very low isoamyl acetate production (Table 4.4–4) and due to its maltose utilization when oxygen was present (Table 4.4–2). The decision between the two similarly performing *Cyberlindnera subsufficiens* strains C6.1 and CBS 5763 was made in favor of C6.1 due to a more pleasant fruitiness. In addition, C6.1 showed increased tolerance towards stress caused by ethanol or low pH (Table 4.4–3).

4.4.5 Response surface methodology (RSM)

To find the optimal fermentation conditions for C6.1 for an up-scaled application to produce a fruity, non-alcoholic beer, RSM was performed. Michel et al. [46] applied RSM to optimize the fermentation conditions of a *Torulaspora delbrueckii* strain for brewing purposes. They found that the pitching rate and fermentation temperature were crucial parameters, which influenced the flavor character of the final beer. The optimal fermentation conditions were shown to be at 21 °C with a high pitching rate of 60×10^6 cells/mL. Especially for non-*Saccharomyces* yeasts, the pitching rate can be crucial since most non-*Saccharomyces* species have comparably smaller cell sizes [46]. Figure 4.4–2 shows an example of the differing cell size between *Cyberlindnera subsufficiens* strain C6.1 (A) and the brewers' yeast strain *Saccharomyces cerevisiae* WLP001 (B) at identical magnification.

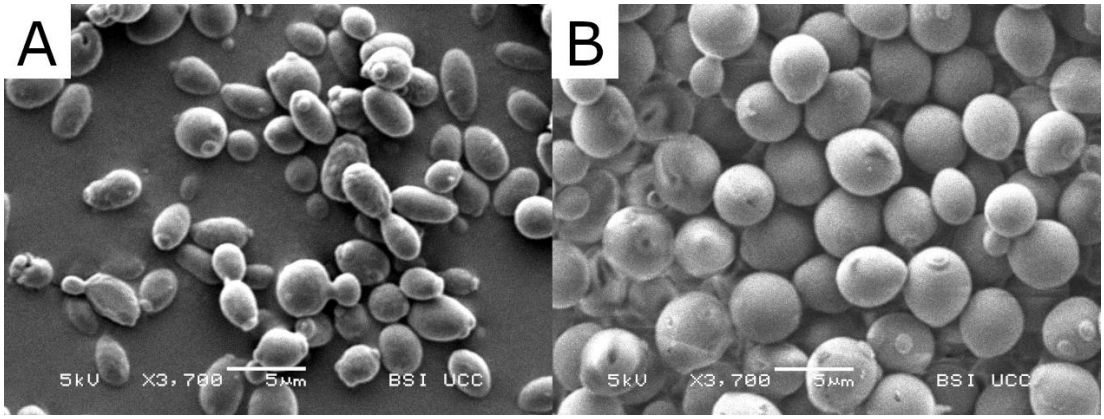


Figure 4.4–2 Scanning electron microscopy (SEM) picture of *Cyberlindnera subsufficiens* strain C6.1 (A) and brewers' yeast strain *Saccharomyces cerevisiae* WLP001 (B) at a magnification of ×3,700. Size of bar 5 µm.

It is also known that temperature and pitching rate has an influence on ester production, though strain-specific differences also play a role [4,6]. Previously reported fermentation temperatures of *Cyberlindnera subsufficiens* and other *Cyberlindnera* spp. range from 20 to 25 °C [12,17,19,20,47]. Consequently, a two-factorial, face-centered central composite design was chosen with the Factor A: Fermentation temperature (17, 22, 27 °C) and Factor B: Pitching rate (10, 35, 60×10^6 cells/mL). The individual experiment runs are listed in Table 4.3–2. The wort extract applied in the RSM trial was the same as that used for the screening, at an extract content of 7 °P (Table 4.3–1). Fermentation was conducted until no change in extract could be measured for two consecutive days. With the measured response values, significant models could be produced. The significant response models, with their respective minima and maxima and a summary of the model statistics are shown in Table 4.4–5. Insignificant response models are not shown and response models with a significant lack of fit will not be discussed in this study but are included in the visualized data for the sake of a complete picture. For a full report on model statistics and response values, refer to the supplementary Data Sheet S1 (Appendix). It was possible to create significant models for 12 responses (Table 4.4–5). However, five also exhibited significant lack of fit (LOF), rendering them unusable for predictions. The aim of the RSM was to investigate the optimal fermentation conditions to create a fruity, non-alcoholic beer.

Table 4.4–5 Analysis of variance (ANOVA) results for response models of the response surface methodology (RSM) trial.

Response	Unit	Minimum	Maximum	Model	*p*-value	LOF *p*-value
Ethanol	% ABV	0.41	0.60	RQuadratic	2.80×10^{-3} **	0.648
Ethyl acetate	mg/L	3.4	9.3	2FI	3.12×10^{-2} *	0.007 **
Isoamyl acetate	mg/L	0.8	2.2	RQuadratic	1.42×10^{-2} *	0.046 *
Acetaldehyde	mg/L	1.9	3.4	RLinear	1.35×10^{-3} **	0.337
n-Propanol	mg/L	3.2	4.5	2FI	9.03×10^{-3} **	0.029 *
Isobutanol	mg/L	3.2	6.7	RQuadratic	4.30×10^{-9} ***	0.145
Isoamyl alcohols	mg/L	7.3	13.3	Quadratic	2.67×10^{-5} ***	0.270
Σ Esters	mg/L	4.2	11.1	RQuadratic	1.48×10^{-2} *	0.018 *
Σ Alcohols	mg/L	13.7	22.9	RQuadratic	3.28×10^{-8} ***	0.339
Glycerol	g/L	0.17	0.37	RQuadratic	4.85×10^{-5} ***	0.034 *
Acceptance	-	1.08	3.38	Linear	1.31×10^{-2} *	0.377
Fruitiness	-	1.13	3.38	Linear	7.31×10^{-3} **	0.484

Model terminology: 'RQuadratic' Reduced Quadratic; '2FI' Two Factor Interaction; 'RLinear' Reduced Linear. 'LOF' Lack of fit. ANOVA significance codes: '***' $p \leq 0.001$, '**' $p \leq 0.01$, '*' $p \leq 0.05$

The 3-dimensional response surface plots of the interactive effects of temperature and pitching rate on the final ethanol content and the fruitiness of the produced NAB are shown in Figure 4.4–3 and Figure 4.4–4.

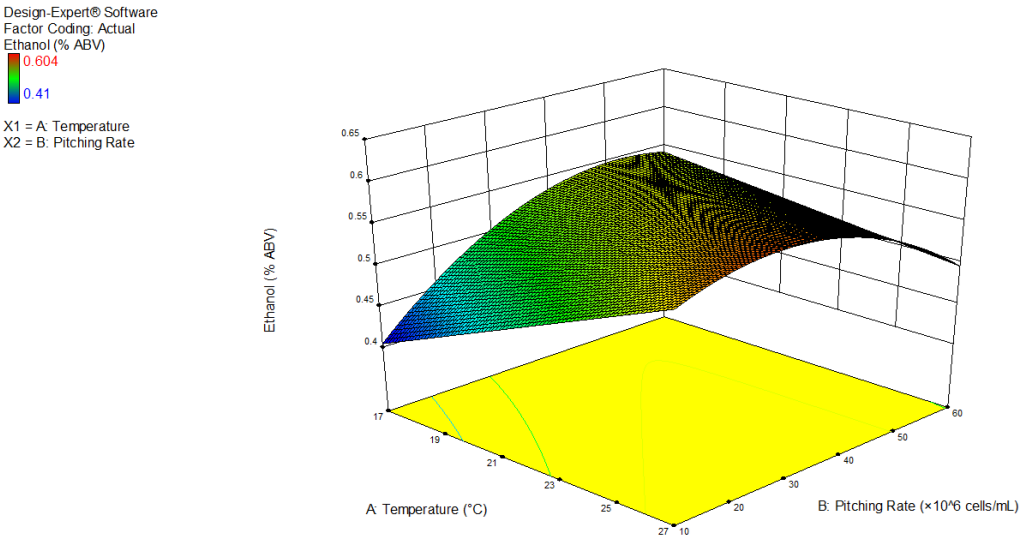


Figure 4.4–3 3-dimensional response surface plot of the interactive effects of temperature and pitching rate on the ethanol content of the produced non-alcoholic beer ($p < 0.01$).

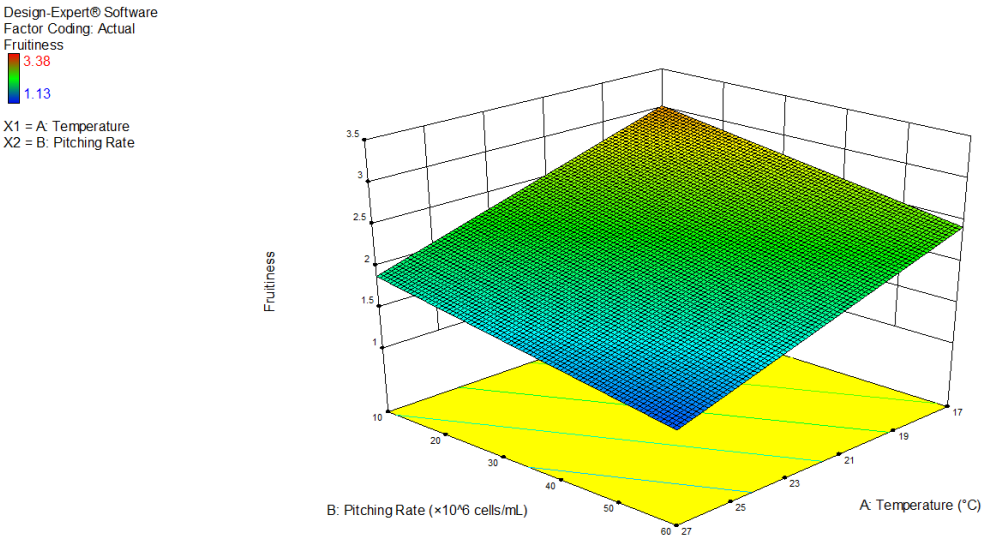


Figure 4.4–4 3-dimensional response surface plot of the effects of temperature and pitching rate on the fruitiness of the produced non-alcoholic beer ($p < 0.01$).

Ethanol content was lowest at a low temperature of 17 °C and low pitching rate (10^7 cells/mL) and went up with increasing temperature and pitching rate, but lowered again at a high pitching rate combined with a high fermentation temperature (Figure 4.4–3). The minium and maximum values were 0.41 and 0.60% ABV. Sugar analysis revealed that at 17 °C and 10^7 cells/mL, about 0.5 g/L of glucose was remaining after fermentation, while it was fully depleted in worts fermented at higher pitching rates and higher temperatures (data not shown). The residual sugar explained the lower final ethanol concentration. Fructose was only fully depleted in the samples were fermented at 27 °C. At 22 °C, fermented samples exhibited residual fructose concentrations between 0.2–0.5 g/L and at 17 °C, fermented samples showed remaining fructose concentrations between 0.2–0.7 g/L. Acetaldehyde concentrations where only dependent on the pitching rate, with increasing amounts of acetaldehyde found at lower pitching rates (Supplementary Figure 4.8–1). This result correlates with other studies that found a decrease in acetaldehyde with increasing pitching rate in wort fermentations with brewers' yeasts [48,49]. However, overdosing yeast ($> 5\times10^7$ cells/mL) can lead to an increase in acetaldehyde again, as observed by Erten et al. [50]. The temperature did not have a significant effect on the acetaldehyde concentration and was therefore excluded from the model ($p = 0.39$; supplementary Data Sheet S1). However, regarding higher alcohols, the fermentation temperature had a stronge effect with increasing amounts of higher alcohols

found at higher temperatures (Figure 4.4–5 and Supplementary Figure 4.8–2) which is consistent with literature [4,5]. Isoamyl acetate concentrations were generally high and ranged from 0.8 to 2.2 mg/L. Although the model was significant ($p < 0.05$), it was unsuitable for value prediction due to a significant lack of fit ($p = 0.046$).

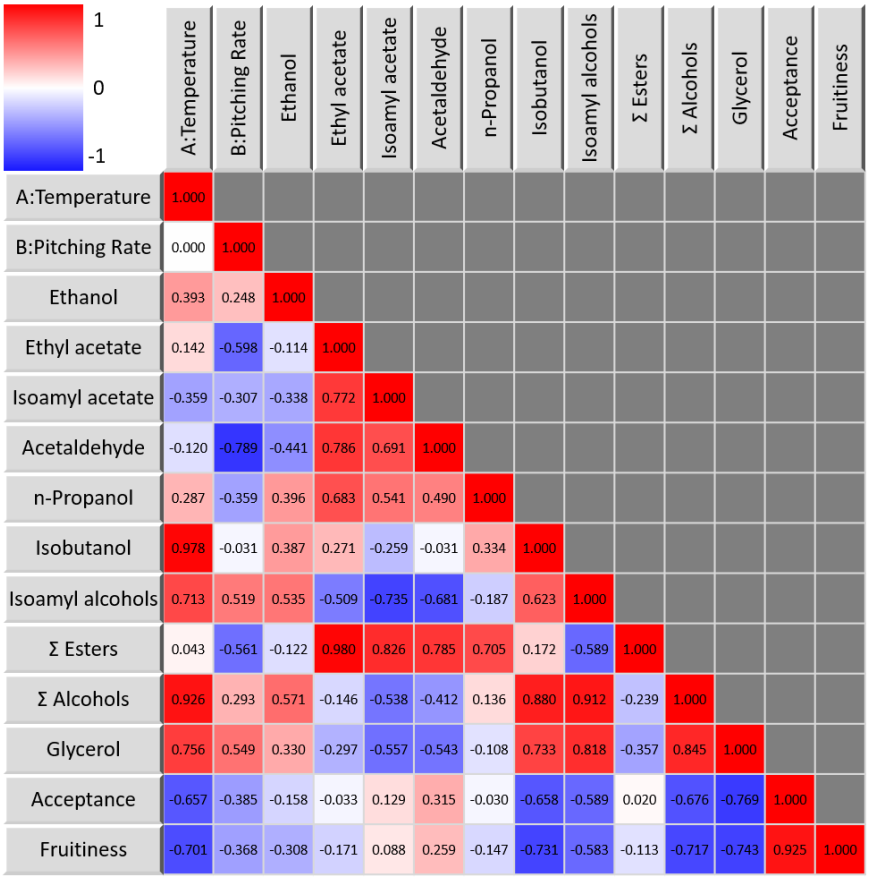


Figure 4.4–5 Map visualizing correlations of response surface methodology (RSM) factors and responses based on the Pearson Correlation Coefficient. 1 signifies strong positive correlation, 0 signifies no correlation and -1 signifies a strong negative correlation.

Interestingly, the production of the esters ethyl acetate and isoamyl acetate did not show a clear correlation to temperature which underlines that the general rule of thumb, that higher fermentation temperatures lead to increased ester production, is not valid for all yeast strains (Figure 4.4–5) [4]. Furthermore, the amount of esters that were quantified in this study did not correlate with the perceived fruitiness of the NAB, which tentatively suggests that the fruity flavor profile was caused by yet unidentified compounds (Figure 4.4–5).

In terms of fruitiness, a low fermentation temperature paired with a low pitching rate led to the highest perceived fruitiness. Indeed, the highest fruitiness was recorded at 17 °C

and 1×10^7 cells/mL and the lowest at 27 °C and 6×10^7 cells/mL, following a linear model. General acceptance showed a strong positive correlation with the fruitiness, indicating that the panel preferred fruity samples (Figure 4.4–5 and Supplementary Figure 4.8–3).

Due to the ideal combination of lowest ethanol content and highest fruitiness and acceptance, the fermentation temperature of 17 °C and pitching rate of 1×10^7 cells/mL were chosen as the optimal fermentation conditions for application to produce a fruity, non-alcoholic beer.

A small-scale fermentation at the optimal conditions (17 °C, 10^7 cells/mL) was conducted to validate the RSM model. Table 4.4–6 shows the predicted mean including 95% prediction intervals (PI) and the measured ("observed") mean with standard deviation.

Table 4.4–6 Response surface methodology (RSM) model validation via predicted value vs. observed value.

Response	95% PI low	Predicted mean	95% PI high	Observed mean	Std. Dev.
Ethanol*	0.33	0.40	0.48	0.53	0.01
Ethyl acetate	0.89	4.74	8.60	6.83	0.59
Isoamyl acetate	0.78	1.63	2.47	2.50	0.10
Acetaldehyde*	2.19	2.97	3.74	1.27	0.29
n-Propanol	2.68	3.28	3.88	3.57	0.06
Isobutanol*	2.91	3.23	3.54	2.80	0.10
Isoamyl alcohols	5.78	7.03	8.29	4.10	0.10
SUM Esters	3.01	7.10	11.19	9.33	0.68
SUM Alcohols*	12.84	13.74	14.64	10.47	0.31
Glycerol	0.13	0.18	0.22	0.27	0.01
Acceptance*	2.12	3.23	4.34	3.75	0.62
Fruitiness*	2.02	3.03	4.05	3.58	0.87

*Significant model with insignificant lack of fit. 'PI' Prediction interval.

Although predicted by a significant model, the observed means for ethanol, acetaldehyde and isobutanol values were not within the 95% prediction interval. Sugar analysis revealed the complete depletion of glucose in the experimental fermentation trial at optimal conditions compared to the RSM model prediciton which explained the increased ethanol production (data not shown). The moderate success in model validation demonstrates the limitations in the application of RSM to optimize fermentations, where small differences in substrate and process conditions can have significant influences on the outcome. Because wort is a very complex substrate, comprising a complex mixture of different sugars, nitrogen sources, minerals and vitamins, among others, any interpretation or the transfer of the RSM results to other substrates (even different wort substrates) should be

made with caution. In particular, a different sugar composition will have a significant effect on the responses when applying maltose-negative yeasts. However, the improved fruitiness and therefore higher acceptance of the NAB produced at low temperature and low pitching rate, the main goal from the optimization, was significant and reproducable (Table 4.4–6).

4.4.6 Pilot-scale brewing

Despite the limited model validation, the fermentation parameters were successfully optimized to enhance the fruity character of the NAB. Therefore, the pilot-scale brewing trial was conducted with the optimized conditions of 17 °C fermentation temperature and a pitching rate of 10^7 cells/mL.

The grain bill of the wort for the pilot-scale brewing trial consisted of 95% pilsner malt and 5% acidulated malt to lower the starting pH of the wort, to account for the reduced pH drop during fermentations with non-*Saccharomyces* yeasts compared to brewers' yeast. A low beer pH is desired to prevent microbial spoilage and to ensure good liveliness of the beer [51,52]. The analytical attributes of the wort produced at pilot-scale are shown in Table 4.4–7.

Table 4.4–7 Attributes of the wort produced on pilot-scale.

Wort attributes	Unit	Value
Extract	°P	7.00 ± 0.01
pH		4.86 ± 0.01
FAN	mg/L	107 ± 3
Glucose	g/L	6.01 ± 0.08
Fructose	g/L	0.80 ± 0.01
Sucrose	g/L	2.13 ± 0.03
Maltose	g/L	31.59 ± 0.44
Maltotriose	g/L	9.32 ± 0.13

To assess the suitability of *Cyberlindnera subsufficiens* C6.1 to produce a fruity NAB, it was compared to two commercial NABs. NAB A was a commercial non-alcoholic beer produced by limited fermentation [29] and NAB B was a non-alcoholic beer produced by "dialysis technology" [30]. The NABs were analyzed for their extract, ethanol, FAN and glycerol content as well as their sugar composition and concentration of volatile fermentation by-products. The results are shown in Table 4.4–8.

Table 4.4–8 Attributes of the non-alcoholic beer (NAB) produced with C6.1 compared to two commercial NABs, NAB A and NAB B.

NAB attributes	Unit	C6.1 NAB	NAB A	NAB B
Extract (real)	°P	6.60 ± 0.01	6.76 ± 0.07	7.05 ± 0.03
Extract (apparent)	°P	6.46 ± 0.02	6.57 ± 0.06	6.86 ± 0.01
Ethanol	% ABV	0.36 ± 0.00	0.50 ± 0.03	0.49 ± 0.04
pH		4.45 ± 0.01	4.29 ± 0.02	4.29 ± 0.04
FAN	mg/L	96 ± 2	86 ± 6	24 ± 0
Glycerol	g/L	0.30 ± 0.02	0.33 ± 0.01	1.40 ± 0.03
Glucose	g/L	2.77 ± 0.05	2.74 ± 0.04	5.61 ± 0.04
Fructose	g/L	1.65 ± 0.03	1.96 ± 0.03	0.19 ± 0.00
Sucrose	g/L	< LOD	< LOD	< LOD
Maltose	g/L	30.27 ± 0.62	30.11 ± 0.50	17.69 ± 0.24
Maltotriose	g/L	8.67 ± 0.24	8.31 ± 0.21	1.84 ± 0.03
Acetaldehyde	mg/L	10.55	2.40	0.70
Ethyl acetate	mg/L	12.00	< 0.10	2.70
Isoamyl acetate	mg/L	0.80	< 0.1	0.70
Isoamyl alcohols	mg/L	4.00	4.80	17.40
n-Propanol	mg/L	2.20	< 0.5	2.50
Isobutanol	mg/L	3.60	1.00	4.90
Diacetyl	mg/L	< 0.01	0.02	0.04
2,3-Pentandione	mg/L	< 0.01	< 0.01	< 0.01
Σ Esters	mg/L	12.8	< 0.1	3.4
Σ Alcohols	mg/L	9.8	5.8	24.8

The C6.1 NAB reached final attenuation after 13 days of fermentation at 17 °C, at an ethanol content of 0.36% ABV. At the end of fermentation, 2.77 g/L glucose were remaining in the wort and sucrose was fully depleted. Compared to the initial sugar concentration of the wort (Table 4.4–7), fructose concentrations in the final beer were significantly higher, at 1.65 g/L, twice as high as the starting concentration in the wort. Since sucrose was fully depleted, it can be assumed that it was converted to glucose and fructose by the yeast's invertase. The high residual fructose could therefore be attributed to the previously observed glucophilic character of the C6.1 strain in the screening and RSM trial. As a result, fructose was not consumed by the yeast due to the permanent presence of glucose until fermentation came to a halt. As expected, maltose and maltotriose consumption was negligible. Despite the limited fermentation, C6.1 produced a relatively high amount of esters, at 12.8 mg/L, the majority of which was ethyl acetate (12 mg/L). NAB A had an ethanol content of 0.50% ABV. Interestingly, the sugar composition was very similar to that of the C6.1 NAB. Regarding fermentation by-

products, however, NAB A exhibited very low concentrations, at about half the amount of higher alcohols and a total lack of the esters ethyl acetate and isoamyl acetate. NAB B had an ethanol content of 0.49% ABV. Owing to its fundamentally different production method, the analyzed attributes were very different from that of the two NABs produced solely by limited fermentation. The low FAN content together with a high glycerol content compared to the other NABs were indicators of a more extensive fermentation, with subsequent removal of ethanol. However, NAB B still exhibited high amounts of monosaccharides which suggested that the production of the NAB either also entailed a limited fermentation, or the dealcoholized beer was blended with wort (or other means of sugar addition). The increased amounts of higher alcohols in NAB B, at 24.8 mg/L, are uncommon for beers dealcoholized via dialysis, since the process commonly reduces their content in the final NAB by 90-95% [37]. Despite the addition of acid malt during the wort production for the C6.1 NAB, the final pH after fermentation was, at 4.45, higher compared to 4.29 in the commercial NABs.

Due to the high amounts of residual sugars, proper pasteurization is essential for non-alcoholic beers produced by limited fermentation to avoid microbial spoilage [1,38,53]. After bottling, C6.1 NAB was therefore pasteurized with approximately 23 PU and the successful pasteurization confirmed with plating the pasteurized NAB on agar to check for microorganism growth, which was found to be negative.

4.4.7 Sensory evaluation

For a holistic evaluation of the C6.1 NAB compared to the two commercial NABs, a sensory trial was conducted with 10 trained and experienced panelists. The panel was asked to describe the flavor of the beer in their own words, followed by an assessment of several intensity attributes. The mean score values of the parameters, wort-like, floral, fruity, citrus-like and tropical aroma as well as sweet taste of the NABs are shown in Figure 4.4–6.

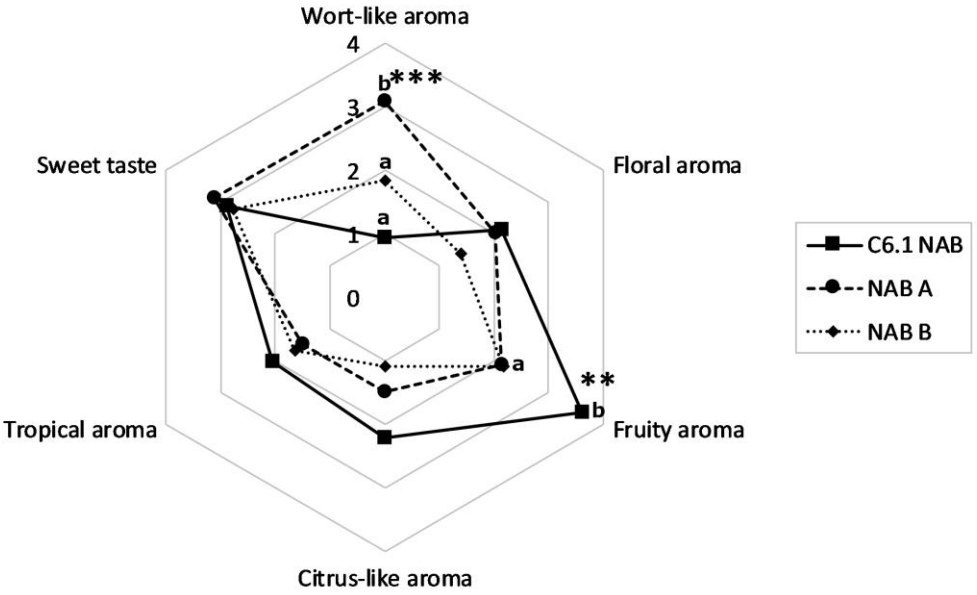


Figure 4.4–6 Spider web with the means of the descriptors from the sensory trial of the NAB produced with *Cyberlindnera subsufficiens* C6.1 and the two commercial NABs. Different letters next to data points indicate a significant difference as per Tukey's post hoc test. Significance codes: '***' $p \leq 0.001$, '**' $p \leq 0.01$.

The NAB produced with C6.1 was described as very fruity with aromas of pear, banana, mango and maracuja together with a slightly wort-like character. NAB A was described as malty, wort-like and hoppy, while NAB B was described as wort-like and caramel-like. The C6.1 NAB was indeed evaluated as being significantly more fruity than the commercial NABs ($p \leq 0.01$), at an average of 3.6 out of 5 compared to 2.1 and 2.2 out of 5, scoring also higher in citrus-like and tropical aroma. Consequently, the wort-like aroma, one of the most criticized flaws of NABs produced by limited fermentation [1,2,52], was least pronounced in the NAB produced with C6.1 with an average of 1 out of 5, followed by NAB B with 1.8 out of 5. NAB A exhibited, at an average of 3.2, a significantly more pronounced wort-like aroma ($p \leq 0.001$). A sweet taste, caused by a high amount of residual sugars, is another major point of criticism for NABs produced by limited fermentation [1,2,52]. All NABs scored similarly in sweet taste without significant differences. NAB B scored lower for "floral" compared to the other NABs. However, the difference was not statistically significant. When the panelists where asked for their favorite sample, 40% chose C6.1 NAB, 40% chose NAB A, and 20% chose NAB B. Similarly, Strejc et al. [3] investigated the production of a non-alcoholic beer (0.5% ABV) by cold contact process (characterized by a low temperature and high pitching rate)

with a mutated lager yeast strain (*Saccharomyces pastorianus*). The strain's targeted mutation resulted in an overproduction of isoamyl acetate and isoamyl alcohols. The authors reported that the fruity flavour of the NAB produced with the mutated strain was "partially able to disguise" the typical wort-like off-flavor [21]. However, the isoamyl acetate concentration of the resulting NAB was, at 0.5 mg/L, lower than the concentration in the C6.1 NAB in this study (Table 4.4–8). Furthermore, the complex mutation and isolation procedure paired with a potentially limited stability of the mutation limits its applicability in practice. Saerens et al. [22] reported the successful production of a NAB at 1,000 L scale with a *Pichia kluyveri* strain, owing to its high production of isoamyl acetate (2-5 mg/L), which reportedly gave the NAB a fruity flavor that was more like that of a regular beer than commercial NABs. In accordance, the results of the sensory indicated that a strong fruity aroma can mask the wort-like off flavor and that the non-*Saccharomyces* yeasts which produce a pronounced fruity character can therefore be a means to produce NAB with improved flavor characteristics.

4.5 Conclusion

The *Cyberlindnera* genus was found to be a promising non-*Saccharomyces* genus for the application in the production of a fruity, non-alcoholic beer. Four of the six investigated species produced a fruity character, despite the limited fermentative capacity which resulted in a low ethanol concentration. It was shown that through optimization of the fermentation parameters of temperature and pitching rate, the fruity character could be enhanced. Process up-scaling with *Cyberlindnera subsufficiens* strain C6.1 produced a NAB which was significantly more fruity compared to two commercial NABs. Owing to the strong fruity aroma, the often-criticized wort-like aroma could successfully be masked. Yeast handling throughout the process (i.e. propagation, yeast pitching, fermentation) proved to be suitable for pilot-scale brewing with potential for application at industrial scale. Further studies should investigate if the masking effect was enhanced by a reduction of wort aldehydes via yeast metabolism.

This study demonstrated the suitability of the non-*Saccharomyces* species *Cyberlindnera subsufficiens* for the production of non-alcoholic beer (< 0.5% ABV) with novel flavor characteristics that can compete with commercial NABs. The successful pilot-scale (60 L) brewing trial gives prospect to future studies with diverse non-*Saccharomyces* yeasts and strengthens their position as a serious and applicable alternative to established methods in non-alcoholic and low alcohol beer brewing.

4.6 Acknowledgements

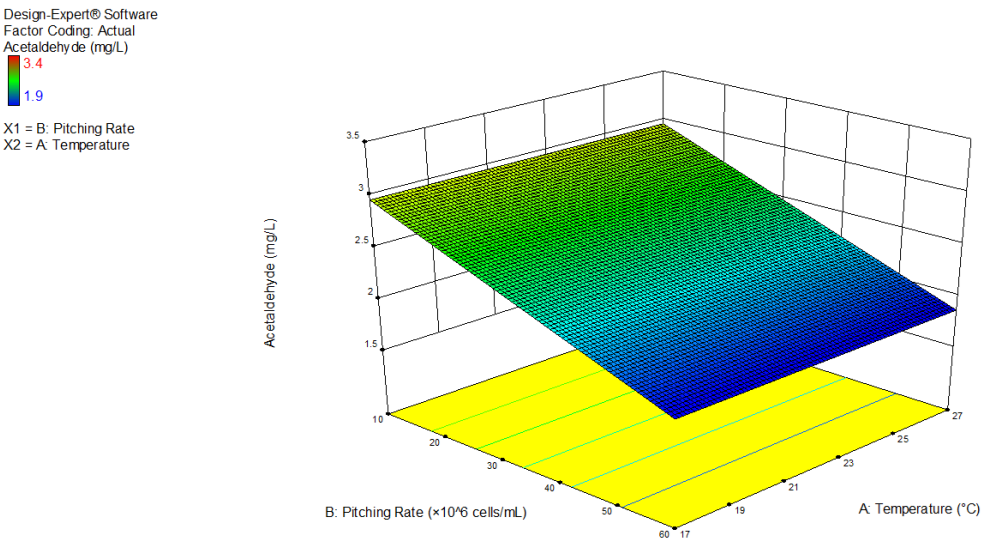
The authors would like to thank Mr. Philippe Lösel for his valuable contribution to this study, Muntons plc for supplying the sprayed wort extract, the Barth-Haas Group for supplying the iso-α-acids extract, and Dr. David De Schutter and Dr. Luk Daenen for their review.

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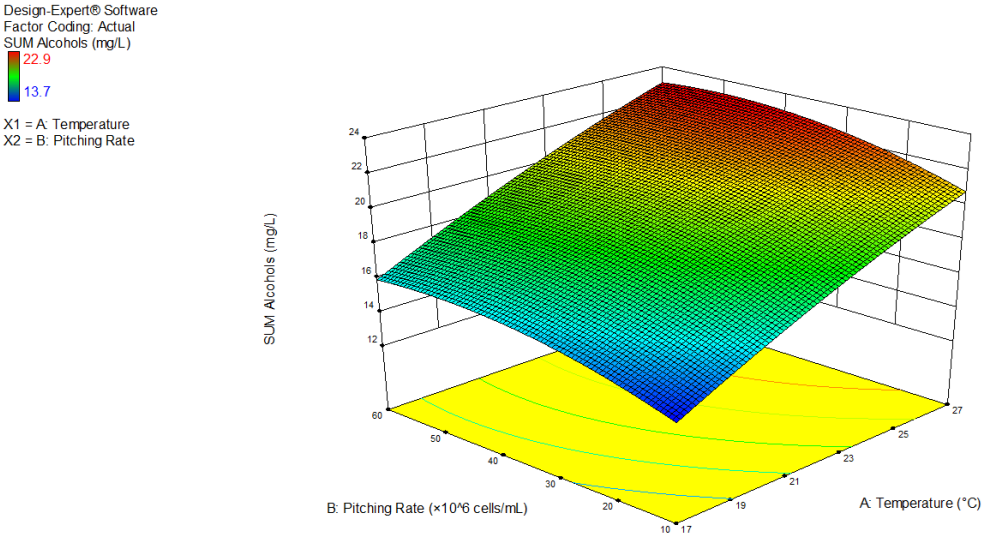
4.7 Author contributions

Conceptualization, K.B., M.M., M.H., M.Z. and E.A.; methodology, K.B. and M.M.; investigation, K.B., J.A., and A.H.; resources, M.H., F.J. and E.A.; formal analysis, K.B.; writing—original draft preparation, K.B.; writing—review and editing, K.B., M.M., M.Z., M.H., K.L. and E.A.; visualization, K.B.; supervision, E.A.; project administration, E.A.; funding acquisition, E.A.

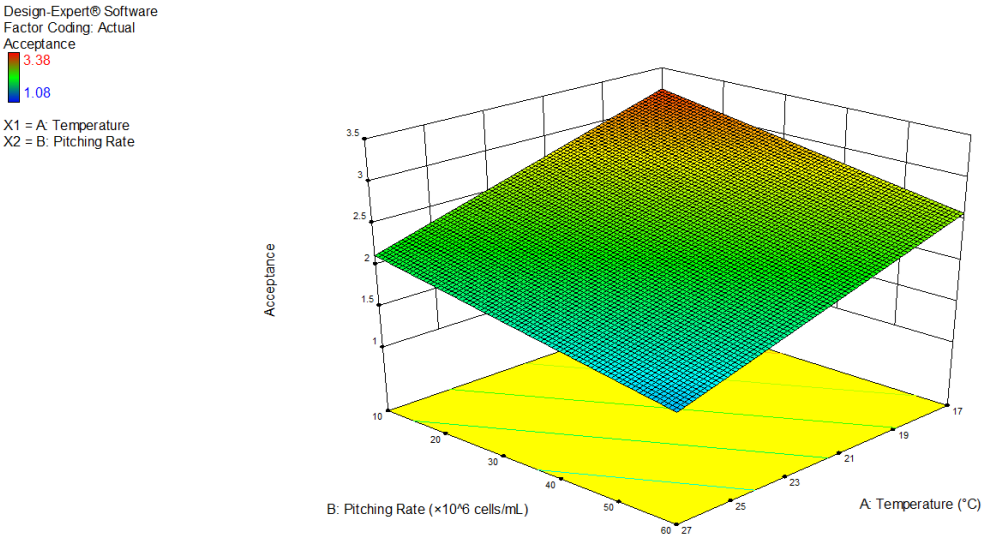
4.8 Supplementary Figures



Supplementary Figure 4.8–1 3-dimensional response surface plot of the effect of pitching rate on the acetaldehyde content of the produced NAB ($p < 0.01$). The factor temperature was excluded from the model due to insignificance ($p = 0.39$; supplementary Data Sheet 1; Appendix).



Supplementary Figure 4.8–2 3-dimensional response surface plot of the interactive effects of temperature and pitching rate on the sum of higher alcohols of the produced NAB ($p < 0.001$).



Supplementary Figure 4.8–3 3-dimensional response surface plot of the effects of temperature and pitching rate on the overall acceptance of the produced NAB ($p < 0.05$).

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Chapter 5:

Investigation into the application of *Lachancea fermentati* strain KBI 12.1 in low alcohol beer brewing

Published as

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5.1 Abstract

In brewing research, non-*Saccharomyces* yeasts have gained attention in recent years, owing to their potential to influence the characteristics and flavor of beer. The *Lachancea* genus possesses an uncommon trait, the production of significant amounts of lactic acid during alcoholic fermentation. This trait could potentially be harnessed for brewing purposes, particularly for the production of low alcohol beer. In this study, the potential of *Lachancea fermentati* strain KBI 12.1 was investigated for the production of low alcohol beer in low gravity wort. KBI 12.1 was characterized for sugar utilization, hop sensitivity, phenolic off-flavor (POF) production, and propagation performance. Lab scale fermentation trials in diluted wort (6.6 °P) were conducted and compared to a brewers' yeast, *Saccharomyces cerevisiae* WLP001. Fermentations were monitored for lactic acid and ethanol production, pH drop, and sugar consumption. In the final beers, amino acid and free amino nitrogen (FAN) content were determined and secondary metabolites were quantified. *Lachancea fermentati* KBI 12.1 showed to be unable to utilize maltotriose. The strain exhibited no POF production, minor hop sensitivity, and excellent propagation performance. Amino acid and FAN consumption were much lower compared to that of the brewers' yeast. In the final beer fermented with KBI 12.1, the lactic acid concentration reached 1.3 g/L, giving the beer a sour taste. During sensory analysis, the beer was additionally described to have a fruity character. In conclusion, *Lachancea fermentati* KBI 12.1 proved to be a suitable strain for brewing purposes, with promising traits with regards to non-alcoholic and low alcohol beer brewing.

5.2 Introduction

A greater appreciation of the role of yeast in determining the character of beer has fueled brewing research, particularly into non-*Saccharomyces* yeasts, in recent years [1]. Non-*Saccharomyces* yeasts have been investigated in sequential and co-fermentation with *Saccharomyces cerevisiae*, as well as in single culture fermentation to create new beers with diverse and innovative flavor profiles [2–4]. In wine research, the use of non-*Saccharomyces* yeasts has been investigated as a tool to increase aroma complexity [5] and to reduce the ethanol content [6,7].

A non-conventional yeast genus that has garnered attention in recent years due to its uncommon metabolic trait of being able to produce lactic acid during alcoholic fermentation is the *Lachancea* genus [8]. In particular, *Lachancea thermotolerans* (formerly *Zygosaccharomyces thermotolerans* [9]) was investigated for its use in reducing pH and enhancing total acidity in wine fermentations [10–13]. As part of the yeast metabolism, lactic acid is formed from pyruvate, the end product of glycolysis. However, the physiological role of lactic acid production and its underlying molecular mechanisms remain poorly understood [14]. A schematic representation of the metabolic pathway for lactic acid production is illustrated in Figure 5.2–1. In connection with beer fermentations, the *Lachancea* genus was first described by Gibson et al. [15] who investigated a *Lachancea fermentati* strain and other non-*Saccharomyces* yeasts for beer flavor modifications. In recent years, four more studies investigated the use of *Lachancea thermotolerans* [16–18] and *Lachancea fermentati* [19] in beer fermentations, proposing that the yeast was suitable for creating 'sour beers' without the use of lactic acid bacteria or the addition of technical lactic acid.

The brewing industry is facing changes with a slowdown in overall market growth and an increase in the non-alcoholic beer and low alcohol beer (NABLAB) sector due to lifestyle trends, demographics, stricter legislation and improved production methods [20]. Besides advances in dealcoholization techniques [21,22], research into the use of non-conventional yeasts with limited ability to ferment wort sugars has been gaining increasing attention in recent years with the aim to reduce or minimize alcohol content and to create novel beers with unique flavor profiles [20]. Non-alcoholic beers produced by limited fermentation or non-conventional yeasts usually lack the desired pH drop, which can lead to a high susceptibility to microbial spoilage and a low liveliness of the beer [23,24]. Therefore, additional acidification is required during the process. However, the

application of lactic acid-producing yeasts has the potential to make additional acidification redundant.

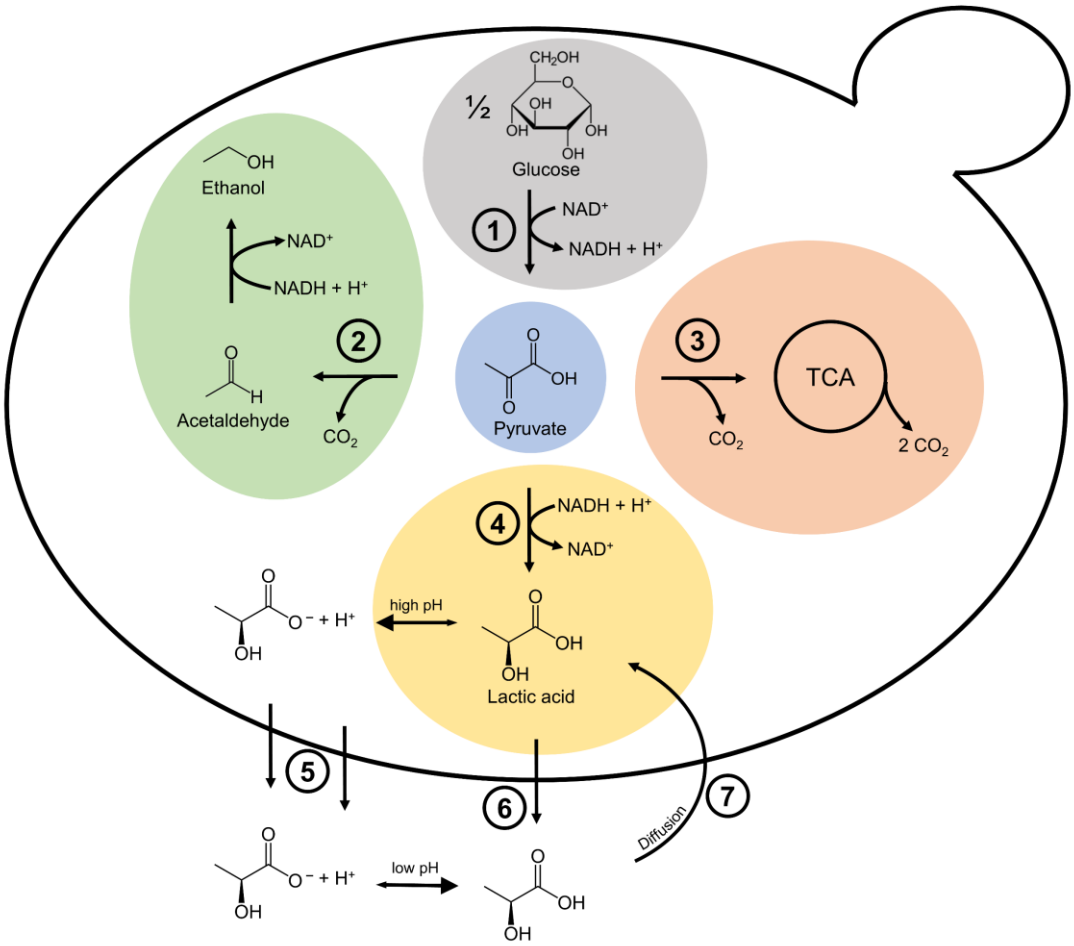


Figure 5.2–1 Relevant metabolic activities for lactic acid production from glucose by yeasts. Adapted from Sauer et al. [37]. **1. Glycolysis**, yielding one mole of ATP (not shown), one mole of pyruvate and one mole of NADH + H^+ (which has to be re-oxidized to NAD^+) from half a mole of glucose**. 2. Alcoholic fermentation.** Pyruvate decarboxylase (PDC) activity, yielding one mole of acetaldehyde and one mole of carbon dioxide per mole of pyruvate. Successive alcohol dehydrogenase (ADH) activity, yielding one mole of ethanol from one mole of acetaldehyde while recycling one mole of NADH + H^+ to NAD^+. **3. Respiration.** Pyruvate dehydrogenase, channeling pyruvate into the oxidative decarboxylation via the tricarboxylic acid (TCA) cycle. Usually suppressed by the Crabtree effect and the lack of oxygen**. 4. Lactic acid fermentation**. Lactate dehydrogenase (LDH), catalyzing the formation of lactic acid from pyruvate while recycling one mole of NADH + H^+ to NAD^+. The relatively high cytoplasmic pH (much higher than the pKa of lactic acid) leads to the deprotonation of lactic acid into lactate + H^+. **5. Lactate/H^+ symport.** At current state of knowledge, the most probable means of lactic acid export [37]. **6. Lactic acid export.** Mechanism currently unclear, but it is believed that the Lactate/H^+ symport is not the only means of transport [63]. **7. Diffusion**. At low extracellular pH, lactic acid is present in its protonated form and is therefore able to cross the cell membrane via diffusion. In the cell, the higher cytoplasmic pH leads to deprotonation with successive symport out of the cell, creating an energy requiring cycle with reaction 5.

The present study investigated the use of *Lachancea fermentati*, strain KBI 12.1, isolated from a kombucha culture, for application in low alcohol beer brewing. After investigating important brewing characteristics such as phenolic off-flavor (POF) production, sensitivity to hop-derived iso-α-acids, flocculation behavior, sugar utilization, and propagation performance, fermentation trials under laboratory conditions were performed [25]. The fermentations were conducted in a diluted wort (6.6 °P) to investigate the performance in a substrate with limited sugar and nutrient availability. During fermentation, extract and pH reduction, cell count, sugar utilization and lactic acid production were monitored. The final beers were analyzed for free amino nitrogen (FAN), amino acids, and secondary metabolites. A sensory trial was conducted by a trained panel.

5.3 Materials and Methods

5.3.1 Materials and yeast strains

All reagents used in this study were at least analytical grade from Sigma-Aldrich (St Louis MO, USA) unless stated otherwise. Malt extract for the flocculation test, hop resistance test and yeast propagation was supplied by Muntons (Spraymalt Light, Muntons plc, Suffolk, UK). Pilsner malt for wort production was sourced from Weyermann (Malzfabrik Weyermann, Bamberg, Germany). The *Saccharomyces cerevisiae* brewers' yeast WLP001 (California Ale Yeast) was sourced from Whitelabs (San Diego, CA, USA). *Lachancea fermentati* KBI 12.1 was isolated from a kombucha culture as described below. Yeast stocks were kept in 50% glycerol at –80 °C. Strains were grown on potato dextrose agar (PDA) plates for 48–72 h at 25 °C and stored at 4 °C.

5.3.2 Yeast isolation

A kombucha culture was grown in a sterilized model tea system (black tea, 7% (w/v) sucrose and 0.5% (w/v) glucose) for 48 hours at 25 °C under aerobic conditions. A sample was diluted and spread on differential agar (WL Nutrient agar) containing 0.01% (v/v) chloramphenicol to suppress bacterial growth. DNA of single colonies was extracted per manufacturers instruction of an extraction kit (Yeast DNA Extraction Kit, Thermo Fisher Scientific, Waltham MA, USA). For identification, the D1/D2 domain of the 26S rRNA gene was amplified, sequenced and compared to publicly available nucleotides on NCBI using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). PCR amplification was performed using the primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') with the temperature protocol: 95 °C / 2 min; 30 cycles of 95 °C / 30 s, 56 °C / 15 s; 72 °C / 60 s; 72 °C / 5 min (TProfessional Basic Gradient, Biometra GmbH, Göttingen, Germany).

5.3.3 Flocculation test

The flocculation test was performed using a slightly modified Helm's assay [26,27]. Essentially, all cells were washed in EDTA and the sedimentation period was extended to 10 min. Wort was composed of 75 g/L spray-dried malt extract (Spraymalt Light,

Muntons plc, Suffolk, UK) adjusted to 30 IBU (30 mg/mL iso-α-acids; from 30% stock solution; Barth-Haas Group, Nürnberg, Germany).

5.3.4 Substrate utilization

The substrate utilization test was performed on a YT MicroPlate (Biolog Inc., Hayward CA, USA) following the instructions from the manufacturer. In short, microtiter wells containing the individual substrates were inoculated with a yeast suspension. After incubation at 25 °C for 72 h, the absorbance was read with the microplate reader (Multiskan FC, Thermo Fischer Scientific) at a wavelength of 595 nm. The absorbance from the substrate-free water control was subtracted from the absorbance of the respective substrates and values were normalized to the absorbance of glucose. The substrate utilization test was performed in duplicate.

5.3.5 Hop sensitivity

Three 100 ml flasks containing sterilized wort (75 g/L Muntons Spraymalt Light; 7.0 °P) were adjusted to 0, 50 and 100 mg/L iso-α-acids (1 mg/L = 1 International Bitterness Unit, IBU), respectively by using an aliquot of a stock solution of 3% iso-α-acids in 96% (v/v) ethanol (Barth-Haas Group, Nürnberg, Germany). Strains were grown in yeast extract peptone dextrose (YPD) broth for 24 h at 25 °C and washed in H_2O before their addition to microtiter plate wells at a concentration of 10^5 cells/mL. The wells contained 200 μL of the respective, IBU adjusted worts. Plates were incubated at 25 °C and absorbance was measured every 40 min at 600 nm without shaking over a period of 96 h (Multiskan FC, Thermo Scientific, Waltham, Massachusetts, USA).

5.3.6 Phenolic off-flavor test

The phenolic off-flavor test was performed according to Meier-Dörnberg et al. [28]. Yeast strains were spread on yeast and mold agar plates (YM-agar) containing only one of the following precursors: either ferulic acid, cinnamic acid or coumaric acid. After three days of incubation at 25 °C, plates were evaluated by sniffing to detect any of the following aromas: clove-like (4-vinylguajacol), Styrofoam-like (4-vinylstyrene) and medicinal-like (4-vinylphenol). *Saccharomyces cerevisiae* LeoBavaricus - TUM 68® (Research Center

Weihenstephan for Brewing and Food Quality, Freising-Weihenstephan, Germany) was used as a positive control.

5.3.7 Propagation

Propagation wort was consisting of 75 g/L spray-dried malt (Spraymalt light, Muntons plc, Suffolk, UK) and 30 g/L glucose (Gem Pack Foods Ltd., Dublin, Ireland), sterilized at 121 °C for 15 min. Single yeast cultures were taken from PDA agar plates and inoculated into 140 ml of this propagation wort in a 250 mL Schott bottle. The bottle was covered with sterile cotton and placed in an incubator with orbital shaker (ES-80 shaker-incubator, Grant Instruments (Cambridge) Ltd, Shepreth, UK) and incubated for 48 h at an orbital agitation of 170 rpm and 25 °C. Viability was measured by staining with Löffler's methylene blue solution (MEBAK 10.11.3.3) and cells were counted using a Thoma Hemocytometer (Blaubrand, Sigma-Aldrich, St. Louis, MO, USA).

5.3.8 Wort production

Wort for the fermentation trials was produced in a 60 L pilot-scale brewing plant consisting of a combined mash-boiling vessel, a lauter tun and whirlpool (FOODING Nahrungsmitteltechnik GmbH, Stuttgart, Germany). Weyermann Pilsner Malt was milled with a two-roller mill ("Derby", Engl Maschinen, Schwebheim, Germany) at a 0.8 mm gap size. Seven kilograms of malt was mashed in with 40 L of brewing water at 50 °C. The following mashing regime was employed: 40 min at 50 °C, 20 min at 62 °C, 20 min at 72 °C and 5 min at 78 °C. The mash was pumped into the lauter tun and lautering was performed, employing three sparging steps of 5 L each. Collected wort (1.039) was boiled for 45 min. Twenty-five grams of Magnum hop pellets (10.5% iso-α-acids) were added at the start of the boil for a calculated IBU content of 10.4. Hot trub precipitates and hop residue were removed in the whirlpool with a rest of 20 min. Wort was pumped back to the boiling vessel, corrected to a final gravity of 6.6 °P extract and heated to 100 °C before filling into sterile 5 L containers which were kept for short-term storage at 2 °C.

5.3.9 Fermentation

Fermentation trials were carried out in 2-litre sterile Duran glass bottles (Lennox Laboratory Supplies Ltd, Dublin, Ireland), equipped with an air lock. Bottles were filled

with 1600 mL of wort. Fermentation temperature was 25 °C. Fermentation was performed until no change in extract could be measured for two consecutive days. Yeast cells for pitching were washed by centrifugation at 4800 *g* for 5 min and resuspended in sterile water to ensure no carryover of sugars from the propagation wort into the fermentation wort. Pitching volume was 30 mL with a pitching rate of 8×10^6 cells/mL.

5.3.10 Beer analyses

Fifty milliliter samples of each fermentation were withdrawn every day. Before sampling, bottles were gently shaken to homogenize the yeast at the bottle base and in suspension. Cell count was performed using the Thoma Hemocytometer (Blaubrand). Yeast was separated by centrifugation at 5000 *g* for 5 min and specific gravity and ethanol content of the supernatant were measured using a density meter DMA 4500M with Alcolyzer Beer ME (Anton-Paar GmbH, Graz, Austria). The pH value was determined using a digital pH meter (Mettler Toledo LLC, Columbus OH, USA).

The cell-free supernatant of the final beers was analyzed using the following methods. Sugars and ethanol were determined by high performance liquid chromatography (HPLC) Agilent 1260 Infinity (Agilent Technologies, Santa Clara CA, USA) equipped a refractive index detector (RID) and a Sugar-Pak I 10 μm, 6.5 mm × 300 mm column (Waters, Milford MA, USA) with 0.1 mM Ca-EDTA as mobile phase and a flow rate of 0.5 mL/min at 80 °C. Differentiation of maltose and sucrose was achieved with a Nova-Pak 4 μm, 4.6 mm × 250 mm column (Waters, Milford MA, USA) with acetonitrile/water 75:25 (v/v) as mobile phase and a flow rate of 1.2 mL/min. Lactic acid was quantified via HPLC (Waters 2690 Separations Module, Waters, Milford MA, USA) with diode array detector (DAD) and a Hi-Plex H 8 μm, 7.7 mm × 300 mm column (Agilent Technologies, Santa Clara CA, USA) with 5 mM H_2SO_4 as mobile phase and a flow rate of 0.5 mL/min at 60 °C.

Free vicinal diketones were quantified by a Clarus 500 gas chromatograph (Perkin-Elmer, Waltham MA, USA) with a headspace unit and Elite-5 60 m × 0.25 mm, 0.5 μm column using a 2,3-hexandione internal standard. Fermentation by products (esters, higher alcohols) were quantified using a Clarus 580 (Perkin-Elmer, Waltham MA, USA) gas chromatograph with a headspace unit and INNOWAX cross-linked polyethylene-glycol 60 m × 0.32 mm 0.5 μm column (Perkin-Elmer, Waltham MA, USA). Vials containing beer samples were equilibrated for 25 min at 60 °C. The samples were injected at 50 °C,

rising to 85 °C after one minute by heating at 7 °C/min. A temperature of 85 °C was maintained for one minute and then elevated to 190 °C at a heating rate of 25 °C/min. Free amino acids content was quantified using the HPLC MEBAK 2.6.4.1 method. Free amino nitrogen (FAN) was measured using a ninhydrin-based dying method, where absorbance is measured at 570 nm against a glycine standard (MEBAK 2.6.4.1). Glycerol was determined via enzymatic assay kit (glucokinase method), following the recommended procedure (K-GCROLGK, Megazyme, Bray Co. Wicklow, Ireland).

5.3.11 Sensory evaluation

Beer samples were tasted and judged by a sensory panel of eleven experienced, DLG-certified (Deutsche Landwirtschafts-Gesellschaft e.V.) panelists. Attributes for the aroma were "fruity", "floral", and "wort-like". Attributes for the flavor were "acidic/sour", and "sweet". Panelists were asked to evaluate the attributes in its intensity on a scale from 0, "nothing", to 10, "extremely". Before the evaluation of the intensity, a descriptive sensory was performed, where the panelists were asked to record the flavors they perceived from the samples. Samples, at a temperature of 20 °C, were provided in dark glasses with a three-digit code.

5.3.12 Statistical analyses

Fermentations and analyses were carried out in triplicate, unless stated otherwise. Statistical analysis was performed using RStudio, Version 1.1.423 with R version 3.4.4 (RStudio Inc, Boston MA, USA; R Core Team, r-project). One-way ANOVA was used to compare means and Tukey's test with 95% confidence intervals was applied for the pairwise comparison of means. Values are given as the mean ± standard deviation.

5.4 Results

5.4.1 Yeast characterization

The results of the sugar utilization test are shown in Table 5.4–1.

Table 5.4–1 Normalized substrate utilization profile by BioLog YT plate test of the investigated yeasts.

Substrate	WLP001 *S. cerevisiae*	KBI 12.1 *L. fermentati*
α-D-Glucose	1.00	1.00
Maltose	0.98 ± 0.09	0.80 ± 0.02
Maltotriose	1.10 ± 0.28	–
Fructose [1]	+	+
Sucrose	1.28 ± 0.02	1.04 ± 0.11
D-Raffinose	0.46 ± 0.06	0.25 ± 0.00
D-Melibiose	–	–
D-Cellobiose	–	0.20 ± 0.01
Gentiobiose	–	0.25 ± 0.03
D-Melezitose	0.93 ± 0.20	0.61 ± 0.09
Palatinose	1.29 ± 0.01	0.77 ± 0.09
Stachyose	0.39 ± 0.06	0.25 ± 0.02
D-Trehalose	0.34 ± 0.10	0.97 ± 0.07
Turanose	1.12 ± 0.29	0.87 ± 0.00
D-Galactose	1.50 ± 0.07	0.67 ± 0.10
α-Methyl-D-Glucoside	0.57 ± 0.00	0.60 ± 0.04
β-Methyl-D-Glucoside	–	0.48 ± 0.10
Maltitol	–	0.80 ± 0.24
2-Keto-D-Gluconic Acid	–	0.18 ± 0.00

[1] Not included in MicroPlate; evaluated by HPLC sugar analysis; fructose was not detected in final beers.
Not listed substrates from the YT MicroPlate were negative.

In terms of wort sugars (maltose, maltotriose, glucose, sucrose, fructose), the substrate utilization test revealed that *L. fermentati* KBI 12.1 was unable to utilize maltotriose. All other wort mono- and disaccharides were utilized. Compared to *S. cerevisiae* WLP001, *L. fermentati* KBI 12.1 was also able to utilize cellobiose, gentiobiose, β-methyl glucoside, 2-keto-D-gluconic acid and maltitol in the substrate utilization test (Table 5.4–1). Melibiose utilization was negative for both strains. Table 5.4–2 summarizes the results of the yeast characterization.

Table 5.4–2 Results of yeast characterization: phenolic off-flavor (POF) performance, flocculation performance, cell count and viability after propagation of the investigated yeasts.

Attribute	WLP001 *S. cerevisiae*	KBI 12.1 *L. fermentati*
POF production	negative	negative
Flocculation (%)	83 ± 3	84 ± 4
Propagation cell count ($\times 10^6$ cells/mL) **	148 ± 9	483 ± 67
Propagation viability (%)	96.0 ± 3.2	99.8 ± 0.3

ANOVA significance codes: '***' $p \leq 0.001$, '**' $p \leq 0.01$, '*' $p \leq 0.05$

The phenolic off-flavor (POF) test revealed that no POF was produced during the plate tests. In terms of flocculation, both strains performed comparably, at 83% for *S. cerevisiae* WLP001 and 84% for *L. fermentati* KBI 12.1. The method defines flocculation values of 85–100% as "very flocculent", 20–80% as "moderately flocculent" and less than 20% as "non-flocculent" yeasts [26], classifying both strains in between "very flocculent" and "moderately flocculent". *S. cerevisiae* WLP001 is described by the supplier as "medium" flocculent.

Regarding hop sensitivity, iso-α-acids concentrations of 50 and 100 IBU led to small, but significant prolongations of lag times (Figure 5.4–1). However, the lag time for *L. fermentati* KBI 12.1 was around 12 hours which was shorter than the 18-hour lag time for the brewers' yeast *S. cerevisiae* WLP001 (Figure 5.4–1). A concentration of 100 IBU resulted in a lower growth compared to 0 and 50 IBU; however, though significant, differences were minor (Figure 5.4–1). For *S. cerevisiae* WLP001, the iso-α-acids concentration had no significant influence on growth. In terms of performance during propagation, *L. fermentati* KBI 12.1 reached a cell count of 4.8×10^8 cells/mL after 48 hours, outperforming *S. cerevisiae* WLP001 which only reached 1.5×10^8 cells/mL (Table 5.4–2). Cell viability of *L. fermentati* KBI 12.1 was higher than that of *S. cerevisiae* WLP001, at 99.8% and 96.0%, respectively.

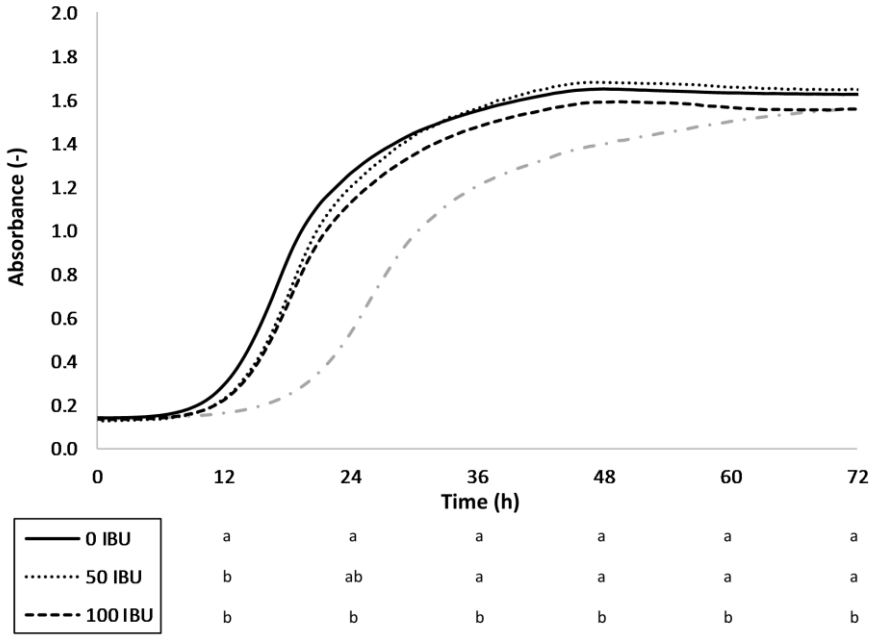


Figure 5.4–1 Hop sensitivity test of *Lachancea fermentati* stain KBI 12.1 grown in wort with 0, 50, and 100 IBU. Different letters under the x-Axis indicate a significant difference ($p \leq 0.05$). Grey dot-dash line shows growth of *S. cerevisiae* WLP001 at 0 IBU for comparison.

5.4.2 Fermentation performance

The composition of the fermentation wort is shown in Table 5.4–3.

Table 5.4–3 **C**omposition of fermentation wort.

Wort composition	Unit	Value
Extract	°P	6.63 ± 0.01
pH		5.73 ± 0.01
Maltose	g/L	26.60 ± 0.25
Maltotriose	g/L	5.09 ± 0.04
Glucose	g/L	5.46 ± 0.01
Sucrose	g/L	1.70 ± 0.04
Fructose	g/L	1.29 ± 0.02
Total amino acids	mg/100 mL	98.31 ± 0.86
Free amino nitrogen	mg/L	110 ± 5

During the fermentation, extract and pH reduction were monitored (Figure 5.4–2). Both strains showed a linear reduction in extract during the first 48 to 72 hours. However, *L. fermentati* KBI 12.1 was a slower fermenter compared to *S. cerevisiae* WLP001. While *L.*

fermentati KBI 12.1 reduced the extract by about 1.8 °P in the first 48 h, *S. cerevisiae* WLP001 reduced the extract by 3.5 °P, nearly double the amount, in the same time. Fermentation ceased for *S. cerevisiae* WLP001 after 5 days, with a final real extract of 2.13 °P. *L. fermentati* KBI 12.1 reached its final real extract of 2.92 °P after 7 days. Both strains produced a desired pH drop in the first 24 hours of fermentation. Values were 4.55 and 4.25 for *S. cerevisiae* WLP001 and *L. fermentati* KBI 12.1, respectively. *L. fermentati* KBI 12.1 reached a final pH value of 3.61 while *S. cerevisiae* WLP001 exhibited a final pH value of 4.18.

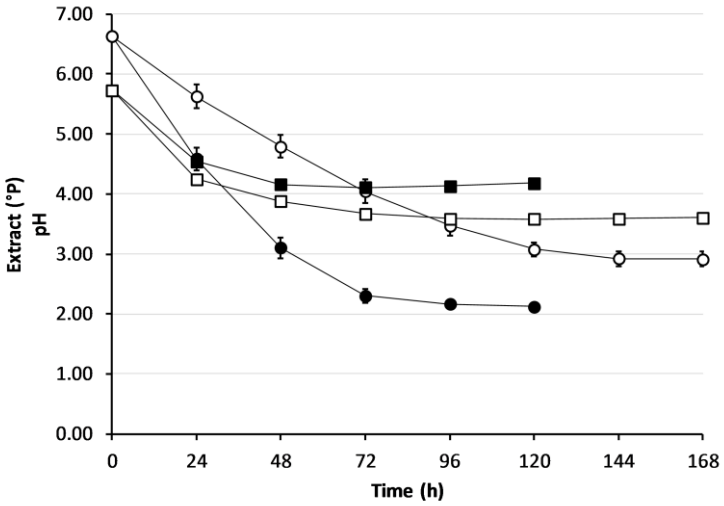


Figure 5.4–2 Drop in real extract for *S. cerevisiae* WLP001 (–●–) and *L. fermentati* KBI 12.1 (–○–), and pH drop for *S. cerevisiae* WLP001 (–■–) and *L. fermentati* KBI 12.1 (–□–) during fermentation.

S. cerevisiae WLP001 reached 4.7×10^7 cells/mL after the first 24 hours of fermentation and the numbers stayed relatively constant with minor fluctuations during the subsequent days of fermentation (Figure 5.4–3). *L. fermentati* KBI 12.1 reached a cell count of 6.6×10^7 cells/mL and numbers fluctuated between 5.1×10^7 cells/mL and 8.5×10^7 cells/mL during the remaining days of fermentation.

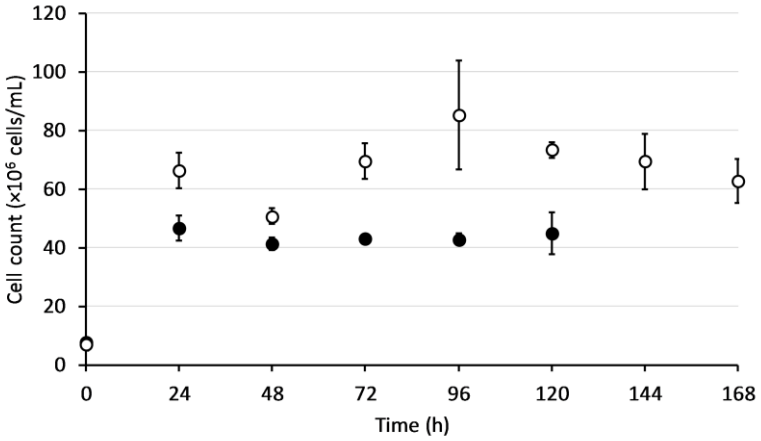


Figure 5.4–3 Cell count of *S. cerevisiae* WLP001 (●) and *L. fermentati* KBI 12.1 (○) during the course of fermentation.

The monosaccharides glucose and fructose were metabolized by *L. fermentati* KBI 12.1 in the first 24 hours (Figure 5.4–4). Maltose and sucrose concentrations gradually decreased to full depletion after 6 days of fermentation. Apart from minor fluctuations, maltotriose concentrations remained constant during the course of fermentation and remained unutilized by the yeast, as expected from the substrate utilization test. Facilitated by the low wort gravity, the final ethanol concentration after 7 days was 2.21% ABV. Lactic acid concentration also gradually increased to a maximum of 1.38 g/L after 6 days. Final lactic acid concentration was 1.30 g/L after 7 days of fermentation (Figure 5.4–4). Besides lactic acid, no other organic acids were detected.

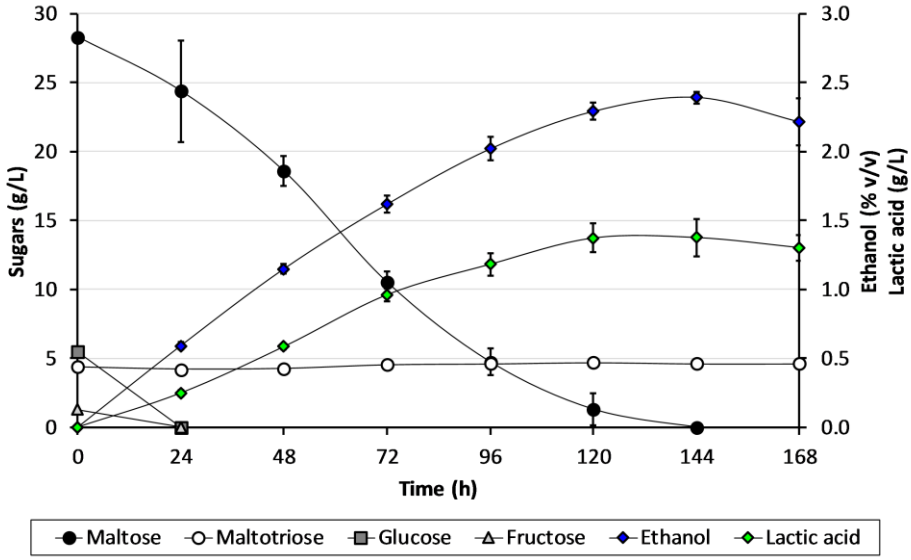


Figure 5.4–4 Sugar consumption and ethanol and lactic acid production by *L. fermentati* KBI 12.1 during fermentation.

Similar to *L. fermentati* KBI 12.1, the monosaccharides glucose and fructose were already depleted after 24 hours of fermentation with *S. cerevisiae* WLP001 (Figure 5.4–5).

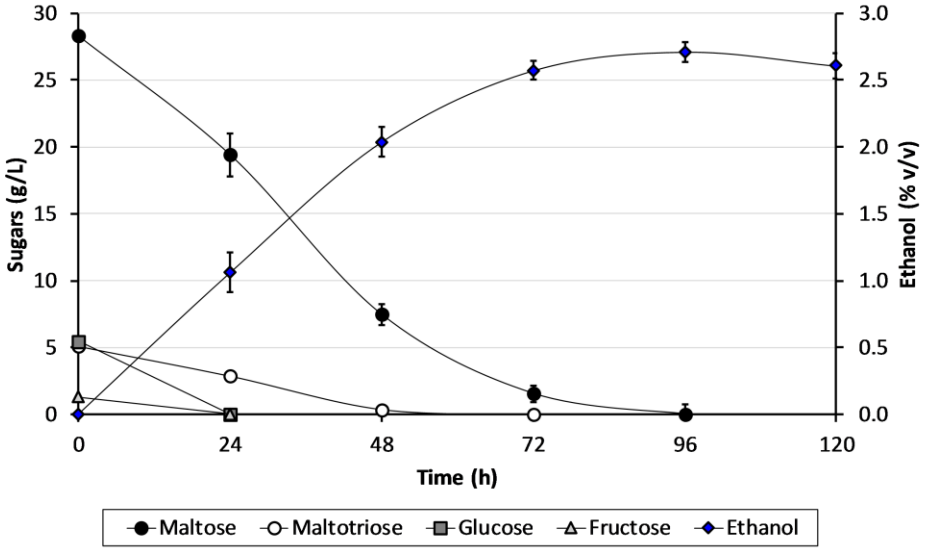


Figure 5.4–5 Sugar consumption and ethanol production by *S. cerevisiae* WLP001 during fermentation.

A more pronounced decrease in maltose and sucrose was observed compared to *L. fermentati* KBI 12.1. Full depletion of maltose was reached after 4 days of fermentation. Maltotriose was depleted after 3 days. Mirroring the faster decrease in fermentable sugars, ethanol concentrations increased rapidly and reached a final concentration of 2.61% ABV after 5 days of fermentation (Figure 5.4–5). In contrast to the fermentation with *L. fermentati* KBI 12.1, lactic acid was not detected at any time.

5.4.3 Nitrogen metabolism and glycerol

In terms of FAN consumption, the final beers fermented with *S. cerevisiae* WLP001 and *L. fermentati* KBI 12.1 contained 48 and 77 mg/L FAN, respectively (Table 5.4–4). Compared to the initial FAN value of the wort of 110 mg/L, the yeasts consumed 56% and 30% of the available FAN, respectively. Regarding amino acid consumption, *L. fermentati* KBI 12.1 only depleted methionine, while *S. cerevisiae* WLP001 depleted six amino acids, namely asparagine, glutamic acid, aspartic acid, leucine, isoleucine and methionine (Figure 5.4–6). A lower uptake of single amino acids by *L. fermentati* KBI 12.1 compared to *S. cerevisiae* WLP001 could be observed. The data suggests that glutamic acid was not assimilated by *L. fermentati* KBI 12.1. In total, *S. cerevisiae* WLP001 consumed 76% of the wort amino acids, while *L. fermentati* KBI 12.1 only consumed half that amount,

with a total of 38%. The glycerol values in the final beers fermented with *S. cerevisiae* WLP001 and *L. fermentati* KBI 12.1 were 0.98 and 1.41 g/L, respectively.

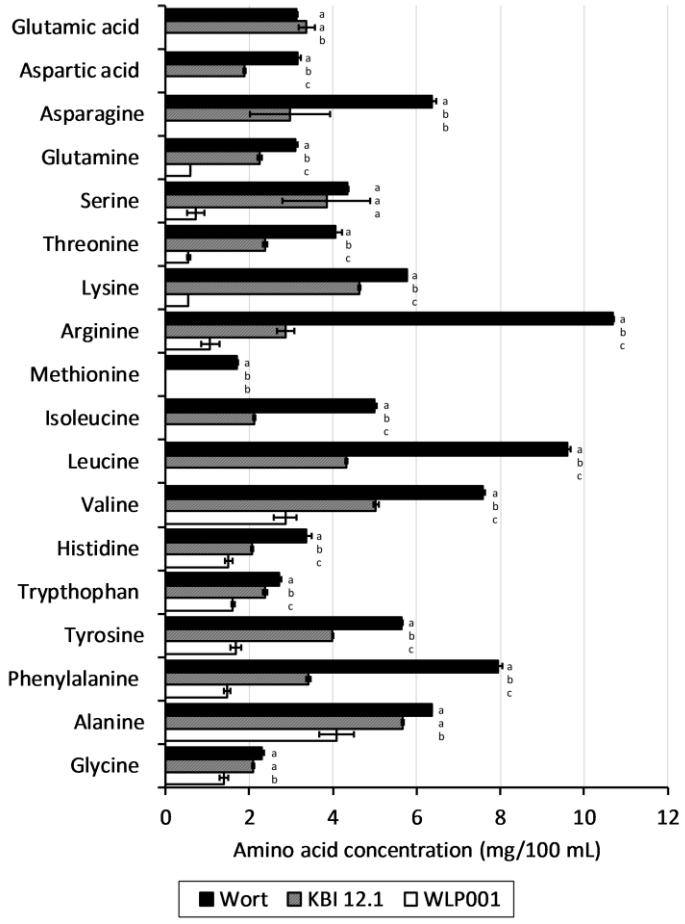


Figure 5.4–6 Amino acid concentration in wort and final beer fermented with *L. fermentati* KBI 12.1; and in the final beer fermented with WLP001. Different letters next to the bars indicate a significant difference ($p \leq 0.05$).

5.4.4 Volatile compounds

The final beers were analyzed for fermentation by-products (Table 5.4–4). In terms of ester production, ethyl acetate concentrations were significantly higher for *L. fermentati* KBI 12.1, at 12.80 mg/L, compared to 4.05 mg/L for *S. cerevisiae* WLP001. Isoamyl acetate values were low for both strains, at 0.20 mg/L for *S. cerevisiae* WLP001 and 0.35 mg/L for *L. fermentati* KBI 12.1. In summary, *L. fermentati* KBI 12.1 produced a threefold amount of esters. Regarding higher alcohols, *S. cerevisiae* WLP001 produced significantly higher amounts of isobutanol and isoamyl-alcohols, at 17.9 and 50.8 mg/L, respectively, compared to *L. fermentati* KBI 12.1 with 12.3 and 34.2 mg/L, respectively. *L. fermentati* KBI 12.1 produced higher amounts of n-propanol compared to *S. cerevisiae*

WLP001; however, this was not statistically significant. Diacetyl values for both strains were approx. 0.04 mg/L, which is below the flavor threshold of 0.1 mg/L [29]. Acetaldehyde concentrations were, at 7.8 and 11.1 mg/L for *S. cerevisiae* WLP001 and *L. fermentati* KBI 12.1 respectively, below and within the lower end of its flavor threshold in beer of 10–25 mg/L [29]. Ethyl formate values were low, at 1.05 and 0.89 mg/L, respectively. Ethyl propionate, ethyl butyrate and ethyl caproate values were also determined but were below the limit of detection of 0.1 mg/L (data not shown).

Table 5.4–4 Analysis of fermentation by-products of final beers.

Analysis of final beer	**WLP001** ***S. cerevisiae***	**KBI 12.1** ***L. fermentati***
Ethanol (% ABV) *	2.61 ± 0.10	2.21 ± 0.17
Final real extract (°P) ***	2.13 ± 0.02	2.92 ± 0.12
pH ***	4.18 ± 0.02	3.61 ± 0.05
FAN (mg/L) ***	48 ± 3	77 ± 2
Glycerol (g/L) ***	0.98 ± 0.03	1.41 ± 0.07
n-Propanol (mg/L)	13.7 ± 3.1	18.5 ± 1.0
Isobutanol (mg/L) *	17.9 ± 1.8	12.3 ± 0.2
Isoamyl alcohols (mg/L) *	50.8 ± 3.0	34.2 ± 0.7
Σ Higher alcohols (mg/L)	82.4 ± 7.9	65.0 ± 0.5
Ethyl acetate (mg/L) *	4.05 ± 0.21	12.80 ± 1.41
Isoamyl acetate (mg/L)	0.20 ± 0.00	0.35 ± 0.07
Σ Esters (mg/L) *	4.25 ± 0.21	13.15 ± 1.48
Diacetyl, total (mg/L)	0.04 ± 0.01	0.04 ± 0.00
Ethyl formate (mg/L)	1.05 ± 0.07	0.89 ± 0.44
Acetaldehyde (mg/L)	7.8 ± 0.4	11.1 ± 3.0

ANOVA significance codes: '***' $p \leq 0.001$, '**' $p \leq 0.01$, '*' $p \leq 0.05$.

5.4.5 Sensory

The results of the sensory analysis are shown in Figure 5.4–7. The beer produced with *L. fermentati* KBI 12.1 was noted to have a fruitier, less wort-like, and more floral aroma compared to the beer produced with *S. cerevisiae* WLP001. However, the intensity of those attributes was generally low, and differences were not statistically significant. In terms of the flavor, the *L. fermentati* KBI 12.1 beer was evaluated as significantly more acidic/sour than that produced with *S. cerevisiae* WLP001 ($p < 0.001$). Consequently, it was also perceived as significantly less sweet ($p < 0.01$). In the descriptive part of the sensory, the panelists described the aroma of the beer from *L. fermentati* KBI 12.1 as fruity, wine-like,

citrus-like, shandy-like and apple-like. The aroma of the *S. cerevisiae* WLP001 beer was described as clean and malty.

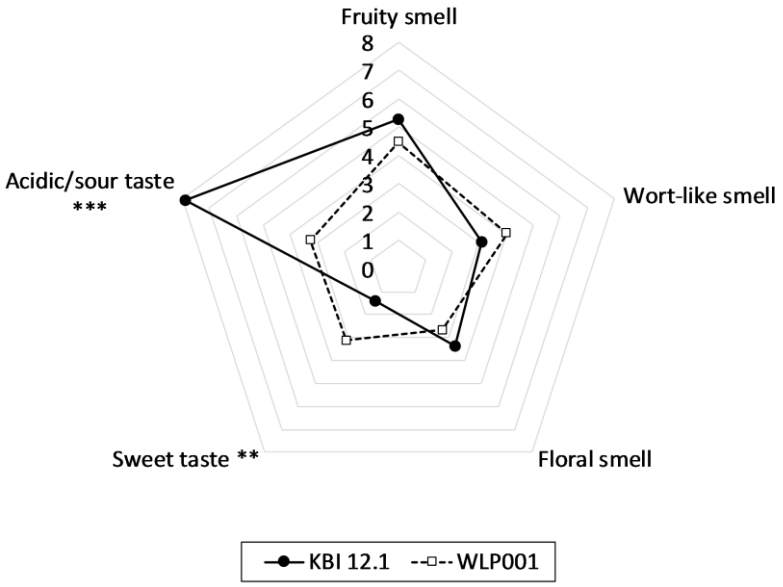


Figure 5.4–7 Spider web diagram of the means of the descriptors from the sensory of the final beers. ANOVA significance codes: '***' $p \leq 0.001$, '**' $p \leq 0.01$.

5.5 Discussion

5.5.1 Yeast characterization

The inability to utilize maltotriose is not an uncommon feature in the *Lachancea* genus. In a study by Domizio et al. [17] from 2016, three investigated *Lachancea thermotolerans* strains were unable to utilize maltotriose. In the well-studied species *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*, the ability to utilize maltose is associated with the presence of permeases that transport the sugars through the cell membrane, and intracellular maltases, that hydrolyze the sugars. While the maltases are capable of hydrolyzing both maltose and maltotriose, several studies indicate, that maltose and maltotriose are transported by different permeases [30,31], therefore suggesting the absence of a maltotriose permease in *L. fermentati* KBI 12.1. Regarding POF, *L. fermentati* KBI 12.1 did not produce any off-flavors, similar to the brewers' yeast *S. cerevisiae* WLP001. Except for some German wheat beers ("Hefeweizen") and some Belgian and specialty beers, the often described as "clove-like" off-flavors are undesirable in most beer styles. Yeast flocculation is a trait desired by brewers for most beer styles and enables easy and efficient collection of the spent yeast after fermentation and maturation of the beer. The similar flocculation behavior of *L. fermentati* KBI 12.1 and the brewers' yeast in the Helm's assay and as observed during the fermentation trials underlines its suitability for brewing applications. A comparable flocculation performance between a *L. fermentati* strain and *S. cerevisiae* WLP001 was also reported by Osburn et al. [19]. In contrast, a *Lachancea thermotolerans* strain, investigated by Domizio et al. [17] for its suitability in brewing applications, was classified as non-flocculent. Hop-derived iso-α-acids had very little impact on *L. fermentati* KBI 12.1, which makes it a suitable yeast for fermenting even highly hopped worts of specialty beers like India Pale Ales (IPA). The reported shorter lag time for *L. fermentati* KBI 12.1 compared to *S. cerevisiae* WLP001 (Figure 5.4–1) was in accordance with the study by Osburn et al. [19], where a *L. fermentati* strain exhibited half the lag time compared to *S. cerevisiae* WLP001. Another important trait is the performance of the yeast during propagation. *L. fermentati* KBI 12.1 produced three times more cells compared to *S. cerevisiae* WLP001, with high viability, again emphasizing its suitability for practical brewing applications (i.e. bottom cropping). Altogether, the yeast characterization indicated the general suitability of *L. fermentati* KBI 12.1 for brewing applications.

5.5.2 Fermentation performance

As a result of the maltotriose gap, the yeasts' inability to ferment maltotriose [32], the final ethanol concentration of the wort fermented with *L. fermentati* KBI 12.1 was lower compared to that of *S. cerevisiae* WLP001, at 2.21% and 2.61% ABV respectively. The low ethanol values were also facilitated by the low wort gravity. Although exhibiting higher cell counts throughout the entire fermentation period, *L. fermentati* KBI 12.1 showed a slower fermentation compared to *S. cerevisiae* WLP001. However, non-*Saccharomyces* species commonly have smaller cells compared to brewers' yeast and thus require significantly higher cell counts to achieve comparable fermentation performances [33]. The fluctuations in the reported total cell count during fermentation, sometimes with high standard deviation, could be attributed to flocculation of the yeast already during fermentation when cell aggregations were visible under the microscope. Premature yeast flocculation can lead to economic losses and undesired changes in beer flavor. However, the usual consequences of premature flocculation, such as a high amount of residual sugars or high diacetyl values were not observed [34]. The lower pH and more extensive pH drop by *L. fermentati* KBI 12.1 could be attributed to the production of lactic acid. The final pH was lower and the range of the pH drop was higher in the present study (5.73 to 3.61) than that in comparable studies (5.35 to 3.74 [19]; 5.66 to 3.77 [17]; 5.47 to 3.88 [18]) which could be attributed to higher lactic acid production and lower amount of buffering substances (i.e. FAN, minerals [35]) in the diluted wort (6.6 °P). Lactic acid production by yeasts is an uncommon metabolic feature and an underexplored trait of the *Lachancea* genus [8,36]. As part of the yeast metabolism, lactic acid is formed from pyruvate, the end product of glycolysis (Figure 5.2–1). The reaction is catalyzed by the enzyme lactate dehydrogenase. This pathway is an alternative means of NADH oxidation to NAD^+, the more common pathway in yeast being via the production of ethanol, catalyzed by pyruvate decarboxylase (Figure 5.2–1) [37]. To date, four studies have reported the production of lactic acid by *Lachancea* yeasts in wort fermentations. Domizio et al. [17] reported a maximum lactic acid concentration of 0.24 g/L produced by a *L. thermotolerans* strain after 10 days of fermentation at 14 °C in a 13.5 °P all-malt wort. In a study by Sheppard et al. (2016) [16,38], a *L. thermotolerans* strain produced 7.3 g/L lactic acid after 25 days of fermentation at 18 °C in a 14 °P Lambic-style wort. Osburn et al. [19] reported a final lactic acid concentration of 0.90 g/L by a strain of *L. fermentati* after one-month fermentation at 21.7 °C (71 °F) of a 11.4 °P wort. Canonico et al. [18] reported a lactic acid concentration of 1.83 g/L by a *L. thermotolerans* strain after 11 days of fermentation

at 19 °C in a 12.3 °P wort. Considering that the present study used a diluted wort of 6.6 °P, the final lactic acid value achieved, at 1.3 g/L, is remarkable. However, the difference in value from previous studies can be attributed to varying fermentation conditions and strain-specific differences [17–19]. For comparison, lactic acid concentrations in commercial sour beers can range between 2 and 9 g/L [39]. Due to the inability of *L. fermentati* KBI 12.1 to ferment maltotriose, the final extract was higher and final ethanol concentrations were correspondingly lower compared to *S. cerevisiae* WLP001. The ability of *Lachancea* to consume maltotriose is not clearly defined at genus or species level. Sheppard et al. [16] investigated two *Lachancea thermotolerans* strains which were able to ferment maltotriose. In contrast, three *Lachancea thermotolerans* strains investigated by Domizio et al. [17] were not able to ferment maltotriose and, therefore, produced less ethanol. The maltotriose content of a wort can be influenced by the mashing regime. Glucose and maltotriose are products of α-amylase activity, while maltose is mostly a product of β-amylase activity [40]. Changes in the mashing procedure with respect to the temperature rests can alter the carbohydrate composition of wort accordingly. Higher α-amylase activity and lower β-amylase activity could potentially lead to a lower amount of fermentable extract, and, in the case of *L. fermentati* KBI 12.1, thus to even lower ethanol values.

Wort FAN and amino acids are important for yeast growth and the production of secondary metabolites [41,42]. A lack of nitrogenous compounds can negatively affect fermentation performance [43]. Interestingly, *L. fermentati* KBI 12.1 only depleted one amino acid (methionine) from the diluted 6.6 °P wort and consumed only half the total amount of amino acids and about half the available FAN compared to the brewers' yeast *S. cerevisiae* WLP001 (Figure 5.4–6). Previous studies have already suggested that non-*Saccharomyces* yeasts may be less demanding concerning amino acids, compared to *Saccharomyces* yeasts [44,45]. A study by Bellut et al. [45] found that six non-*Saccharomyces* species only consumed between 11–27% of the available amino acids. However, fermentation with those species was less extensive given their inability to consume maltose and final ethanol values were reported as low. Estela-Escalante et al. (2016) [44] found that a strain of *Candida zemplinina* consumed a far lower amount of FAN, compared to a *Saccharomyces cerevisiae* brewers' yeast (S-23), corresponding with the findings in the present study. The data indicated that the non-*Saccharomyces* yeast, *Lachancea fermentati* KBI 12.1, requires a lower concentration of FAN and free amino acids. Therefore, the yeast strain is well suited for fermentation of diluted worts of 6.6 °P and potentially even

lower extract values. Concerning *S. cerevisiae* WLP001, although depleting six amino acids and consuming a considerably larger amount of FAN, no negative impact on the fermentation performance or the taste of the final beer were observed.

5.5.3 Fermentation by-products & sensory

Glycerol is produced and accumulated by yeast cells as a by-product of the sugar metabolism, but it is also produced for its protective properties against hyperosmotic stress [46,47]. In beer, glycerol can potentially contribute positively to the mouthfeel and body and is usually found at concentrations between 1 and 3 g/L [48]. Glycerol production by *L. fermentati* KBI 12.1 was, at 1.41 g/L, 44% higher than that of *S. cerevisiae* WLP001 (0.98 g/L). These findings are consistent with the findings by Domizio et al. [17], where three *L. thermotolerans* strains produced around 1.4 g/L, while a *S. cerevisiae* brewers' yeast only produced around 0.8 g/L. However, glycerol production was found to be influenced by the original wort gravity. *L. thermotolerans* strains, in the study by Sheppard et al. [16], produced between 1.5 and 2.9 g/L glycerol depending on the original gravity of the wort, with higher production at higher original gravity values. Glycerol production by *L. fermentati* in wort has not been described in literature prior to this study.

Ester concentrations in the beer produced by *L. fermentati* KBI 12.1 were significantly higher than in the beer produced with *S. cerevisiae* WLP001. This finding is consistent with the results from Canonico et al. [18], who found similar ester levels in a beer produced by a *L. thermotolerans* strain which also was higher in comparison to a brewers' yeast strain. Meilgaard et al. [29] reported the flavor thresholds of ethyl acetate and isoamyl acetate in beer to be 33 and 1.2 mg/L, respectively, neither of which was reached in either beer. However, esters can have synergistic effects and thus can have an influence on the flavor, even below their individual flavor thresholds [49,50]. The minimal reported flavor threshold for diacetyl, known for its butter- or butterscotch-like flavor, is 0.1 mg/L [51]. Aside from Bohemian Pilsners and some English ales, diacetyl is undesirable at concentrations above the flavor threshold [51]. Final beers produced with *L. fermentati* KBI 12.1 had values below the threshold and diacetyl was not detected during the sensory evaluation, which highlights the suitability of *L. fermentati* KBI 12.1 for beer brewing. Acetaldehyde has a flavor threshold in beer of 10–25 mg/L [29]. Values above the threshold can result in green apple-like, solvent-like off-flavors. Although many tasters

can detect acetaldehyde at low levels, it was not picked up for the beer produced with *L. fermentati* KBI 12.1 which had a concentration of 11.1 mg/L [52].

The perceived fruitier aroma of the *L. fermentati* KBI 12.1 beer during the sensory evaluation could be attributed to the significantly higher ester concentrations, although the fruitier aroma did not exhibit statistical significance in the sensory. However, in the descriptive part of the sensory, the *L. fermentati* KBI 12.1 beer was associated to words describing a fruity character (fruity, apple, citrus), while the *S. cerevisiae* WLP001 beer was described as clean.

The lactic acid production by *L. fermentati* KBI 12.1 led to a strong acidic/sour taste of the beer. The flavor threshold of lactic acid in beer is reported to be around 80 mg/L, which is far below the measured value of 1.3 g/L [53]. Consequently, *L. fermentati* KBI 12.1 was perceived as less sweet compared to the control beer produced with *S. cerevisiae* WLP001. The residual maltotriose in the *L. fermentati* KBI 12.1 beer seemingly had no impact on that perception. However, the sweetening power of maltotriose is, at around a quarter of that of sucrose, very low [54].

5.6 Conclusion

The yeast characterization with respect to flocculation, hop sensitivity, POF and yeast propagation confirmed the suitability of *Lachancea fermentati* KBI 12.1 for brewing purposes: the strain showed flocculation characteristics comparable to brewers' yeast, only marginal hop sensitivity at high IBU values, no phenolic off-flavors, and a good performance during propagation. While the utilization of maltotriose is a desirable characteristic in brewing in terms of minimal extract losses, and a most efficient brewing process, the inability to ferment maltotriose can be a useful trait in low alcohol beer brewing. Maltotriose is the second most abundant wort sugar [55]. The use of *L. fermentati* KBI 12.1, unable to ferment maltotriose, could be combined with high temperature mashing to further decrease fermentability of the worts and thus decrease final ethanol content [40,56].

During the fermentation trials, *L. fermentati* KBI 12.1 was found to quickly ferment the wort, with only slight delay compared to the brewers' yeast. During the fermentation, the strain produced significant amounts of lactic acid which is a trait that could be harnessed for the production of non-alcoholic and low alcohol beers. Stopping the fermentation based on a certain residual extract content and/or lactic acid content could introduce a way to produce lower alcohol beers with *L. fermentati* KBI 12.1. The acidity from the lactic acid could counteract the often-criticized sweetness from the residual wort sugars [57,58]. In fact, a certain ratio of sugars to acids ('brix/acid ratio') is desired during the production of juice blends and beverages [59]. A ratio of roughly 10–15 is intended. Above those values, the beverage tends to be too sweet, below those values, the beverage tends to be too sour [59]. In the present study, the right ratio would have been reached between 48 and 72 hours of fermentation (1.15–1.62% ABV). Sheppard et al. [16] reported, that a *Lachancea thermotolerans* strain produced significant amounts of lactic acid (2.4 g/L) while producing little ethanol (0.2% ABV) at the very beginning of fermentation (day two) in a 14 °P wort fermented at 18 °C. Further trials with *L. fermentati* KBI 12.1 should investigate the temperature- and extract-dependency of the lactic acid production. With a similar fermentation performance to the study of Sheppard et al. [16], significant lactic acid concentrations for an optimal brix/acid ratio could be reached by *L. fermentati* KBI 12.1, without reaching high alcohol concentrations. However, the right ratio is dependent on the beverage matrix and the types of sugars and acids present. In addition, the low pH due to the lactic acid production, means that the requirement for additional acidification,

desired in non-alcoholic beers to ensure microbial stability and to impart a liveliness, is unnecessary.

Another positive trait of *L. fermentati* KBI 12.1 is its higher glycerol production compared to a brewers' yeast. The lack of mouthfeel and body are often criticized characteristics of non-alcoholic and low alcohol beers, hence the application of a yeast with increased glycerol production could potentially moderate those flavor defects [57]. However, the flavor threshold of glycerol in beer is reported to be approximately 10 g/L [48].

In addition, no high concentrations of undesirable fermentation by-products (i.e. diacetyl, acetaldehyde) were detected during analysis or during sensory evaluation, and a trained panel gave the *L. fermentati* KBI 12.1 beer fruity attributes.

Regarding safety, *L. fermentati* KBI 12.1 was isolated from a food source (kombucha) and the species *Lachancea fermentati* is listed in the 2012 IDF/EFFCA "Inventory of Microbial Species with technological beneficial role in fermented food products" [60,61], due to its history of use in wine fermentations [62].

To conclude, *Lachancea fermentati* KBI 12.1 was found to be a suitable yeast for beer production, with promising traits and potential with regards to non-alcoholic and low alcohol beer brewing.

5.7 Acknowledgements

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Chapter 6:

Lachancea fermentati strains isolated from kombucha: fundamental insights, and practical application in low alcohol beer brewing

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6.1 Abstract

With a growing interest in non-alcoholic and low alcohol beer (NABLAB), researchers are looking into non-conventional yeasts to harness their special metabolic traits for their production. One of the investigated species is *Lachancea fermentati*, which possesses the uncommon ability to produce significant amounts of lactic acid during alcoholic fermentation, resulting in the accumulation of lactic acid while exhibiting reduced ethanol production. In this study, four *Lachancea fermentati* strains isolated from individual kombucha cultures were investigated. Whole genome sequencing was performed, and the strains were characterized for important brewing characteristics (e.g., sugar utilization) and sensitivities towards stress factors. A screening in wort extract was performed to elucidate strain-dependent differences, followed by fermentation optimization to enhance lactic acid production. Finally, a low alcohol beer was produced at 60 L pilot-scale. The genomes of the kombucha isolates were diverse and could be separated into two phylogenetic groups, which were related to their geographical origin. Compared to a *Saccharomyces cerevisiae* brewers' yeast, the strains' sensitivities to alcohol and acidic conditions were low, while their sensitivities towards osmotic stress were higher. In the screening, lactic acid production showed significant, strain-dependent differences. Fermentation optimization by means of response surface methodology (RSM) revealed an increased lactic acid production at a low pitching rate, high fermentation temperature, and high extract content. It was shown that a high initial glucose concentration led to the highest lactic acid production (max. 18.0 mM). The data indicated that simultaneous lactic acid production and ethanol production occurred as long as glucose was present. When glucose was depleted and/or lactic acid concentrations were high, the production shifted towards the ethanol pathway as the sole pathway. A low alcohol beer (< 1.3% ABV) was produced at 60 L pilot-scale by means of stopped fermentation. The beer exhibited a balanced ratio of sweetness from residual sugars and acidity from the lactic acid produced (13.6 mM). However, due to the stopped fermentation, high levels of diacetyl were present, which could necessitate further process intervention to reduce concentrations to acceptable levels.

6.2 Introduction

Humans have utilized yeasts for the preparation of their foods and beverages long before they even knew of their existence, and beer brewing has been a human activity ever since the Neolithic period [1]. But it was not until the introduction of brewing with pure culture yeast by Emil Christian Hansen that brewers started to consciously select yeasts for specific purposes [1]. The species *Saccharomyces cerevisiae* especially, has been harnessed as a trustworthy workhorse in the production of beer, and production volumes have been growing to almost two billion hectoliters in 2018 [2].

However, emerging lifestyle trends, demographics and stricter legislation have led to a slowdown in beer volume growth over the past years, while the non-alcoholic and low alcohol beer (NABLAB) sector has seen a strong and steady growth, which is forecast to continue [3]. There are two fundamentally different approaches when it comes to NABLAB production: physical dealcoholization by means of thermal or membrane methods to remove the ethanol after its formation [4], and biological methods like stopped fermentation to limit ethanol production in the first place [5].

Another old, biological method for NABLAB production has seen a revival in recent years: the application of non-*Saccharomyces* yeasts (also called non-conventional yeasts) with limited ability to ferment wort sugars, resulting in a low ethanol production. This method was already mentioned in 1929 [6], and the proposed species, *Saccharomycodes ludwigii*, has been investigated thoroughly [7–16]. However recently, research into other non-*Saccharomyces* species to produce NABLAB has gained momentum [3]. Researchers have been looking into isolating yeasts from non-cereal environments, to take advantage of their inability to consume the most abundant wort sugars maltose and maltotriose. Such environments include, for example, grapes and wine [13,17], honey [9], glaciers in Italy and the Antarctica [14,18], Japanese miso [11,19] and, more recently, kombucha [15,20].

To date, more than 27 yeast genera have been found in kombucha cultures with up to 25 different species inhabiting a single culture [21–24]. One of the yeast genera associated with kombucha fermentation is the *Lachancea* genus, among which, *Lachancea fermentati* was first recorded by Marsh et al. [22] and has since been reported to be the most abundant *Lachancea* species in kombucha [21]. *L. fermentati* has mostly been associated with grape must and kefir [25] but the species was recently proposed as a novel brewing species to

create sour beer or low alcohol beer [20,26]. The proposed applications are motivated by the fact that strains of the genus possess the uncommon ability to produce significant amounts of lactic acid during alcoholic fermentation. The production of high amounts of lactic acid by yeasts is an underexplored trait of both the *Lachancea* and *Saccharomyces* genus [27–29]. Lactic acid production is facilitated by the enzyme lactic acid dehydrogenase (LDH), which catalyzes the formation of lactic acid from pyruvate, the product of glycolysis. From a metabolic view-point, this pathway is an alternative, simultaneous means of NADH recycling to NAD^+, with the more common pathway in yeast being via the production of ethanol [29].

Lactic acid production in *Lachancea fermentati* has received little attention, but has been associated with *Lachancea thermotolerans*, where it has been shown to be highly strain-dependent [25]. Osburn et al. [26] proposed the use of lactic acid-producing species like *Lachancea fermentati* to produce single-culture sour beer, making the use of lactic acid bacteria for souring redundant. Bellut et al. [20] proposed the use of *Lachancea fermentati* to produce low alcohol beer by stopping fermentation of a diluted wort and exploiting its lactic acid production to counteract residual wort sweetness.

In this study, we investigated four *Lachancea fermentati* strains isolated from four individual kombucha cultures. To better understand the variation in these four strains, whole-genome sequencing of the isolates and the CBS 707 type strain was performed. The strains were characterized for important brewing characteristics like sugar consumption, flocculation behavior, and susceptibility to stress factors like ethanol, low pH and high osmotic pressure. A screening in wort fermentations was performed to show differences in lactic acid production, sugar consumption and the production of volatile fermentation by-products. Further investigation involved an assessment of fermentation conditions and their impact on lactic acid production. Fermentation parameters studied were wort extract, fermentation temperature, and pitching rate, and results were evaluated via response surface methodology (RSM). Alterations of the sugar profile was investigated as another tool to enhance lactic acid production in wort. Finally, a low alcohol beer (< 1.3% ABV) was produced by stopped fermentation at 60 L pilot scale and sensory evaluation was conducted with a trained panel.

6.3 Materials and Methods

6.3.1 Yeast strains

The *Lachancea fermentati* strains KBI 1.2, KBI 3.2, KBI 5.3, and KBI 12.1 (Table 6.4–1) were isolated from four individual kombucha cultures according to Bellut et al. [21]. CBS 707, the *Lachancea fermentati* type strain, was sourced from the CBS collection (Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands). The brewers' yeast WLP001 (California Ale Yeast) was sourced from White Labs (San Diego CA, USA).

6.3.2 Genomics

6.3.2.1 DNA content by flow cytometry

Flow cytometry was used to estimate the ploidy of the yeast strains essentially as described by Haase and Reed [30]. Cells were grown overnight in YPD medium (1% yeast extract, 2% peptone, 2% glucose), and approximately 1×10^7 cells were washed with 1 mL of 50 mM citrate buffer. Cells were fixed with ice-cold 70% ethanol, and incubated overnight at -20 °C. Cells were then washed with 50 mM citrate buffer (pH 7.2), resuspended in 50 mM citrate buffer containing 0.25 mg/mL RNAse A and incubated overnight at 37 °C. Proteinase K was then added to a concentration of 1 mg/mL, and cells were incubated for 1 hour at 50 °C. Cells were then stained with SYTOX Green (2 μM; Life Technologies, USA), and their DNA content was determined using a FACSAria IIu cytometer (Becton Dickinson, USA). DNA contents were estimated by comparing fluorescence intensities. In addition to the *L. fermentati* strains, analysis was also performed on *S. cerevisiae* haploid (CEN.PK113-1A) and diploid (CEN.PK) reference strains. Measurements were performed on duplicate independent yeast cultures, and 100,000 events were collected per sample during flow cytometry.

6.3.2.2 DNA extraction and sequencing

DNA was extracted from pellets using the Sigma GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis MO, USA). After DNA isolation, DNA was quantified using the Qubit High Sensitivity DNA assay (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and shotgun metagenomic libraries were prepared using the Nextera

XT library preparation kit (Illumina, San Diego CA, USA) as described by the manufacturer. The final libraries were sequenced on an Illumina NextSeq using a 300 cycle V2 Mid-Output kit as per Illumina guidelines. The raw sequencing reads generated in this study have been submitted to NCBI-SRA under BioProject number PRJNA587400 in the NCBI BioProject database.

6.3.2.3 Bioinformatics

The 150 bp paired-end reads were quality-analyzed with FastQC [31] and trimmed and filtered with Trimmomatic [32]. Reads were aligned to a reference genome of *L. fermentati* CBS 6772 (NCBI Accession GCA_900074765.1) using SpeedSeq [33]. Variant analysis was performed on aligned reads using FreeBayes [34]. Prior to variant analysis, alignments were filtered to a minimum MAPQ of 50 with SAMtools [35]. The median coverage over 1,000 bp windows was calculated with mosdepth [36] and visualized in R.

In addition, to test if any of the strains were interspecies hybrids, the trimmed reads were also aligned to a concatenated reference genome consisting of the assembled genomes of the twelve *Lachancea* species available at GRYC[1]. The median coverage over 1,000 bp windows was again calculated with mosdepth and was visualized in R using modified scripts from sppIDer [37].

For phylogenetic analysis, consensus genotypes of the *L. fermentati* strains were called from the identified variants using BCFtools [38]. A genome assembly of *L. kluyveri* CBS 3082 was retrieved from GRYC[1]. Multiple sequence alignment of the consensus genotypes and genome assemblies was performed with the NASP pipeline [39] using *L. fermentati* CBS 6772 as the reference genome. A matrix of single nucleotide polymorphisms (SNP) in the 7 strains was extracted from the aligned sequences. The SNPs were filtered so that only sites that were present in all 7 strains and with a minor allele frequency greater than 15% (one strain) were retained. The filtered matrix contained 11,517 SNPs at 6,330 sites. A maximum likelihood phylogenetic tree was estimated using IQ-TREE [40]. IQ-TREE was run using the 'GTR+ASC' model and 1,000 ultrafast bootstrap replicates [41]. The resulting maximum likelihood tree was visualized in FigTree and rooted with *L. kluyveri* CBS 3082. Haplotype phasing was attempted using WhatsHap (0.14.1) [42], and by

[1] http://gryc.inra.fr

dividing haplotypes based on similarity to the reference genome as described by Ortiz-Merino et al. [43].

6.3.3 Strain characterization

6.3.3.1 API sugar utilization test

Substrate utilization test API ID 32C (BioMérieux, Marcy-l'Étoile, France) was used to analyze the biochemical spectrum of all *Lachancea fermentati* strains. Preparation of inoculum and inoculation of the strips was performed according to the manufacturers' instructions. Colonies for the inoculum were grown on YPD agar plates for 48 h at 27 °C. After inoculation, API ID 32C strips were incubated for 2 days at 28 °C. The samples were evaluated visually by turbidity of the wells, differentiating positive (+), negative (-), and weak (w) growth.

6.3.3.2 Scanning Electron Microscopy

Yeast cultures for scanning electron microscopy (SEM) were prepared following the protocol for cultured microorganisms by Das Murtey and Ramasamy [44]. Single colonies were taken from YPD agar plate and grown in YPD broth for 24 h at 25 °C. One milliliter of sample was centrifuged at 900 *g* for 2 min for pellet formation and resuspended in 5% glutaraldehyde solution prepared in 0.1 M phosphate buffer (pH 7.2) for fixation. After 30 min, the sample was centrifuged, the supernatant was discarded, and the pellet was washed twice in 0.1 M phosphate buffer. Consequently, the pellet was resuspended in 1% osmium tetroxide prepared in 0.1 M phosphate buffer. After 1 h, cells were again washed twice in 0.1 M phosphate buffer. The sample was then dehydrated through ethanol series of 35%, 50%, 75%, 95%, absolute ethanol, and hexamethyldisilazane (HDMS) for 30 minutes per step (last two ethanol steps twice), centrifuging and discarding the supernatant for each change. Lastly, the second HDMS was discarded and the sample left drying overnight in a desiccator.

The dehydrated yeast sample was mounted onto plain aluminum stubs using carbon double surface adhesive and coated with a 5 nm gold-palladium (80:20) layer using a Gold Sputter Coater (BIO-RAD Polaron Division, SEM coating system, England) and

observed under a constant accelerating voltage of 5 kV under a JEOL scanning electron microscope type 5510 (JEOL, Tokyo, Japan).

6.3.3.3 Antifungal susceptibility test

Antifungal susceptibility was investigated using an agar-based method where a strip of inert material impregnated with a predefined concentration gradient of a single antifungal agent is used to directly quantify antifungal susceptibility in terms of an MIC (minimal inhibitory concentration) value, which corresponds to the growth inhibition in an elliptical zone. Antifungals tested were Amphotericin B, Fluconazole, Itraconazole, Voriconazole, Caspofungin and Flucytosine, covering a wide range of antifungal mechanisms of action. Strips and RPMI agar plates were sourced from Liofilchem (Roseto degli Abruzzi, Italy). Yeast cultures were grown on Sabouraud dextrose agar for 48 h at 27 °C. Well-isolated colonies were homogenized in sterile saline solution (0.85% NaCl) to obtain a turbidity equivalent to 0.5 McFarland standard. A sterile swab was soaked in the inoculum and used to streak the entire agar surface three times, rotating the plate 60° each time to ensure even distribution of the inoculum. The soaking and streaking procedure was repeated a second time. Strips were carefully applied on dry agar surface and plates were incubated at 35 °C. Plates were read after 24, 48 and 72 hours following the Etest antifungal reading guide [45]. The test was carried out in duplicate. If MICs differed between the duplicates, the higher MIC was reported.

6.3.3.4 Phenolic off-flavor test

The phenolic off-flavor test was performed according to Meier-Dörnberg et al. [46]. Yeast strains were spread on yeast and mold agar plates (YM-agar) containing only one of the following precursors: either ferulic acid, cinnamic acid or coumaric acid. After three days of incubation at 25 °C, plates were evaluated by a trained panel by sniffing to detect any of the following aromas: clove-like (4-vinylguajacol), Styrofoam-like (4-vinylstyrene) and medicinal-like (4-vinylphenol). *Saccharomyces cerevisiae* LeoBavaricus - TUM 68® (Research Center Weihenstephan for Brewing and Food Quality, Freising-Weihenstephan, Germany) was used as a positive control.

6.3.3.5 Flocculation test

The flocculation test was performed using a slightly modified Helm's assay [47,48]. Essentially, all cells were washed in EDTA and the sedimentation period was extended to 10 min. Wort was composed of 100 g/L spray-dried malt extract (Spraymalt Light, Muntons plc, Suffolk, UK) adjusted to 15 IBU (15 mg/mL iso-α-acids; from 30% stock solution; Barth-Haas Group, Nürnberg, Germany).

6.3.3.6 Stress tests

Stress tests were performed on microplates through the repeated measurement of absorbance over a time period of 96 hours (Multiskan FC, Thermo Scientific, Waltham, Massachusetts, USA).

The substrate for the hop sensitivity test was 75 g/L sterile-filtered wort adjusted to 0, 50 and 100 mg/L iso-α-acids (1 mg/L = 1 International Bitterness Unit, IBU), respectively by using an aliquot of a stock solution of 3% iso-α-acids in 96% (v/v) ethanol (Barth-Haas Group, Nürnberg, Germany).

For testing ethanol sensitivity, the sterile-filtered wort extract was adjusted to 0%, 2.5%, 5%, 7.5% and 10% ABV with an aliquot of 100% (v/v) ethanol.

For testing pH sensitivity by lactic acid, the sterile-filtered wort was adjusted to pH ranges from 5.5 (no addition of acid) to 3.0 in steps of 0.5 with aliquots of 80% lactic acid (corresponding to lactic acid concentrations of 0; 1.7; 3.1; 6.1; 16.3; 48.4 mM).

For testing pH sensitivity by HCl, the sterile-filtered wort was adjusted to pH ranges from 5.5 (no addition of acid) to 1.5 in steps of 0.5 with aliquots of 2 M HCl.

Osmotic stress was tested by adjusting the sterile-filtered wort extract (100 g/L Muntons Spraymalt Light) to sorbitol concentrations of 0, 50, 100, 150, and 200 g/L, respectively.

For inoculation, strains were grown in sterilized wort for 24 h at 25 °C under aerobic conditions. The microtiter plate wells were inoculated with a concentration of 10^5 cells/mL. The wells contained 200 µL of the respective wort substrates. Plates were incubated at 25 °C and absorbance was measured every 30 min at 600 nm without shaking over a time period of 96 h (Multiskan FC, Thermo Scientific, Waltham, Massachusetts, USA).

6.3.4 Fermentation trial

Single colonies of the respective strains were taken from YPD agar plates after 72 h growth at 25 °C and transferred into a 250 mL sterile Duran glass bottle (Lennox Laboratory Supplies Ltd, Dublin, Ireland) containing 150 mL propagation wort consisting of 75 g/L spray-dried malt and 30 g/L glucose (Gem Pack Foods Ltd., Dublin, Ireland), sterilized at 121 °C for 15 min. The bottles were covered with sterile cotton and placed in an incubator with orbital shaker (ES-80 shaker-incubator, Grant Instruments (Cambridge) Ltd, Shepreth, UK) and incubated for 48 h at an orbital agitation of 170 rpm at 25 °C. Cell count was performed using a Thoma Hemocytometer with a depth of 0.1 mm (Blaubrand, Sigma-Aldrich, St. Louis, MO, USA).

Fermentation wort was prepared by dissolving 100 g/L spray-dried malt extract in 1 L of brewing water and sterilized at 121 °C for 15 min followed by filtration through sterile grade 1V Whatman filter (Whatman plc, Maidstone, UK) to remove hot trub built up during sterilization. Iso-α-acids were added to the wort at a concentration of 15 mg/L (15 IBU).

Fermentation trials were carried out in 250 mL sterile Duran glass bottles, equipped with an air lock. Bottles were filled with 150 mL of wort. Yeast cells for pitching were washed by centrifugation at 900 *g* for 5 min and resuspended in sterile water to ensure no carryover of sugars or acids from the propagation wort into the fermentation wort. Pitching rate was 10^7 cells/mL. Fermentation temperature was 25 °C. Fermentation was performed until no change in extract could be measured for two consecutive days.

6.3.5 Lactic acid production optimization of KBI 12.1

6.3.5.1 Response surface methodology (RSM)

To investigate lactic acid production performance by KBI 12.1 at different fermentation parameters, response surface methodology (RSM) was performed using DesignExpert 9 software (StatEase, Minneapolis MN, USA). A three factorial, face-centered, central composite design with duplicate factorial points and 6 replications of the center point was chosen. The predictor factors were extract (5, 10, 15 °P), temperature (16, 22, 28 °C), and pitching rate (5, 32.5, 60×10^6 cells/mL).

Spray-dried malt extract served as substrate. Wort preparation, propagation and inoculation was carried out as outlined in 6.3.4. Sterilized and filtered wort extract of 15 °P was used as the base and diluted with sterile water when necessary. Fermentation volume was 150 mL in 250 mL Duran glass bottles equipped with an air lock. Fermentation was performed until no change in extract could be measured for two consecutive days.

6.3.5.2 Spiked glucose trial

Wort preparation, propagation and inoculation was carried out as outlined in 6.3.4. The 7 °P wort was produced from 75 g wort extract in 1 L of water. The 7 °P wort plus 3% glucose was produced from 75 g wort extract and 30 g glucose in 1 L of water. The 10 °P wort was produced by dilution of the 15 °P wort from 6.3.5.1 with water. Fermentation volume was 150 mL in 250 mL Duran glass bottles equipped with an air lock. Pitching rate was 5×10^6 cells/mL and fermentation temperature was 25 °C. Fermentation was performed until no change in extract could be measured for two consecutive days.

6.3.6 Pilot brew

Wort for the pilot brew was produced in a 60 L pilot-scale brewing plant consisting of a combined mash-boiling vessel, a lauter tun and whirlpool (FOODING Nahrungsmitteltechnik GmbH, Stuttgart, Germany). Weyermann Pilsner Malt was milled with a two-roller mill ("Derby", Engl Maschinen, Schwebheim, Germany) at a 0.8 mm gap size. Seven kilograms of crushed malt was mashed in with 30 L of brewing water at 50 °C. To increase the amount of glucose, 7 g of Amylo™ 300 (Kerry Group, Tralee, Ireland) were added at the begin of mashing (1 g/kg of malt). The following mashing regime was employed: 20 min at 50 °C, 60 min at 65 °C and 5 min at 78 °C. The mash was pumped into the lauter tun and lautering was performed after a 15 min lauter rest, employing four sparging steps of 5 L hot brewing water each. Boil volume was 50 L at a gravity of 1.038 (9.9 °P). At the start of the boil, 15 g of Magnum hop pellets (10.5% iso-α-acids) were added for a calculated IBU content of 6.5. Total boiling time was 45 min. After boiling, gravity was adjusted to 1.034 (8.5 °P) with hot brewing water, and hot trub precipitates and hop residue were removed in the whirlpool with a rest of 20 min. Clear wort was pumped through a heat exchanger and filled into 60 L fermentation vessels at a temperature of 25 °C.

Yeast was pitched at a pitching rate of 5×10^6 cells/mL. Fermentation temperature was 25 ± 1 °C (uncontrolled). Samples were taken every 12 h. After 36 h, 30 liters of the young beer were filtered through a plate filter (Seitz K 200; Pall GmbH, Dreieich, Germany) to stop fermentation by removing the yeast, and filled into a 50 L keg. The remaining young beer was left in the fermenter to reach final attenuation. To carbonate the kegged beer, the keg was repeatedly topped up with CO_2 at a pressure of 1 bar at 2 °C. Ten days after stopping fermentation, the carbonated beer was filled into 330 mL brown glass bottles with a counter-pressure hand-filler (TOPINCN, Shenzen, China) and capped. Bottles were pasteurized in a pilot retort (APR-95; Surdry, Abadiano, Vizcaya, Spain) with spray water at 65 °C for 10 min resulting in approximately 23 pasteurization units (PU). Beer bottles were stored in a dark place at 2 °C for further analysis and sensory evaluation.

6.3.7 Sensory

The low alcohol *Lachancea* beer produced at pilot scale (bottled beer) was tasted and judged by a sensory panel of 15 experienced panelists. The panelists were asked to evaluate the intensity of fruitiness in aroma, the sweetness/acidity ratio (0 "too sweet"; 5 "just right"; 10 "too sour") and the general acceptability of the low alcohol beer on a scale from 0, "not acceptable", to 10, "extremely acceptable". Samples were served at a temperature of 12 °C.

6.3.8 Analytics

6.3.8.1 HPLC analyses

The cell-free supernatant of fermented samples was analyzed using the following methods. Sugars and ethanol were determined by high performance liquid chromatography (HPLC) Agilent 1260 Infinity (Agilent Technologies, Santa Clara CA, USA) equipped a refractive index detector (RID) and a Sugar-Pak I 10 µm, 6.5 mm × 300 mm column (Waters, Milford MA, USA) with 50 mg/L Ca-EDTA as mobile phase and a flow rate of 0.5 mL/min at 80 °C. Differentiation of maltose and sucrose was achieved with a Nova-Pak 4 µm, 4.6 mm × 250 mm column (Waters, Milford MA, USA) with acetonitrile/water 78:22 (v/v) as mobile phase and a flow rate of 1.0 mL/min. Lactic acid was quantified via HPLC (DIONEX UltiMate 3000, Thermo Scientific, Waltham MA, USA) with diode array detector (DAD) and a Hi-Plex H 8 µm, 7.7 mm × 300 mm column

(Agilent Technologies, Santa Clara CA, USA) with 5 mM H_2SO_4 as mobile phase and a flow rate of 0.5 mL/min at 60 °C. Quantification was achieved by external standards in a calibration range of 0.5 to 30 mM.

6.3.8.2 Volatiles analysis by GC-MS

Analysis of volatiles in the cell-free supernatant of the fermented samples was carried out as follows. Analytes were extracted using liquid-liquid extraction with Methyl-tert-butyl ether directly in the vial. Analysis was performed using a mid-polarity column (Zebron ZB-1701, GC Cap. Column 30 m × 0.25 mm × 0.25 μm; Phenomenex, Torrance CA, USA) installed in a GC 7890B (Agilent Technologies, Santa Clara CA, USA) coupled with a quadrupole detector 5977B (Agilent Technologies, Santa Clara CA, USA). The system was controlled by ChemStation (Agilent Technologies, Santa Clara CA, USA). The GC-method was set up as described by Pinu and Villas-Boas [49] with only minor modifications. Samples were analyzed in Selected Ion Monitoring (SIM) mode. Quantifications were performed using external calibration lines.

6.3.8.3 Free amino nitrogen

Free amino nitrogen (FAN) was measured using a ninhydrin-based dying method, where absorbance is measured at 570 nm against a glycine standard (ASBC Method Wort-12 A).

6.3.8.4 Statistical analysis

Fermentations and analyses were carried out in triplicate, unless stated otherwise. Statistical analysis was performed using RStudio, Version 1.1.463 with R version 3.5.2 (RStudio Inc, Boston MA, USA; R Core Team, r-project). One-way ANOVA was used to compare means and Tukey's test with 95% confidence intervals was applied for the pairwise comparison of means. Principal component analysis (PCA) was performed with the R packages FactoMineR and Factoshiny [50]. Values are given as the mean ± standard deviation.

6.4 Results

The *Lachancea fermentati* strains investigated in this study were isolated from four individual kombucha cultures. KBI 1.2 and KBI 3.2 originate from the Conterminous United States, while KBI 12.1 originates from Hawaii, and KBI 5.3 originates from a kombucha culture from Australia. They were identified as *Lachancea fermentati* strains via sequencing and comparing the D1/D2 region of the large subunit rDNA to the public NCBI nucleotide database[2] [20]. The country of origin of the strain CBS 707 is unknown. CBS 6772 was isolated from a spoiled strawberry beverage in South Korea (Table 6.4–1).

6.4.1 Genomics

To better understand the variation in these four strains, whole-genome sequencing of the four *L. fermentati* kombucha isolates and the CBS707 type strain was performed. These were sequenced with 150 bp paired-end Illumina sequencing to an average coverage ranging from 115× to 139×. Reads were aligned to the reference genome of *L. fermentati* CBS 6772, and single nucleotide polymorphisms (SNPs) were called with FreeBayes. A total of 370,027 variable sites were observed across the five strains compared to the reference genome. Interestingly, a high number of SNPs (> 250,000) were observed in the three kombucha isolates originating from the United States (Table 6.4–1). This corresponds to a nucleotide sequence divergence around 2.4–2.7% in the 10.3 Mbp genome. The majority of these SNPs were heterozygous (>2% heterozygosity), suggesting that not only were these strains non-haploid, but possessed divergent genotypes.

Table 6.4–1 The ploidy and amount of homozygous and heterozygous single nucleotide polymorphisms (SNPs) observed in the *Lachancea fermentati* strains in comparison to the CBS 6772 reference genome.

Strain name	Origin	Measured ploidy	Homozygous SNPs	Heterozygous SNPs	Total SNPs
CBS 6772	Spoiled strawberry soft-drink, South Korea	-	-	-	-
CBS 707	Sediment of peppermint, Unknown	2	20,281	838	21,119
KBI 1.2	Kombucha, USA (Florida)	2	43,937	237,929	281,866
KBI 3.2	Kombucha, USA (Arizona)	2	44,797	235,170	279,967
KBI 5.3	Kombucha, Australia	1	21,245	965	22,210
KBI 12.1	Kombucha, USA (Hawaii)	2	45,237	205,790	251,027

[2] https://blast.ncbi.nlm.nih.gov/Blast.cgi

Allele frequency peaks at 0, 0.5 and 1, suggested that these strains were diploid (Figure 6.4–1B). The heterozygosity was considerably higher than what was observed, for example, in any of the recently sequenced 1,011 *S. cerevisiae* strains [51] or 14 *Kluyveromyces marxianus* strains [43]. The kombucha isolate originating from Australia, KBI 5.3, and the CBS707 type strain had around 20,000 SNPs compared to the reference genome. Here, the majority of the SNPs were homozygous, suggesting that the strains were either haploid or homozygous diploids (Figure 6.4–1B). The average pairwise nucleotide diversity (π) in this limited set of strains was 0.0126, which is comparable to what has been observed for *Kluyveromyces marxianus* [43] and slightly higher than for a wild population of *Lachancea quebecensis*. The three heterozygous kombucha isolates also contained several regions where heterozygosity was lost (Supplementary Figure 6.9–1). Common regions, where loss of heterozygosity (LOH) was observed in all three strains, could be found across chromosome G and on the right arm of chromosome F. In addition, KBI 12.1 had large LOH regions on the left arms of both chromosome F and H.

Flow cytometry was used to confirm the ploidy of the strains. The natural ploidy of *L. fermentati* and other members of the *Lachancea* genus appears to vary, with reports of both haploid and diploid strains [27,52–55]. Here, the three heterozygous kombucha isolates appeared diploid, while KBI 5.3 appeared haploid (Figure 6.4–1A). Despite the lack of heterozygous SNPs, the CBS707 type strain also appeared diploid. This is in line with what has previously been reported for the strain [52]. Read coverage also suggested that CBS 707 also harbored an extra third copy of chromosome C, while no aneuploidy was observed in any of the kombucha isolates (Supplementary Figure 6.9–2). Fluorescence intensities of the *L. fermentati* strains during flow cytometry were slightly lower than those of haploid and diploid *S. cerevisiae* references, as can be expected based on the smaller genome size of *L. fermentati.* Phylogenetic analysis based on the single nucleotide variants that were observed in the four kombucha isolates and the CBS 707 type strain, separated the three heterozygous kombucha isolates (KBI 1.2, KBI 3.2 and KBI 12.1) into a separate clade from the one containing CBS 707, CBS 6772 and KBI 5.3 (Figure 6.4–1C). Because of the high heterozygosity, which can skew the results, we attempted to separate the two haplotypes both using variant phasing with WhatsHap and by assigning the haplotypes based on similarity to the reference genome as described by Ortiz-Merino et al. [43]. In both cases, one haplotype could be found together with CBS 707, CBS 6772 and KBI 5.3, while the other haplotype formed a separate clade (Supplementary Figure 6.9–3). It is therefore likely that the heterozygous kombucha isolates have emerged through breeding

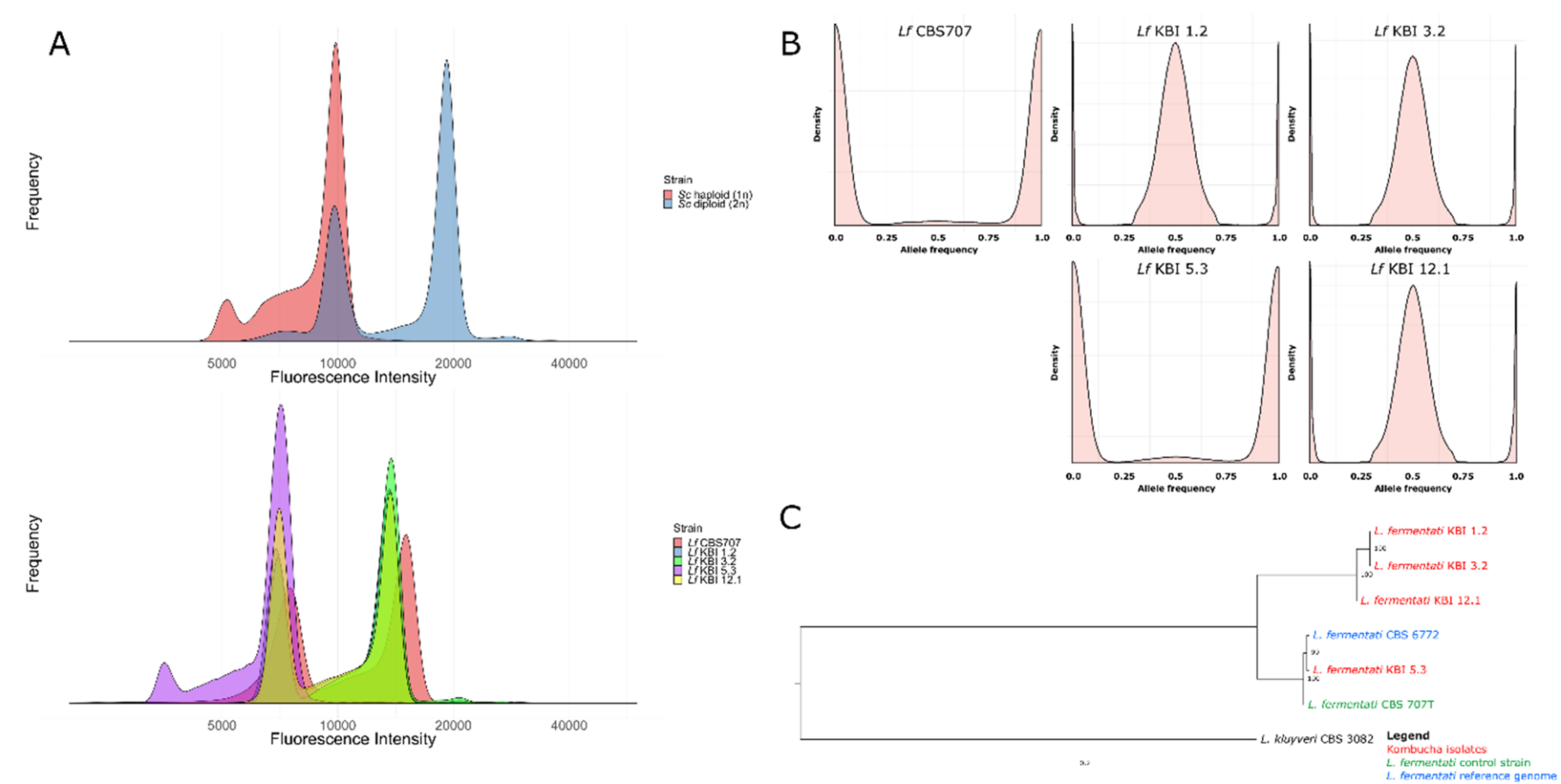


Figure 6.4–1 (A) Fluorescence intensity of SYTOX Green-stained haploid and diploid *S. cerevisiae* control strains and the five *L. fermentati* strains during flow cytometry. (B) The allele frequency distributions of the single nucleotide variants observed in the five sequenced *L. fermentati* strains. Peaks only at 0 and 1 suggest a single allele at each site, while peaks at 0, 0.5 and 1 suggest two alleles at each site. (C) A maximum likelihood phylogenetic tree based on SNPs at 6,330 sites in the six *L. fermentati* and one *L. kluyveri* genomes (rooted with *L. kluyveri* as outgroup). Numbers at nodes indicate bootstrap support values. Branch lengths represent the number of substitutions per site.

between strains from two different *L. fermentati* populations. To ensure that the heterozygous strains were not interspecific hybrids, sequencing reads were also aligned to a concatenated reference genome consisting of the genomes of 12 species in the *Lachancea* genus. Reads aligned almost exclusively to the *L. fermentati* genome, confirming that they were not interspecific hybrids (Supplementary Figure 6.9–4).

6.4.2 Yeast characterization

6.4.2.1 API sugar utilization, flocculation and POF test

The API sugar utilization test was performed to investigate intraspecific differences. The results are shown in Table 6.4–2, alongside the results from the flocculation test and phenolic off-flavor test.

Table 6.4–2 API sugar utilization test. – negative; + positive; w weak. Substrates negative for all strains are not included in the table. Different letters in superscripts indicate a significant difference of the means within a row ($p \leq 0.05$). The full table of substrates is included in Supplementary Data Sheet 1 (Appendix).

Substrate/Assay	CBS 707	KBI 1.2	KBI 3.2	KBI 5.3	KBI 12.1
Control	–	–	–	–	–
D-Galactose	+	+	+	+	+
Cycloheximide (Actidione)	+	+	+	+	+
D-Saccharose	+	+	+	+	+
Lactic acid	–[1]	w	w	–	w
D-Cellobiose	+	w	w	–	+
D-Raffinose	+	+	+	+	+
D-Maltose	+	+	+	+	+
D-Trehalose	+	+	+	+	+
Potassium 2-Ketogluconate	w	w	w	w	w
Methyl-αD-Glucopyranoside	+	+	+	+	+
D-Mannitol	+	+	+	w	+
D-Sorbitol	+	+	+	+	+
Palatinose	+	+	+	+	+
D-Melezitose	w	+	w	w	+
Potassium Gluconate	w	–	w	–	w
D-Glucose	+	+	+	+	+
L-Sorbose	w	+	w	+	+
Esculin ferric citrate	w	+	+	w	+
Flocculation (%)	15 ± 2 [a]	88 ± 10 [b]	28 ± 1 [a]	25 ± 8 [a]	20 ± 6 [a]
Definition	non-flocculent	very flocculent	moderately flocculent	moderately flocculent	moderately flocculent
Phenolic off-flavor	negative	negative	negative	negative	negative

[1] Deviation from literature which states a positive reaction [56].

The sugar utilization pattern showed minor differences. The type strain CBS 707 and KBI 5.3 showed no growth with lactic acid as substrate, whereas KBI 1.2, KBI 3.2 and KBI 12.1, exhibited weak growth. However, Kurtzman et al. [56] reported positive growth for CBS 707. Esculin ferric citrate was positive for KBI 1.2, KBI 3.2 and KBI 12.1 and weak for CBS 707 and KBI 5.3. The color reaction resulting from a positive reaction to esculin ferric citrate is associated with β-glucosidase activity [57]. However, cellobiose, a β-1,4-linked sugar, was not metabolized by KBI 5.3 and only weakly by KBI 1.2 and KBI 5.3 despite showing weak or positive reactions to esculin ferric citrate. In a study on *Lachancea fermentati* wine strains, Porter [58] reported that from 10 tested strains, 80% showed β-glucosidase activity.

According to the modified Helm's assay, CBS 707, KBI 3.2, KBI 5.3 and KBI 12.1 showed low flocculation between 15 and 28%, with no statistically significant difference ($p \leq 0.05$). KBI 1.2 showed, with 88%, the highest flocculation behavior. Flocculation of *Lachancea fermentati* strains has also been reported in other studies and its degree was shown to be strain-dependent [58–60]. However, yeast flocculation assays like the Helm's assay can deviate from observations on flocculation behavior in practice and can be difficult to reproduce [61]. In a previous study by Bellut et al. [20], KBI 12.1 exhibited high flocculation > 80%. In fact, from observations during fermentation trials in this study, KBI 12.1 shows a more flocculent behavior than the results of the Helm's assay suggest here, with flocculation more comparable to that of the brewers' yeast WLP001 as previously reported [20]. All strains showed negative POF behavior.

6.4.2.2 Scanning electron microscopy (SEM)

To visualize the different yeast strains and to investigate differences in cell morphology, scanning electron microscopy (SEM) was performed. The SEM pictures of the strains can be seen in Figure 6.4–2.

The SEM confirmed inter- and intraspecific differences in cell morphology that had been suspected from observations under the light microscope. The almost rod-shaped cells of the type strain CBS 707 were longer and thinner than the other *Lachancea fermentati* KBI strains. Bud scars appeared to be mostly located at or near the ends of the rod-shape. The KBI strains seemed to have a rounder shape compared to the type strain. KBI 12.1 appeared to exhibit the highest proportion of oval or spherical shaped cells of the *Lachancea fermentati* strains, while cells of WLP001 showed a substantially more

pronounced spherical shape. Regarding cell size, the cells of the brewers' yeast were larger compared to the *Lachancea fermentati* cells. The cell size is related to the total surface area of the cell, which determines import and export rates of nutrients and fermentation products [62]. The difference in cell size can therefore have a strong effect on fermentation performance and must be considered when choosing pitching rates.

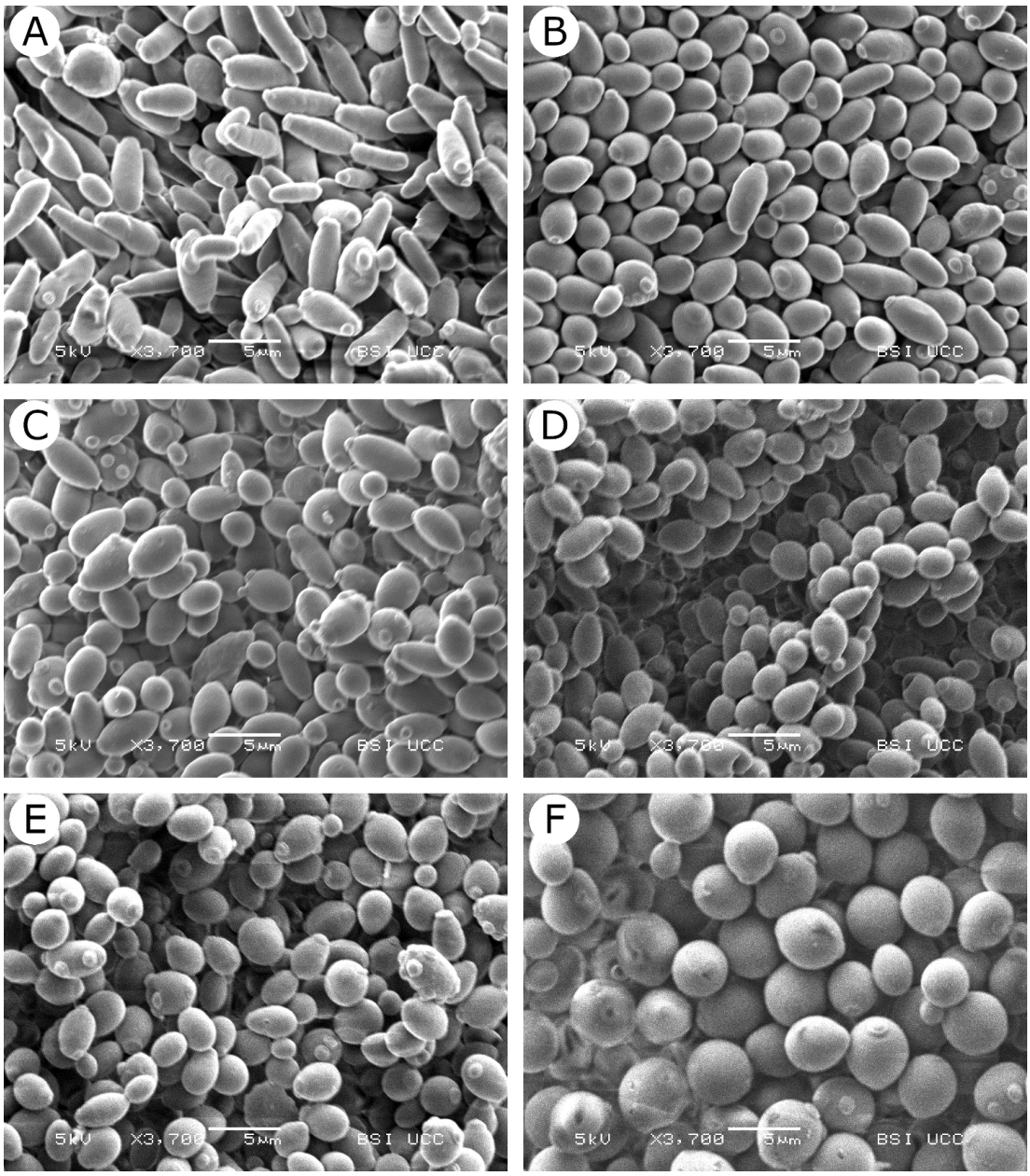


Figure 6.4–2 Scanning electron microscopy (SEM) pictures of the yeast strains (A) CBS 707, (B) KBI 1.2, (C) KBI 3.2, (D) KBI 5.3, (E) KBI 12.1, and (F) WLP001 at same magnification of ×3,700. Size of horizontal bar: 5 µm.

6.4.2.3 Stress tests

During fermentation, yeast strains applied in brewing must deal with several stress factors. Iso-α-acid concentrations of 100 and more mg/L are no longer a rarity (e.g., strong India Pale Ales (IPAs)). Ethanol, another stressor, accumulates during fermentation, especially in high gravity brewing, which by itself involves another stress factor: osmotic stress (here simulated with sorbitol). Sour beers are also gaining popularity and yeasts are required to ferment wort with a low pH and high initial lactic acid concentration [63]. Additionally, strains of the *Lachancea* genus can possess the ability to produce significant amounts of lactic acid during alcoholic fermentation. The stress tests were performed to investigate inter- and intraspecific differences. Table 6.4–3 shows the results of the relative growth in wort in microtiter analyses at a snapshot at 48 h after pitching with, and without the stressor in different concentrations.

Table 6.4–3 Relative growth in percent in wort after 48 h with and without stressor in different concentrations based on OD_{600} measurements. Bold values are significantly different from the previous value within a stress test for the individual strain ($p \leq 0.05$).

Stress factor (Unit)	Concen-tration	CBS 707	KBI 1.2	KBI 3.2	KBI 5.3	KBI 12.1	WLP001
Hops (IBU)	0	100	100	100	100	100	100
	50	**107**	103	99	100	105	**95**
	100	105	99	99	101	105	98
Ethanol (% ABV)	0	100	100	100	100	100	100
	2.5	**90**	98	97	95	96	95
	5	**77**	87	**87**	**86**	**85**	**76**
	7.5	**5**	**53**	**70**	**66**	**71**	**16**
	10	**0**	**0**	**25**	**8**	**4**	**0**
Sorbitol (g/L)	0	100	100	100	100	100	100
	50	89	89	90	92	**86**	99
	100	**64**	**77**	**78**	**78**	**72**	91
	150	**35**	**52**	**54**	**62**	**46**	83
	200	26	**35**	**32**	**41**	35	**70**
Lactic acid (pH)	5.5	100	100	100	100	100	100
	5	**96**	99	99	99	97	100
	4.5	95	**97**	**97**	**98**	98	101
	4	96	98	98	98	98	104
	3.5	95	97	95	**95**	98	100
	3	**84**	**87**	**83**	**85**	**86**	**53**
HCl (pH)	5.5	100	100	100	100	100	100
	5	101	100	102	101	99	**105**
	4.5	102	101	100	102	100	104
	4	101	102	100	102	98	108
	3.5	98	**98**	**97**	**98**	97	105
	3	**93**	**88**	**87**	**91**	**89**	**85**
	2.5	**71**	**71**	**69**	**79**	**72**	**0**
	2	**1**	**5**	**1**	**1**	**2**	1
	1.5	1	**1**	1	1	1	1

The concentration of iso-α-acids did not have an influence of the growth of the strains which is in accordance with previous reports on various non-*Saccharomyces* species [15]. However, Michel et al. [64] reported the presence of 90 IBU to affect *Torulaspora delbrueckii* strains, resulting in a slightly prolonged lag phase and slightly decreased slope of the growth curve.

Among the *L. fermentati* strains, the KBI strains exhibited a greater tolerance towards higher ethanol concentrations in the wort compared to CBS 707, which showed a small but significant growth impairment already at 2.5% ABV, manifesting as a 10% decreased relative growth. At 7.5% ABV, CBS 707 showed almost full growth inhibition (5% relative growth remaining) while the KBI strains still showed relative growth between 53 and 71%. At an ABV of 10% in the wort, growth of CBS 707 and KBI 1.2 was fully inhibited, while KBI 3.2, KBI 5.3 and KBI 12.1 still exhibited little growth, at 4 to 25%, with KBI 3.2 being the most ethanol tolerant strain. In accordance, Porter et al. [60] observed full inhibition of a *L. fermentati* strain at 10% ABV during a growth test on agar while it still exhibited growth at 7% ABV. The brewers' yeast WLP001 showed significant inhibition at 5% ABV with 24% decreased relative growth. Full growth inhibition was reached at 10% ABV. Overall, WLP001 showed a greater sensitivity towards ethanol compared to the KBI strains.

During osmotic stress, at the presence of high concentrations of sorbitol, the *Lachancea fermentati* strains showed a greater growth impairment compared to WLP001 with only 26 to 41% remaining relative growth at 200 g/L sorbitol compared to 70% for WLP001. Intraspecific differences in growth inhibition among the *Lachancea fermentati* were generally low, CBS 707 tentatively showing greater sensitivity.

In the presence of lactic acid, all yeast strains were resilient against concentrations of up to 16.3 mM (pH 3.5). Although statistically significant, growth impairment at lactic acid concentrations between 1.7 and 16.3 mM showed to be very low with a maximum decrease in relative growth by 5%. Only at extreme lactic acid concentrations of 48.4 mM (pH 3), did the *L. fermentati* strains show slight growth impairment of 13 to 17%, while WLP001 exhibited a growth impairment of 47%.

When the wort pH was adjusted with HCl, the strains showed less sensitivity compared to the pH adjustment with lactic acid. For example, at pH 3, WLP001 still exhibited 85% growth compared to 53% at pH 3 when adjusted with lactic acid. However, at pH 2.5 and

lower, WLP001 growth was fully inhibited while the *L. fermentati* strains still exhibited relative growth between 72 and 79%. Full growth inhibition of the *L. fermentati* strains was reached at pH 2. Intraspecific differences among the *Lachancea fermentati* strains were small.

Differences in growth impairment by the different acids at same pH can be explained with the chemical property of weak acids. The presence of a weak acid like lactic acid leads to an increased stress for the yeast cell. The lower the extracellular pH, the more lactic acid is present in its protonated form, especially at a pH below the pK_a of the respective acid (lactic acid pK_a: 3.86) and can therefore enter the cell via passive diffusion. Inside the cell, at a higher intercellular pH, lactic acid is deprotonated. Consequently, the cell must export the proton as well as the anion, creating an energy-requiring cycle. At high concentrations, this mechanism can lead to the dissipation of the proton motive force, leading to cell death [29,65].

6.4.2.4 Antifungal susceptibility

While *Candida* species are the lead cause for fungemia, cases of non-pathogenic species such as *Saccharomyces cerevisiae* acting as opportunistic pathogens in immunocompromised hosts have been reported [66,67] and one case of fungemia caused by *Lachancea fermentati* in an immunocompromised host has also been recorded [68]. Also, given the fact that *Lachancea* species are capable of growth at human body temperature (37 °C) [56], it is reasonable to investigate potential resistances against antifungal agents. The minimal inhibitory concentration (MIC) of a range of antifungal agents was tested by Etest. The results are shown in Table 6.4–4. All strains showed to be susceptible to all classes of antifungal agents with only small intra- and interspecific differences.

Table 6.4–4 Minimal inhibitory concentration (MIC) of selected antifungal agents after 24 hours of incubation. Values in μg/mL.

Antifungal agent	Range	CBS 707	KBI 1.2	KBI 3.2	KBI 5.3	KBI 12.1	WLP001
Amphotericin B	0.002 – 32	0.032	0.094	0.094	0.125	0.094	1
Caspofungin	0.002 – 32	1	1.5	1.5	1.5	1.5	0.5
Flucytosine	0.002 – 32	0.094	0.064	0.125	0.125	0.064	0.023
Fluconazole	0.016 – 256	12	12	12	12	12	24
Itraconazole	0.002 – 32	0.5	0.75	1	1	0.75	1
Voriconazole	0.002 – 32	0.094	0.125	0.125	0.125	0.125	0.125

6.4.3 Fermentation trials

6.4.3.1 Fermentation of wort

Fermentation trials were conducted to investigate strain performances in terms of ethanol and lactic acid production and the concentration of fermentation by-products. Spray-dried wort extract from barley malt served as the substrate for all fermentations. Table 6.4–5 shows the analytical parameters of the fermentation wort including extract, pH, free amino nitrogen (FAN) and sugar concentration.

Table 6.4–5 Analysis of fermentation wort.

Extract	°P	9.40 ± 0.00
pH		4.99 ± 0.01
FAN	mg/L	99 ± 1
Fructose	g/L	1.78 ± 0.02
Glucose	g/L	8.53 ± 0.05
Sucrose	g/L	1.02 ± 0.01
Maltose	g/L	40.64 ± 0.25
Maltotriose	g/L	11.94 ± 0.07

6.4.3.1.1 Analysis of fermented samples

Fermentation was carried out until no change in extract was measurable for two consecutive days. For CBS 707, KBI 1.2, KBI 5.3 and WLP001, final attenuation was reached after 11 days of fermentation at 25 °C. KBI 3.2 and KBI 12.1 reached final attenuation after 13 days of fermentation. Table 6.4–6 shows the analytical results of the fermentation trials.

The *L. fermentati* strains reached final attenuations of 70% and lower, owing to their inability to consume maltotriose. KBI 12.1 exhibited, at 55%, the lowest attenuation. Sugar analysis revealed that KBI 12.1 had only used up 76% of maltose while the other strains had depleted it by the end of fermentation. Only WLP001 consumed maltotriose, at 81%, while the *L. fermentati* strains did not consume any maltotriose. At the end of fermentation, slightly higher values for maltotriose than the initial values were detected in some of the worts fermented with the *L. fermentati* strains. Glucose and sucrose were completely consumed by all strains by the end of fermentation. In the wort fermented with CBS 707, a small amount of fructose remained.

Table 6.4–6 Analysis of fermented worts. Sugars are given in percent consumption of the initial amount. 100% consumption indicates a concentration below the limit of detection (LOD). Values are shown as means ± standard deviation. Different letters in superscripts indicate a significant difference of the means within a row ($p \leq 0.05$).

	Attribute	Unit	CBS 707	KBI 1.2	KBI 3.2	KBI 5.3	KBI 12.1	WLP001
	Attenuation		70% ± 0% ^{b}	70% ± 0% ^{b}	68% ± 1% ^{b}	70% ± 0% ^{b}	55% ± 2% ^{a}	85% ± 1% ^{c}
	app. Extract	°P	2.83 ± 0.03 ^{b}	2.83 ± 0.03 ^{b}	2.99 ± 0.14 ^{b}	2.79 ± 0.01 ^{b}	4.27 ± 0.22 ^{c}	1.38 ± 0.06 ^{a}
	real Extract	°P	4.14 ± 0.01 ^{b}	4.17 ± 0.02 ^{b}	4.27 ± 0.11 ^{b}	4.12 ± 0.01 ^{b}	5.31 ± 0.18 ^{c}	2.94 ± 0.08 ^{a}
	Ethanol	% ABV	3.73 ± 0.01 ^{b}	3.73 ± 0.04 ^{b}	3.63 ± 0.12 ^{b}	3.76 ± 0.03 ^{b}	2.96 ± 0.11 ^{a}	4.42 ± 0.02 ^{c}
	pH		4.24 ± 0.02 ^{d}	4.27 ± 0.01 ^{de}	4.13 ± 0.02 ^{c}	4.31 ± 0.01 ^{e}	3.95 ± 0.01 ^{a}	4.07 ± 0.02 ^{b}
	Lactic acid	mM	2.41 ± 0.02 ^{e}	1.55 ± 0.03 ^{c}	1.82 ± 0.03 ^{d}	1.33 ± 0.01 ^{b}	3.47 ± 0.12 ^{f}	0.94 ± 0.02 ^{a}
	FAN	mg/L	82 ± 4 ^{bc}	82 ± 2 ^{bc}	80 ± 4 ^{bc}	73 ± 3 ^{b}	83 ± 1 ^{c}	52 ± 4 ^{a}
Sugar consumption	Fructose		92% ± 0% ^{a}	100% ^{b}	100% ^{b}	98% ± 4% ^{b}	100% ^{b}	100% ^{b}
	Glucose		100%	100%	100%	100%	100%	100%
	Sucrose		100%	100%	100%	100%	100%	100%
	Maltose		100% ^{b}	100% ^{b}	98% ± 2% ^{b}	100% ^{b}	76% ± 4% ^{a}	100% ^{b}
	Maltotriose		-4% ± 1% ^{a}	-8% ± 1% ^{a}	-5% ± 4% ^{a}	-10% ± 1% ^{a}	3% ± 1% ^{b}	81% ± 1% ^{c}
Fermentation by-products	Diacetyl	mg/L	< LOD	< LOD	0.02 ± 0.02 ^{a}	< LOD	0.02 ± 0.00 ^{a}	< LOD
	Ethyl acetate	mg/L	14.35 ± 0.20 ^{d}	13.72 ± 0.28 ^{d}	14.01 ± 0.68 ^{d}	9.06 ± 0.21 ^{b}	11.70 ± 0.82 ^{c}	7.06 ± 0.50 ^{a}
	3-Methylbutyl acetate	mg/L	0.48 ± 0.06 ^{a}	0.36 ± 0.08 ^{a}	0.40 ± 0.03 ^{a}	0.43 ± 0.13 ^{a}	0.29 ± 0.05 ^{a}	0.30 ± 0.04 ^{a}
	2-Phenylethyl acetate	mg/L	0.08 ± 0.01 ^{a}	0.52 ± 0.03 ^{c}	0.57 ± 0.02 ^{c}	0.13 ± 0.02 ^{a}	0.44 ± 0.04 ^{b}	0.08 ± 0.02 ^{a}
	Σ Esters	mg/L	16.14 ± 0.31 ^{c}	16.02 ± 0.40 ^{c}	16.10 ± 0.64 ^{c}	10.84 ± 0.38 ^{a}	13.61 ± 0.99 ^{b}	9.52 ± 0.47 ^{a}
	Σ Alcohols	mg/L	119.98 ± 7.87 ^{d}	77.83 ± 1.64 ^{ab}	86.35 ± 4.81 ^{bc}	81.42 ± 4.72 ^{bc}	65.61 ± 2.34 ^{a}	93.39 ± 6.19 ^{c}

Ethanol concentrations correlated with attenuation. The brewers' yeast WLP001 exhibited the highest concentration, at 4.4% ABV, followed by four of the *L. fermentati* strains at around 3.7% ABV. KBI 12.1 produced only 3.0% ABV.

Lactic acid concentrations reached 0.94 mM in the sample fermented with WLP001. Li and Liu [67] reported similar values produced by a lager yeast, at 1.03 mM. The *Lachancea* yeasts exhibited significantly higher final lactic acid values. KBI 12.1 exhibited the highest lactic acid concentration, at 3.47 mM, followed by CBS 707, KBI 3.2, KBI 1.2, and KBI 5.3, at 2.41, 1.82, 1.55, and 1.33 mM, respectively. However, these values were still below the reported flavor threshold of lactic acid in beer of 4.44 mM (400 mg/L) [70].

FAN consumption by the *L- fermentati* strains was relatively low with 70 or 80% of the initial amount remaining by the end of fermentation. By comparison, WLP001 consumed half of the amount of FAN in the wort. The pattern of a low FAN consumption of non-*Saccharomyces* yeasts compared to brewers' yeasts has been observed in previous studies [15,17]. It has mostly been attributed to a less intensive fermentation due to limited sugar consumption, however, in this study, fermentation and sugar consumption did not differ to an extent that would account for the reduced FAN uptake, suggesting an alternative cause.

When detected, diacetyl values were, at 0.02 mg/L, low and below the flavor threshold of 0.10 mg/L for light beers [71]. Ethyl acetate values were significantly higher in the *L. fermentati* strains compared to WLP001, up to double the concentration. 3-Methylbutyl acetate (isoamyl acetate) concentrations were similar among all strains. 2-Phenylethyl acetate concentrations were significantly higher in KBI 1.2, KBI 3.2 and KBI 12.1 compared to the other strains. CBS 707 produced the highest amount of higher alcohols, at 120 mg/L, and KBI 12.1 produced the lowest amount, at 66 mg/L. Figure 6.4–3 illustrates the relative amounts of volatile fermentation by-products produced by the different yeast strains.

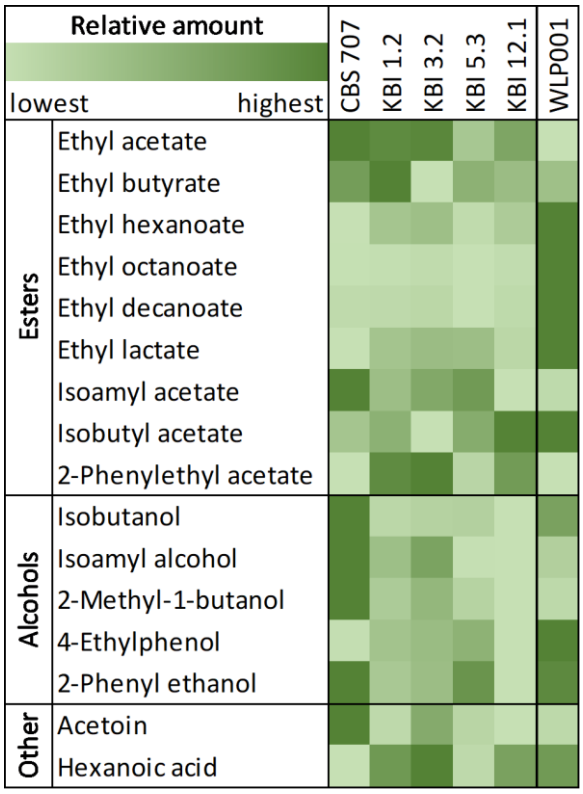


Figure 6.4–3 Heatmap of relative amounts of volatile compounds in the fermented worts. A full table of relative and quantified compounds can be found in Supplementary Data Sheet 1 (Appendix).

WLP001 produced higher amounts of the higher ethyl esters (i.e. ethyl hexanoate, ethyl octanoate, ethyl decanoate) while CBS 707 produced more higher alcohols compared to the other strains. Interestingly, despite the increased lactic acid production by the *Lachancea* strains, ethyl lactate concentrations were higher in the wort fermented with WLP001. None of the volatile fermentation by-products were detected in concentrations above their individual flavor thresholds (Supplementary Data Sheet 1; Appendix).

6.4.3.2 Lactic acid production optimization with KBI 12.1

While the *L. fermentati* strains produced significantly higher amounts of lactic acid compared to the *S. cerevisiae* control, the values were still below the reported flavor threshold for beer of 4.44 mM (400 mg/L) [70]. Therefore, we applied response surface methodology (RSM) and conducted a trial in wort extract with spiked glucose to enhance lactic acid production of strain KBI 12.1, which was chosen as the highest lactic acid producer from the screening (Table 6.4–6).

6.4.3.2.1 Response surface methodology

Non-*Saccharomyces* yeasts can require a significantly higher pitching rate to show good fermentation performance compared to brewers' yeast due to their typically smaller cell size. A study by Michel et al. [62] using RSM to optimize fermentation conditions of a *Torulaspora delbrueckii* strain in wort showed that high sensorial desirability of the produced beer was achieved at a high pitching rate of 60×10^6 cells/mL. Furthermore, the fermentation temperature can have significant influences on the production of fermentation by-products across yeast genera, e.g., a higher temperature resulting in increased ester production [62,72,73].

To investigate the influences of the fermentation parameters: pitching rate, temperature and starting extract, on the production of lactic acid, response surface methodology (RSM) was applied. A three factorial, face-centered, central composite design was chosen to investigate the lactic acid production by KBI 12.1 in wort extract in the range of extract content between 5 and 15 °P, a pitching rate between 5 and 60×10^6 cells/mL, and a fermentation temperature between 16 and 28 °C. The detailed experiment design and model statistics are shown in Supplementary Data Sheet 2 (Appendix).

Figure 6.4–4 shows the response surface as a 3D model of the lactic acid production at 5, 10, and 15 °P, as a function of the fermentation temperature and pitching rate.

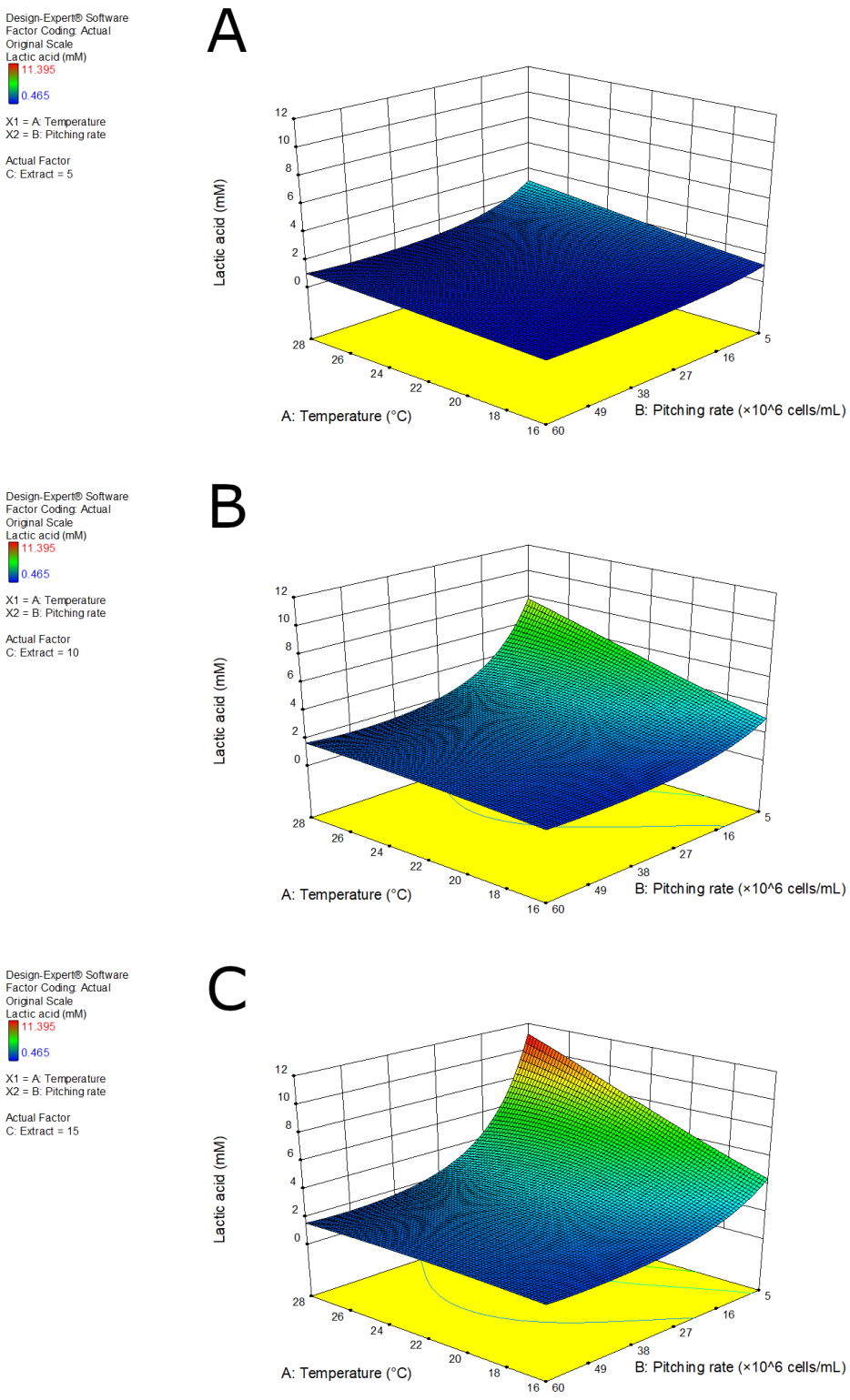


Figure 6.4–4 3D response surface model of response factor lactic acid as a function of fermentation temperature and pitching rate at 5 °P (A), 10 °P (B), and 15 °P (C). Model details and statistics in Supplementary Data Sheet 2 (Appendix).

Increasing extract content enhanced the effect of the temperature and pitching rate parameters. Additionally, a low pitching rate had a very strong positive effect on the lactic acid production. Lactic acid also increased with an increasing fermentation temperature. The highest lactic acid concentration achieved was 11.4 mM at a pitching rate of 5×10^6 cells/mL and a fermentation temperature of 28 °C. A full table of the results of the response factors can be found in Supplementary Data Sheet 2 (Appendix).

Results indicate, that in order to boost lactic acid production, a low pitching rate should be used in combination with a high fermentation temperature. Furthermore, in the favored conditions, a higher initial extract led to higher lactic acid concentrations. The fact that the samples with high lactic acid production did not reach final attenuation (Supplementary Data Sheet 2; Appendix) was suggested to be caused by end-product inhibition through the mechanism described in 6.4.2.3. Combined with the knowledge that glucose is commonly taken up at the beginning of fermentation before high amounts of maltose, sucrose or maltotriose are consumed [74], it was hypothesized that the increased lactic acid production in the worts with higher extract during the RSM trial was attributed to a higher amount of glucose.

6.4.3.2.2 Added glucose trial

To investigate the hypothesis that lactic acid production can be boosted by the presence of higher amounts of glucose at the beginning of fermentation, a trial with a glucose-spiked wort sample was conducted. Table 6.4–7 shows the analytical results of the three worts used in this trial before and after fermentation with KBI 12.1.

The addition of glucose to the 7 °P wort led to a significant increase in final lactic acid concentration ($p < 0.01$) of 246%, from 5.2 to 18.0 mM, while the final ethanol content of 2.6% ABV remained unchanged (Figure 6.4–5). The pH of the glucose spiked wort sample was correspondingly low, at 3.46. On the other hand, increasing the extract content from 7 °P to 10 °P (without the addition of glucose) did not have an influence on the final lactic acid concentration ($p > 0.05$) but resulted in a significantly higher final ethanol content ($p < 0.001$) (Figure 6.4–5).

Table 6.4–7 Analysis of worts before and after fermentation with KBI 12.1. Values are given as means ± standard deviation. Different letters in superscripts indicate a significant difference between the fermented samples ($p \leq 0.05$). 'n.d.' not determined.

Attribute	Unit	7 °P		7 °P + 3% glucose		10 °P	
		wort	fermented	wort	fermented	wort	fermented
Attenuation	%	-	65 ± 2 [c]	-	47 ± 0 [a]	-	60 ± 1 [b]
app. Extract	°P	7.35 ± 0.01	2.57 ± 0.12	9.66 ± 0.02	5.08 ± 0.03	9.99 ± 0.01	3.95 ± 0.09
real Extract	°P	7.35 ± 0.01	3.50 ± 0.08	9.66 ± 0.02	5.98 ± 0.02	9.99 ± 0.01	5.15 ± 0.08
Ethanol	% ABV	-	2.61 ± 0.04 [a]	-	2.59 ± 0.02 [a]	-	3.45 ± 0.03 [b]
pH		4.83 ± 0.01	3.81 ± 0.01 [b]	4.88 ± 0.01	3.46 ± 0.09 [a]	4.80 ± 0.01	3.91 ± 0.01 [b]
FAN	mg/L	83 ± 1	n.d.	83 ± 7	n.d.	88 ± 2	n.d.
Lactic acid	mM	-	5.19 ± 0.11 [a]	-	18.00 ± 4.64 [b]	-	5.10 ± 0.26 [a]
Fructose	g/L	1.28 ± 0.01	< LOD	1.56 ± 0.01	< LOD	2.09 ± 0.01	< LOD
Glucose	g/L	6.05 ± 0.01	< LOD	34.59 ± 0.10	< LOD	8.52 ± 0.05	< LOD
Sucrose	g/L	0.78 ± 0.01	< LOD	0.78 ± 0.00	< LOD	1.10 ± 0.01	< LOD
Maltose	g/L	31.06 ± 0.38	2.03 ± 0.77	31.12 ± 0.06	26.49 ± 0.59	43.67 ± 0.25	5.43 ± 0.70
Maltotriose	g/L	9.05 ± 0.11	9.90 ± 0.08	9.03 ± 0.02	8.46 ± 0.13	12.70 ± 0.02	13.56 ± 0.11

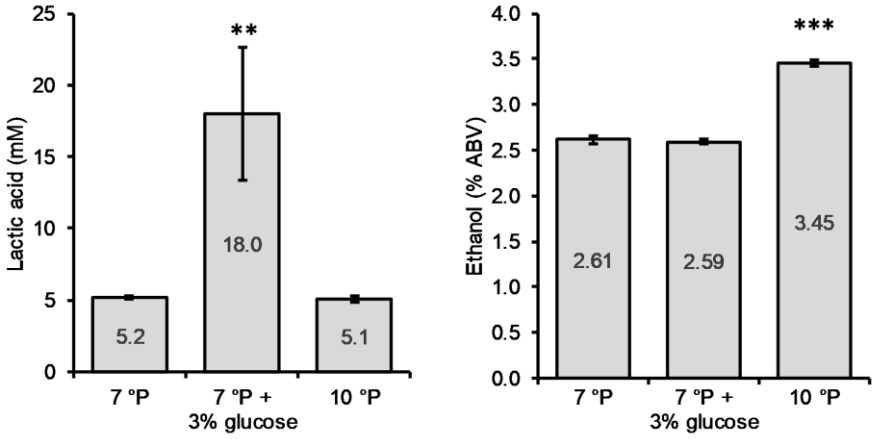


Figure 6.4–5 Final lactic acid and ethanol concentrations of 7 °P wort extract, 7 °P wort extract + 3% glucose, and 10 °P wort extract after fermentation with KBI 12.1. '**' $p \leq 0.01$; '***' $p \leq 0.001$.

The monosaccharides fructose and glucose were depleted by the end of fermentation while maltose was never fully depleted (Table 6.4–7). Especially the fermented sample with spiked glucose, resulting in high lactic acid production, exhibited high residual maltose concentrations by the end of fermentation which is an indication for a premature inhibition by low pH and/or high lactic acid concentration.

The results indicate that lactic acid production by KBI 12.1 can indeed be modulated by the amount of glucose present at the start of fermentation. Within the investigated range of 7 to 10 °P, a higher amount of glucose resulted in increased lactic acid production, but without an increased ethanol production.

6.4.4 Pilot-scale brewing trial

The results from the lactic acid optimization experiment gave valuable insights for the process development of a scaled-up brewing trial. The RSM results indicated that a low pitching rate and high fermentation temperature are favorable for increased lactic acid production, while the spiked glucose trial indicated that lactic acid production can be boosted by the initial glucose concentration of the wort. Considering these insights, amyloglucosidase was added during the mashing process of wort production to increase the amount of glucose relative to maltose. At the same time, a low pitching rate, at 5×10^6 cells/mL, together with a high fermentation temperature (25 °C) was chosen to increase lactic acid production on the process side. The aim was to create a low alcohol beer (LAB) by stopping the fermentation prematurely, at a point where the produced lactic acid is in balance with the sweetness of the residual wort sugars. For that reason, samples were taken every 12 hours until the fermentation was stopped by filtering out the yeast by means of a plate filter. Figure 6.4–6 illustrates fermentation progress as well as results from volatile fermentation by-products analysis and sensory evaluation of the produced LAB (36 h).

The ethanol concentration of the beer at interruption of fermentation after 36 hours had reached 1.26% ABV. The lactic acid concentration reached 13.6 mM (= 1.23 g/L) at a final pH of 3.56. Final apparent extract of the LAB was 6.23 °P. The cell count showed a constant growth in the first 24 hours, after which it slowed down to a cell concentration at time of filtration of 43×10^6 cells/mL. Glucose was fully depleted after 36 hours of fermentation while 0.17 mM (0.24 g/L) of fructose remained. Maltose only saw a small decrease and maltotriose was left untouched. The analysis of the beer that was left in the fermenter to reach final attenuation (216 h) showed only a small further increase in lactic acid to a concentration of 16.1 mM, while doubling in ethanol concentration to a final value of 2.57% ABV. At final attenuation, only about 55% of the maltose was consumed, with maltotriose concentrations unchanged. Analysis of volatile fermentation by-products of the stopped fermentation LAB revealed a low ester concentration of 6.5 mg/L (Figure

6.4–6B). At 0.27 mg/L, the diacetyl value was as well above its flavor threshold for light beers at 0.1 mg/L [71]. Diacetyl, an unwanted buttery flavor compound, is a fermentation by-product which, at the end of fermentation, is often at concentrations higher than its flavor threshold. In that case, a diacetyl rest is applied to allow yeast to reduce diacetyl to concentrations below the flavor threshold. In this study, the yeast was separated from the young beer before final attenuation was reached, and therefore reduction of the diacetyl concentration was not possible.

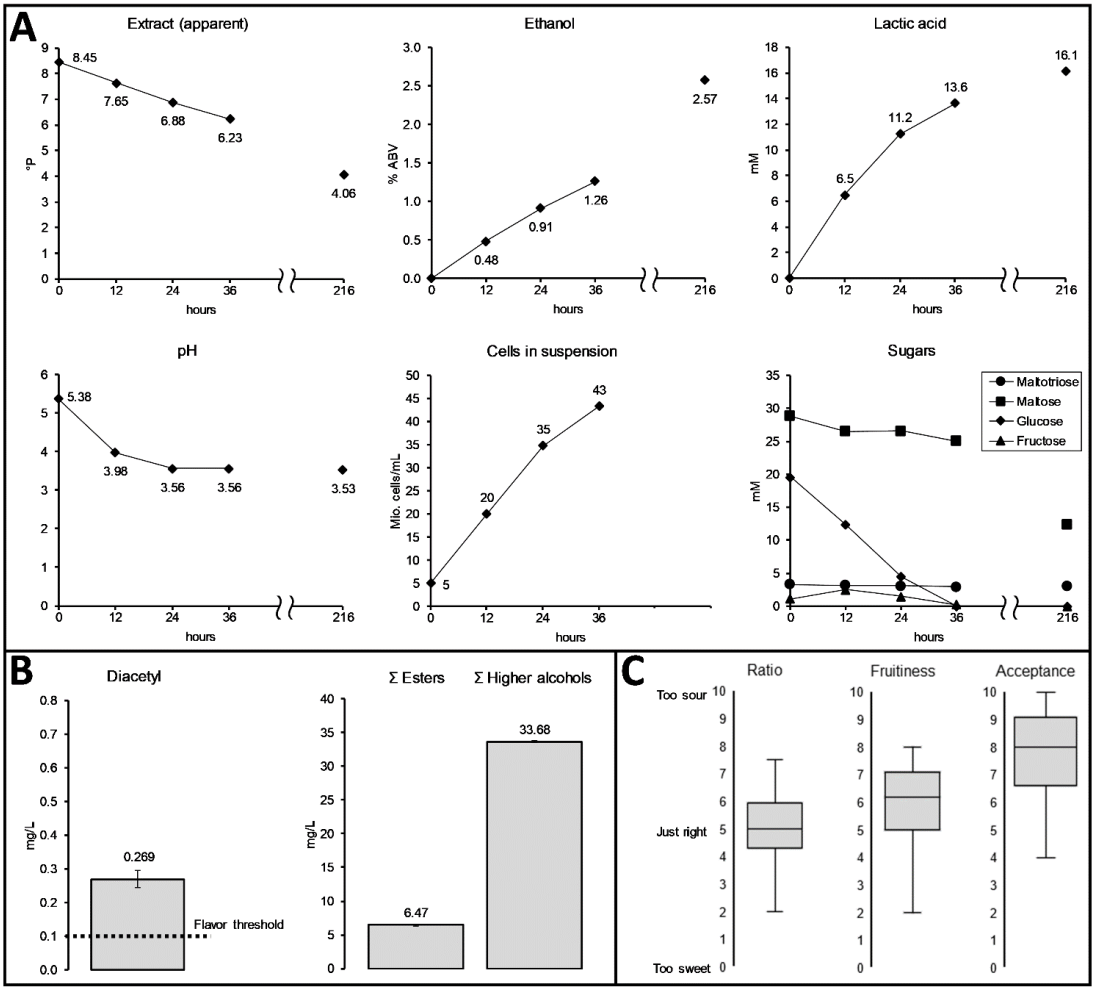


Figure 6.4–6 Analyses of pilot-scale (60 L) fermentation (A) of low alcoholic beer with KBI 12.1. Fermentation by-products (B) and sensory data (C) of the low alcoholic beer corresponds to the fermentation data at 36 h (finished low alcohol beer). Values at 216 h show the beer at final attenuation reached without the interuption of fermentation.

The results of the sensory evaluation indicated that a balanced ratio between residual sweetness from maltose and maltotriose and acidity from lactic acid was reached (Figure 6.4–6C). Fifty percent (interquartile range IQR; 50% of total reported values) of the panelists rated the sweetness/acidity ratio between 4.2–6.0 at a scale from 0 to ten, with

0 “too sweet”, 5 “just right”, and 10 “too sour”. Values corresponded to residual sugars of 17.0 mM (12.4 g/L) maltose, 6.2 mM (3.1 g/L) maltotriose, and 0.17 mM (0.24 g/L) fructose and a lactic acid concentration of 13.6 mM (1.23 g/L). The fruitiness was rated medium to high with the IQR ranging from 5–7 out of 10. Overall acceptability was rated with an IQR ranging from 6.5–9.0 out of 10.

6.5 Discussion

In this study, we investigated four *Lachancea fermentati* strains isolated from kombucha. Genome analysis was performed to gain fundamental insights, to elucidate intraspecific differences due to their origin, and in an attempt to link the strains' genotypes to their phenotype in wort fermentations. The strains were characterized by, e.g., their sugar utilization and stress sensitivities to evaluate their suitability in beer brewing. Screening in wort was performed to investigate intraspecific differences and to determine the best lactic acid producer. Subsequently, the fermentation parameters temperature, pitching rate, and glucose concentration were investigated to enhance lactic acid production. Finally, a low alcohol beer was produced at pilot-scale under optimized conditions.

The results from the genome analysis showed that the four kombucha isolates were diverse and generally separated into two groups, relating to their origin. The diploid isolates KBI 1.2, KBI 3.2, and KBI 12.1 exhibited high heterozygosity, an indication for intraspecific hybrids. Potentially, the isolates share a common ancestor based on patterns in loss of heterozygosity. This hypothesis is supported by the geographically close origin of KBI 1.2 and KBI 3.2, the USA. Due to the remote geographical origin of KBI 12.1 (Hawaii), its close phylogenetic relationship to KBI 1.2 and KBI 3.2, in contrast to KBI 5.3, calls for the assumption that an exchange of kombucha cultures between the Conterminous United States and Hawaii has taken place at some point. In fact, the exchange of kombucha cultures between kombucha brewers, and kombucha brewer communities has been common practice in the United States [75]. Unlike the isolates from the USA, KBI 5.3, which originates from Australia, showed a closer phylogenetic relationship to CBS 6772, which originates from South Korea, and CBS 707, whose country of origin is unknown. Unfortunately, to date, very limited sequence data is available for comparison.

Generally, compared to the extensively studied species *Saccharomyces cerevisiae*, the species *L. fermentati* or even the *Lachancea* genus has not been investigated thoroughly. Consequently, only with the initial assumption of a strong degree of homology between the yeast species, assumptions about the *Lachancea fermentati* metabolism can be made.

The greater resistance to low pH conditions of the *Lachancea* strains, compared to the brewers' yeast during the stress test, could tentatively be connected to their tendency to produce significant amounts of lactic acid during alcoholic fermentation. The strains must

constantly export lactate and H^+ out of the cell to maintain proton motive force and for this reason may be pre-adapted to high concentrations of H^+-ions. In addition, the acidic kombucha environment has also likely selected for strains with enhanced tolerance to high acid concentrations and low pH values [21].

Beside obtaining fundamental insights into the strains' characteristics, we aimed to optimize the lactic acid production by *L. fermentati* during fermentation. The observed values of lactic acid production (between 1.33 to 3.47 mM) in wort extract of the investigated *L. fermentati* strains were low compared to previously reported values. Osburn et al. [26] reported lactic acid production of 10 mM by a *L. fermentati* strain in a 11.4 °P wort at a pitching rate of approximately 5×10^6 cells/mL and 21.7 °C. In a previous study with KBI 12.1, Bellut et al. [20] reported the production of 14.4 mM of lactic acid in a 6.6 °P wort at a pitching rate of 8×10^6 cells/mL and 25 °C. However, the aforementioned studies used different fermentation conditions (e.g., pitching rate, temperature) and substrates, which has a significant influence on the lactic acid production, as we have shown in this study. Bellut et al. [76] already reported significant differences in lactic acid production by KBI 12.1 in different substrates from cereals, pseudocereals and pulses which could not be traced back to the sugar spectrum or free amino acid spectrum, further underlining the poor state of knowledge regarding factors that modulate lactic acid production in *Lachancea fermentati*.

Whole genome sequencing was performed to connect observations in the phenotype to the genotype of the individual strains. KBI 5.3 carried a mutation (397C>T) in the gene LAFE_0A07888G, resulting in a premature stop codon (Gln133*) (Supplementary Data Sheet 1; Appendix). LAFE_0A07888G is a gene with high similarity to the *JEN1* gene in *S. cerevisiae*. *JEN1* encodes for the monocarboxylate transporter Jen1 that was shown to be a lactic acid exporter [77], enhancing lactic acid yield in *S. cerevisiae* strains transformed with bacterial lactic acid dehydrogenases [78,79]. The nonsense mutation in the *JEN1*-homologue of KBI 5.3 could tentatively have been the reason for the significantly low lactic acid production in comparison to the other *L. fermentati* strains. However, besides Jen1p, at least one other lactic acid transporter exists [78], which could tentatively explain the remaining, albeit low, lactic acid production. In addition, a single nucleotide deletion (230delT) was also observed in LAFE_0E15192G in the strain KBI 5.3 (Supplementary Data Sheet 1; Appendix). LAFE_0E15192G shows some similarities with *S. cerevisiae* YML054C *CYB2*, a cytochrome b2 (L-lactate cytochrome-c oxidoreductase) component

of the mitochondrial intermembrane space which is required for lactate utilization (and repressed by glucose and anaerobic conditions) [29]. This frameshift mutation could tentatively explain the inability to grow in lactic acid as the sole substrate in the API test. However, these effects should be tested in future studies by reverse engineering.

As the RSM optimization and added glucose trial have shown, lactic acid production by KBI 12.1 is highly dependent on the pitching rate, fermentation temperature and initial glucose concentration. Lactic acid production by the strain KBI 12.1 varied from 0.5 to 18.0 mM based on the fermentation conditions and substrate composition. It was shown that, in order to increase lactic acid production by *Lachancea fermentati* KBI 12.1 in wort, the glucose concentration should be as high as possible, the pitching rate low (5×10^6 cells/mL) and the fermentation temperature high (≥ 25°C).

The high lactic acid production in samples with a low pitching rate suggests that lactic acid production mostly took place during the growth phase at the beginning of fermentation. In contrast, under the same conditions, but at high pitching rates, little lactic acid was produced. This hypothesis was supported by fermentation data from the scaled-up brewing trial where 84% of total lactic acid was already produced in the first 36 hours of fermentation, while the cells in suspension grew from 5 to 43×10^6 cells/mL. However, in the case of the scaled-up fermentation, the lactic acid production also correlated with the consumption and depletion of glucose.

On a molecular level, the metabolization of pyruvate via lactic acid dehydrogenase is an additional means of NADH recycling, with pyruvate decarboxylase and alcohol dehydrogenase being the more common pathway. NADH is produced during glycolysis, the yeasts' ATP-generating pathway under anaerobic conditions, and has to be recycled to NAD^+ (Figure 6.5–1). However, while ethanol can leave the cell by passive diffusion, lactic acid has to be actively transported out of the cell at the expense of ATP. This is due to the fact that at high intracellular pH, lactic acid dissociates into lactate and a proton. In order to maintain proton motive force and intracellular pH, this proton has to be exported via the plasma membrane H^+-ATPase, with the expense of one ATP per proton. In the worst case, lactate export is also ATP-dependent, though the exact mechanisms are still unknown [80–82]. Abbott et al. [80] confirmed that the lactic acid export requires energy in the form of ATP, which was shown by a full ATP depletion during anaerobic homolactate fermentation with a *S. cerevisiae* strain. Outside of the cell, at a low extracellular pH, the lactic acid is again present in its protonated form and can thus

permeate the cell membrane via passive diffusion, creating an energy-requiring cycle [29]. Available evidence suggests therefore that the recycling of NADH via the lactic acid pathway seems to be more expensive for the cell than the ethanol pathway. Why the lactic acid pathway is chosen in the first place, at least at the beginning of fermentation, is still unknown and highlights the need for more research in this area. Presumably, the simultaneous recycling of NADH via the lactic acid and the ethanol pathway, resulting in an accumulation of lactic acid, developed as a strategy to compete with other microbes, comparable to the "make-accumulate-consume" strategy for ethanol in *S. cerevisiae* [83,84]. In a study on *S. cerevisiae*, Pacheco et al. [78] found that when glucose is present, the produced lactic acid is exported out of the cell via Jen1 and Ady2, but when glucose (acting as the single carbon source) is depleted, the transporters are also actively involved in lactic acid consumption.

There is a general consensus that all maltose transport systems in *S. cerevisiae* so far characterized mediate the transport into the yeast cells against a concentration gradient in symport with protons. This proton import is balanced by proton export via the plasma membrane H^+-ATPase, at the expense of one ATP per proton. This means, that the uptake of one molecule of maltose comes at the expense of one molecule of ATP [85]. Consequently, while glucose enters the cell via facilitated diffusion, maltose has to be actively imported into the cell via proton symport. The consequent export of the proton at the expense of ATP lowers the net ATP yield from maltose to 1.5 ATP per glucose molecule, instead of 2 ATP per molecule of glucose which entered the cell via facilitated diffusion. Figure 6.5–1 illustrates the simplified cellular mechanisms involved in lactic acid production and proton motive force maintenance in *Lachancea fermentati* in anaerobic wort fermentations assuming fundamental homology to *S. cerevisiae*.

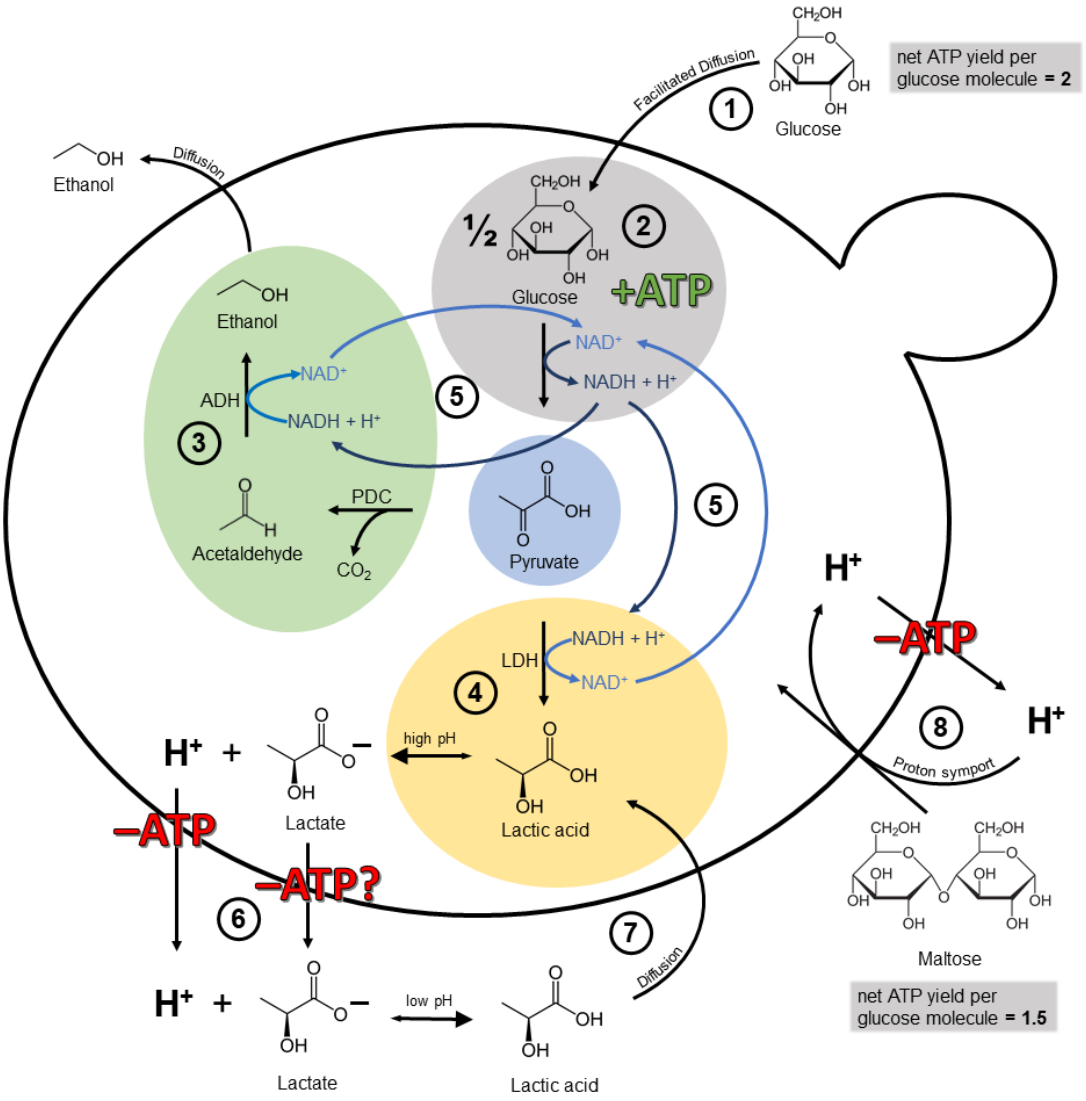


Figure 6.5–1 Simplified illustration of the cellular mechanisms involved in lactic acid production and self-inhibition in *Lachancea fermentati* in anaerobic wort fermentations under the assumption of fundamental homology to *Saccharomyces cerevisiae*. Adapted from Sauer at al. [29] and Bellut et al. [20].
1 Glucose transport into the cell by facilitated diffusion. The net ATP yield per glucose molecule is 2.
2 Glycolysis, yielding one molecule of ATP per molecule pyruvate formed and one molecule of NADH which has to be recycled to NAD^+.
3 Ethanol production via pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). The ethanol can leave the cell by passive diffusion.
4 Lactic acid production via lactic acid dehydrogenase (LDH). At high intracellular pH, the lactic acid dissociates into lactate and H^+.
5 Both, ethanol formation and lactic acid formation are a means to recycle NADH to NAD^+.
6 At the very least, the H^+ has to be exported out of the cell at the expense of one molecule of ATP. In the worst-case scenario, ATP-dependent mechanisms may be involved in both proton and anion export [80,81].
7 At low extracellular pH, lactic acid is present in its protonated form and can enter the cell again via passive diffusion, creating an energy-requiring cycle with 6.
8 Maltose transport into the cell is facilitated via proton symport. Consequently, the proton has to be exported out of the cell at the expense of ATP. For that reason, the net ATP yield per glucose molecule from maltose is 1.5 instead of 2.

The results from this study indicate that at the beginning of fermentation, at relatively high pH, low lactic acid concentration and the presence of glucose, *L. fermentati* KBI 12.1 can afford to simultaneously recycle NADH via the lactic acid pathway. A shift towards the ethanol pathway as the sole means of NADH recycling seemed to occur once glucose was depleted and the proton motive force maintenance became more costly due to a reduced net ATP yield from maltose compared to glucose, combined with an increasing stress caused by increased lactic acid concentrations (Figure 6.5–1). However, although our results give first indications on the underlying mechanisms for lactic acid production modulation in *L. fermentati* in wort fermentations, more research on ATP utilization and redox balance is necessary to draw conclusions.

It was possible to create a low alcohol beer (1.26% ABV) with KBI 12.1 by interrupting the fermentation after 36 hours. The panelists evaluated the ratio of residual sweetness to acidity caused by lactic acid as balanced, giving a good indication for future applications. However, by removing the yeast from the wort prematurely, significant amounts of diacetyl were left in the young beer which can negatively affect the flavor of the beer, limiting the use of this strain for stopped fermentation. The high diacetyl concentration could potentially be tackled post-fermentation with an enzyme treatment by immobilized α-acetolactate decarboxylase [86]. In a previous study, Bellut et al. [20] produced a low alcohol beer with KBI 12.1 from a 6.6 °P wort. However, the ethanol concentration was, at 2.6% ABV, considerably higher after final attenuation was reached, compared to 1.26% ABV after the interruption of fermentation in this study. Due to the consumption of all fermentable sugars and a high lactic acid production (14.4 mM), the taste of the beer was also characterized as sour. However, diacetyl was below its flavor threshold since the fermentation came to a halt naturally.

To conclude, while the exact mechanisms for lactic acid production in *Lachancea fermentati* remain unknown, we have elucidated influencing factors and were able to shine some light on the KBI 12.1 strain's behavior in wort fermentations regarding an enhanced lactic acid production and its consequent induction of a premature fermentation inhibition. We showed that the strain can afford the energy-expensive lactic acid production until a high concentration is reached (here up to 18 mM) only as long as glucose is present in the wort. A low alcohol beer could be produced which had a balanced profile between sweetness from residual sugars and acidity from the produced lactic acid. However, due to the premature cessation of fermentation, diacetyl was present above its flavor threshold.

Future application trials should focus on finding the ideal extract value and ideal sugar spectrum of the wort to facilitate high lactic acid concentrations in balance with residual sweetness while still reaching final attenuation. To validate the hypothesis of the influence of the mutated *JEN1*- and *CYB2*-similar genes in KBI 5.3 leading to a reduced lactic acid production, gene knock-out experiments in the strains without the mutation could lead to further insights regarding the modulation of lactic acid production in *Lachancea fermentati*.

6.6 Data Availability Statement

The Illumina reads generated in this study have been submitted to NCBI-SRA under BioProject number PRJNA587400 in the NCBI BioProject database. (Date of Publication February 2020).

6.7 Author contributions

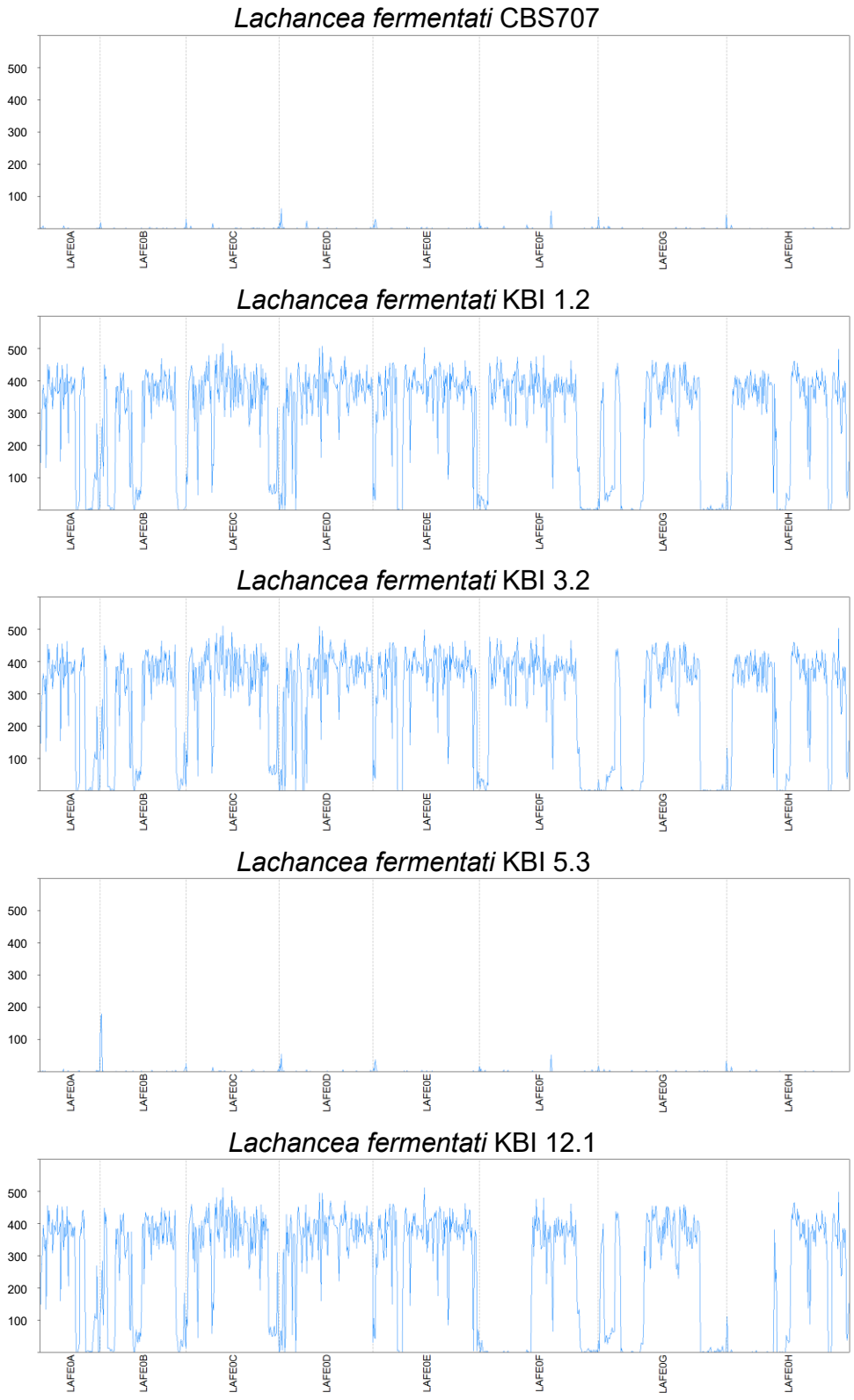
KB performed the fermentation experiments, analyses and statistics described in this study. KK performed flow cytometry and bioinformatic analysis of the whole genome sequence data. KB, KK, and EA designed the experiments. KB and KK wrote the manuscript. EA supervised this study. All authors read and approved the final manuscript.

6.8 Acknowledgements

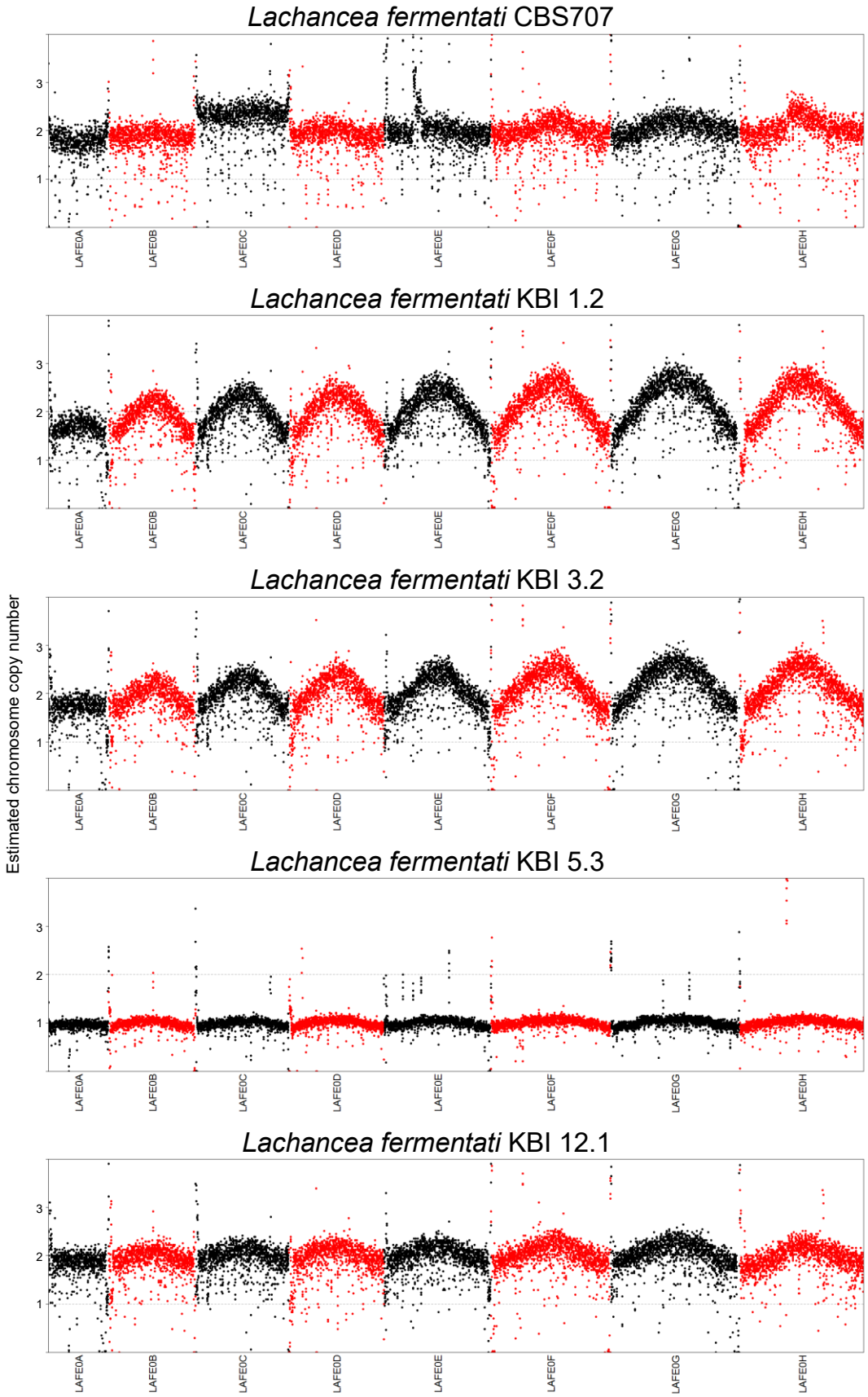
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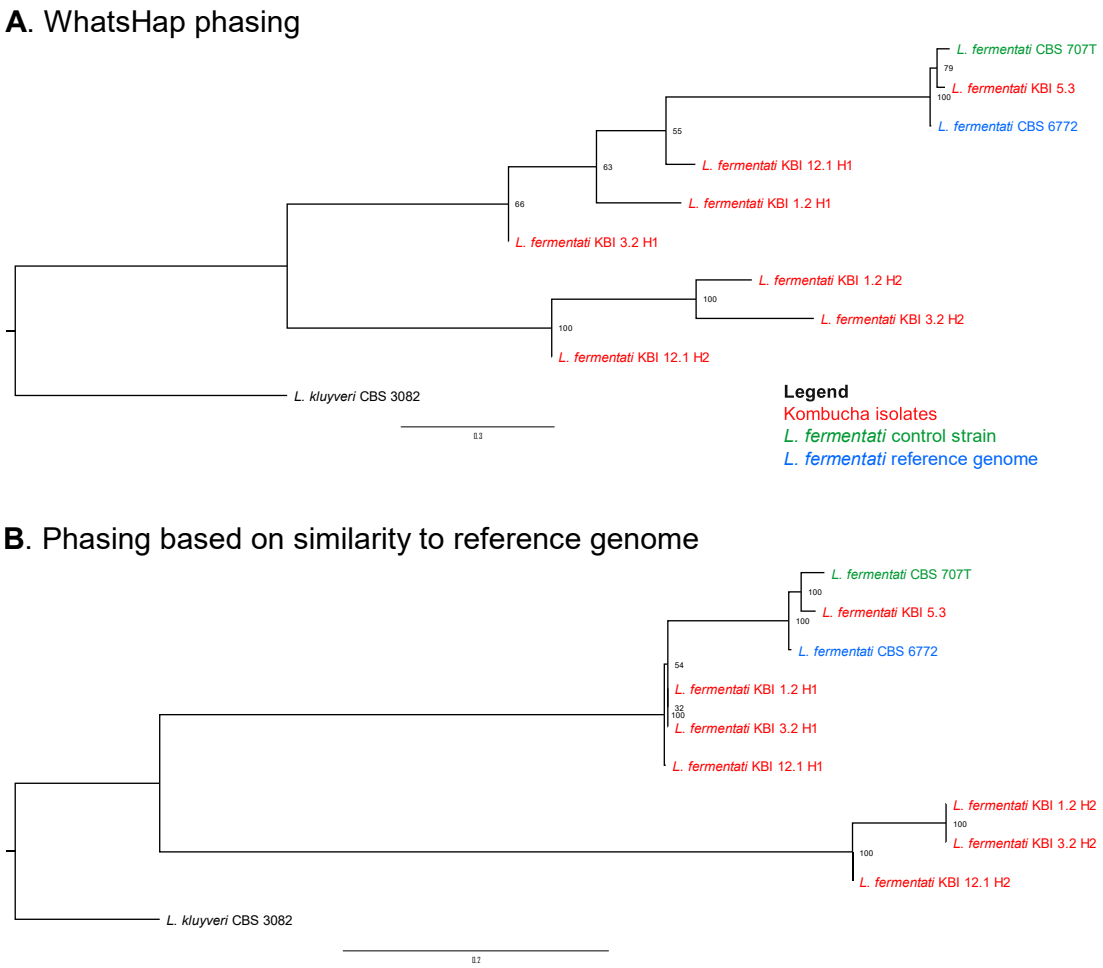
6.9 Supplementary Figures



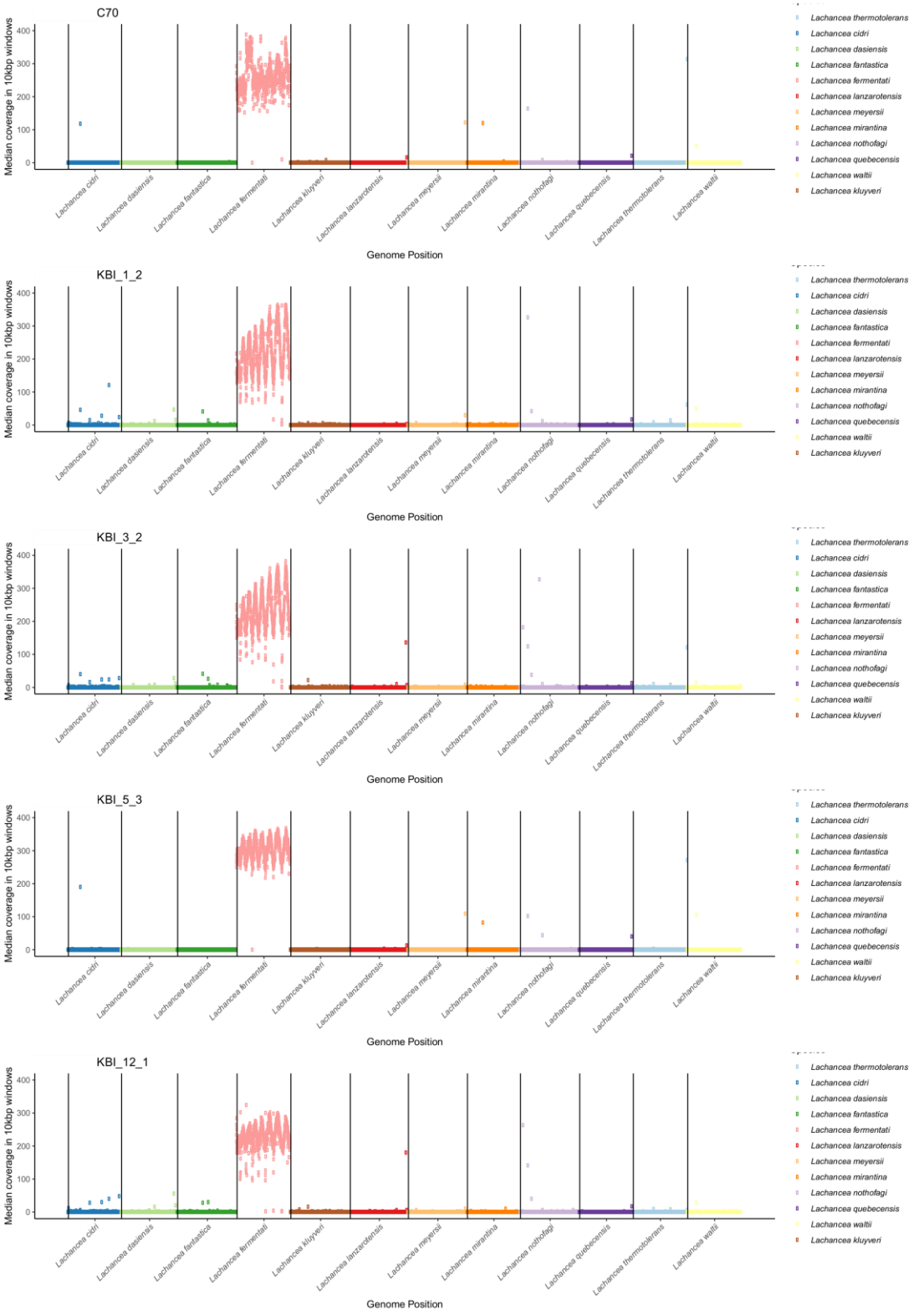
Supplementary Figure 6.9–1 The number of heterozygous single nucleotide polymorphisms (in 10 kbp windows) in the five sequenced *Lachancea fermentati* strains compared to the *L. fermentati* CBS 6772 (NCBI Accession GCA_900074765.1) reference genome. Values close to zero indicate regions lacking heterozygosity.



Supplementary Figure 6.9–2 Estimated chromosome copy numbers of the five sequenced *Lachancea fermentati* strains based on the sequencing coverage (median coverage in 1 kbp windows) of reads aligned to the *L. fermentati* CBS 6772 (NCBI Accession GCA_900074765.1) reference genome.



Supplementary Figure 6.9–3 Maximum likelihood phylogenetic trees based on phased single nucleotide polymorphisms (SNPs) at 6330 sites in the six *L. fermentati* and one *L. kluyveri* genomes (rooted with *L. kluyveri* as outgroup). Numbers at nodes indicate bootstrap support values. Branch lengths represent the number of substitutions per site. SNPs in (A) were phased with WhatsHap based on reads containing two or more heterozygous SNPs, while SNPs in (B) were phased based on similarity to the reference genome as described by Ortiz-Merino et al. [44].



Supplementary Figure 6.9–4 The median coverage in 10 kbp windows of sequencing reads from the five sequenced *Lachancea fermentati* strains aligned to a concatenated reference genome consisting of 12 species in the *Lachancea* genus. Reads align exclusively to *L. fermentati*, ruling out that any of the strains were interspecific hybrids.

For Supplementary Data Sheets 1 and 2, refer to Appendix.

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Chapter 7:

General discussion

7.1 General discussion

In recent years, non-alcoholic and low alcohol beer (NABLAB) has been on the rise. From 2015 to 2018, three of the four biggest brewing companies in the world, which together amount to 48% of the world's beer production (as of 2018 [1]), have introduced their flagship beers as non-alcoholic versions (0.0% ABV). Carlsberg launched their flagship brew as the non-alcoholic version "Carslberg 0.0" in 2015 [2]. In 2017, Heineken launched "Heineken 0.0" in the Netherlands, and sales expanded into 16 European markets and the United States by 2019 [3]. In 2018 alone, AB InBev launched 12 new non-alcoholic beer (NAB) products, adding to their wide range of non-alcoholic versions of popular brands like Budweiser, Hoegaarden, and Leffe [4]. In addition to the big brewers, small and middle-sized breweries expand their product portfolio to satisfy the growing NABLAB demand. NAB with an alcohol content below 0.05% ABV is usually achieved with thermal dealcoholization methods, which requires substantial investment into dealcoholization equipment [5]. Via limited fermentation, NAB below 0.5% ABV can be achieved without the necessity of special equipment. In fact, the application of maltose-negative yeasts with limited fermentation capacity in wort (e.g. *Saccharomycodes ludwigii*) is an easy adjustment to make for breweries of all sizes. In this thesis, alternative non-*Saccharomyces* yeasts were investigated for their suitability and applicability to produce NABLAB with improved and novel flavor characteristics.

The literature review (**Chapter 2**) quantified the growing interest in NABLAB by consumers, as well as a strong growth of the NABLAB sector within the beer market, which is forecast to continue. Consumer and sensory studies revealed recurring disappointment in connection with non-alcoholic beer (NAB) consumption, owed to its taste deficits in comparison to regular beer [6–8]. It was found that with non-*Saccharomyces* yeasts fermented end products often revealed a fruity character in sensory evaluations. This opens up the chance to produce NABs with novel, atypical flavor characteristics. Those NABs could create a new category within NABLABs, distinguishable from common methods and therefore lacking reference points, which would offer an opportunity to avoid consumer disappointment. In fact, consumer studies on NAB suggested that it should be treated as a category in its own right and that any comparison to regular beer, especially regarding the taste, should be avoided [7]. Regarding available literature on non-*Saccharomyces* yeasts in wort fermentations, the old concept of using

Saccharomycodes ludwigii for NAB brewing has seen revival in the application of a wide range of non-*Saccharomyces* species. However, recent studies were still predominantly conducted on laboratory scale and were often lacking sensory evaluation [9].

When applying non-*Saccharomyces* yeasts for brewing purposes, several boxes have to be checked. The sugar utilization pattern of the yeast is a first indicator for its suitability in NABLAB brewing. The composition of fermentable sugars in all barley malt wort usually amounts to approximately 64–70% maltose, 13–19% maltotriose, 10–14% glucose, 2–4% sucrose, and 2–3% fructose [10–13]. Non-*Saccharomyces* yeasts for NABLAB brewing should be selected for their inability to consume maltose and maltotriose, the most abundant wort sugars, which would naturally limited fermentation and, consequently, ethanol production. Therefore, the sugar utilization pattern can give an indication of the strain's ability for NABLAB brewing. However, the outcome of sugar utilization patterns should be interpreted in concert with the assay procedure. "False-positive" results are common, when the assay is performed under aerobic conditions where the yeast shows positive results for maltose utilization which can differ significantly under anaerobic conditions during fermentation, also described as the Kluyver effect [14,15]. Consequently, only maltose-positive strains under anaerobic conditions should be disregarded for NAB brewing based on the outcome of the sugar utilization test. Sucrose utilization can also differ widely amongst genera and species [13,14,16]. For NAB brewing in a diluted 7 °P wort, sucrose utilization can make a difference of around 0.09–0.15% ABV in the final product, which should not be neglected [13,16]. Missing sucrose utilization also entails an increased contamination risk with other microbes during fermentation or in the end product. However, pasteurization is essential in any case when substantial amounts of residual sugars remain in the final product as it is the case for NABs produced via biological methods. Ultimately, for the screening of non-*Saccharomyces* strains in wort for their application in NAB brewing, 7 °P should be the maximum extract content to not exceed a final ethanol concentration of 0.5% ABV [13,16–18].

Other attributes that can be checked prior to fermentation trials in wort include phenolic off-flavor (POF) production, yeast flocculation, and the resistance to diverse stress factors including hop iso-α-acids, ethanol, pH, weak acids (i.e. lactic acid), and osmotic stress. POF production should be negative, unless it is desired to suit the beer style (e.g. Bavarian style wheat beer, some Belgian beers). Yeast flocculation can give an indication about yeast handling in terms of potential bottom cropping for re-pitching. However, most non-

Saccharomyces yeasts tend to show low flocculation [12,15]. Desired resistance to stressors depends on the intended application of the strain, e.g. if the strain is required to ferment highly hopped worts (e.g. India Pale Ales), or soured worts (e.g. sour beer production).

Similar to Michel et al. [18], who developed a screening system for non-*Saccharomyces* yeasts in brewing applications, the proof-of-concept study with five non-*Saccharomyces* species isolated from kombucha applied a screening system to evaluate the strains' suitability for NAB brewing (**Chapter 3**). The study with five pre-selected non-*Saccharomyces* species isolated from kombucha was performed to investigate if non-*Saccharomyces* species are suitable for producing NAB and if they can compete with commercially applied NAB strain *Saccharomycodes ludwigii*. In summary, all non-*Saccharomyces* strains performed comparably to *S. ludwigii*. It was shown that the applied maltose-negative strains of the species *Hanseniaspora valbyensis, H. vineae, Torulaspora delbrueckii, Zygosaccharomyces bailii,* and *Z. kombuchaensis* performed comparably to the commercially applied *Saccharomycodes ludwigii* strain TUM SL 17. The non-*Saccharomyces* strains exhibited excellent propagation performance and reached high cell numbers ranging from twice to over eight times the amount of *S. ludwigii* cells. All strains were able to ferment a 6.6 °P wort to a maximum ethanol content of 0.5% ABV in the same time (± 1 day) as *S. ludwigii*. The sensory evaluation showed a strong wort-like flavor for all end products. However, some NABs produced with the kombucha strains were additionally granted atypical flavors like "black tea" and "white wine". In summary, all NABs produced by the non-*Saccharomyces* yeasts, including *S. ludwigii*, were statistically indifferentiable. None of the strains were able to mask or reduce the wort-like off flavor, however, neither did they underperform compared to commercially applied *S. ludwigii*, indicating their suitability for NAB brewing. The study showed, that a wide range of non-*Saccharomyces* yeasts can be applied in NAB brewing. However, there was much room for improving the flavor characteristics and decrease the wort-like character, suggesting a continued search for the ideal species or strain.

Following the results from this proof-of-concept study, with the study on *Cyberlindnera* (**Chapter 4**), a genus was investigated whose species are particularly known for their high ester production [19–22]. The objective was to harness this increased ester production to produce a NAB with reduced wort-like off-flavor due to an increased fruity character. The study followed the previous study's approach of a basic strain characterization and screening to identify the best performing species/strain. Subsequently, the fermentation

conditions were optimized by means of response surface methodology (RSM) to enhance the fruity character. Finally, a NAB was produced on pilot-scale (60 L) and the end product was compared to commercial NABs in a sensory evaluation. It was shown that four out of the six investigated strains produced a pleasant fruity character in wort while exhibiting low ethanol production due to missing maltose utilization. Fermentation by-product analysis revealed that the type of ester production was just as important as the quantity. A high ethyl acetate production led to an unpleasant solvent-like aroma in one fermented sample. The samples with a pleasant fruity aroma exhibited only moderate ethyl acetate concentrations and higher concentrations of isoamyl acetate. Fermentation optimization by means of RSM showed that the fruity character could be accentuated at low pitching rates and low fermentation temperatures. A NAB (0.36% ABV) with the strain *Cyberlindnera subsufficiens* C6.1 was successfully produced on 60 L pilot-scale and compared to two commercially available NABs. In a sensory evaluation, the C6.1 NAB was significantly more fruity and significantly less wort-like compared to the commercial NABs. This study underlines the potential of non-*Saccharomyces* yeasts with the example of *Cyberlindnera subsufficiens* to produce NABs with novel flavor characteristics which are free from the often-criticized wort-like off flavor. A recent example for the successful commercialization of a non-*Saccharomyces* yeast in NABLAB brewing by a very similar approach is NEER™ by Chr. Hansen [23]. The product provides a direct-pitch solution with a non-*Saccharomyces* strain to produce NABLAB in an easy-to-apply manner. It is presumably based on a patent by Saerens and Swiegers [17], and utilizes a *Pichia kluyveri* strain which produces high amounts of fruity esters while producing little alcohol.

Regarding low alcohol beers (LAB) produced with non-*Saccharomyces* species, beers with an ethanol content between 0.5 and 3.5% ABV, a different approach than just limited fermentation with a diluted wort was pursued in this thesis. Once again, a special metabolic trait was harnessed to create a novel LAB type: Species of the *Lachancea* genus possess the for yeasts' uncommon ability to produce significant amounts of lactic acid during alcoholic fermentation. Previously investigated to reduce pH and enhance total acidity in wine fermentations [24–27], the *Lachancea* species *L. thermotolerans* and *L. fermentati* recently made their way into brewing research for flavor modification [28], or the production of a single culture sour beer [29–31].

The objective of the first study on *Lachancea fermentati* strain KBI 12.1 isolated from kombucha (**Chapter 5**) was to investigate its suitability to produce a low alcohol beer in

a diluted wort; but more importantly, to introduce the idea of harnessing its significant lactic acid production to counteract residual sweetness in LAB produced via stopped fermentation. Unlike the previously applied non-*Saccharomyces* strains, *Lachancea fermentati* can utilize maltose, which leads to a considerably higher final attenuation in wort fermentations compared to maltose-negative strains. However, *Lachancea fermentati* is still unable to utilize maltotriose.

Like in the previous studies, this study again included the investigation of fundamental brewing characteristics (i.e. sugar utilization, flocculation, hop sensitivity, propagation performance), which underlined the strain's suitability for brewing applications. In laboratory scale fermentations of a 6.6 °P wort, ethanol concentrations at final attenuation were, at 2.2% ABV, 15% lower compared to a brewers' yeast, owing to its inability to utilize maltotriose. The sensory evaluation showed, that the significant lactic acid production (1.3 g/L) led to a sour taste of the final product. In order to achieve a LAB with a more balanced sweetness-acidity ratio, the idea was introduced to stop fermentation of *Lachancea fermentati* KBI 12.1 at a point where the produced lactic acid would counteract the residual sweetness from residual sugars before final attenuation was reached. This approach would result in a further decreased final ethanol concentration while the residual sugars would balance the lactic acid produced. Additionally, the lactic acid production significantly reduces the pH of the beer, which is favorable for resistance against microbial spoilage [32]. Recent studies have suggested the use of *Lachancea fermentati*, *Lachancea thermotolerans* and other lactic acid-producing yeasts to create single culture sour beers, with the latter already being used in commercial brewing [29,30,33]. Additionally, the global yeast company Lallemand recently introduced SOURVISIAE® to the North American market [34]. The strain is a genetically modified *Saccharomyces cerevisiae* brewers' yeast that produces high amounts of lactic acid and is able to produce single culture sour beer. However, to harness the lactic acid production by yeasts to counteract residual sweetness in the production of LAB is a completely new approach.

To proceed with this approach, it was important to gain more fundamental insights about which factors modulate lactic acid production by *Lachancea fermentati*. Lactic acid production by yeasts is a generally underexplored topic even for *Saccharomyces cerevisiae*, let alone the *Lachancea* genus. Therefore, a study on four *Lachancea fermentati* strains, including comprehensive fundamental investigation and practical application to produce a LAB, was conducted (**Chapter 6**). The origin of the four investigated *L. fermentati* were

individual kombucha cultures. In addition to whole genome sequencing (WGS) and genome analysis, the fundamental part of the study included a more comprehensive stress test in an attempt to find connections between the strains' genotypes and their phenotypes. It was no surprise that barely any connection between the strains' genotype and their varying lactic acid production could be established due to the very limited availability of reference genomes and studies on *Lachancea fermentati*. During the fermentation optimization with the best lactic acid producer KBI 12.1, for the first time for the *Lachancea* genus, fermentation parameters to enhance lactic acid production were identified. It was shown that a high initial glucose concentration, high temperature and low pitching rate yielded the highest lactic acid concentrations. Comparable to the study on *Cyberlindera subsufficiens*, the findings from the fermentation optimization were used for recipe and process development for the pilot-scale brewing trial. The production of a LAB (1.26% ABV) via stopped fermentation with *L. fermentati* KBI 12.1 was shown to be applicable on pilot-scale and yielded a well-balanced product. The results indicated that the lactic acid production can easily be modulated with the sugar profile of the wort and fermentation conditions. Due to the high impact of glucose in enhancing lactic acid production, with a lower starting extract content comprising mostly or totally of glucose, NAB production at or below 0.5% ABV could be feasible and should be investigated. In the first study on *Lachancea fermentati* KBI 12.1 (**Chapter 5**), it was impossible to identify the importance of glucose in the lactic acid modulation due to its low initial amount and because sampling was done every 24 h instead of every 12 h, as done in the second study. This underlines the importance of in-depth investigation and singling out the individual fermentation parameters to optimize and customize fermentation to match the desired end product.

A challenge when using diluted wort (e.g. 7 °P) is the consequently diluted free amino nitrogen (FAN) and free amino acids (FAA) content. However, during the studies making up this thesis, FAN was never found to be a limiting factor when using non-*Saccharomyces* yeasts to produce NABLABs. It was concurrently shown that the non-*Saccharomyces* species in this study consumed far less FAN (and FAA) during fermentation compared to brewers' yeast. On the one hand, this observation was owed to the generally less intensive fermentation but was also observed in the production of LAB, where fermentation intensity (i.e. final attenuation) was similar to that of brewers' yeast. These observations are in accordance with existing literature where it was found that non-

Saccharomyces yeasts consume less FAN compared to brewers' yeast and may be less demanding in terms of FAN and FAA content of the wort [35].

Response surface methodology (RSM) showed to be a valuable tool for fermentation optimization to boost certain characteristics. However, because wort is a very complex substrate, prediction about total values should be made with caution. RSM was rather applied to estimate how adjusting process parameters would enhance or decrease the prevalence of certain fermentation by-products or aromas (e.g. fruity aroma, lactic acid). It proved to be an invaluable tool for recipe development of the subsequent pilot-scale brewing trials. In both cases when RSM was applied to optimize fermentation conditions, a low pitching rate (5–10×10^6 cells/mL) showed to be favorable despite the substantially smaller cell size compared to brewers' yeast. This is in contrast with findings by Michel et al. [36] who found a high pitching rate (6×10^7 cells/mL) to be favorable in an optimization study on *Torulaspora delbrueckii* in beer production.

In regard to the safety aspect of *Lachancea fermentati*, with WGS, groundwork has been established in this thesis. Pariza et al. [37] introduced a decision tree for safety assessment of strains to be used in food applications. The required hurdles include whole genome sequencing and analysis for e.g., genetic elements encoding virulence factors and/or toxins associated with pathogenicity; recorded history of safe consumption of isolation source (if isolated from food); and a comprehensive peer-reviewed safety evaluation to affirm safety for food use by an authoritative group of qualified scientific experts. Besides the Qualified Presumption of Safety (QPS) list, which covers only a limited amount of species, the "2012 Inventory of Microbial Species with technological beneficial role in fermented food products" by the International Dairy Federation (IDF) and European Food and Feed Cultures Association (EFFCA) became a de facto reference for food cultures in practical use [38–40]. The IDF/EFFCA inventory, which covers a wide range of food matrices including dairy, meat, fish, vegetables, cereals, beverages, and vinegar, lists the species *Lachancea fermentati* due to its usage in wine fermentations [41,42]. Of the *Cyberlindnera* species, only *C. jadinii* and *C. mrakii* are named in the inventory, owed to their recorded usage in dairy and wine, respectively [41]. *Cyberlindnera subsufficiens* is not named on the list. Regarding biogenic amines, the European Food Safety Authority (EFSA) evaluated alcoholic fermentation not to be of concern for biogenic amines production due to a lack of evidence about massive formation of biogenic amines by yeast [43].

The results from this thesis lay the foundation for the application of a wider variety of non-*Saccharomyces* species in commercial NABLAB brewing. This thesis illustrated the development of NABLABs, starting with the isolation of non-*Saccharomyces* strains, their identification, characterization for brewing applications, screening in wort, fermentation optimization, and recipe development all the way to pilot-scale brewing. This process can generally be applied and adjusted to all non-*Saccharomyces* species as it was applied to twelve different non-*Saccharomyces* species from six different genera throughout the studies making up this thesis. It was shown that strain-specific metabolic traits can be harnessed and accentuated to improve technical and sensorial properties of the NABLABs produced. NAB with *Cyberlindnera sufficiens* C6.1 and LAB with *Lachancea fermentati* KBI 12.1 was successfully produced at 60 L pilot-scale with the prospect of further upscaling. In order to accentuate the yeast flavors in the pilot-scale brewing trial, a very basic recipe in terms of malt and hop addition was applied. Only pilsner malt, a very pale malt with little flavor, and small amounts of bittering hops were used. The big variety in malt products and bittering, flavoring, and aroma hops on the market give great leeway for recipe improvements.

To conclude, this thesis showed that selected non-*Saccharomyces* species are capable of performing well in brewers' wort. It was shown that fermentation conditions and substrate can be optimized to accentuate desired characteristics. Special metabolic traits of non-*Saccharomyces* yeasts can be harnessed to create novel NABLAB types, i.e. high production of fruity esters by *Cyberlindnera subsufficiens* to create a fruity NAB, and the exploitation of lactic acid production by *Lachancea fermentati* to create LAB with a balanced taste profile by the means of stopped fermentation. Additionally, it was shown that the right non-*Saccharomyces* strain (here *Cyberlindnera subsufficiens* C6.1) has the potential to produce a better NAB than comparable products on the market. When brewing with adjuncts, the sugar profile of the resulting wort is the most important factor and has to be considered in concert with the applied strain's sugar utilization patterns. The pilot-scale brewing trials have shown that NABLAB production with non-*Saccharomyces* yeasts is applicable in practice, which gives prospect to further scale-up to industrial scale and strengthens their position as a serious alternative to established NABLAB production methods.

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Appendix

List of publications and presentations

First author publications in peer-reviewed Journals

Bellut, K.; Michel, M.; Zarnkow, M.; Hutzler, M.; Jacob, F.; De Schutter, D. P.; Daenen, L.; Lynch, K. M.; Zannini, E.; Arendt, E. K. Application of Non-*Saccharomyces* Yeasts Isolated from Kombucha in the Production of Alcohol-Free Beer. *Fermentation* **2018**, *4*, 1–13. DOI:10.3390/fermentation4030066.

Bellut, K.; Arendt, E. K. Chance and Challenge: Non-*Saccharomyces* yeasts in nonalcoholic and low alcohol beer brewing: A Review. *J. Am. Soc. Brew. Chem.* **2019**, *77*, 77–91. DOI:10.1080/03610470.2019.1569452.

Bellut, K.; Michel, M.; Hutzler, M.; Zarnkow, M.; Jacob, F.; De Schutter, D. P.; Daenen, L.; Lynch, K. M.; Zannini, E.; Arendt, E. K. Investigation into the application of *Lachancea fermentati* strain KBI 12.1 in low alcohol beer brewing. *J. Am. Soc. Brew. Chem.* **2019**, *77*, 157–169. DOI:10.1080/03610470.2019.1629227.

Bellut, K.; Michel, M.; Zarnkow, M.; Hutzler, M.; Jacob, F.; Lynch, K. M.; Arendt, E. K. On the suitability of alternative cereals, pseudocereals and pulses in the production of alcohol-reduced beers by non-conventional yeasts. *Eur. Food Res. Technol.* **2019**, *245*, 2549–2564. DOI:10.1007/s00217-019-03372-3.

Bellut, K.; Michel, M.; Zarnkow, M.; Hutzler, M.; Jacob, F.; Atzler, J. J.; Hoehnel, A.; Lynch, K. M.; Arendt, E. K. Screening and application of *Cyberlindnera* yeasts to produce a fruity, non-alcoholic beer. *Fermentation* **2019**, *5*, 1–25.

Bellut, K.; Krogerus, K.; Arendt, E. K. *Lachancea fermentati* strains isolated from kombucha: fundamental insights, and practical application in low alcohol beer brewing. *Front. Microbiol.* (peer review). Date of submission: 20/01/20

Other publications

Peyer, L. C.; Bellut, K.; Lynch, K. M.; Zarnkow, M.; Jacob, F.; De Schutter, D. P.; Arendt, E. K. Impact of buffering capacity on the acidification of wort by brewing-relevant lactic acid bacteria. *J. Inst. Brew.* **2017**, *123*, 497–505. DOI:10.1002/jib.447.

Bellut, K.; Lynch, K. M.; Arendt, E. K. Alcoholic beverages. In *Handbook of Molecular Gastronomy: Scientific Foundations and Culinary Applications*; Lavelle, C., Herve, T., Kelly, A. L., Burke, R., Eds.; **2020**; ISBN 1-4665-9478-0 (in press).

Oral presentations

Bellut, K.; Michel, M.; Zarnkow, M.; Hutzler, M.; Jacob, F.; De Schutter, D. P.; Daenen, L.; Arendt, E. K. (2018). From kombucha to beer – The application of non-*Saccharomyces* yeasts isolated from kombucha in the production of a non-alcoholic beer. **13th International Trends in Brewing**, Ghent, Belgium, April 2018

Bellut, K.; Michel, M.; Zarnkow, M.; Hutzler, M.; Jacob, F.; De Schutter, D. P.; Daenen, L.; Lynch, K. M.; Zannini, E.; Arendt, E. K. (2018). Application of non-*Saccharomyces* yeasts in the production of alcohol-free beer. **Young Scientists Symposium on Malting, Brewing and Distilling**, Bitburg, Germany, September 2018

Bellut, K. and Arendt, E. K. (2019). Chance and Challenge: Non-*Saccharomyces* yeasts in nonalcoholic and low alcohol beer brewing. **37th Congress of the European Brewery Convention**, Antwerp, Belgium, June 2019

Poster presentations

Peyer, L. C.; Bellut, K.; Zarnkow, M.; Jacob, F.; Arendt, E. K. (2017). Strategies to improve lactic acid production from brewing-relevant lactic acid bacteria strains in wort substrates. **36th Congress of the European Brewery Convention**, Ljubljana, Slovenia, May 2017

Chapter 4: Supplementary Data Sheet

Data Sheet S1

Run	Factor 1 A:Temperature °C	Factor 2 B:Pitching Rate ×10^6 cells/mL	Response 1 Ethanol % ABV	Response 2 Ethyl acetate mg/L	Response 3 Isoamyl acetate mg/L	Response 4 Acetaldehyde mg/L	Response 5 n-Propanol mg/L	Response 6 Isobutanol mg/L	Response 7 Isoamyl alcohols mg/L	Response 8 SUM Esters mg/L	Response 9 SUM Alcohols mg/L	Response 10 Glycerol g/L	Response 11 Acceptance	Response 12 Fruitiness
1	22	60	0.526	4.5	1.5	2.0	3.6	4.6	11.4	6.00	19.6	0.240	2.46	2.13
2	22	10	0.468	8.1	2.2	3.4	4.00	4.8	8.8	10.3	17.6	0.192	1.96	2.00
3	17	35	0.509	6.4	1.7	2.6	3.5	3.4	8.5	8.1	15.4	0.196	2.83	2.25
4	27	35	0.556	5.5	0.8	2.2	3.3	6.7	12.9	6.3	22.9	0.296	1.71	1.42
5	22	35	0.570	4.2	1.1	1.9	3.7	4.5	11.2	5.5	19.4	0.234	2.17	2.04
6	22	35	0.572	3.5	0.9	2.2	3.7	4.6	11.4	4.8	19.7	0.250	2.96	2.96
7	17	60	0.538	4.9	1.8	2.3	3.7	3.6	9.1	6.7	16.4	0.233	2.29	2.21
8	22	35	0.533	4.3	1.2	2.2	3.5	4.6	11.9	4.4	20.00	0.236	2.25	2.25
9	22	35	0.604	3.9	0.9	2.00	3.7	4.7	11.3	5.3	19.7	0.239	2.33	2.08
10	22	35	0.545	3.4	0.9	2.6	3.6	4.6	11.9	4.3	20.1	0.234	2.88	2.42
11	17	10	0.410	4.7	1.3	2.9	3.2	3.2	7.3	6.00	13.7	0.170	3.38	3.38
12	27	10	0.575	9.3	1.8	3.2	4.5	6.7	9.7	11.1	20.9	0.250	2.42	1.88
13	27	60	0.497	3.4	0.8	1.9	3.4	6.2	13.3	4.2	22.9	0.367	1.08	1.13

Response Values

Data Sheet S1

Design Summary

File Version	9.0.3.1		
Study Type	Response Surface	Runs	13
Design Type	Central Composite	Blocks	No Blocks
Design Model	Quadratic		

Factor	Name	Units	Type	Subtype	Minimum	Maximum	Coded	Values	Mean	Std. Dev.
A	Temperature	°C	Numeric	Continuous	17	27	FALSE	1.000=27	22	3.535534
B	Pitching Rate	×10^6 cells/mL	Numeric	Continuous	10	60	FALSE	1.000=60	35	17.67767

Design Summary

Response	Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio	Trans	Model
R1	Ethanol	% ABV	13	Polynomial	0.41	0.604	0.531	0.051329	1.473171	None	RQuadratic
R2	Ethyl acetate	mg/L	13	Polynomial	3.4	9.3	5.084615	1.832051	2.735294	None	2FI
R3	Isoamyl lacetate	mg/L	13	Polynomial	0.8	2.2	1.3	0.460072	2.75	None	RQuadratic
R4	Acetaldehyde	mg/L	13	Polynomial	1.9	3.4	2.415385	0.493028	1.789474	None	RLinear
R5	n-Propanol	mg/L	13	Polynomial	3.2	4.5	3.646154	0.328165	1.40625	None	2FI
R6	Isobutanol	mg/L	13	Polynomial	3.2	6.7	4.784615	1.13273	2.09375	None	RQuadratic
R7	Isoamyl alcohols	mg/L	13	Polynomial	7.3	13.3	10.66923	1.817225	1.821918	None	Quadratic
R8	SUM Esters	mg/L	13	Polynomial	4.2	11.1	6.384615	2.20675	2.642857	None	RQuadratic
R9	SUM Alcohols	mg/L	13	Polynomial	13.7	22.9	19.1	2.698456	1.671533	None	RQuadratic
R10	Glycerol	g/L	13	Polynomial	0.17	0.367	0.241308	0.048922	2.158824	None	RQuadratic
R11	Acceptance		13	Polynomial	1.08	3.38	2.363077	0.590203	3.12963	None	Linear
R12	Fruitiness		13	Polynomial	1.13	3.38	2.165385	0.573623	2.99115	None	Linear

Data Sheet S1

Use your mouse to right click on individual cells for definitions.
Response 1 Ethanol
Backward Elimination Regression with Alpha to Exit = 0.100

Forced Ter Intercept

F p-value Removed	Value	Prob > F	R-Squared	MSE
A^2	0.69694	0.43138	0.855236	0.000654
B-Pitching	3.090284	0.116816	0.840823	0.000629

Hierarchical terms added after Backward elimination regression
B

ANOVA for Response Surface Reduced Quadratic model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.026583	4	0.006646	10.56461	0.002801	significant
A-Temper	0.004874	1	0.004874	7.74717	0.0238	
B-Pitching	0.001944	1	0.001944	3.090284	0.116816	
AB	0.010609	1	0.010609	16.86462	0.003406	
B^2	0.009157	1	0.009157	14.55637	0.005124	
Residual	0.005033	8	0.000629			
Lack of Fit	0.002014	4	0.000503	0.667069	0.647792	not significant
Pure Error	0.003019	4	0.000755			
Cor Total	0.031616	12				

The Model F-value of 10.56 implies the model is significant. There is only
a 0.28% chance that an F-value this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case A, AB, B++2+- are significant model terms.
Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fit F-value" of 0.67 implies the Lack of Fit is not significant relative to the pure
error. There is a 64.78% chance that a "Lack of Fit F-value" this large could occur due
to noise. Non-significant lack of fit is good -- we want the model to fit.

Std. Dev.	0.025081	R-Squared	0.840823
Mean	0.531	Adj R-Squa	0.761234
C.V. %	4.723397	Pred R-Squ	0.668207
PRESS	0.01049	Adeq Preci	11.55521

The "Pred R-Squared" of 0.6682 is in reasonable agreement with the "Adj R-Squared" of 0.7612;
i.e. the difference is less than 0.2.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your
ratio of 11.555 indicates an adequate signal. This model can be used to navigate the design space.

Coefficient Factor	Standard Estimate	95% CI df	95% CI Error	Low	High	VIF
Intercept	0.555571	1	0.00948	0.533711	0.577432	
A-Tempera	0.0285	1	0.010239	0.004888	0.052112	1
B-Pitching	0.018	1	0.010239	-0.00561	0.041612	1
AB	-0.0515	1	0.012541	-0.08042	-0.02258	1
B^2	-0.05324	1	0.013954	-0.08542	-0.02106	1

Final Equation in Terms of Coded Factors:

Ethanol =
0.555571
0.0285 * A
0.018 * B
-0.0515 * AB
-0.05324 * B^2

The equation in terms of coded factors can be used to make predictions about the response for
given levels of each factor. By default, the high levels of the factors are coded as +1 and the
low levels of the factors are coded as -1. The coded equation is useful for identifying the
relative impact of the factors by comparing the factor coefficients.

Final Equation in Terms of Actual Factors:

Ethanol =
-0.01662
0.02012 * Temperature
0.015747 * Pitching Rate
-0.00041 * Temperature * Pitching Rate
-8.52E-05 * Pitching Rate^2

The equation in terms of actual factors can be used to make predictions about the response for
given levels of each factor. Here, the levels should be specified in the original units for
each factor. This equation should not be used to determine the relative impact of each factor
because the coefficients are scaled to accommodate the units of each factor and the intercept
is not at the center of the design space.

ANOVA of R1 Ethanol

Data Sheet S1

Use your mouse to right click on individual cells for definitions.
Response 5 Ethyl acetate
Backward Elimination Regression with Alpha to Exit = 0.100

Forced Ter Intercept

F	p-value			
Removed	Value	Prob > F	R-Squared	MSE
A-Tempera	0.460872	0.514293	0.608889	1.750306

Hierarchical terms added after Backward elimination regression
A

ANOVA for Response Surface 2FI model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	24.52417	3	8.174722	4.670452	0.031188	significant
A-Temper	0.806667	1	0.806667	0.460872	0.514293	
B-Pitching	14.415	1	14.415	8.235702	0.018483	
AB	9.3025	1	9.3025	5.314784	0.046587	
Residual	15.75276	9	1.750306			
Lack of Fit	15.10076	5	3.020151	18.52854	0.007187	significant
Pure Error	0.652	4	0.163			
Cor Total	40.27692	12				

The Model F-value of 4.67 implies the model is significant. There is only
a 3.12% chance that an F-value this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case B, AB are significant model terms.
Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fit F-value" of 18.53 implies the Lack of Fit is significant. There is only a
0.72% chance that a "Lack of Fit F-value" this large could occur due to noise.
Significant lack of fit is bad -- we want the model to fit.

Std. Dev.	1.322991	R-Squared	0.608889
Mean	5.084615	Adj R-Squa	0.478518
C.V. %	26.0195	Pred R-Squ	0.142197
PRESS	34.54966	Adeq Preci	8.380304

The "Pred R-Squared" of 0.1422 is not as close to the "Adj R-Squared" of 0.4785 as one might
normally expect; i.e. the difference is more than 0.2. This may indicate a large block effect
or a possible problem with your model and/or data.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your
ratio of 8.380 indicates an adequate signal. This model can be used to navigate the design space.

Coefficient Factor	Standard Estimate	95% CI df	95% CI Error	Low	High	VIF
Intercept	5.084615	1	0.366932	4.254558	5.914673	
A-Tempera	0.366667	1	0.540109	-0.85514	1.588478	1
B-Pitching	-1.55	1	0.540109	-2.77181	-0.32819	1
AB	-1.525	1	0.661496	-3.02141	-0.02859	1

Final Equation in Terms of Coded Factors:

Ethylacetat =
5.084615
0.366667 * A
-1.55 * B
-1.525 * AB

The equation in terms of coded factors can be used to make predictions about the response for
given levels of each factor. By default, the high levels of the factors are coded as +1 and the
low levels of the factors are coded as -1. The coded equation is useful for identifying the
relative impact of the factors by comparing the factor coefficients.

Final Equation in Terms of Actual Factors:

Ethylacetat =
-3.75272
0.500333 * Temperature
0.2064 * Pitching Rate
-0.0122 * Temperature * Pitching Rate

The equation in terms of actual factors can be used to make predictions about the response for
given levels of each factor. Here, the levels should be specified in the original units for
each factor. This equation should not be used to determine the relative impact of each factor
because the coefficients are scaled to accommodate the units of each factor and the intercept
is not at the center of the design space.

ANOVA of R2 Ethyl acetate

Data Sheet S1

Use your mouse to right click on individual cells for definitions.
Response 6 Isoamyl lacetate
Backward Elimination Regression with Alpha to Exit = 0.100

Forced Ter Intercept

F Removed	p-value Value	Prob > F	R-Squared	MSE
A^2	0.184654	0.680322	0.762773	0.08608
B-Pitching	3.104524	0.116099	0.756515	0.077307

Hierarchical terms added after Backward elimination regression
B

ANOVA for Response Surface Reduced Quadratic model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1.921548	4	0.480387	6.214052	0.01415	significant
A-Temper	0.326667	1	0.326667	4.225602	0.07386	
B-Pitching	0.24	1	0.24	3.104524	0.116099	
AB	0.5625	1	0.5625	7.276227	0.027181	
B^2	0.792381	1	0.792381	10.24986	0.012583	
Residual	0.618452	8	0.077307			
Lack of Fit	0.538452	4	0.134613	6.730655	0.045869	significant
Pure Error	0.08	4	0.02			
Cor Total	2.54	12				

The Model F-value of 6.21 implies the model is significant. There is only
a 1.42% chance that an F-value this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case AB, B□++2□+- are significant model terms.
Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fit F-value" of 6.73 implies the Lack of Fit is significant. There is only a
4.59% chance that a "Lack of Fit F-value" this large could occur due to noise.
Significant lack of fit is bad -- we want the model to fit.

Std. Dev.	0.278041	R-Squared	0.756515
Mean	1.3	Adj R-Squa	0.634772
C.V. %	21.38773	Pred R-Squ	-0.31196
PRESS	3.332368	Adeq Preci	7.055863

A negative "Pred R-Squared" implies that the overall mean is a better predictor of your
response than the current model.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your
ratio of 7.056 indicates an adequate signal. This model can be used to navigate the design space.

Coefficient Factor	Standard Estimate	95% CI df	95% CI Error	Low	High	VIF
Intercept	1.071429	1	0.105089	0.829092	1.313765	
A-Tempera	-0.23333	1	0.11351	-0.49509	0.02842	1
B-Pitching	-0.2	1	0.11351	-0.46175	0.061754	1
AB	-0.375	1	0.13902	-0.69558	-0.05442	1
B^2	0.495238	1	0.154687	0.138528	0.851948	1

Final Equation in Terms of Coded Factors:

Iso-Amylac =
1.071429
-0.23333 * A
-0.2 * B
-0.375 * AB
0.495238 * B^2

The equation in terms of coded factors can be used to make predictions about the response for
given levels of each factor. By default, the high levels of the factors are coded as +1 and the
low levels of the factors are coded as -1. The coded equation is useful for identifying the
relative impact of the factors by comparing the factor coefficients.

Final Equation in Terms of Actual Factors:

Iso-Amylac =
1.038762
0.058333 * Temperature
0.002533 * Pitching Rate
-0.003 * Temperature * Pitching Rate
0.000792 * Pitching Rate^2

The equation in terms of actual factors can be used to make predictions about the response for
given levels of each factor. Here, the levels should be specified in the original units for
each factor. This equation should not be used to determine the relative impact of each factor
because the coefficients are scaled to accommodate the units of each factor and the intercept
is not at the center of the design space.

ANOVA of R3 Isoamyl acetate

Data Sheet S1

Use your mouse to right click on individual cells for definitions.
Response 7 Acetaldehyde
Backward Elimination Regression with Alpha to Exit = 0.100

Forced Ter Intercept

F	p-value			
Removed	Value	Prob > F	R-Squared	MSE
A-Tempera	0.392987	0.544779	0.636515	0.106026

ANOVA for Response Surface Reduced Linear model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1.815	1	1.815	18.11832	0.001351	significant
B-Pitching	1.815	1	1.815	18.11832	0.001351	
Residual	1.101923	11	0.100175			
Lack of Fit	0.813923	7	0.116275	1.614927	0.336826	not significant
Pure Error	0.288	4	0.072			
Cor Total	2.916923	12				

The Model F-value of 18.12 implies the model is significant. There is only
a 0.14% chance that an F-value this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case B is a significant model term.
Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fit F-value" of 1.61 implies the Lack of Fit is not significant relative to the pure
error. There is a 33.68% chance that a "Lack of Fit F-value" this large could occur due
to noise. Non-significant lack of fit is good -- we want the model to fit.

Std. Dev.	0.316504	R-Squared	0.622231
Mean	2.415385	Adj R-Squa	0.587888
C.V. %	13.10367	Pred R-Squ	0.466805
PRESS	1.555288	Adeq Preci	8.860742

The "Pred R-Squared" of 0.4668 is in reasonable agreement with the "Adj R-Squared" of 0.5879;
i.e. the difference is less than 0.2.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your
ratio of 8.861 indicates an adequate signal. This model can be used to navigate the design space.

Coefficient Factor	Estimate	df	95% CI Error	95% CI Low	High	VIF
Intercept	2.415385	1	0.087782	2.222177	2.608592	
B-Pitching	-0.55	1	0.129212	-0.83439	-0.26561	1

Final Equation in Terms of Coded Factors:

Acetaldehy =
2.415385
-0.55 * B

The equation in terms of coded factors can be used to make predictions about the response for
given levels of each factor. By default, the high levels of the factors are coded as +1 and the
low levels of the factors are coded as -1. The coded equation is useful for identifying the
relative impact of the factors by comparing the factor coefficients.

Final Equation in Terms of Actual Factors:

Acetaldehy =
3.185385
-0.022 * Pitching Rate

The equation in terms of actual factors can be used to make predictions about the response for
given levels of each factor. Here, the levels should be specified in the original units for
each factor. This equation should not be used to determine the relative impact of each factor
because the coefficients are scaled to accommodate the units of each factor and the intercept
is not at the center of the design space.

ANOVA of R4 Acetaldehyde

Data Sheet S1

Use your mouse to right click on individual cells for definitions.
Response 8 n-Propanol
Backward Elimination Regression with Alpha to Exit = 0.100

Forced Ter Intercept

F	p-value			
Removed	Value	Prob > F	R-Squared	MSE
A-Tempera	2.533153	0.145941	0.706746	0.042108

Hierarchical terms added after Backward elimination regression
A

ANOVA for Response Surface 2FI model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.913333	3	0.304444	7.230041	0.009029	significant
A-Temper	0.106667	1	0.106667	2.533153	0.145941	
B-Pitching	0.166667	1	0.166667	3.958051	0.077861	
AB	0.64	1	0.64	15.19892	0.003627	
Residual	0.378974	9	0.042108			
Lack of Fit	0.346974	5	0.069395	8.674359	0.028601	significant
Pure Error	0.032	4	0.008			
Cor Total	1.292308	12				

The Model F-value of 7.23 implies the model is significant. There is only
a 0.90% chance that an F-value this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case AB is a significant model term.
Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fit F-value" of 8.67 implies the Lack of Fit is significant. There is only a
2.86% chance that a "Lack of Fit F-value" this large could occur due to noise.
Significant lack of fit is bad -- we want the model to fit.

Std. Dev.	0.205203	R-Squared	0.706746
Mean	3.646154	Adj R-Squa	0.608995
C.V. %	5.62793	Pred R-Squ	0.142388
PRESS	1.108298	Adeq Preci	9.956706

The "Pred R-Squared" of 0.1424 is not as close to the "Adj R-Squared" of 0.6090 as one might
normally expect; i.e. the difference is more than 0.2. This may indicate a large block effect
or a possible problem with your model and/or data.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your
ratio of 9.957 indicates an adequate signal. This model can be used to navigate the design space.

Coefficient Factor	Standard Estimate	95% CI df	95% CI Error	Low	High	VIF
Intercept	3.646154	1	0.056913	3.517408	3.7749	
A-Tempera	0.133333	1	0.083774	-0.05618	0.322843	1
B-Pitching	-0.16667	1	0.083774	-0.35618	0.022843	1
AB	-0.4	1	0.102601	-0.6321	-0.1679	1

Final Equation in Terms of Coded Factors:

n-Propanol =
3.646154
0.133333 * A
-0.16667 * B
-0.4 * AB

The equation in terms of coded factors can be used to make predictions about the response for
given levels of each factor. By default, the high levels of the factors are coded as +1 and the
low levels of the factors are coded as -1. The coded equation is useful for identifying the
relative impact of the factors by comparing the factor coefficients.

Final Equation in Terms of Actual Factors:

n-Propanol =
0.828821
0.138667 * Temperature
0.063733 * Pitching Rate
-0.0032 * Temperature * Pitching Rate

The equation in terms of actual factors can be used to make predictions about the response for
given levels of each factor. Here, the levels should be specified in the original units for
each factor. This equation should not be used to determine the relative impact of each factor
because the coefficients are scaled to accommodate the units of each factor and the intercept
is not at the center of the design space.

ANOVA of R5 n-Propanol

Data Sheet S1

Use your mouse to right click on individual cells for definitions.
Response 9 Isobutanol
Backward Elimination Regression with Alpha to Exit = 0.100

Forced Ter Intercept

F p-value				
Removed	Value	Prob > F	R-Squared	MSE
B^2	0.017259	0.899175	0.994593	0.011892
B-Pitching	1.437946	0.264782	0.99458	0.010432

Hierarchical terms added after Backward elimination regression
B

ANOVA for Response Surface Reduced Quadratic model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	15.31347	4	3.828368	366.999	4.30E-09	significant
A-Temper	14.72667	1	14.72667	1411.743	2.76E-10	
B-Pitching	0.015	1	0.015	1.437946	0.264782	
AB	0.2025	1	0.2025	19.41227	0.002269	
A^2	0.369304	1	0.369304	35.40261	0.000342	
Residual	0.083452	8	0.010432			
Lack of Fit	0.063452	4	0.015863	3.172619	0.144777	not significant
Pure Error	0.02	4	0.005			
Cor Total	15.39692	12				

The Model F-value of 367.00 implies the model is significant. There is only
a 0.01% chance that an F-value this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case A, AB, A□++2□+- are significant model terms.
Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fit F-value" of 3.17 implies the Lack of Fit is not significant relative to the pure
error. There is a 14.48% chance that a "Lack of Fit F-value" this large could occur due
to noise. Non-significant lack of fit is good -- we want the model to fit.

Std. Dev.	0.102135	R-Squared	0.99458
Mean	4.784615	Adj R-Squa	0.99187
C.V. %	2.134653	Pred R-Squ	0.97471
PRESS	0.389391	Adeq Preci	56.57174

The "Pred R-Squared" of 0.9747 is in reasonable agreement with the "Adj R-Squared" of 0.9919;
i.e. the difference is less than 0.2.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your
ratio of 56.572 indicates an adequate signal. This model can be used to navigate the design space.

Coefficient Factor	Standard Estimate	95% CI df	95% CI Error	Low	High	VIF
Intercept	4.628571	1	0.038603	4.539552	4.717591	
A-Tempera	1.566667	1	0.041696	1.470515	1.662819	1
B-Pitching	-0.05	1	0.041696	-0.14615	0.046152	1
AB	-0.225	1	0.051067	-0.34276	-0.10724	1
A^2	0.338095	1	0.056823	0.207062	0.469128	1

Final Equation in Terms of Coded Factors:

iso-Butano =
4.628571
1.566667 * A
-0.05 * B
-0.225 * AB
0.338095 * A^2

The equation in terms of coded factors can be used to make predictions about the response for
given levels of each factor. By default, the high levels of the factors are coded as +1 and the
low levels of the factors are coded as -1. The coded equation is useful for identifying the
relative impact of the factors by comparing the factor coefficients.

Final Equation in Terms of Actual Factors:

iso-Butano =
2.964762
-0.21871 * Temperature
0.0376 * Pitching Rate
-0.0018 * Temperature * Pitching Rate
0.013524 * Temperature^2

The equation in terms of actual factors can be used to make predictions about the response for
given levels of each factor. Here, the levels should be specified in the original units for
each factor. This equation should not be used to determine the relative impact of each factor
because the coefficients are scaled to accommodate the units of each factor and the intercept
is not at the center of the design space.

ANOVA of R6 Isobutanol

Data Sheet S1

Use your mouse to right click on individual cells for definitions.
Response 10 Isoamyl alcohols
Backward Elimination Regression with Alpha to Exit = 0.100

Forced Ter Intercept

ANOVA for Response Surface Quadratic model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	38.52896	5	7.705791	49.09328	2.67E-05	significant
A-Temper	20.16667	1	20.16667	128.481	9.31E-06	
B-Pitching	10.66667	1	10.66667	67.9569	7.52E-05	
AB	0.81	1	0.81	5.160477	0.057336	
A^2	0.851264	1	0.851264	5.423371	0.052706	
B^2	3.68555	1	3.68555	23.48049	0.001866	
Residual	1.098736	7	0.156962			
Lack of Fit	0.646736	3	0.215579	1.907775	0.269736	not significant
Pure Error	0.452	4	0.113			
Cor Total	39.62769	12				

The Model F-value of 49.09 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case A, B, B□++2□+- are significant model terms.
Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fit F-value" of 1.91 implies the Lack of Fit is not significant relative to the pure error. There is a 26.97% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Std. Dev.	0.396185	R-Squared	0.972274
Mean	10.66923	Adj R-Squa	0.952469
C.V. %	3.713338	Pred R-Squ	0.83757
PRESS	6.436727	Adeq Preci	23.53048

The "Pred R-Squared" of 0.8376 is in reasonable agreement with the "Adj R-Squared" of 0.9525; i.e. the difference is less than 0.2.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 23.530 indicates an adequate signal. This model can be used to navigate the design space.

Coefficient Factor	Standard Estimate	95% CI df	95% CI Error	Low	High	VIF
Intercept	11.45862	1	0.164507	11.06962	11.84762	
A-Tempera	1.833333	1	0.161742	1.450875	2.215792	1
B-Pitching	1.333333	1	0.161742	0.950875	1.715792	1
AB	0.45	1	0.198092	-0.018414	0.918414	1
A^2	-0.555172	1	0.238393	-1.118882	0.008537	1.169761
B^2	-1.155172	1	0.238393	-1.718882	-0.591463	1.169761

Final Equation in Terms of Coded Factors:

iso-Amylalc =
11.45862
1.833333 * A
1.333333 * B
0.45 * AB
-0.555172 * A^2
-1.155172 * B^2

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels of the factors are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Final Equation in Terms of Actual Factors:

iso-Amylalc =
-8.714989
1.21777 * Temperature
0.103513 * Pitching Rate
0.0036 * Temperature * Pitching Rate
-0.022207 * Temperature^2
-0.001848 * Pitching Rate^2

The equation in terms of actual factors can be used to make predictions about the response for given levels of each factor. Here, the levels should be specified in the original units for each factor. This equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor and the intercept is not at the center of the design space.

ANOVA of R7 Isoamyl alcohols

Data Sheet S1

Use your mouse to right click on individual cells for definitions.
Response 11 SUM Esters
Backward Elimination Regression with Alpha to Exit = 0.100

Forced Ter Intercept

F Removed	p-value Value	Prob > F	R-Squared	MSE
A-Tempera	0.0565	0.818924	0.773852	1.887915
A^2	0.711932	0.423314	0.772027	1.665259

Hierarchical terms added after Backward elimination regression
A

ANOVA for Response Surface Reduced Quadratic model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	44.03597	4	11.00899	6.115703	0.014805	significant
A-Temper	0.106667	1	0.106667	0.059255	0.813803	
B-Pitching	18.375	1	18.375	10.20766	0.012709	
AB	14.44	1	14.44	8.021692	0.022073	
B^2	11.1143	1	11.1143	6.174205	0.037828	
Residual	14.40095	8	1.800119			
Lack of Fit	13.26895	4	3.317238	11.72169	0.017565	significant
Pure Error	1.132	4	0.283			
Cor Total	58.43692	12				

The Model F-value of 6.12 implies the model is significant. There is only
a 1.48% chance that an F-value this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case B, AB, B□++2□+- are significant model terms.
Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fit F-value" of 11.72 implies the Lack of Fit is significant. There is only a
1.76% chance that a "Lack of Fit F-value" this large could occur due to noise.
Significant lack of fit is bad -- we want the model to fit.

Std. Dev.	1.341685	R-Squared	0.753564
Mean	6.384615	Adj R-Squa	0.630346
C.V. %	21.01435	Pred R-Squ	0.089694
PRESS	53.19548	Adeq Preci	8.773218

The "Pred R-Squared" of 0.0897 is not as close to the "Adj R-Squared" of 0.6303 as one might
normally expect; i.e. the difference is more than 0.2. This may indicate a large block effect
or a possible problem with your model and/or data.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your
ratio of 8.773 indicates an adequate signal. This model can be used to navigate the design space.

Factor	Coefficient Estimate	Standard df	95% CI Error	95% CI Low	High	VIF
Intercept	5.528571	1	0.507109	4.359175	6.697968	
A-Tempera	0.133333	1	0.547741	-1.129759	1.396426	1
B-Pitching	-1.75	1	0.547741	-3.013092	-0.486908	1
AB	-1.9	1	0.670843	-3.446966	-0.353034	1
B^2	1.854762	1	0.746445	0.133457	3.576066	1

Final Equation in Terms of Coded Factors:

SUM Esters =
5.528571
0.133333 * A
-1.75 * B
-1.9 * AB
1.854762 * B^2

The equation in terms of coded factors can be used to make predictions about the response for
given levels of each factor. By default, the high levels of the factors are coded as +1 and the
low levels of the factors are coded as -1. The coded equation is useful for identifying the
relative impact of the factors by comparing the factor coefficients.

Final Equation in Terms of Actual Factors:

SUM Esters =
-0.676762
0.558667 * Temperature
0.056667 * Pitching Rate
-0.0152 * Temperature * Pitching Rate
0.002968 * Pitching Rate^2

The equation in terms of actual factors can be used to make predictions about the response for
given levels of each factor. Here, the levels should be specified in the original units for
each factor. This equation should not be used to determine the relative impact of each factor
because the coefficients are scaled to accommodate the units of each factor and the intercept
is not at the center of the design space.

ANOVA of R8 SUM Esters

Data Sheet S1

Use your mouse to right click on individual cells for definitions.
Response 12 SUM Alcohols
Backward Elimination Regression with Alpha to Exit = 0.100

Forced Ter Intercept

F	p-value			
Removed	Value	Prob > F	R-Squared	MSE
AB	1.288416	0.293707	0.992383	0.095078

ANOVA for Response Surface Reduced Quadratic model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	86.59195	4	21.64799	219.7637	3.28E-08	significant
A-Temper	74.90667	1	74.90667	760.4294	3.23E-09	
B-Pitching	7.481667	1	7.481667	75.95158	2.35E-05	
A^2	0.411954	1	0.411954	4.18203	0.075089	
B^2	2.420764	1	2.420764	24.57485	0.001111	
Residual	0.788046	8	0.098506			
Lack of Fit	0.480046	4	0.120011	1.558591	0.338862	not significant
Pure Error	0.308	4	0.077			
Cor Total	87.38	12				

The Model F-value of 219.76 implies the model is significant. There is only
a 0.01% chance that an F-value this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case A, B, B²A²+- are significant model terms.
Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fit F-value" of 1.56 implies the Lack of Fit is not significant relative to the pure
error. There is a 33.89% chance that a "Lack of Fit F-value" this large could occur due
to noise. Non-significant lack of fit is good -- we want the model to fit.

Std. Dev.	0.313856	R-Squared	0.990981
Mean	19.1	Adj R-Squa	0.986472
C.V. %	1.643226	Pred R-Squ	0.97205
PRESS	2.442257	Adeq Preci	47.7792

The "Pred R-Squared" of 0.9721 is in reasonable agreement with the "Adj R-Squared" of 0.9865;
i.e. the difference is less than 0.2.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your
ratio of 47.779 indicates an adequate signal. This model can be used to navigate the design space.

Coefficient Factor	Standard Estimate	95% CI df	95% CI Error	Low	High	VIF
Intercept	19.71034	1	0.130322	19.40982	20.01087	
A-Tempera	3.533333	1	0.128131	3.237862	3.828805	1
B-Pitching	1.116667	1	0.128131	0.821195	1.412138	1
A^2	-0.38621	1	0.188854	-0.82171	0.049291	1.169761
B^2	-0.93621	1	0.188854	-1.37171	-0.50071	1.169761

Final Equation in Terms of Coded Factors:

SUM Alcoh =
19.71034
3.533333 * A
1.116667 * B
-0.38621 * A^2
-0.93621 * B^2

The equation in terms of coded factors can be used to make predictions about the response for
given levels of each factor. By default, the high levels of the factors are coded as +1 and the
low levels of the factors are coded as -1. The coded equation is useful for identifying the
relative impact of the factors by comparing the factor coefficients.

Final Equation in Terms of Actual Factors:

SUM Alcoh =
-6.71159
1.386391 * Temperature
0.149522 * Pitching Rate
-0.01545 * Temperature^2
-0.0015 * Pitching Rate^2

The equation in terms of actual factors can be used to make predictions about the response for
given levels of each factor. Here, the levels should be specified in the original units for
each factor. This equation should not be used to determine the relative impact of each factor
because the coefficients are scaled to accommodate the units of each factor and the intercept
is not at the center of the design space.

ANOVA of R9 SUM Alcohols

Data Sheet S1

Use your mouse to right click on individual cells for definitions.
Response 13 Glycerol
Backward Elimination Regression with Alpha to Exit = 0.100

Forced Ter Intercept

F p-value				
Removed	Value	Prob > F	R-Squared	MSE
B^2	0.708442	0.427777	0.948741	0.00021

ANOVA for Response Surface Reduced Quadratic model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.0271	4	0.006775	33.4317	4.85E-05	significant
A-Temper	0.016433	1	0.016433	81.08938	1.85E-05	
B-Pitching	0.008664	1	0.008664	42.75377	0.000181	
AB	0.000729	1	0.000729	3.597356	0.094451	
A^2	0.001274	1	0.001274	6.286304	0.036527	
Residual	0.001621	8	0.000203			
Lack of Fit	0.001442	4	0.00036	8.046822	0.033954	significant
Pure Error	0.000179	4	4.48E-05			
Cor Total	0.028721	12				

The Model F-value of 33.43 implies the model is significant. There is only
a 0.01% chance that an F-value this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case A, B, A²+2²+- are significant model terms.
Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fit F-value" of 8.05 implies the Lack of Fit is significant. There is only a
3.40% chance that a "Lack of Fit F-value" this large could occur due to noise.
Significant lack of fit is bad -- we want the model to fit.

Std. Dev.	0.014235	R-Squared	0.943553
Mean	0.241308	Adj R-Squa	0.91533
C.V. %	5.899305	Pred R-Squ	0.776166
PRESS	0.006429	Adeq Preci	20.4641

The "Pred R-Squared" of 0.7762 is in reasonable agreement with the "Adj R-Squared" of 0.9153;
i.e. the difference is less than 0.2.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your
ratio of 20.464 indicates an adequate signal. This model can be used to navigate the design space.

Coefficient Factor	Standard Estimate	95% CI df	95% CI Error	Low	High	VIF
Intercept	0.232143	1	0.005381	0.219735	0.24455	
A-Tempera	0.052333	1	0.005812	0.038932	0.065735	1
B-Pitching	0.038	1	0.005812	0.024598	0.051402	1
AB	0.0135	1	0.007118	-0.00291	0.029914	1
A^2	0.019857	1	0.00792	0.001594	0.03812	1

Final Equation in Terms of Coded Factors:

Glycerol =
0.232143
0.052333 * A
0.038 * B
0.0135 * AB
0.019857 * A^2

The equation in terms of coded factors can be used to make predictions about the response for
given levels of each factor. By default, the high levels of the factors are coded as +1 and the
low levels of the factors are coded as -1. The coded equation is useful for identifying the
relative impact of the factors by comparing the factor coefficients.

Final Equation in Terms of Actual Factors:

Glycerol =
0.41627
-0.02826 * Temperature
-0.00086 * Pitching Rate
0.000108 * Temperature * Pitching Rate
0.000794 * Temperature^2

The equation in terms of actual factors can be used to make predictions about the response for
given levels of each factor. Here, the levels should be specified in the original units for
each factor. This equation should not be used to determine the relative impact of each factor
because the coefficients are scaled to accommodate the units of each factor and the intercept
is not at the center of the design space.

ANOVA of R10 Glycerol

Data Sheet S1

Use your mouse to right click on individual cells for definitions.
Response 15 Acceptance
Backward Elimination Regression with Alpha to Exit = 0.100

Forced Ter Intercept

ANOVA for Response Surface Linear model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	2.424833	2	1.212417	6.907398	0.013055	significant
A-Temper	1.804017	1	1.804017	10.27787	0.009397	
B-Pitching	0.620817	1	0.620817	3.536926	0.089425	
Residual	1.755244	10	0.175524			
Lack of Fit	1.200564	6	0.200094	1.44295	0.376637	not significant
Pure Error	0.55468	4	0.13867			
Cor Total	4.180077	12				

The Model F-value of 6.91 implies the model is significant. There is only
a 1.31% chance that an F-value this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case A is a significant model term.
Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fit F-value" of 1.44 implies the Lack of Fit is not significant relative to the pure
error. There is a 37.66% chance that a "Lack of Fit F-value" this large could occur due
to noise. Non-significant lack of fit is good -- we want the model to fit.

Std. Dev.	0.418956	R-Squared	0.580093
Mean	2.363077	Adj R-Squa	0.496112
C.V. %	17.72927	Pred R-Squ	0.260999
PRESS	3.089081	Adeq Preci	8.64553

The "Pred R-Squared" of 0.2610 is not as close to the "Adj R-Squared" of 0.4961 as one might
normally expect; i.e. the difference is more than 0.2. This may indicate a large block effect
or a possible problem with your model and/or data.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your
ratio of 8.646 indicates an adequate signal. This model can be used to navigate the design space.

Coefficient Factor	Standard Estimate	95% CI df	95% CI Error	Low	High	VIF
Intercept	2.363077	1	0.116198	2.104173	2.621981	
A-Tempera	-0.548333	1	0.171038	-0.92943	-0.167237	1
B-Pitching	-0.321667	1	0.171038	-0.702763	0.05943	1

Final Equation in Terms of Coded Factors:

Acceptance =
2.363077
-0.548333 * A
-0.321667 * B

The equation in terms of coded factors can be used to make predictions about the response for
given levels of each factor. By default, the high levels of the factors are coded as +1 and the
low levels of the factors are coded as -1. The coded equation is useful for identifying the
relative impact of the factors by comparing the factor coefficients.

Final Equation in Terms of Actual Factors:

Acceptance =
5.226077
-0.109667 * Temperature
-0.012867 * Pitching Rate

The equation in terms of actual factors can be used to make predictions about the response for
given levels of each factor. Here, the levels should be specified in the original units for
each factor. This equation should not be used to determine the relative impact of each factor
because the coefficients are scaled to accommodate the units of each factor and the intercept
is not at the center of the design space.

ANOVA of R11 Acceptance

Data Sheet S1

Use your mouse to right click on individual cells for definitions.
Response 16 Fruitiness
Backward Elimination Regression with Alpha to Exit = 0.100

Forced Ter Intercept

ANOVA for Response Surface Linear model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	2.472033	2	1.236017	8.371319	0.007311	significant
A-Temper	1.938017	1	1.938017	13.12584	0.004667	
B-Pitching	0.534017	1	0.534017	3.616799	0.086367	
Residual	1.47649	10	0.147649			
Lack of Fit	0.92049	6	0.153415	1.103705	0.483958	not significant
Pure Error	0.556	4	0.139			
Cor Total	3.948523	12				

The Model F-value of 8.37 implies the model is significant. There is only
a 0.73% chance that an F-value this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case A is a significant model term.
Values greater than 0.1000 indicate the model terms are not significant.

The "Lack of Fit F-value" of 1.10 implies the Lack of Fit is not significant relative to the pure
error. There is a 48.40% chance that a "Lack of Fit F-value" this large could occur due
to noise. Non-significant lack of fit is good -- we want the model to fit.

Std. Dev.	0.384251	R-Squared	0.626065
Mean	2.165385	Adj R-Squa	0.551278
C.V. %	17.74517	Pred R-Squ	0.394806
PRESS	2.389624	Adeq Preci	9.390267

The "Pred R-Squared" of 0.3948 is in reasonable agreement with the "Adj R-Squared" of 0.5513;
i.e. the difference is less than 0.2.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your
ratio of 9.390 indicates an adequate signal. This model can be used to navigate the design space.

Coefficient Factor	Estimate	95% CI df	95% CI Error	Low	High	VIF
Intercept	2.165385	1	0.106572	1.927927	2.402842	
A-Tempera	-0.568333	1	0.15687	-0.917861	-0.218805	1
B-Pitching	-0.298333	1	0.15687	-0.647861	0.051195	1

Final Equation in Terms of Coded Factors:

Fruitiness =
2.165385
-0.568333 * A
-0.298333 * B

The equation in terms of coded factors can be used to make predictions about the response for
given levels of each factor. By default, the high levels of the factors are coded as +1 and the
low levels of the factors are coded as -1. The coded equation is useful for identifying the
relative impact of the factors by comparing the factor coefficients.

Final Equation in Terms of Actual Factors:

Fruitiness =
5.083718
-0.113667 * Temperature
-0.011933 * Pitching Rate

The equation in terms of actual factors can be used to make predictions about the response for
given levels of each factor. Here, the levels should be specified in the original units for
each factor. This equation should not be used to determine the relative impact of each factor
because the coefficients are scaled to accommodate the units of each factor and the intercept
is not at the center of the design space.

ANOVA of R12 Fruitiness

Data Sheet S1

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding
A	Temperatu	17	17	27	0	Actual
B	Pitching Ra	10	10	60	0	Actual

Predicted Response	Predicted Mean	CI for ^1Median	Mean Observed	99% of Std Dev	Population SE Mean	95% CI low	95% CI high	95% TI low	95% TI high
Ethanol	0.404333	0.404333	-	0.025081	0.021721	0.354245	0.454422	0.253402	0.555265
Extract (rea	6.161282	6.161282	-	0.022781	0.014592	6.12877	6.193794	6.041354	6.28121
Extract (ap	6.016667	6.016667	-	0.021798	0.015413	5.9818	6.051534	5.89604	6.137293
pH	4.5	4.5	-	0.039291	0.022685	4.449455	4.550545	4.297653	4.702347
Ethyl aceta	4.742949	4.742949	-	1.322991	1.075012	2.311102	7.174795	-2.834174	12.32007
Isoamyl lac	1.625	1.625	-	0.278041	0.24079	1.069737	2.180263	-0.048165	3.298165
Acetaldehy	2.965385	2.965385	-	0.316504	0.15621	2.621569	3.3092	1.420678	4.510091
n-Propanol	3.279487	3.279487	-	0.205203	0.16674	2.902295	3.656679	2.104235	4.454739
Isobutanol	3.225	3.225	-	0.102135	0.088451	3.021031	3.428969	2.610382	3.839618
Isoamyl lal	7.031609	7.031609	-	0.396185	0.352188	6.198817	7.864401	4.530362	9.532856
SUM Esters	7.1	7.1	-	1.341685	1.161933	4.420577	9.779423	-0.97386	15.17386
SUM Alcoh	13.73793	13.73793	-	0.313856	0.230685	13.20597	14.26989	11.92571	15.55016
Glycerol	0.175167	0.175167	-	0.014235	0.012328	0.146738	0.203596	0.089502	0.260832
FAN	68.71731	68.71731	-	3.340216	2.714132	62.57752	74.8571	49.587	87.84761
Acceptance	3.233077	3.233077	-	0.418956	0.268347	2.635163	3.830991	1.027529	5.438624
Fruitiness	3.032051	3.032051	-	0.384251	0.246118	2.483667	3.580436	1.009205	5.054898

Point Prediction

Data Sheet S1

Confirmation Report

Two-sided	Confidence	95%	n =	1			
Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding	
A	Temperatu	17	17	27	0	Actual	
B	Pitching Ra	10	10	60	0	Actual	

Predicted Response	Predicted Mean	^1Median	Observed	Std Dev	n	SE Pred	95% PI low	Data Mean	95% PI high
Ethanol	0.404333	0.404333	-	0.025081	1	0.033179	0.327822	0.53	0.480845
Ethyl acetate	4.742949	4.742949	-	1.322991	1	1.704687	0.886679	7.5	8.599218
Isoamyl lacetate	1.625	1.625	-	0.278041	1	0.367813	0.776822	2.6	2.473178
Acetaldehyde	2.965385	2.965385	-	0.316504	1	0.352954	2.188539	1.6	3.742231
n-Propanol	3.279487	3.279487	-	0.205203	1	0.264406	2.681359	3.6	3.877615
Isobutanol	3.225	3.225	-	0.102135	1	0.135112	2.913432	2.7	3.536568
Isoamyl alcohols	7.031609	7.031609	-	0.396185	1	0.530093	5.778139	3.9	8.28508
SUM Esters	7.1	7.1	-	1.341685	1	1.774883	3.007113	10.1	11.19289
SUM Alcohols	13.73793	13.73793	-	0.313856	1	0.389514	12.83971	10.2	14.63615
Glycerol	0.175167	0.175167	-	0.014235	1	0.018832	0.131741	0.28	0.218593
Acceptance	3.233077	3.233077	-	0.418956	1	0.497528	2.124515	3	4.341639
Fruitiness	3.032051	3.032051	-	0.384251	1	0.456314	2.015319	4	4.048783

Confirmation (Model Validation)

Chapter 6: Supplementary Data Sheets

Supplementary Data Sheet 1

Substrate	CBS 707	KBI 1.2	KBI 3.2	KBI 5.3	KBI 12.1
Control	-	-	-	-	-
D-Galactose	+	+	+	+	+
Cycloheximide (Actidione)	+	+	+	+	+
D-Saccharose	+	+	+	+	+
N-Acetyl-Glucosamine	-	-	-	-	-
Lactic acid	-	w	w	-	w
L-Arabinose	-	-	-	-	-
D-Cellobiose	+	w	w	-	+
D-Raffinose	+	+	+	+	+
D-Maltose	+	+	+	+	+
D-Trehalose	+	+	+	+	+
Potassium 2-Ketogluconate	w	w	w	w	w
Methyl-αD-Glucopyranoside	+	+	+	+	+
D-Mannitol	+	+	+	w	+
D-Lactose	-	-	-	-	-
Inositol	-	-	-	-	-
D-Sorbitol	+	+	+	+	+
D-Xylose	-	-	-	-	-
D-Ribose	-	-	-	-	-
Glycerol	-	-	-	-	-
L-Rhamnose	-	-	-	-	-
Palatinose	+	+	+	+	+
Erythritol	-	-	-	-	-
D-Melibiose	-	-	-	-	-
Sodium Glucuronate	-	-	-	-	-
D-Melezitose	w	+	w	w	+
Potassium Gluconate	w	-	w	-	w
Levulinic acid	-	-	-	-	-
D-Glucose	+	+	+	+	+
L-Sorbose	w	+	w	+	+
Glucosamine	-	-	-	-	-
Esculin ferric citrate	w	+	+	w	+

Page 1 API substrate test

Supplementary Data Sheet 1

	Dp	31.9	7.3	27.9	3.8	25.9	24.6	46.7	8.2	19.3	11.8	42.5	3.4	49.5	23.9	19.2	26.0	41.6	1.8	25.1	26.1
Relative Precision (%)		2	6	4	19	3	4	5	11	6	7	5	7	8	4	7	14	9	32	4	11
Absolute Precision		0.02	0.06	0.04	0.19	0.03	0.04	0.05	0.11	0.06	0.07	0.05	0.07	0.08	0.04	0.07	0.14	0.09	0.32	0.04	0.11
Customer ID	**Compound** *Unit*	**Ethyl acetate** *Relative*	**Diacetyl** *Relative*	**Isobutanol** *Relative*	**Isobutyl acetate** *Relative*	**Isoamyl alcohol** *Relative*	**2-methyl-1-butanol** *Relative*	**Ethyl butyrate** *Relative*	**Acetoin** *Relative*	**Ethyl Lactate** *Relative*	**Isoamyl acetate** *Relative*	**Ethyl hexanoate** *Relative*	**Linalool** *Relative*	**Ethyl octanoate** *Relative*	**2-phenyl ethanol** *Relative*	**2-phenylethyl acetate** *Relative*	**4-ethylphenol** *Relative*	**Ethyl decanoate** *Relative*	**Nerol** *Relative*	**?1-Propanol** *Relative*	**?Acetal** *Relative*
KBI 1.2 1		1.82	1.45	1.65	0.77	1.92	1.82	0.07	0.44	1.16	1.05	1.11	0.81	0.52	1.57	3.23	0.46	0.88	0.94	1.97	0.13
KBI 1.2 2		1.88	1.46	1.60	1.04	1.84	1.76	0.07	0.50	1.15	1.43	1.13	0.99	0.64	1.47	3.50	0.42	1.04	0.97	2.11	0.14
KBI 1.2 3		1.83	1.43	1.63	0.66	1.87	1.80	0.06	0.54	1.21	0.92	1.03	1.19	0.49	1.51	3.59	0.31	0.70	1.51	2.06	0.12
KBI 3.2 1		1.78	1.34	1.62	0.78	1.97	1.94	0.04	0.61	1.10	1.37	1.14	1.20	0.96	1.55	3.60	0.43	0.95	1.05	2.39	0.11
KBI 3.2 2		1.94	1.47	1.78	0.60	2.21	2.15	0.03	0.52	1.35	1.26	1.08	1.32	0.85	1.74	3.72	0.39	1.14	1.03	2.16	0.11
KBI 3.2 3		1.90	1.52	1.76	0.74	2.14	2.10	0.03	0.57	1.25	1.21	1.46	1.28	1.05	1.71	3.87	0.40	1.23	1.27	2.63	0.12
KBI 5.3 1		1.32	1.14	1.74	1.13	1.62	1.69	0.05	0.46	1.24	1.83	0.49	1.34	0.21	2.31	0.78	0.44	0.35	0.89	1.64	0.13
KBI 5.3 2		1.28	1.08	1.87	0.75	1.68	1.77	0.05	0.49	1.34	1.24	0.62	1.28	0.26	2.35	0.97	0.46	0.32	0.86	1.57	0.12
KBI 5.3 3		1.28	1.12	1.63	0.62	1.53	1.60	0.04	0.54	1.08	1.04	0.61	1.19	0.22	2.07	0.78	0.35	0.25	0.80	1.59	0.12
KBI 12.1 1		1.62	1.36	1.52	0.73	1.66	1.57	0.05	0.50	1.09	0.85	0.84	1.37	0.81	1.23	2.93	0.39	0.70	1.05	1.94	0.10
KBI 12.1 2		1.69	1.29	1.43	1.48	1.56	1.48	0.05	0.58	1.01	1.11	1.10	1.26	0.81	1.15	3.09	0.35	0.72	1.24	3.85	0.13
KBI 12.1 3		1.50	1.21	1.43	0.58	1.57	1.48	0.04	0.36	0.98	0.80	0.90	1.44	0.68	1.15	2.62	0.33	1.08	1.44	1.87	0.12
CBS 707 1		1.92	1.39	3.09	0.74	2.31	2.70	0.06	0.55	0.94	1.30	0.39	1.34	0.35	2.43	0.47	0.31	0.50	0.69	1.88	0.11
CBS 707 2		1.89	1.45	2.95	0.76	2.28	2.61	0.05	0.59	0.84	1.63	0.50	1.30	0.40	2.39	0.51	0.33	0.34	0.67	1.95	0.12
CBS 707 3		1.93	1.64	3.44	0.83	2.53	2.96	0.06	0.76	1.08	1.62	0.47	1.43	0.33	2.65	0.40	0.44	1.06	0.54	1.97	0.16
WLP001 1		1.01	0.99	2.52	1.15	1.71	1.55	0.02	0.46	1.76	0.88	2.85	1.36	15.17	2.30	0.34	0.52	13.81	1.61	2.23	0.14
WLP001 2		1.13	1.14	2.85	0.67	1.81	1.74	0.03	0.51	1.60	1.10	2.57	1.36	12.17	2.61	0.64	0.39	6.92	1.31	1.57	0.15
WLP001 3		1.06	1.13	2.45	0.97	1.69	1.55	0.08	0.51	1.68	0.89	2.43	1.25	12.34	2.27	0.43	0.52	10.25	1.37	1.53	0.22

Supplementary Data Sheet 1

	Dp	22.6	18.3	13.9	8.5	24.8	36.3	7.9	11.2	26.4	12.2	25.4	14.9	6.1	23.3	26.4	7.0	17.6	28.5	34.0	21.3
Relative Precision (%)		5	6	10	9	8	3	13	12	11	9	4	6	10	4	7	14	5	10	4	12
Absolute Precision		0.05	0.06	0.10	0.09	0.08	0.03	0.13	0.12	0.11	0.09	0.04	0.06	0.10	0.04	0.07	0.14	0.05	0.10	0.04	0.12
Customer ID	**Compound**	**?Acetic acid**	**?Propanoic acid**	**?2-Methylpropanoic acid**	**?Butanoic acid**	**?3-Ethoxy-1-propanol**	**?2,3-Butanediol**	**?2-Furanmethanol**	**?2-Methylbutanoic acid**	**?3-Methylbutanoic acid**	**?2-Acetylfuran**	**?1,3-Propylene diacetate**	**?3-(Methylthio)-1-propanol**	**Hexanoic acid**	**?1-Methylhydantoin**	**?Furaneol**	**?2-Acetylpyrrole**	**?Pyranone**	**?4-Ethoxy-4-oxobutanoic acid**	**?1-(2-Furyl)-1,2-ethanediol**	**?Benzenacetic acid**
KBI 1.2 1	*Unit*	*Relative*	*Relative*	*Relative*	*Relative*	*Relative*	*Relative*	*Relative*	*Relative*	*Relative*	*Relative*	*Relative*	*Relative*	*Relative*	*Relative*	*Relative*	*Relative*	*Relative*	*Relative*	*Relative*	*Relative*
		0.35	0.57	0.80	0.92	0.96	1.42	2.02	0.92	0.17	1.98	2.09	1.33	1.16	2.18	1.54	1.97	2.27	0.21	2.67	0.50
KBI 1.2 2		0.33	0.68	0.86	0.95	0.88	1.37	2.00	0.99	0.18	2.05	1.99	1.42	1.08	2.07	1.49	1.97	2.19	0.23	2.59	0.55
KBI 1.2 3		0.33	0.59	0.91	0.99	0.98	1.36	1.99	1.01	0.19	2.13	2.03	1.44	1.14	2.00	1.46	1.98	2.19	0.21	2.45	0.60
KBI 3.2 1		0.28	0.77	1.14	1.00	0.92	1.41	2.01	1.42	0.22	2.17	1.44	2.03	1.11	2.01	1.62	1.86	2.00	0.32	2.69	0.63
KBI 3.2 2		0.30	0.61	1.06	0.93	0.97	1.35	2.02	1.32	0.20	1.92	1.63	1.97	1.19	2.13	1.65	2.00	2.14	0.38	2.56	0.55
KBI 3.2 3		0.30	0.84	1.52	1.24	0.97	1.36	2.46	2.01	0.30	2.73	1.69	2.38	1.62	2.09	1.73	2.63	2.08	0.40	2.51	0.81
KBI 5.3 1		0.38	0.85	1.99	0.95	0.92	1.23	2.08	2.59	0.36	2.07	0.47	1.70	0.61	2.01	1.37	1.92	2.07	0.22	2.60	0.66
KBI 5.3 2		0.36	0.68	2.08	0.94	0.95	1.13	2.08	2.59	0.35	2.11	0.34	1.68	0.63	1.83	1.21	1.92	1.86	0.24	2.43	0.63
KBI 5.3 3		0.37	0.84	1.98	0.94	0.84	1.15	2.20	2.54	0.35	2.15	0.43	1.66	0.57	1.97	1.39	1.96	1.96	0.23	2.67	0.63
KBI 12.1 1		0.25	0.61	0.68	0.77	1.46	1.05	1.76	0.50	0.12	1.90	2.65	1.44	0.87	1.96	1.25	1.61	2.08	0.22	2.66	0.48
KBI 12.1 2		0.27	1.88	0.90	1.09	1.78	1.11	2.33	0.67	0.17	2.44	2.73	1.85	1.33	2.15	1.61	2.16	2.22	0.35	3.02	0.83
KBI 12.1 3		0.25	0.66	0.75	0.82	1.42	0.97	2.03	0.59	0.13	2.10	2.78	1.51	0.98	2.03	1.32	2.00	2.21	0.22	2.64	0.52
CBS 707 1		0.29	0.81	2.40	0.74	7.17	1.35	1.84	1.94	0.29	1.80	0.14	2.71	0.42	1.88	1.37	1.65	1.80	0.26	2.48	0.67
CBS 707 2		0.28	0.88	3.00	0.94	8.69	1.41	2.16	2.46	0.37	2.26	0.20	3.16	0.49	1.95	1.53	2.05	1.87	0.25	2.49	0.76
CBS 707 3		0.32	0.81	2.24	0.72	6.51	1.23	2.10	1.91	0.30	1.80	-0.18	2.68	0.72	1.92	1.45	1.94	1.86	0.29	2.44	0.61
WLP001 1		0.11	0.85	0.32	1.03	0.74	2.41	2.01	0.36	0.33	2.35	2.55	2.40	1.34	2.07	7.56	2.04	2.30	0.16	2.26	0.60
WLP001 2		0.15	0.67	0.27	0.81	0.47	2.46	1.85	0.28	0.26	2.10	1.59	1.97	0.92	1.88	7.31	1.87	2.11	0.10	2.18	0.44
WLP001 3		0.23	0.70	0.30	0.95	0.64	2.57	1.95	0.34	0.30	2.27	2.19	2.26	1.09	1.91	7.28	2.00	2.16	0.13	2.16	0.52

Dp		31.9	7.3	27.9	3.8	25.9	24.6	46.7	8.2	19.3	11.8	42.5	3.4	49.5
Relative Precision (%)		2	6	4	19	3	4	5	11	6	7	5	7	8
Absolute Precision		0.2	0.06	0.4	0.007	0.549	0.308	0.474	0.024	0.017	0.022	0.001	0.027	0.003
LOD		2	0.2	0.02	0.004	0.012	0.031	0.339	0.021	0.037	0.008	0.004	0.130	0.020
	Compound	**Ethyl acetate**	**Diacetyl**	**Isobutanol**	**Isobutyl acetate**	**Isoamyl alcohol**	**2-methyl-1-butanol**	**Ethyl butyrate**	**Acetoin**	**Ethyl Lactate**	**Isoamyl acetate**	**Ethyl hexanoate**	**Linalool**	**Ethyl octanoate**
Customer ID	*Unit*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*
KBI 1.2 1		14	0.009	17.0	0.031	30.995	15.118	1.007	0.087	0.321	0.333	0.017	<LOD	0.034
KBI 1.2 2		14	<LOD	16.5	0.041	29.710	14.639	0.995	0.101	0.319	0.450	0.017	0.139	0.038
KBI 1.2 3		14	<LOD	16.8	0.026	30.253	14.911	0.925	0.108	0.334	0.291	0.016	0.222	0.033
KBI 3.2 1		13	0.034	16.7	0.031	31.888	16.108	0.720	0.125	0.304	0.430	0.017	0.229	0.048
KBI 3.2 2		15	<LOD	18.3	0.024	35.662	17.826	0.583	0.105	0.375	0.396	0.016	0.275	0.045
KBI 3.2 3		14	0.019	18.2	0.030	34.643	17.471	0.655	0.115	0.345	0.381	0.021	0.260	0.051
KBI 5.3 1		9	<LOD	17.9	0.045	26.077	14.031	0.832	0.093	0.345	0.570	0.009	0.287	0.024
KBI 5.3 2		9	<LOD	19.3	0.030	27.122	14.663	0.853	0.100	0.370	0.391	0.010	0.262	0.026
KBI 5.3 3		9	<LOD	16.8	0.025	24.642	13.276	0.762	0.109	0.298	0.329	0.010	0.224	0.025
KBI 12.1 1		12	0.019	15.6	0.030	26.784	12.996	0.791	0.102	0.302	0.268	0.013	0.298	0.044
KBI 12.1 2		12	0.011	14.7	0.058	25.125	12.304	0.831	0.118	0.280	0.351	0.017	0.252	0.044
KBI 12.1 3		11	0.018	14.7	0.024	25.396	12.227	0.708	0.070	0.271	0.252	0.014	0.328	0.040
CBS 707 1		14	<LOD	31.9	0.030	37.374	22.503	0.929	0.112	0.260	0.409	0.007	0.284	0.029
CBS 707 2		14	0.025	30.4	0.030	36.809	21.681	0.780	0.119	0.232	0.510	0.009	0.267	0.030
CBS 707 3		14	<LOD	35.6	0.033	40.901	24.640	0.949	0.156	0.300	0.508	0.009	0.321	0.028
WLP001 1		7	<LOD	26.0	0.046	27.585	12.866	0.541	0.092	0.488	0.280	0.039	0.292	0.475
WLP001 2		8	0.002	29.5	0.027	29.297	14.430	0.616	0.103	0.442	0.348	0.036	0.294	0.389
WLP001 3		7	<LOD	25.3	0.039	27.304	12.871	1.137	0.103	0.466	0.283	0.034	0.247	0.394

Supplementary Data Sheet 1

	Dp	23.9	19.2	26.0	41.6	1.8	ND	ND	ND	ND	ND	ND	ND	ND
	Relative Precision (%)	4	7	14	9	32	ND	ND	ND	ND	ND	ND	ND	ND
	Absolute Precision	0.403	0.010	0.001	0.004	0.015	ND	ND	ND	ND	ND	ND	ND	ND
	LOD	0.004	0.010	0.005	0.007	0.020	0.010	0.037	0.004	0.021	0.019	0.14	0.098	0.028
	Compound	**2-phenyl ethanol**	**2-phenylethyl acetate**	**4-ethylphenol**	**Ethyl decanoate**	**Nerol**	**Myrcene**	**Limonene**	**alpha-terpinol**	**Linalyl acetate**	**Geraniol**	**Citronellyl acetate**	**4-ethylguaiacol**	**Geranylacetate**
Customer ID	*Unit*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*
KBI 1.2 1		16.383	0.492	0.008	0.028	0.047	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
KBI 1.2 2		15.362	0.533	0.007	0.034	0.048	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
KBI 1.2 3		15.726	0.545	0.006	0.021	0.074	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
KBI 3.2 1		16.216	0.548	0.007	0.030	0.052	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
KBI 3.2 2		18.154	0.565	0.007	0.038	0.051	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
KBI 3.2 3		17.883	0.587	0.007	0.041	0.062	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
KBI 5.3 1		24.140	0.125	0.008	<LOD	0.044	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
KBI 5.3 2		24.587	0.153	0.008	<LOD	0.043	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
KBI 5.3 3		21.603	0.124	0.007	<LOD	0.040	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
KBI 12.1 1		12.870	0.448	0.007	0.020	0.052	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
KBI 12.1 2		12.016	0.471	0.007	0.021	0.061	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
KBI 12.1 3		12.039	0.400	0.007	0.035	0.070	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
CBS 707 1		25.370	0.077	0.006	0.013	0.035	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
CBS 707 2		24.943	0.084	0.006	<LOD	0.034	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
CBS 707 3		27.752	0.068	0.008	0.034	0.028	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
WLP001 1		24.037	0.058	0.008	0.457	0.078	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
WLP001 2		27.313	0.103	0.007	0.246	0.064	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
WLP001 3		23.704	0.072	0.008	0.352	0.067	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

Supplementary Data Sheet 1

CHROM	POS	REF	ALT	KBI_12_1	KBI_1_2	KBI_5_3	KBI_3_2	CBS707	EFFECT	IMPACT	CDS_CHANG	PROT_CHAN	GENE	DESCRIPTION	Column1	Column2	Column3	Column4	Column7	Column8	Column9	Column10
LAFE0A	686693	ATGGC	GTGGT	0/0	0/0	1/1	0/0	0/0	stop_gained	intragenic_variant	non_coding_transcript_exon_variant	HIGH	MODIFIER	MODIFIER	c.393_397del ATGGCmsGT GGT	n.686693_686698delATGGCmsGTGGT	n.1761_1765delATGGCmsGTGGT	p.Gln133*	LAFE_0A078886G	gene0	gene0	similar to uniprot|P36035 Saccharomyces cerevisiae YKL217W JEN1 Monocarboxylate/proton symporter of the plasma membrane transport activity is dependent on the pH gradient across the membrane mediates high-affinity uptake of carboxylic acids such as lactate, pyruvate, and short-chain fatty acids; also transports ketone body acetoacetate; expression of this structure mimics that of monocarboxylates expression and localization are tightly regulated with transcription repression mRNA degradation and protein endocytosis and degradation all occurring in the presence of glucose
LAFE0E	1321555	CAG	CG	0/0	0/0	1/1	0/0	0/0	frameshift_variant	HIGH	c.230delT	p.Leu77fs	LAFE_0E151920	some similarities with uniprot|P00175 Saccharomyces cerevisiae YKL043C CYB2 Cytochrome b2 (L-lactate cytochrome-c oxidoreductase) component of the mitochondrial intermembrane space required for lactate utilization expression is repressed by glucose and anaerobic conditions							

Supplementary Data Sheet 2

Run	Factor 1 A:Temperature °C	Factor 2 B:Pitching rate Mio. cells/mL	Factor 3 C:Extract °P	Response 1 Lactic acid mM	Response 2 Ethanol % ABV	Response 3 pH	Response 4 real Extract °P	Response 5 app Extract °P	Attenuation %
1	16	5	15	4.8	2.50	4.08	10.78	9.89	34%
2	22	32.5	5	1.1	1.73	3.91	2.19	1.53	69%
3	22	32.5	10	2.2	2.33	4.05	6.28	5.42	46%
4	16	5	5	1.1	1.34	3.85	1.72	1.21	76%
5	28	60	15	1.5	2.03	4.36	11.31	10.58	29%
6	28	32.5	10	2.0	2.67	4.08	5.90	4.92	51%
7	28	60	5	1.0	1.77	4.04	2.35	1.68	66%
8	22	60	10	1.7	2.86	4.05	5.60	4.54	55%
9	28	5	5	3.0	1.64	3.81	2.51	1.88	62%
10	22	32.5	10	2.3	2.00	4.05	6.75	6.01	40%
11	22	32.5	15	2.7	2.20	4.19	11.16	10.37	31%
12	28	5	15	11.3	1.52	3.92	12.26	11.72	22%
13	22	32.5	10	2.4	2.17	4.05	6.71	5.91	41%
14	22	60	10	1.4	2.65	4.06	5.82	4.84	52%
15	22	32.5	10	2.3	2.32	4.02	6.38	5.53	45%
16	28	5	5	3.2	1.72	3.78	2.37	1.71	66%
17	28	32.5	10	1.7	2.08	4.17	6.76	6.00	40%
18	22	32.5	10	2.0	2.36	4.03	6.00	5.13	49%
19	28	5	15	11.4	1.43	3.91	12.37	11.86	21%
20	16	60	5	0.5	1.77	4.05	2.17	1.49	70%
21	16	60	15	1.4	1.94	4.27	11.70	11.01	27%
22	22	32.5	15	2.5	2.25	4.23	11.16	10.36	31%
23	22	32.5	5	1.0	1.61	3.92	2.01	1.39	72%
24	16	5	15	4.2	2.21	4.10	11.27	10.48	30%
25	16	60	5	0.5	1.47	4.05	1.91	1.34	73%
26	28	60	15	1.7	2.30	4.32	11.00	10.18	32%
27	22	5	10	6.1	2.21	3.94	6.61	5.79	42%
28	28	60	5	1.0	1.81	4.08	2.25	1.56	69%
29	22	5	10	4.8	2.48	3.97	6.18	5.27	47%
30	22	32.5	10	2.2	2.45	4.04	6.07	5.16	48%
31	16	60	15	1.3	1.98	4.27	11.63	10.92	27%
32	16	5	5	1.3	1.55	3.88	1.97	1.37	73%
33	16	32.5	10	1.2	2.32	4.09	6.43	5.58	44%
34	16	32.5	10	1.1	2.43	4.09	6.24	5.35	47%

Supplementary Data Sheet 2

Response	1 Lactic acid					
Transform:	Natural Log	Constant:	0			
ANOVA for Response Surface Reduced Quartic model						
Analysis of variance table [Partial sum of squares - Type III]						
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	16.97441427	13	1.3057242	185.2256	6.86E-18	significant
A-Temperature	0.193433649	1	0.1934336	27.43983	3.98E-05	
B-Pitching rate	1.593138462	1	1.5931385	225.9972	2.31E-12	
C-Extract	4.938201597	1	4.9382016	700.5163	4.85E-17	
AB	0.261843279	1	0.2618433	37.14419	5.89E-06	
AC	0.079479807	1	0.0794798	11.27473	0.003132	
BC	0.33787252	1	0.3378725	47.92944	1.01E-06	
A^2	0.433527539	1	0.4335275	61.49873	1.58E-07	
B^2	0.145041024	1	0.145041	20.57502	0.000201	
C^2	0.199231406	1	0.1992314	28.26228	3.34E-05	
ABC	0.071019294	1	0.0710193	10.07455	0.00477	
A^2B	0.000280013	1	0.00028	0.039722	0.844038	
AB^2	0.044446769	1	0.0444468	6.305066	0.020741	
A^2B^2	0.073984937	1	0.0739849	10.49525	0.004107	
Residual	0.140987478	20	0.0070494			
Lack of Fit	0.013506097	1	0.0135061	2.012967	0.172156	not significant
Pure Error	0.127481381	19	0.0067095			
Cor Total	17.11540175	33				

The Model F-value of 185.23 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, AC, BC, A^2, B^2, C^2, ABC, AB^2, A^2B^2 are significant model terms.

The "Lack of Fit F-value" of 2.01 implies the Lack of Fit is not significant relative to the pure error. There is a 17.22% chance that a "Lack of Fit F-value" this large could occur due to noise.

Std. Dev.	0.08396055	R-Squared	0.9917625
Mean	0.686561422	Adj R-Square	0.9864082
C.V. %	12.22913886	Pred R-Squa	0.972661
PRESS	0.467918418	Adeq Precisi	57.232168

The "Pred R-Squared" of 0.9727 is in reasonable agreement with the "Adj R-Squared" of 0.9864; i.e. the difference is less than 0.2.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 57.232 indicates an adequate signal. This model can be used to navigate the design space.

Coefficient Factor	Standard Estimate	95% CI df	95% CI Error	Low	High	VIF
Intercept	0.805768282	1	0.0342768	0.734268	0.877268	
A-Temperature	0.219905462	1	0.0419803	0.132336	0.307475	5
B-Pitching rate	-0.631097944	1	0.0419803	-0.718667	-0.543529	5
C-Extract	0.496900473	1	0.0187741	0.457738	0.536063	1
AB	-0.127926561	1	0.0209901	-0.171711	-0.084142	1
AC	-0.070480408	1	0.0209901	-0.114265	-0.026696	1
BC	-0.145317007	1	0.0209901	-0.189102	-0.101532	1

Supplementary Data Sheet 2

A^2	-0.425013499	1	0.0541963	-0.538065	-0.311962	3.431373
B^2	0.245832789	1	0.0541963	0.132781	0.358884	3.431373
C^2	-0.288119916	1	0.0541963	-0.401171	-0.175068	3.431373
ABC	-0.066623613	1	0.0209901	-0.110408	-0.022839	1
A^2B	-0.009354357	1	0.0469354	-0.10726	0.088551	5
AB^2	0.117854212	1	0.0469354	0.019949	0.21576	5
A^2B^2	0.330811205	1	0.1021137	0.117806	0.543817	12.52941

Final Equation in Terms of Coded Factors:

Ln(Lactic acid) =

0.805768282
0.219905462 * A
-0.631097944 * B
0.496900473 * C
-0.127926561 * AB
-0.070480408 * AC
-0.145317007 * BC
-0.425013499 * A^2
0.245832789 * B^2
-0.288119916 * C^2
-0.066623613 * ABC
-0.009354357 * A^2B
0.117854212 * AB^2
0.330811205 * A^2B^2

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels of the factors are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Final Equation in Terms of Actual Factors:

Ln(Lactic acid) =

-1.852009932
0.02776173 * Temperature
-0.383921392 * Pitching rate
0.358168851 * Extract
0.03351161 * Temperature * Pitching rate
0.00027522 * Temperature * Extract
0.000719779 * Pitching rate * Extract
0.00133566 * Temperature^2
0.005634742 * Pitching rate^2
-0.011524797 * Extract^2
-8.08E-05 * Temperature * Pitching rate * Extract
-0.000799264 * Temperature^2 * Pitching rate
-0.000508671 * Temperature * Pitching rate^2
1.22E-05 * Temperature^2 * Pitching rate^2

The equation in terms of actual factors can be used to make predictions about the response for given levels of each factor. Here, the levels should be specified in the original units for each factor. This equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor and the intercept is not at the center of the design space.

Supplementary Data Sheet 2

Response 1 Lactic acid Transform: Natural Log Constant: 0

Diagnostics Case Statistics

Internally Run Order	Externally Actual Value	Influence on Predicted Value	Studentized Residual	Studentized Leverage	Cook's Residual	Fitted Value Residual	Standard Distance	DFFITS	Order
1	1.56024767	1.490119202	0.070128467	0.4875	1.166735	1.177987758	0.09249	1.148897	10
2	0.10526051	0.020747893	0.084512617	0.3	1.203088	1.217508215	0.044309	0.797046	26
3	0.78572636	0.805768282	-0.02004192	0.166666667	-0.26149	-0.255305487	0.000977	-0.114176	30
4	0.13015068	0.197970653	-0.067819968	0.4875	-1.128328	-1.136526346	0.086501	-1.10846	1
5	0.40812823	0.453139117	-0.045010892	0.4875	-0.748851	-0.740342652	0.038102	-0.72206	16
6	0.66834206	0.600660245	0.067681817	0.5	1.140018	1.149115181	0.092832	1.149115	19
7	-0.0110609	0.024180229	-0.035241176	0.4875	-0.586311	-0.576440876	0.023357	-0.562206	7
8	0.51819838	0.420503126	0.097695252	0.5	1.645558	1.724906754	0.193419	1.724907	23
9	1.08653971	1.137056712	-0.050517004	0.4875	-0.840457	-0.83403674	0.047994	-0.81344	3
10	0.85185887	0.805768282	0.04609059	0.166666667	0.60135	0.591495724	0.005166	0.264525	33
11	1.00686243	1.014548839	-0.007686409	0.3	-0.109421	-0.106681971	0.000367	-0.06984	28
12	2.42036813	2.413778081	0.006590047	0.4875	0.109639	0.106895315	0.000817	0.104256	11
13	0.8683602	0.805768282	0.062591916	0.166666667	0.816646	0.809579978	0.009527	0.362055	32
14	0.32280787	0.420503126	-0.097695252	0.5	-1.645558	-1.724906754	0.193419	-1.724907	24
15	0.83377831	0.805768282	0.028010028	0.166666667	0.365451	0.357392642	0.001908	0.159831	29
16	1.16158709	1.137056712	0.024530376	0.4875	0.408114	0.399447536	0.011317	0.389583	4
17	0.53297843	0.600660245	-0.067681817	0.5	-1.140018	-1.149115181	0.092832	-1.149115	20
18	0.68662596	0.805768282	-0.119142319	0.166666667	-1.554467	-1.615860579	0.03452	-0.722635	34
19	2.43317466	2.413778081	0.019396581	0.4875	0.322703	0.315354036	0.007076	0.307566	12
20	-0.5996568	-0.669694041	0.070037204	0.4875	1.165216	1.176343036	0.09225	1.147293	5
21	0.35767444	0.307680933	0.049993511	0.4875	0.831747	0.825082499	0.047004	0.804707	13
22	0.91828873	1.014548839	-0.096260104	0.3	-1.37032	-1.403114472	0.057483	-0.918554	27
23	0.04018179	0.020747893	0.019433896	0.3	0.276653	0.270165552	0.002343	0.176865	25
24	1.44597736	1.490119202	-0.044141838	0.4875	-0.734392	-0.725648229	0.036645	-0.707728	9
25	-0.7657179	-0.669694041	-0.096023832	0.4875	-1.597559	-1.667105992	0.173407	-1.625937	6
26	0.52413664	0.453139117	0.07099752	0.4875	1.181193	1.19367329	0.094797	1.164195	15
27	1.80599105	1.682699015	0.123292036	0.5	2.076705	2.285480312	0.30805	2.28548	22
28	0.03343478	0.024180229	0.009254548	0.4875	0.153969	0.150159332	0.001611	0.146451	8
29	1.55940698	1.682699015	-0.123292036	0.5	-2.076705	-2.285480312	0.30805	-2.28548	21
30	0.80825999	0.805768282	0.002491705	0.166666667	0.03251	0.031687307	1.51E-05	0.014171	31
31	0.28367405	0.307680933	-0.024006882	0.4875	-0.399405	-0.390853782	0.010839	-0.381202	14
32	0.23980399	0.197970653	0.04183334	0.4875	0.695986	0.686730178	0.032912	0.669771	2
33	0.18979357	0.160849321	0.02894425	0.5	0.487531	0.478035266	0.016978	0.478035	18
34	0.13190507	0.160849321	-0.02894425	0.5	-0.487531	-0.478035266	0.016978	-0.478035	17

* Exceeds limits

Box-Cox Power Transformation

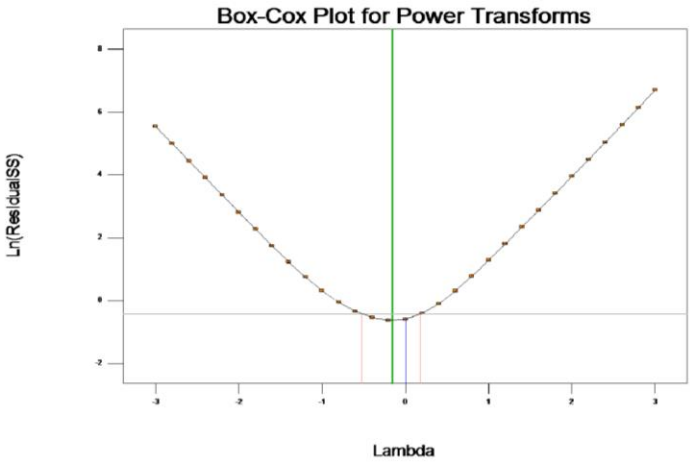
Constant k	95% CI Low	95% CI High	Best Lambda	Rec. Transform
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Supplementary Data Sheet 2

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Ln(Lactic acid)

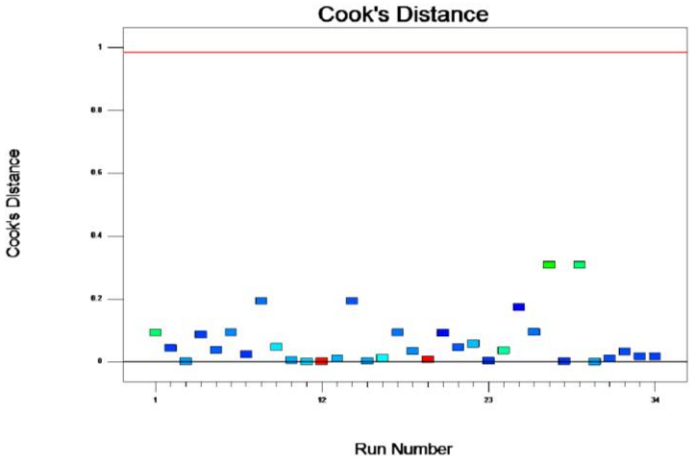
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Recommend transform:
Log
(Lambda = 0)



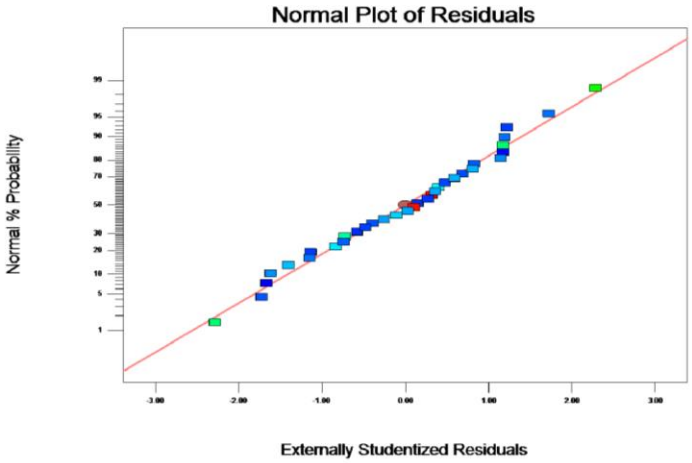
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Ln(Lactic acid)

Color points by value of
Ln(Lactic acid):
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-0.766



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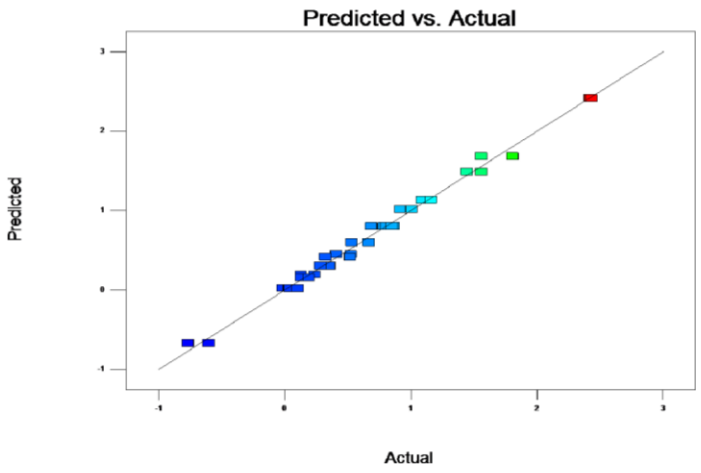
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Ln(Lactic acid):
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-0.766



Supplementary Data Sheet 2

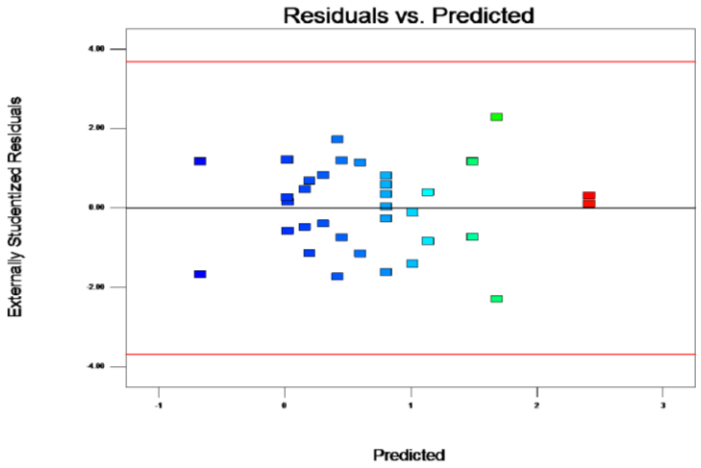
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Ln(Lactic acid)

Color points by value of
Ln(Lactic acid):
2.433
-0.766



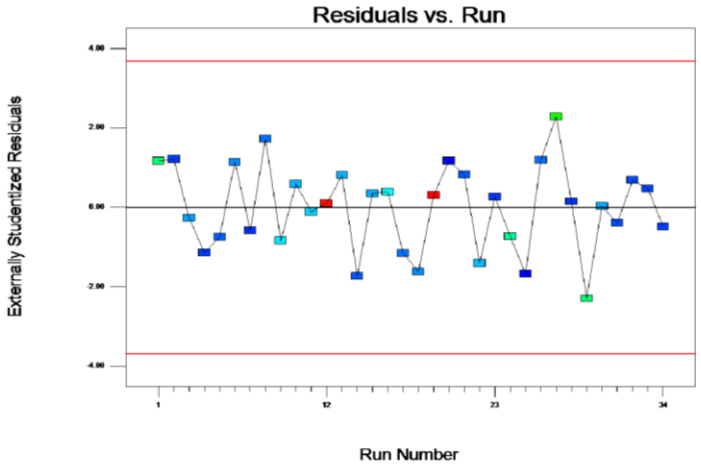
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Chapter 3

Bellut, K.; Michel, M.; Zarnkow, M.; Hutzler, M.; Jacob, F.; De Schutter, D. P.; Daenen, L.; Lynch, K. M.; Zannini, E.; Arendt, E. K. Application of Non-*Saccharomyces* Yeasts Isolated from Kombucha in the Production of Alcohol-Free Beer. *Fermentation* **2018**, *4*, 1–13.

Chapter 4

Bellut, K.; Michel, M.; Zarnkow, M.; Hutzler, M.; Jacob, F.; Atzler, J. J.; Hoehnel, A.; Lynch, K. M.; Arendt, E. K. Screening and application of *Cyberlindnera* yeasts to produce a fruity, non-alcoholic beer. *Fermentation* **2019**, *5*, 1–25.

Chapter 5

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Chapter 6

Awaiting publication.

Appendix

Bellut, K.; Michel, M.; Zarnkow, M.; Hutzler, M.; Jacob, F.; Lynch, K. M.; Arendt, E. K. On the suitability of alternative cereals, pseudocereals and pulses in the production of alcohol-reduced beers by non-conventional yeasts. *Eur. Food Res. Technol.* **2019**, *245*, 2549–2564.

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Article

Application of Non-*Saccharomyces* Yeasts Isolated from Kombucha in the Production of Alcohol-Free Beer

Konstantin Bellut [1], Maximilian Michel [2], Martin Zarnkow [2], Mathias Hutzler [2], Fritz Jacob [2], David P. De Schutter [3], Luk Daenen [3], Kieran M. Lynch [1], Emanuele Zannini [1] and Elke K. Arendt [1,4,*]

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Abstract: Alcohol-free beer (AFB) is no longer just a niche product in the beer market. For brewers, this product category offers economic benefits in the form of a growing market and often a lower tax burden and enables brewers to extend their product portfolio and promote responsible drinking. Non-*Saccharomyces* yeasts are known for their flavor-enhancing properties in food fermentations, and their prevailing inability to ferment maltose and maltotriose sets a natural fermentation limit and can introduce a promising approach in the production of AFB ($\leq$0.5% v/v). Five strains isolated from kombucha, *Hanseniaspora valbyensis*, *Hanseniaspora vineae*, *Torulaspora delbrueckii*, *Zygosaccharomyces bailii* and *Zygosaccharomyces kombuchaensis* were compared to a commercially applied AFB strain *Saccharomycodes ludwigii* and a *Saccharomyces cerevisiae* brewer's yeast. The strains were characterized for their sugar utilization, phenolic off-flavors, hop sensitivity and flocculation. Trial fermentations were analyzed for extract reduction, ethanol formation, pH drop and final beers were analyzed for amino acids utilization and fermentation by-products. The performance of non-*Saccharomyces* strains and the commercial AFB strain were comparable during fermentation and production of fermentation by-products. An experienced sensory panel could not discriminate between the non-*Saccharomyces* AFB and the one produced with the commercial AFB strain, therefore indicating their suitability in AFB brewing.

Keywords: non-alcoholic beer; non-conventional yeast; non-*Saccharomyces*; brewing; fermentation; yeast characterization; sensory

1. Introduction

In many countries nowadays, alcohol-free beer (AFB) is no longer just a niche product in the beer market. For brewers, this product category offers economic benefits in the form of a steadily growing market and often a lower tax burden. At the same time, consumer preference for low-alcohol and alcohol-free beer is increasing due to greater interest in health, concern about weight, and considering the encouragement of responsible drinking, especially when driving. Furthermore, consumers benefit from the health effects of alcohol-free beers, which lie in the healthy beer components

(antioxidants, soluble fiber, vitamins, and minerals), lower energy intake and absence of negative aspects of alcohol consumption [1].

The terminology of alcohol-free beer and the corresponding alcohol limits are not uniform. The classifications of alcohol-free beers are defined in the statutory regulations of the individual countries. In many European countries such as Germany, Switzerland, Austria, Finland, and Portugal, the term "alcohol-free" describes a maximum alcohol limit of 0.5% (*v*/*v*) ethanol. In Denmark and in the Netherlands the term "alcohol-free" may be applied to beers with <0.1% (*v*/*v*) [2]. In the UK, the term "alcohol-free" can be applied to beer with <0.05% (*v*/*v*) alcohol and the term "de-alcoholised" when the alcohol content is <0.5% (*v*/*v*) [3]. In the USA and China, the limit of <0.5% (*v*/*v*) is described by the term "non-alcoholic". Other countries like Spain or France are more tolerant towards the term "alcohol-free" with limits of 1.0% and 1.2% (*v*/*v*), respectively [2].

The strategies to produce alcohol-free beers can be divided into two main groups: physical and biological processes. The physical processes, divided into thermal and membrane-based methods, are based on the removal of alcohol from regular beer and require considerable investments into special equipment [4]. In the case of thermal processes, the beer is heated to evaporate the ethanol, whereby also volatile aroma components are partly or completely evaporated. During membrane-based processes, ethanol (as well as aroma components) is removed mainly by its molecular size. Both cases can lead to less aromatic beers with reduced body and a significant acidity [2]. The most widespread biological approaches are based on limited ethanol formation by the yeast during the beer fermentation. Limited fermentation is usually performed in traditional brewery equipment and hence does not require additional investment. However, the beers are often perceived as sweet because of the interruption of the fermentation; fermentable sugars are not or only partly metabolized by the yeast, and the aromatic secondary metabolites are formed only in small quantities or have not yet been generated due to the short fermentation time. In the field of limited fermentation different approaches are being pursued to improve the taste impression, which include the reduction of worty taste caused by strecker aldehydes [5,6], the use of immobilized yeasts [7], and the use of alternative yeast strains or yeast mutants [8]. The use of non-*Saccharomyces* yeasts (other than *Saccharomycodes ludwigii*) for the production of AFB has not been studied to a great extent, though changing the yeast is an easy adjustment for breweries to make. By using yeast strains which are unable to ferment the most abundant wort sugars maltose and maltotriose, a natural fermentation limit is set. It is unnecessary to stop the fermentation by cooling or yeast separation, since the fermentation will naturally come to a halt by the depletion of the fermentable sugars. However, the challenge is to discover non-*Saccharomyces* yeasts, that are able to produce flavors that can mask the wort-like off-flavors created by residual wort sugars and aldehydes [5,6].

There are few published studies on the application of non-*Saccharomyces* yeasts in the production of alcohol-free beer [9]. Mostly known as spoilage yeasts for beer or other beverages, they can form a range of flavors which could potentially benefit the alcohol-free beer [10–12]. In a recent patent application, Saerens and Swiegers [13] used *Pichia kluyveri* to produce a low-alcohol or alcohol-free beer with a flavor profile very close to a beer of at least 4% (*v*/*v*) alcohol. Another patent by Li et al. [14] suggests the use of *Candida shehatae* to produce an alcohol-free beer. Sohrabvandi et al. [15] investigated the use of *Zygosaccharomyces rouxii* in a successive application after *Saccharomyces cerevisiae* in order to produce an alcohol-free beer. A significant alcohol reduction could be shown, however, the taste was compromised. De Francesco et al. [3] investigated strains of *Z. rouxii* and *Saccharomycodes ludwigii* for the production of low-alcohol beers. In contrast to the results from Sohrabvandi et al. [15], *Z. rouxii* strains were found unsuitable to produce low alcohol beer due to the production of a high concentration of ethanol, however, *S. ludwigii* was identified as a yeast species with great potential for the production of low-alcohol and alcohol-free beer.

In this study, five non-*Saccharomyces* yeast strains isolated from kombucha, namely *Hanseniaspora valbyensis* KBI 22.1, *Hanseniaspora vineae* KBI 7.1, *Torulaspora delbrueckii* KBI 22.2, *Zygosaccharomyces bailii* KBI 25.2 and *Zygosaccharomyces kombuchaensis* KBI 5.4, were investigated for their application in

the production of an alcohol-free beer. Kombucha is effervescent, slightly sweet, and slightly acidic fermented tea, comprising a SCOBY, a Symbiotic Culture of Bacteria and Yeast, consisting of ethanol fermenting yeast and bacteria originating from the acetic acid bacteria family, which are enclosed in a thick cellulose containing pellicle [16]. The yeasts were characterized, and fermentation performance and final beer quality were compared to two commercially applied yeast strains. *Saccharomyces cerevisiae* WLP001 (California Ale Yeast®) is a broadly applied, maltose-positive brewer's yeast and *Saccharomycodes ludwigii* TUM SL 17 is a maltose-negative yeast, which is commercially applied in AFB brewing [17].

The objective of this study was to compare the selected non-*Saccharomyces* yeasts to the commercially applied AFB strain TUM SL 17 and to evaluate their general applicability in AFB brewing.

2. Materials and Methods

2.1. Materials

All reagents used in this study were at least analytical grade from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Malt extract used for the flocculation test, hop resistance test, and propagation was supplied by Muntons (Spraymalt Light, Muntons plc, Suffolk, UK). Pilsner malt for wort production was sourced from Weyermann® (Malzfabrik Weyermann, Bamberg, Germany).

2.2. Yeast Strains

The yeast strains investigated in this study were isolated from kombucha. DNA of the isolates was extracted using an extraction kit (Yeast DNA Extraction Kit, Thermo Fisher Scientific, Waltham, MA, USA). To amplify the D1/D2 domain of the 26S rRNA gene the primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) were used. PCR was performed using the temperature protocol: 95 °C/2 min; 30 cycles of 95 °C/30 s, 56 °C/15 s; 72 °C/60 s; 72 °C/5 min.

Stocks were kept in glycerol at −80 °C. Table 1 lists the yeast strains that were used in this study. Strains were grown on PDA agar plates for 72 h at 25 °C and stored in a sterile environment at 2–4 °C. During this study, strains were subcultured at intervals of two weeks. The strains were chosen from a collection of 64 isolated strains by their performance in a pre-screening in wort (data not shown).

Table 1. Yeast strain designation, species and origin of yeast strains used in this study.

Strain Designation	Species	Origin
KBI 5.4	*Zygosaccharomyces kombuchaensis*	UCC Culture Collection (Kombucha, Australia)
KBI 7.1	*Hanseniaspora vineae*	UCC Culture Collection (Kombucha, USA)
KBI 22.1	*Hanseniaspora valbyensis*	UCC Culture Collection (Kombucha, Australia)
KBI 22.2	*Torulaspora delbrueckii*	UCC Culture Collection (Kombucha, Australia)
KBI 25.2	*Zygosaccharomyces bailii*	UCC Culture Collection (Kombucha, USA)
TUM SL 17	*Saccharomycodes ludwigii*	FZW BLQ [1], Weihenstephan, Germany
WLP001	*Saccharomyces cerevisiae*	California Ale Yeast®, Whitelabs, San Diego, CA, USA
TUM 68 [2]	*Saccharomyces cerevisiae*	FZW BLQ [1], Weihenstephan, Germany

[1] Research Centre Weihenstephan for Brewing and Food Quality, Technische Universität München [2] only used as positive control for POF test.

2.3. Flocculation Test

Flocculation of the yeast strains was evaluated using a slightly modified Helm's assay [18,19]. Essentially, all cells were washed in EDTA and the sedimentation period was extended to 10 min to allow slowly flocculating strains to show their potential. Fermentation wort was 75 g spray-dried malt extract (Spraymalt Light, Muntons plc, Suffolk, UK) in 1000 mL brewing water with 30 IBU (30 mg/mL iso-α-acids; from 30% stock solution; Barth-Haas Group, Nürnberg, Germany). Cultures recovered from fermentation were washed with 5 mM EDTA (pH 7) to break the cell aggregates. Flocculation

was assayed by first washing the yeast pellets with 3.7 mM $CaSO_4$ solution and resuspending them in flocculation solution containing 3.7 mM $CaSO_4$, 6.8 g/L sodium acetate and 4.05 g/L acetic acid (pH 4.5). Yeast cells in control tubes were resuspended in 5 mM EDTA (pH 7) without undergoing the flocculation step with $CaSO_4$. After a sedimentation period of 10 min, samples were taken from just below the meniscus and dispersed in 5 mM EDTA. The absorbance at 600 nm was measured (Helios Gamma Spectrophotometer, Thermo Fisher Scientific, Waltham MA, USA), and percentage of flocculation was determined from the difference in absorbance between the control and flocculation tubes.

2.4. Sugar Utilization

Substrate utilization tests YT MicroPlate™ (Biolog Inc., Hayward, CA, USA) were used to analyze the biochemical spectrum of the yeast isolates. The yeast strains were cultured on Sabouraud agar for 72 h at 25 °C. Individual colonies were taken from the surface using sterile inoculation loops and suspended in 20 mL of sterile water. Colonies were gradually added to increase the turbidity until 46 ± 1%. From this yeast solution, 100 µL were added to each of the 96 wells of the YT MicroPlate™. After incubation at 25 °C for 72 h, the YT MicroPlate™ was read with the Microplate reader (Multiskan FC, Thermo Fischer Scientific) at a wavelength of 590 nm. Results are shown as "+" for a significant increase in optical density (OD) compared to the OD of the water control and a "−" for showing no difference. The substrate utilization test was carried out in duplicate.

2.5. Hop Resistance

Three 100 mL flasks containing sterile filtered wort (75 g Muntons Spraymalt Light in 1000 mL brewing water) were adjusted to 0, 50, and 100 mg/L iso-α-acids respectively by using an aliquot of a stock solution of 3% iso-α-acids in 96% (*v*/*v*) ethanol (Barth-Haas Group, Nürnberg, Germany). The pure grown yeast cells were added to a total cell count of 10^5 cells/mL. Optical density (OD_{600}) was measured every 40 min at 25 °C without shaking over a time period of 96 h (Multiskan FC, Thermo Scientific, Waltham, MA, USA).

2.6. Phenolic Off-Flavor Test

The phenolic off-flavor (POF) test was conducted according to Meier-Dörnberg et al. [20]. Yeast strains were spread on yeasts and mold agar plate (YM-agar) containing one of the following precursors: ferulic acid, cinnamic acid or coumaric acid. After three days of incubation at 25 °C, plates were evaluated by sniffing to detect any of the following aromas: ferulic acid becomes 4-vinylguaiacol (clove-like), cinnamic acid becomes 4-vinylstyrene (Styrofoam-like), and coumaric acid becomes 4-vinylphenol (medicinal-like). TUM 68 (Research Center Weihenstephan for Brewing and Food Quality, Freising-Weihenstephan, Germany) was used as a positive control.

2.7. Propagation

Propagation wort was prepared by dissolving 75 g spray-dried malt (Muntons Spraymalt light, Muntons plc, Suffolk, UK) and 30 g glucose (Gem Pack Foods Ltd., Dublin, Ireland) in 1000 mL brewing water, followed by sterilization (15 min, 121 °C). Investigated pure yeast strains were inoculated into a 140 mL of sterile propagation wort. The flask was covered with sterile cotton and placed in an incubator with orbital shaker (ES-80 shaker-incubator, Grant Instruments (Cambridge) Ltd., Shepreth, UK) and incubated for 48 h at an orbital agitation of 170 rpm and 25 °C. Viability was measured by staining with Löffler's methylene blue solution (MEBAK 10.11.3.3) and cells were counted with a Hemocytometer (Blaubrand, Thoma pattern, Sigma-Aldrich, St. Louis, MO, USA).

2.8. Wort Production

Wort for fermentation trials was produced on a 60 L pilot-scale brewing plant comprising of a combined mash-boiling vessel, a lauter tun and a whirlpool tank. Weyermann® Pilsner Malt was milled with a two-roller mill fitted with a 0.8 mm gap size between the rollers. Seven kg of malt were mashed in with 40 L of brewing water. The following mashing regime was employed: 40 min at 50 °C, 20 min at 62 °C, 20 min at 72 °C and 5 min at 78 °C for mashing off. The heating rate was 1 °C/min between the temperature rests. The mash was pumped in the lauter tun and lautering was performed using three sparging steps of 5 L each. Collected wort was boiled for 45 min. 25 g Magnum hop pellets (10.5% iso-α-acids) were added at the start of the boil for a calculated IBU content of 10.4. Hot trub precipitates and hop residue were removed by means of the whirlpool with a rest of 20 min. Wort was pumped back to the boiling vessel, corrected to a specific gravity of 6.6 °P extract by the addition of brewing water, and heated to 100 °C before filling into sterile 5 L containers, which were kept for short-term storage at 2 °C.

2.9. Fermentation

Fermentation trials were carried out in 2-L sterile Duran glass bottles (Lennox Laboratory Supplies Ltd., Dublin, Ireland), equipped with an air lock to control CO_2 under sterile conditions. Bottles were filled with 1600 mL wort. Respective fermentation temperature was 25 °C, a temperature that suits most non-*Saccharomyces* species [21]. Fermentation was performed until no change in extract could be measured for 24 h. Yeast cells for pitching were washed by centrifugation at 5000 *g* for 5 min and resuspension in sterile water. Supernatant was discarded to ensure no carryover of sugars from the propagation wort into the fermentation wort and yeast cells were resuspended in sterile water. The pitching volume was 30 mL with a pitching rate of 8×10^6 CFU/mL at a viability of at least 96% for all fermentations.

2.10. Analyses of the Produced Beers

50 mL samples of each fermentation were withdrawn every day. Cell count was performed using the Hemocytometer (Blaubrand, Thoma pattern). Yeast was separated by centrifugation at 5000 *g* for 5 min and specific gravity and ethanol content of the supernatant were measured using a density meter DMA 4500 M with Alcolyzer Beer ME (Anton-Paar GmbH, Graz, Austria). The pH value was determined using a digital pH meter (Mettler Toledo LLC, Columbus, OH, USA).

Analyses of the final beers were performed by the following methods. Sugars and ethanol were determined by high performance liquid chromatography HPLC Agilent 1260 Infinity (Agilent Technologies, Santa Clara, CA, USA) equipped a refractive index detector (RID) and a Sugar-Pak I 10 µm, 6.5 mm × 300 mm column (Waters, Milford, MA, USA) with 0.1 mM Ca-EDTA as mobile phase and a flow rate of 0.2 mL/min. Differentiation of maltose and sucrose was achieved with a Nova-Pak 4 µm, 4.6 mm × 250 mm column (Waters) with acetonitrile/water 75:25 (*v*/*v*) as mobile phase and a flow rate of 1.2 mL/min.

Free vicinal diketones were quantified by a Clarus 500 gas chromatograph (Perkin-Elmer, Waltham, MA, USA) with a headspace unit and Elite-5 60 m × 0.25 mm, 0.5 µm column using a 2,3-hexanedione internal standard. The final concentrations of fermentation by-products (e.g., acetaldehyde, ethyl acetate, n-propanol, i-butanol, isoamyl acetate, amyl alcohols) were quantified using a gas chromatograph with a headspace unit and INNOWAX cross-linked polyethylene-glycol 60 m × 0.32 mm 0.5 µm column (Perkin-Elmer). The amino acid content was quantified using the HPLC MEBAK 2.6.4.1 method. Free amino nitrogen (FAN) was measured using a ninhydrin-based dying method where absorbance is measured at 570 nm against glycine (MEBAK 2.6.4.1). Free vicinal diketones, fermentation by-products and amino acids were quantified in duplicate.

2.11. Sensory Evaluation

All beer samples were tasted and judged by a sensory panel of 11 panelists with long-standing experience in the sensory analysis of beer. "Fruity", "floral", and "wort-like" were chosen as attributes for the smell. "Acidic/sour" and "sweet" were chosen as attributes for the taste and the panelists were additionally asked to evaluate the "body". Panelists were asked to evaluate the attributes in its intensity on a scale from 0, nothing, to 10, extremely. Before the evaluation of the intensity, a descriptive sensory was performed where the panelists were asked to record the flavors they perceived from the samples. Samples were given in dark glasses with a three-digit code.

2.12. Statistical Analyses

Fermentations and analyses were carried out in triplicate, unless stated otherwise. The data was statistically analyzed using RStudio, Version 1.1.423 with R version 3.4.4 (RStudio Inc., Boston, MA, USA; R Core Team, r-project). For the analysis of sensory data and constructing the multidimensional sensory profile, the R package "SensoMineR" was used [22]. One-way ANOVA was used to compare means and Tukey's test with 95% confidence intervals was applied for the pairwise comparison of means. The statistical significance value for both ANOVA and multiple comparison analysis was set at $p = 0.05$. Values are given as means ± standard deviation.

3. Results and Discussion

3.1. Yeast Characterization

When characterizing non-*Saccharomyces* yeasts for their suitability in alcohol-free beer production, several key attributes should be investigated. The first attribute is the ability to utilize the sugars in the wort, as for all-malt beers the average composition of fermentable wort sugars is 12% glucose and fructose (0.8–2.8%), 5% sucrose, 65% maltose, and 17.5% maltotriose [23]. For its suitability to produce alcohol-free beers it should not be able to ferment maltose. Considering the sugars that are important for brewing (glucose, fructose, sucrose, maltose, maltotriose), all strains were capable of fermenting glucose and fructose (Table 2).

Table 2. Substrate utilization profile by BioLog YT plate test, phenolic off-flavor (POF) performance and flocculation performance of the investigated yeasts. Different superscripts of values within a row indicate a significant difference ($p \leq 0.05$).

Attribute	WLP001	TUM SL 17	KBI 22.1	KBI 7.1	KBI 22.2	KBI 25.2	KBI 5.4
Maltose	+	−	−	−	−	−	−
Maltotriose	+	−	−	−	−	−	−
Glucose	+	+	+	+	+	+	+
Fructose [1]	+	+	+	+	+	+	+
Sucrose	+	+	−	−	+	+	+
Melibiose	−	−	−	−	+	−	−
Raffinose	+	+	−	−	+	−	+
Cellobiose	−	+	+	+	−	−	−
POF	−	−	−	−	−	−	−
Flocculation (%)	83 ± 3^{d}	60 ± 7^{c}	11 ± 8^{a}	41 ± 4^{b}	17 ± 0^{a}	45 ± 0^{bc}	44 ± 3^{bc}

[1] By HPLC sugar analysis; fructose was not detected in final beers.

All investigated strains except KBI 22.1 and KBI 7.1 (*Hanseniaspora* spp.) were able to ferment sucrose. The inability to ferment sucrose by KBI 22.1 and KBI 7.1 can be traced to the absence of the enzyme invertase, which converts sucrose into glucose and fructose [24]. In kombucha (source of investigated yeasts), where sucrose is the main or only sugar source, the conversion of sucrose by yeast invertase is required for *Acetobacter* spp. to subsequently produce acetic acid [25]. Looking at the main sugars of wort, only the control strain WLP001 was able to ferment maltose and maltotriose.

The disability to ferment maltose and maltotriose indicates the absence of a maltose transporter and the enzyme maltase [26,27]. The sugar fermentation patterns were confirmed by the sugar analysis of the final beers.

The second criterion for a yeast to be applied in brewing is its capability of growing in the presence of hop-derived iso-α-acids. The resistance against iso-a-acids and their induced weak organic acid stress were studied for the *Saccharomyces* species but it has barely been investigated for non-*Saccharomyces* species [28–30]. All investigated strains were able to grow in wort with 0, 50 and 100 IBU (international bitterness units). Figure 1 shows the exemplary growth of the investigated strains at 50 IBU (due to all strains exhibiting the same behavior at different IBU values, the rest of the data is not shown).

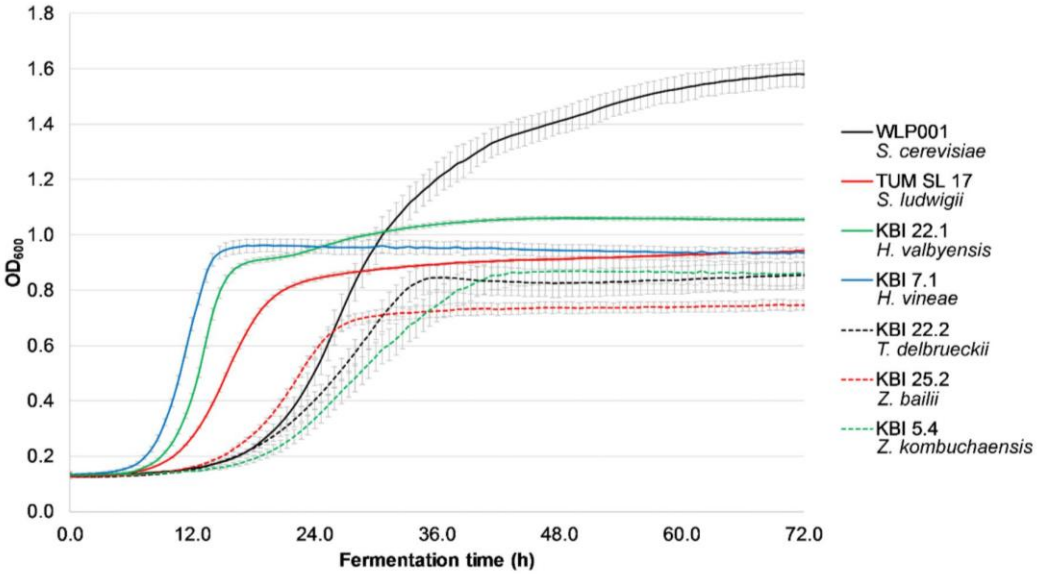


Figure 1. Growth curves of investigated yeast strains in 7 °P wort with 50 IBU.

KBI 7.1, KBI 22.1 and TUM SL 17 had the shortest lag time with log phases starting between 8 and 13 h after inoculation, followed by the rest of the investigated strains with log phases between 19 and 23 h. However, all the investigated yeast strains were able to grow in high iso-α-acid concentrations and are therefore able to ferment even highly hopped worts. The presence of iso-α-acids did not have any influence on the growth of the investigated yeast strains. This is in contrast to a study by Michel et al. [29], where the presence of 90 IBU resulted in a longer log phase as well as a lower slope during log phase compared with 50 and 0 IBU with several *Torulaspora delbrueckii* strains.

None of the investigated yeast strains, except the positive control TUM 68, showed any positive POF behavior on plate when exposed to precursors, suggesting the absence of a functional *POF1* gene [31] (Table 2). Those results were consistent with the sensory of the final beers where no panelist detected any phenolic off-flavors. POF are produced by decarboxylation of ferulic acid, coumaric acid and cinnamic acid, which are present in beer wort. Ferulic acid becomes 4-vinylguaiacol, which is described as having a clove-like flavor [32]. Apart from the wheat beer style this flavor is usually unwanted [33]. Coumaric acid is decarboxylated to 4-vinylphenol, having a solvent-like flavor, and cinnamic acid becomes 4-vinylstyrene, which has a Styrofoam-like flavor [34].

In terms of flocculation, a prerequisite for bulk sedimentation of yeast during brewery fermentation, the control yeast WLP001 performed as most flocculent of all the investigated strains. The method defines flocculation values of 85–100% as "very flocculent", 20–80% as "moderately flocculent" and less than 20% as "non-flocculent" yeasts [18]. By that definition WLP001

was with 83.3% at the very upper scale of moderately flocculent yeasts. KBI 22.1 and KBI 22.2 fell with 11.0% and 17.0%, respectively into the category of non-flocculent yeasts, while the rest qualified as moderately flocculent (Table 2). The most common mechanism of yeast flocculation is generally accepted to be the lectin-mediated adhesion of adjacent yeast cells to form large cell aggregations [35]. The flocculation characteristics of yeast are strongly strain-dependent and largely defined by which members of the *FLO* genes, which encode for lectin proteins, are functional in each strain. Rossouw et al. [36] showed that for 17 out of 18 investigated, non-*Saccharomyces* strains the flocculation phenotypes were calcium-dependent, thus indicating a *FLO*-dependency much like in *Saccharomyces cerevisiae*.

3.2. Fermentation Performance

The aim of propagation is to get a high quantity of yeast cells with high viability and vitality. After propagation for 48 h, cell counts ranged from 7.1×10^7 cells/mL for TUM SL 17 to 6.5×10^8 cells/mL for KBI 22.1, as illustrated in Figure 2.

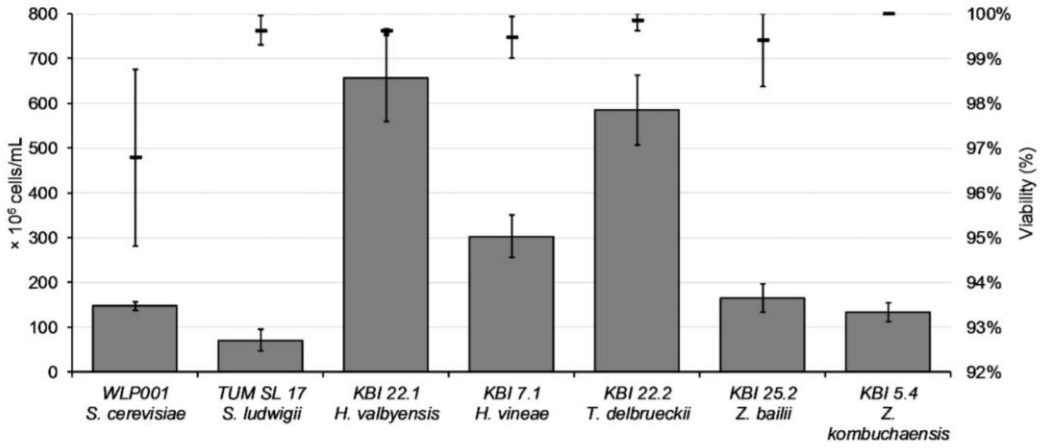


Figure 2. Cell count (bars) and viability (lines) of investigated strains after propagation for 48 h in 10 °P propagation wort (7% wort extract spiked with 3% glucose).

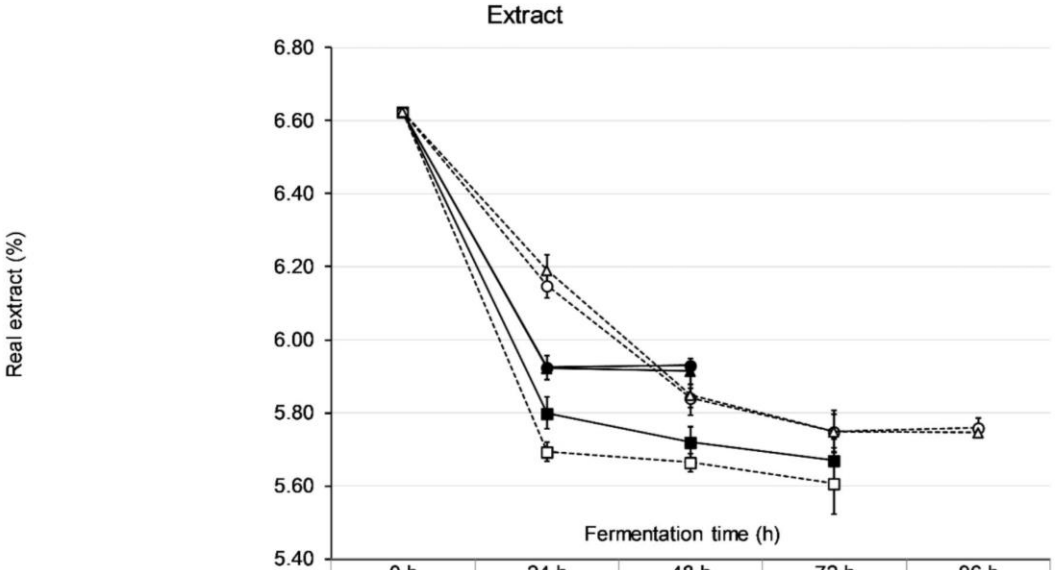
Except for WLP001 with a viability value around 97%, viability values after propagation were over 99%. The composition of the wort used for the fermentation trials is shown in Table 3.

Table 3. Wort composition of fermentation wort.

Wort Composition	Unit	Value
Extract	°P	6.63 ± 0.01
pH	-	5.73 ± 0.01
Maltose	g/L	26.60 ± 0.25
Maltotriose	g/L	5.09 ± 0.04
Glucose	g/L	5.46 ± 0.01
Sucrose	g/L	1.70 ± 0.04
Fructose	g/L	1.29 ± 0.02
Total amino acids	mg/100 mL	98.31 ± 0.86
Free amino nitrogen	mg/L	110 ± 5

The wort was fermented until no change in extract was measurable for 24 h. KBI 22.2 showed the steepest decrease in extract with a drop in extract of nearly 1 °P extract in the first 24 h followed by TUM SL 17 (0.8 °P) and the *Hanseniaspora* spp. KBI 22.1 and KBI 7.1 (0.7 °P) (Figure 3). The *Zygosaccharomyces*

spp., KBI 25.2 and KBI 5.4 followed a lesser decrease in extract with a linear decrease of about 0.45 °P per 24 h for the first 48 h. Consequently, KBI 22.2 reached an ethanol concentration of 0.42% (*v*/*v*) after 24 h, while KBI 25.2 and KBI 5.4 produced only 0.21% (*v*/*v*) and 0.20% (*v*/*v*), respectively. Fermentation ceased fastest for KBI 22.1 and KBI 7.1 after 24 h when fructose and glucose were depleted while sucrose remained untouched. TUM SL 17 and KBI 22.2 reached their final extract after 48 h of fermentation. KBI 25.2 and KBI 5.4, demonstrating the slowest metabolism, ceased fermentation after 72 h. WLP001 fermented the wort to a final extract (real) of 2.13 °P after 96 h (data not shown).



		0 h	24 h	48 h	72 h	96 h
TUM SL 17	*S. ludwigii*	6.63	5.80	5.72	5.67	
KBI 22.1	*H. valbyensis*	6.63	5.93	5.93		
KBI 7.1	*H. vineae*	6.63	5.92	5.91		
KBI 22.2	*T. delbrueckii*	6.63	5.69	5.66	5.61	
KBI 25.2	*Z. bailii*	6.63	6.15	5.84	5.75	5.76
KBI 5.4	*Z. kombuchaensis*	6.63	6.19	5.85	5.75	5.75

Figure 3. Drop in real extract for the investigated maltose-negative yeast strains.

The pH value dropped during the first 24 h of fermentation by values ranging from 0.7 for KBI 5.4 to 1.0 for KBI 22.2 with only marginal changes thereafter (data not shown). Due to the high starting pH of the wort of 5.7, the beers, except WLP001, did not reach pH values below 4.5, which are desired in order to serve as one of the microbial hurdles for beer spoiling bacteria to overcome [37]. However, lower pH values can be reached with a lower starting pH of the wort, which can be adjusted; i.e., by lactic acid, the use of sour malt or biological acidification. Visual evaluation of the finished beers matched the analyzed flocculation behavior from Table 2. KBI 22.1 and KBI 22.2 showed the highest turbidity and cells in suspension while TUM SL 17 and WLP001 were the clearest beers with a layer of flocculated yeast at the bottom.

Sugar analysis of the final beers revealed a complete depletion of all fermentable sugars by WLP001. Consistent with the sugar utilization patterns from Table 2, the other investigated strains showed a complete depletion of monosaccharides. Sucrose was not fermented by the *Hanseniaspora* spp. KBI 22.1 and KBI 7.1 but was depleted by the other strains as predicted before.

Analyses of the ethanol content of the final beers showed all investigated maltose-negative strains at or below 0.5% (*v*/*v*). WLP001, the maltose-positive control reached an ethanol content of 2.61% (*v*/*v*). The maltose-negative control, TUM SL 17, together with KBI 22.2 showed an ethanol content of 0.50% (*v*/*v*), followed by KBI 5.4 and KBI 25.2 with 0.48% (*v*/*v*) and 0.42% (*v*/*v*), respectively. The

least ethanol content showed KBI 7.1 and KBI 22.1 with 0.34% (*v*/*v*) and 0.35% (*v*/*v*), respectively. The lower ethanol production by KBI 7.1 and KBI 22.1 was due to the inability to ferment sucrose, which reflected in a higher final gravity of the beers (Table 4). Corresponding to a lower degree of fermentation, pH values for the alcohol-free beers are higher, ranging between 4.61 (KBI 5.4), and 4.84 (KBI 22.1).

Table 4. Analysis of final beers after fermentation with investigated yeasts.

Beer Analyses	WLP001	TUM SL 17	KBI 22.1	KBI 7.1	KBI 22.2	KBI 25.2	KBI 5.4
	S. cerevisiae	*S. ludwigii*	*H. valbyensis*	*H. vineae*	*T. delbrueckii*	*Z. bailii*	*Z. kombuchaensis*
Ethanol (%*v*/*v*)	2.61 ± 0.10^{d}	0.50 ± 0.01^{c}	0.35 ± 0.01^{ab}	0.34 ± 0.02^{a}	0.50 ± 0.01^{c}	0.42 ± 0.07^{abc}	0.48 ± 0.01^{bc}
Final real extract (°P)	2.13 ± 0.02	5.67 ± 0.06	5.93 ± 0.00	5.91 ± 0.04	5.61 ± 0.09	5.76 ± 0.03	5.75 ± 0.01
pH	4.18 ± 0.02^{a}	4.76 ± 0.04^{cd}	4.84 ± 0.02^{e}	4.78 ± 0.03^{de}	4.69 ± 0.02^{c}	4.71 ± 0.02^{cd}	4.61 ± 0.02^{b}
FAN (mg/L)	48 ± 3^{a}	90 ± 6^{b}	91 ± 0^{b}	91 ± 0^{b}	83 ± 0^{b}	83 ± 17^{b}	93 ± 1^{b}

Different superscripts of values within a row indicate a significant difference ($p \leq 0.05$).

3.3. Amino Acid Metabolism

The amino acid (AA) catabolism is very important for the formation of higher alcohols in the final beer. AA are important for the formation of higher alcohols such as propanol, isobutanol, and isoamyl alcohol via the Ehrlich pathway [38]. The AA are transaminated to α-keto acids and decarboxylated to form the respective aldehyde, which are further reduced to higher alcohols [10]. AA analysis revealed a substantial AA consumption only by WLP001 with a consumption of 76.4% of AA and depleting six AA namely aspartic and glutamic acid, asparagine, methionine, leucine, and isoleucine (Table 5), owing to its longer fermentation time and higher sugar uptake. WLP001 also formed higher concentrations of higher alcohols (4 times higher) than the other strains, as seen in Table 6. Adequate levels of amino acids and free amino nitrogen (FAN) in wort are necessary for a "healthy" fermentation [39–41]. Only one depletion of methionine for KBI 22.1 revealed that, for the low-alcohol strains, every AA was available in the wort in sufficient amounts. The high amount of residual amino acids and FAN after fermentation indicated that the diluted wort (6.64 °P) used in this study held a sufficient amount of amino acids and free amino nitrogen for a healthy fermentation. Generally, AA consumption was strain dependent with TUM SL 17 and KBI 22.2 being on the higher end with 26.6% and 25.5% of consumption, respectively. KBI 5.4 consumed with 11.2% the lowest amount of AA (Table 5). KBI 7.1 formed serine, which is shown at a significantly higher value after fermentation in Table 5.

3.4. Volatile Compounds

Analysis of the volatile fraction of the beers fermented with the different yeasts showed mostly only small differences in higher alcohols, esters, and diacetyl (Table 6). Regarding higher alcohols, n-propanol, isobutanol, and isoamyl alcohol contents were significantly higher for the maltose-positive control WLP001, owing to the extensive fermentation compared to the low-alcohol strains, which showed no significant differences amongst each other for n-propanol and isobutanol. Small, yet significant differences could be found for isoamyl alcohol values with KBI 22.1 exhibiting highest (16.5 mg/L) and KBI 22.2 exhibiting lowest (10.4 mg/L) values amongst the strains. The odor threshold for isoamyl alcohol, which is considered to have a fruity, brandy-like aroma, is reported to lay between 50–70 mg/L [10]. All the investigated low-alcohol yeasts produced a fifth to a third of the odor threshold of isoamyl alcohols. In sum, the low-alcohol strains produced an average of 21 mg/L of higher alcohols compared to 82 mg/L by WLP001. Other major contributors to the aroma of beer are acetate esters [11]. Volatile esters are the product of an enzyme-catalyzed condensation reaction between acyl-CoA—a product of the sugar and lipid metabolism—and a higher alcohol, originating from the nitrogen metabolism [42,43]. Ethyl acetate represents approximately one third of all esters in beers [44]. Sum of acetate ester concentration was low in all the beers ranging from 0.77 mg/L for KBI 22.2 to 6.00 mg/L for KBI 7.1 (Table 6).

Table 5. Amino acid analysis of wort and final beers fermented with the investigated strains; values in mg/100 mL.

Amino Acid	Wort	WLP001	TUM SL 17	KBI 22.1	KBI 7.1	KBI 22.2	KBI 25.2	KBI 5.4
		S. cerevisiae	*S. ludwigii*	*H. valbyensis*	*H. vineae*	*T. delbrueckii*	*Z. bailii*	*Z. kombuchaensis*
Aspartic acid	3.16 ± 0.08^{b}	$<0.5^{a}$	2.45 ± 0.08^{b}	3.10 ± 0.13^{b}	3.29 ± 0.49^{b}	2.73 ± 0.51^{b}	3.31 ± 0.05^{b}	3.24 ± 0.04^{b}
Glutamic acid	3.13 ± 0.02^{b}	$<0.5^{a}$	3.91 ± 0.03^{c}	3.21 ± 0.15^{b}	2.91 ± 0.01^{b}	3.04 ± 0.11^{b}	3.26 ± 0.02^{b}	3.14 ± 0.05^{b}
Asparagine	6.36 ± 0.12^{d}	$<0.5^{a}$	1.04 ± 0.07^{ab}	4.14 ± 0.15^{bcd}	2.63 ± 0.81^{abc}	5.58 ± 0.89^{cd}	5.23 ± 0.05^{cd}	4.14 ± 1.24^{bcd}
Serine	4.34 ± 0.04^{bc}	0.73 ± 0.21^{a}	2.21 ± 0.10^{ab}	2.99 ± 0.06^{abc}	6.00 ± 0.25^{c}	3.91 ± 0.40^{abc}	3.64 ± 0.03^{abc}	5.08 ± 1.53^{bc}
Glutamine	3.12 ± 0.03^{d}	0.59 ± 0.01^{a}	1.23 ± 0.01^{ab}	1.34 ± 0.06^{b}	2.28 ± 0.08^{c}	2.81 ± 0.34^{cd}	2.76 ± 0.00^{cd}	2.64 ± 0.01^{cd}
Histidine	3.37 ± 0.12^{c}	1.51 ± 0.09^{a}	2.23 ± 0.20^{ab}	2.98 ± 0.06^{bc}	3.01 ± 0.10^{bc}	3.18 ± 0.31^{c}	3.05 ± 0.02^{c}	3.26 ± 0.03^{c}
Glycine	2.31 ± 0.05^{b}	1.39 ± 0.11^{a}	2.33 ± 0.12^{b}	2.27 ± 0.04^{b}	2.57 ± 0.26^{b}	2.27 ± 0.01^{b}	2.03 ± 0.03^{b}	2.06 ± 0.05^{b}
Threonine	4.07 ± 0.17^{c}	0.55 ± 0.04^{a}	1.62 ± 0.03^{ab}	2.25 ± 0.06^{b}	3.75 ± 0.20^{c}	3.70 ± 0.54^{c}	3.71 ± 0.01^{c}	3.78 ± 0.09^{c}
Alanine	6.36 ± 0.02^{bc}	4.10 ± 0.42^{a}	7.10 ± 0.10^{c}	6.55 ± 0.23^{bc}	5.82 ± 0.06^{bc}	5.86 ± 0.52^{bc}	5.20 ± 0.01^{ab}	5.21 ± 0.07^{ab}
Arginine	10.70 ± 0.04^{b}	1.07 ± 0.22^{a}	7.99 ± 0.14^{b}	9.73 ± 0.28^{b}	8.16 ± 0.37^{b}	7.31 ± 3.36^{ab}	8.22 ± 0.02^{b}	9.39 ± 0.15^{b}
Tyrosine	5.65 ± 0.02^{b}	1.68 ± 0.14^{a}	5.02 ± 0.01^{b}	5.06 ± 0.17^{b}	5.35 ± 0.16^{b}	5.19 ± 0.49^{b}	4.63 ± 0.04^{b}	5.09 ± 0.09^{b}
Valine	7.59 ± 0.05^{b}	2.86 ± 0.28^{a}	6.09 ± 0.03^{b}	5.90 ± 0.27^{b}	6.42 ± 0.01^{b}	6.69 ± 0.95^{b}	6.50 ± 0.02^{b}	6.57 ± 0.01^{b}
Methionine	1.72 ± 0.02^{c}	$<0.5^{a}$	0.79 ± 0.06^{abc}	$<0.5^{a}$	0.93 ± 0.02^{abc}	1.17 ± 0.54^{abc}	1.07 ± 0.05^{abc}	1.28 ± 0.06^{bc}
Isoleucine	5.00 ± 0.04^{b}	$<0.5^{a}$	3.81 ± 0.03^{b}	3.52 ± 0.16^{b}	4.04 ± 0.00^{b}	4.23 ± 0.82^{b}	4.38 ± 0.03^{b}	4.31 ± 0.08^{b}
Tryptophan	2.71 ± 0.05^{c}	1.62 ± 0.03^{a}	2.47 ± 0.03^{bc}	2.36 ± 0.05^{b}	2.46 ± 0.04^{bc}	2.67 ± 0.10^{c}	2.44 ± 0.04^{bc}	2.67 ± 0.06^{c}
Phenylalanine	7.95 ± 0.09^{b}	1.48 ± 0.08^{a}	6.10 ± 0.00^{b}	4.94 ± 1.27^{b}	6.04 ± 0.00^{b}	7.11 ± 0.93^{b}	5.48 ± 0.06^{b}	6.58 ± 0.07^{b}
Leucine	9.61 ± 0.07^{b}	$<0.5^{a}$	6.79 ± 0.09^{b}	6.75 ± 0.04^{b}	6.89 ± 0.04^{b}	8.19 ± 1.48^{b}	6.92 ± 0.05^{b}	7.91 ± 0.17^{b}
Lysine	5.77 ± 0.02^{bc}	0.55 ± 0.00^{a}	3.57 ± 0.08^{b}	6.24 ± 0.99^{bc}	5.20 ± 0.02^{bc}	5.00 ± 0.76^{bc}	5.56 ± 0.02^{bc}	5.46 ± 0.06^{bc}
Total AA	98.31 ± 0.86^{f}	23.25 ± 1.60^{a}	72.14 ± 0.21^{b}	80.10 ± 2.69^{cd}	82.98 ± 1.17^{de}	73.24 ± 0.33^{bc}	82.85 ± 0.34^{de}	87.32 ± 0.69^{e}
AA consumption (%)	-	76.4	26.6	18.5	15.6	25.5	15.7	11.2

Different superscripts of values within a row indicate a significant difference ($p \leq 0.05$).

Table 6. Analysis of fermentation by-products of final beers fermented with investigated yeasts; values in mg/L.

Component	WLP001	TUM SL 17	KBI 22.1	KBI 7.1	KBI 22.2	KBI 25.2	KBI 5.4
	S. cerevisiae	*S. ludwigii*	*H. valbyensis*	*H. vineae*	*T. delbrueckii*	*Z. bailii*	*Z. kombuchaensis*
N-Propanol	13.7 ± 3.1^{b}	2.6 ± 0.9^{a}	2.1 ± 0.1^{a}	2.2 ± 0.0^{a}	2.9 ± 0.5^{a}	2.7 ± 0.1^{a}	2.1 ± 0.0^{a}
Isobutanol	17.9 ± 1.8^{b}	6.4 ± 0.1^{a}	4.8 ± 0.1^{a}	4.6 ± 0.3^{a}	4.9 ± 0.1^{a}	5.7 ± 0.1^{a}	7.1 ± 0.5^{a}
Isoamyl alcohols	50.8 ± 3.0^{c}	12.1 ± 0.4^{ab}	16.5 ± 1.1^{b}	13.4 ± 0.1^{ab}	10.4 ± 0.3^{a}	14.8 ± 0.2^{ab}	12.9 ± 0.5^{ab}
Σ Higher alcohols (HA)	82.4 ± 7.9^{b}	21.1 ± 0.4^{a}	23.3 ± 1.1^{a}	20.2 ± 0.4^{a}	18.1 ± 0.1^{a}	23.1 ± 0.0^{a}	22.0 ± 1.0^{a}
Ethyl acetate	4.05 ± 0.21^{b}	0.80 ± 0.01^{a}	0.90 ± 0.05^{a}	6.00 ± 0.14^{c}	0.77 ± 0.02^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}
Isoamyl acetate	0.20 ± 0.00	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Σ Esters (E)	4.25 ± 0.21^{b}	0.80 ± 0.01^{a}	0.90 ± 0.05^{a}	6.00 ± 0.14^{b}	0.77 ± 0.02^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}
Diacetyl, total	0.04 ± 0.01^{a}	0.03 ± 0.00^{a}	0.21 ± 0.03^{b}	0.05 ± 0.01^{a}	0.06 ± 0.01^{a}	0.03 ± 0.00^{a}	0.15 ± 0.04^{b}
Ethyl formate	1.05 ± 0.07	1.01 ± 0.13	0.78 ± 0.06	0.76 ± 0.03	0.90 ± 0.05	0.56 ± 0.03	0.72 ± 0.07
Acetaldehyde	7.8 ± 0.4^{c}	8.5 ± 0.7^{c}	3.3 ± 0.4^{a}	4.1 ± 0.4^{ab}	9.1 ± 0.4^{c}	4.9 ± 1.3^{ab}	6.8 ± 2.6^{bc}

Different superscripts of values within a row indicate a significant difference ($p \leq 0.05$).

Ethyl acetate production by KBI 7.1 was with 6.00 mg/L the highest of all investigated strains and outperformed even the maltose-positive control yeast WLP001 with 4.05 mg/L, which is described by the supplier to have a clean taste and has been reported to produce low concentrations of esters in previous studies [45]. Threshold values for ethyl acetate in beer range from 21–30 mg/L which is usually higher than the amount found in alcohol-free beers [11]. However, synergistic effects of different volatile aroma compounds could contribute to the overall flavor, as suggested by Sterckx et al. [46]. The concentration of isoamyl acetate was below the detection level of 0.1 mg/L in all alcohol-free beers. The concentrations of ethyl formate (light estery, fruity, solvent) were with 1 mg/L and lower far below their individual threshold of 150 mg/L [47]. The concentration of ethyl propionate, ethyl butyrate and ethyl caproate, did not reach higher than the LOD of 0.01 mg/L in either of the beers (data not shown). Diacetyl levels were strain dependent with KBI 22.1 and KBI 5.4 producing values above the flavor threshold in light beers of 0.1 mg/L with 0.21 mg/L and 0.15 mg/L, respectively, while diacetyl production of the other strains stayed below the threshold [48]. Diacetyl is known for its undesired buttery flavor, which usually undergoes reduction during maturation of the beer [49]. Acetaldehyde is the most important aldehyde of beer and is formed in the metabolic pathway leading from carbohydrate to ethanol. Its level varies during fermentation and aging and in beers, it usually lies in the range 2–20 mg/L, while its threshold lies between 10–25 mg/L [44,47]. Acetaldehyde concentrations were below the threshold for all beers produced (Table 6). The overall flavor of beer depends on the relative contents of all the flavor-active compounds [44]. The presence of different esters can have a synergistic effect on the individual flavors, which means that esters can also have a positive effect on beer flavor, even at amounts below their individual threshold concentrations [50].

3.5. Sensory

To evaluate and compare the flavor of the beers, a panel of 11 trained and experienced beer tasters judged the beers by individual description of the aroma, followed by the evaluation of the intensity descriptors "fruity", "wort-like" and "floral" smell, "sweet" and "acidic/sour" taste, and the body of the beer. Each descriptor was given a value on a scale from 0 (nothing) to 10 (extremely). A spider web graph of the means for the descriptors is shown in Figure 4.

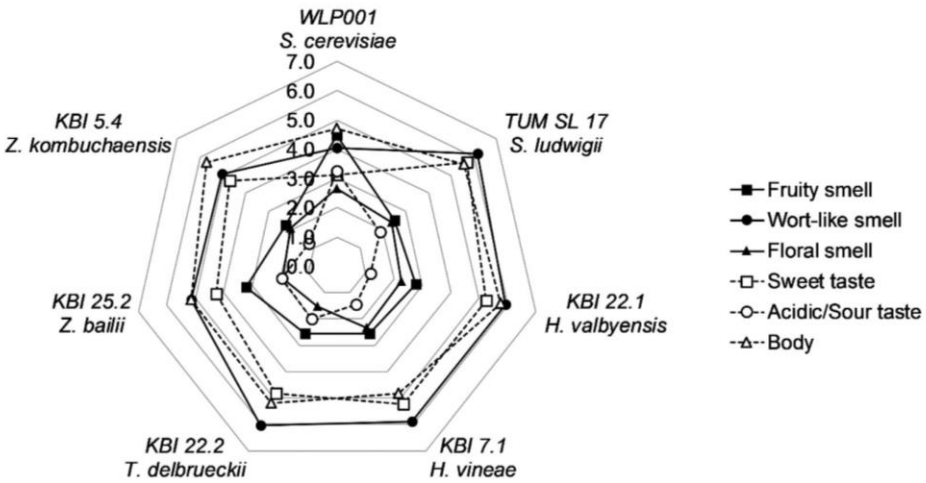


Figure 4. Spider web of the means of the descriptors from the sensory of the final beers.

WLP001 showed to have a less wort-like and fruitier smell and a less sweet, but more acidic/sour taste, owing to the longer fermentation time and higher extract consumption. The body of the beers was evaluated as being a little lower compared to the alcohol-free beers. Floral smell and acidic/sour taste were generally described to be low in intensity. Overall, the differences between the alcohol-free

beers were small. KBI 25.2 was described to have a slightly fruitier smell and lower wort-like smell and sweet taste amongst the alcohol-free beers. However, analysis of variance (ANOVA) revealed no significant difference ($p \leq 0.05$) between the AFB. ANOVA analysis between all beer samples revealed significant differences in acidic/sour taste ($p < 0.001$) and differences in sweet taste ($p < 0.1$) and fruity smell ($p < 0.1$).

To create a multidimensional sensory profile of all beers, a principal component analysis (PCA) was conducted. PCA is a tool used to transform and combine a large amount of data into new components, based on variation and correlation within a data set. As descriptors, wort-like and fruity smell were selected as well as sweet and acidic/sour taste and body. If descriptors do not discriminate the products, they cause distortion in the PCA. Hence the descriptor "flora smell", having a P value for the F-test of the product effect greater than the default value of 0.5, was excluded from the PCA [22]. The Variables factor map (Figure 5) presents the observed variables projected into the plane, spanned by the first two principal components. It shows the structural relationship between the variables and helps to name the components. The projection of a variable vector onto the component axis allows to directly read the correlation between the variable and the component.

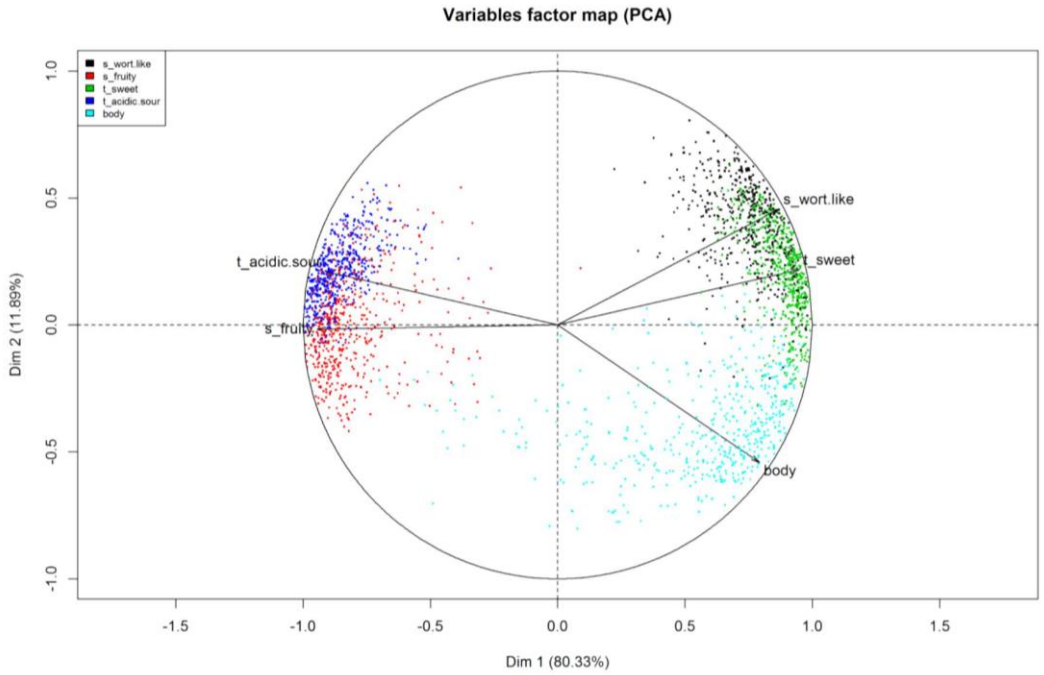


Figure 5. Variables factor map of the principal component analysis (PCA) of the sensory of the final beers. Criteria for descriptors to be included in the PCA was a p value of the F-test of below 0.5. For the descriptors "s_" stands for smell, and "t_" stands for taste.

The variables factor map should be interpreted in terms of angles, either between each variable or between a variable and the component axes. Narrow angles reflect positively linked variables (i.e., sweet taste and wort-like smell). Right angles depict variables that are unrelated to each other (i.e., body and wort-like smell) and obtuse angles represent negative relationships (i.e., wort-like and fruity smell). The first principal component described about 80% of the total variation and showed an almost perfect correlation to the variable fruity smell and a very strong correlation to sweet and acidic/sour taste and wort-like smell. The second principal component explained an additional 12% of the total variation with a correlation to the body of the beers. Combined, the first two principal components explained about 92% of the total variance of the data.

In the PCA graph (Figure 6), confidence ellipses (α = 0.05) around each beer were added to visualize the uncertainty as for the position of the beer given by the panel. Well separated confidence ellipses indicate a great discriminant power of the panel.

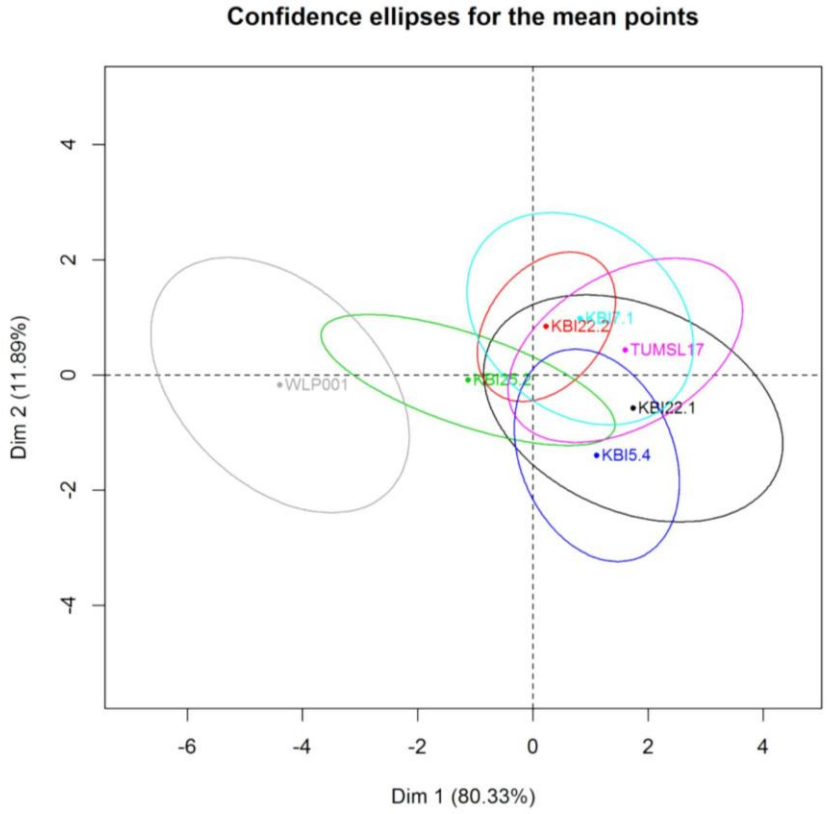


Figure 6. Mean points with confidence ellipses (α = 0.05) of the PCA from the sensory of the final beers.

As expected, WLP001 could be well discriminated from the other beers in the direction of a fruitier smell, more acidic/sour taste and away from a sweet taste and wort-like smell. The alcohol-free beers were not well discriminated, but were all located in the direction of a sweeter taste and wort-like smell. However, a tendency of KBI 25.2 separating from the group of alcohol-free beers in the direction of WLP001 could be observed. The highest means for body by KBI 5.4 and KBI 22.1 also reflected in the PCA. The results of the sensory reflect the marginal differences of the alcohol-free beers between each other from the analyses of secondary metabolites. However, the significantly higher ester content of the beer fermented with KBI 7.1 did not show in the sensory analyses of the different beers.

In the descriptive part of the sensory, the panelists gave all the alcohol-free beers attributes like: "wort-like", "bread-like", and "honey-like". TUM SL 17 was described using at least one of those attributes, by 90% of the panelists. KBI 22.1 was additionally given a "cereal-like" character and half of the panel detected the diacetyl flavor as expected from the metabolites analysis (Table 6). KBI 7.1 was also described with attributes like "black tea" and "caramel". KBI 25.2 was given additional attributes like "slightly grassy", "fruity" and "white wine". The elevated diacetyl values for KBI 5.4 were again detected by 50% of the panelists. The problem of wort-like off-flavor in alcohol-free beers is very common. Aldehydes are reported to be the cause, with 3-methylthiopropionaldehyde seemingly being the key compound responsible for the worty off-flavor [51,52]. Wort aldehydes form mainly during mashing and boiling, but are also partially formed during fermentation by the yeast. They can originate from oxo-acids via the anabolic process, and from exogenous amino acids via the catabolic

pathway [53]. Ethanol plays a significant role in the reduction of the worty character of the beer. As a flavor component, it contributes directly to the flavor of beer, giving rise to a warming character and influencing the partitioning of flavor components between the liquid beer, foam, and the headspace above the beer [54]. Additionally, Perpète and Collin [6] reported, that aldehyde retention caused by its solubilization in ethanol leads to a lower perception of the worty taste. In regular beers the retention of aldehydes is 32–39% as opposed to 8–12% retention in alcohol-free beers [4]. It is also known that yeast metabolism reduces wort aldehydes to less flavor active ones [55]. The absence of ethanol, the lack of aldehyde reduction due to shortened fermentation times and the higher level of mono and disaccharides such as maltose intensify undesirable worty flavors [6]. The results of the sensory indicate, that none of the investigated maltose-negative strains were able to mask the worty off-flavors. However, they neither stood out negatively compared to TUM SL 17, which is already commercially applied in the production of alcohol-free beers. KBI 25.2 showed the highest potential of non-*Saccharomyces* yeasts to become a serious alternative in the brewing of alcohol-free beer with an improved sensorial profile.

3.6. Concluding Remarks

This study on the application of five non-*Saccharomyces* yeasts in the production of AFB, gave a comprehensive overview of their suitability and characteristics. After ruling out undesirable traits during characterization, such as POF production and hop sensitivity, the non-*Saccharomyces* yeasts showed excellent performance during propagation, outperforming TUM SL 17 in cell numbers and showing very high viability rates. In fermentation trials the non-*Saccharomyces* yeasts exhibited a comparable performance, and analysis of volatile compounds revealed only marginal differences. The AFB fermented with the commercial AFB yeast (TUM SL 17) could not be discriminated from the alcohol-free beers fermented with the investigated non-*Saccharomyces* yeasts, which indicates the potential of their application in alcohol-free beer brewing. All fermentations were performed at 25 °C to be able to compare the strains. Twenty-five degrees Celsius most likely was not the optimum for each of the yeast strains in terms of fermentation performance or production of secondary metabolites, but it allows an indication of the suitability of the investigated strains in alcohol-free beer production.

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Screening and Application of *Cyberlindnera* Yeasts to Produce a Fruity, Non-Alcoholic Beer

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Abstract: Non-alcoholic beer (NAB) is enjoying growing demand and popularity due to consumer lifestyle trends and improved production methods. In recent years in particular, research into the application of non-*Saccharomyces* yeasts to produce NAB via limited fermentation has gained momentum. Non-*Saccharomyces* yeasts are known to produce fruity aromas, owing to a high ester production. This trait could be harnessed to mask the often-criticized wort-like off-flavor of NAB produced via limited fermentation. Six *Cyberlindnera* strains were characterized and screened in wort extract. Four of the six strains produced a pleasant, fruity aroma while exhibiting low ethanol production. The strain *Cyberlindnera subsufficiens* C6.1 was chosen for fermentation optimization via response surface methodology (RSM) and a pilot-scale (60 L) brewing trial with subsequent sensory evaluation. A low fermentation temperature and low pitching rate enhanced the fruitiness and overall acceptance of the NAB. The NAB (0.36% ABV) produced on pilot-scale was significantly more fruity and exhibited a significantly reduced wort-like off-flavor compared to two commercial NABs. This study demonstrated the suitability of *Cyberlindnera subsufficiens* to produce a fruity NAB, which can compete with commercial NABs. The outcome strengthens the position of non-*Saccharomyces* yeasts as a serious and applicable alternative to established methods in NAB brewing.

Keywords: brewing; *Cyberlindnera*; NABLAB; non-alcoholic beer; non-conventional yeast; non-*Saccharomyces* yeast; response surface methodology

1. Introduction

While the overall market growth of beer is slowing down, non-alcoholic and low alcohol beer (NABLAB) is growing in volume and popularity, owed to stricter legislation, lifestyle trends and improved production methods [1]. The increasing interest has fueled research in NABLAB production methods, especially in recent years, aimed at overcoming taste deficits compared to regular beer and consequently improving consumer acceptance. The two major production methods, physical dealcoholization and limited fermentation, both compromise the taste of the beer. Dealcoholized beer is often criticized for its lack of body and aromatic profile, a consequence of the removal of volatile esters and higher alcohols in conjunction with ethanol. Apart from a sweet taste due to residual sugars, one of the main points of criticism of NAB produced by limited fermentation is its wort-like off-flavor caused by aldehydes present in the wort [2]. In regular beer, ethanol significantly increases aldehyde retention, reducing the perceptibility of the wort-like flavor. However, in NAB produced by limited fermentation, the low ethanol content and higher levels of mono- and disaccharides intensify this undesired off-flavor [3].

It is known that esters, which yeast produce as a by-product of alcoholic fermentation, are extremely important for the flavor profile of beer [4,5]. The lack thereof, as well as their overproduction, can significantly compromise the flavor. Aside from strain-specific differences, the process parameters such as the fermentation temperature, pitching rate and wort gravity have been shown to have a significant influence on ester formation [4,6]. In non-alcoholic beers, ester concentrations are lower compared to regular beer, independent of the production method [7,8]. While physical dealcoholization removes esters that were previously produced, limited fermentation adversely affects the production of substantial amounts in the first place.

Non-*Saccharomyces* yeasts are known for their important contribution to the flavor profile of fermented foods and beverages and have therefore been investigated for their targeted application in bioflavoring and, not least, NABLAB brewing [1,9,10]. Species that have been mentioned in the context of NABLAB production belong to the genera *Cyberlindnera*, *Hanseniaspora*, *Lachancea*, *Mrakia*, *Pichia*, *Torulaspora*, *Saccharomycodes*, *Scheffersomyces* and *Zygosaccharomyces* [1,11–16]. In particular, the *Cyberlindnera* species are known for their high ester production, which was shown in studies with *Cyberlindnera saturnus* (formerly *Williopsis saturnus*), *C. mrakii* (formerly *Williopsis saturnus* var. *mrakii*) and *C. subsufficiens* (formerly *Williopsis saturnus* var. *subsufficiens*) [17–20]. Furthermore, it has been proposed to use yeasts with high production of flavor compounds (i.e., esters, higher alcohols) to mask the wort-like flavor of NAB produced by limited fermentation. However, research in that direction is sparse [21,22]. In addition, such yeasts are capable of reducing aldehydes to their correspondent alcohol, which can also enhance the reduction of the often-criticized wort-like off-flavor [23,24].

In this study, six strains of the genus *Cyberlindnera* were investigated to create a fruity NAB. After identification, the strains were characterized for their substrate utilization, flocculation behavior and stress responses. A screening in diluted wort extract was performed to investigate the strains' potential to produce a pronounced fruity flavor without the production of high concentrations of ethanol. Interspecific differences in sugar consumption and the production of volatile fermentation by-products was investigated by means of high-performance liquid chromatography (HPLC) and gas chromatography (GC). The most promising strain was studied further to determine the optimal fermentation conditions to enhance the fruity flavor, which was performed by means of response surface methodology (RSM). Finally, a non-alcoholic beer was produced on pilot-scale (60 L), and its analytical attributes, aroma, and taste compared to two commercial NABs were examined.

2. Materials and Methods

2.1. Materials

All reagents used in this study were at least analytical grade from Sigma-Aldrich (St Louis MO, USA) unless stated otherwise. The wort extract applied in this study was spray-dried wort from 100% barley malt (Spraymalt Light, Muntons plc, Suffolk, UK). For the pilot-scale brewing, pilsner malt and acidulated malt were sourced from Weyermann (Malzfabrik Weyermann, Bamberg, Germany).

2.2. Yeast Strains

Strain Origin and Identification

Strain 837A was isolated from a brewery cellar, NT Cyb originates from a dried fermentation starter for rice wine, strain C6.1 originates from a coconut, and L1 from "Lulo", the fruit of *Solanum quitoense*. The type strains CBS 1707 and CBS 5763 originate from soil samples. For identification, the D1/D2 domain of the 26S rRNA gene was amplified, sequenced and compared to publicly available sequences in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The DNA of the yeast isolates was extracted using an extraction kit (Yeast DNA Extraction Kit, Thermo Fisher Scientific, Waltham MA, USA). To amplify the D1/D2 domain

of the 26S rRNA gene, the primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) were used. Polymerase chain reaction (PCR) was performed using the temperature protocol: 95 °C/2 min; 30 cycles of 95 °C/30 s, 56 °C/15 s; 72 °C/60 s; 72 °C/5 min. Stock cultures were kept in 50% (*v/v*) glycerol at −80 °C.

2.3. Yeast Characterization

2.3.1. Flocculation Assay and Phenolic Off-Flavor (POF) Test

The flocculation test was performed using a slightly modified Helm's assay [25,26]. Essentially, all cells were washed in ethylenediaminetetraacetic acid (EDTA) and the sedimentation period was extended to 10 min. Wort was composed of 75 g/L spray-dried malt extract (Spraymalt Light, Muntons plc, Suffolk, UK) adjusted to 15 International Bitterness Units (IBU) (15 mg/mL iso-α-acids; from 30% stock solution; Barth-Haas Group, Nürnberg, Germany).

The phenolic off-flavor test was performed according to Meier-Dörnberg et al. [27]. In short, yeast strains were spread on yeast and mold agar plates (YM-agar) containing only one of the following precursors: either ferulic acid, cinnamic acid or coumaric acid. After three days of incubation at 25 °C, plates were evaluated by a trained panel by sniffing to detect any of the following aromas: clove-like (4-vinylguajacol), Styrofoam-like (4-vinylstyrene) and medicinal-like (4-vinylphenol). *Saccharomyces cerevisiae* LeoBavaricus—TUM 68®(Research Center Weihenstephan for Brewing and Food Quality, Freising-Weihenstephan, Germany) was used as a positive control.

2.3.2. Substrate Utilization

To analyze substrate utilization by the *Cyberlindnera* strains, the test kit API ID 32C (BioMérieux, Marcy-l'Étoile, France) was used. Preparation of the inoculum and inoculation of the strips were performed according to the manufacturers' instructions. Colonies for the inoculum were grown on yeast extract peptone dextrose (YPD) agar plates for 48 h at 27 °C. After inoculation, API ID 32C strips were incubated for 2 days at 28 °C. The samples were evaluated visually for turbidity in the wells, differentiating positive (+), negative (−), and weak (w) growth.

2.3.3. Stress Tests

Stress tests were performed via the measurement of yeast growth in a microplate, through the repeated measurement of absorbance over a time period of 96 h (Multiskan FC, Thermo Scientific, Waltham, MA, USA). The substrate for the hop sensitivity test was sterile-filtered wort extract (75 g/L Muntons Spraymalt Light) adjusted to 0, 50 and 100 mg/L iso-α-acids (1 mg/L = 1 International Bitterness Unit, IBU), respectively, by using an aliquot of a stock solution of 3% iso-α-acids in 96% (*v/v*) ethanol (Barth-Haas Group, Nürnberg, Germany). For testing ethanol sensitivity, the sterile-filtered wort extract was adjusted to 0%, 2.5%, 5% and 7.5% ABV with an aliquot of 100% (*v/v*) ethanol. For testing pH sensitivity, the sterile-filtered wort extract was adjusted to the following pHs with 2 M HCl: 5.5 (control without addition of HCl): 5.0, 4.0 and 3.0. For inoculation, strains were grown in sterilized wort extract for 24 h at 25 °C under aerobic conditions. The microtiter plate wells were inoculated with a concentration of 10^5 cells/mL. The wells contained 200 µL of the respective wort substrates. Plates were incubated at 25 °C, and absorbance was measured every 30 min at 600 nm without shaking over a time period of 96 h (Multiskan FC, Thermo Scientific, Waltham, MA, USA). Stress tests were performed in triplicate.

2.4. Yeast Screening

2.4.1. Propagation

Single colonies of the respective strains were taken from yeast extract peptone dextrose (YPD) agar plates after 72 h growth at 25 °C and transferred into a 250 mL sterile Duran glass bottle (Lennox

Laboratory Supplies Ltd, Dublin, Ireland) containing 150 mL propagation wort consisting of 75 g/L spray-dried malt (Spraymalt light, Muntons plc, Suffolk, UK) and 30 g/L glucose (Gem Pack Foods Ltd., Dublin, Ireland), sterilized at 121 °C for 15 min. The bottles were covered with sterile cotton and placed in an incubator with orbital shaker (ES-80 shaker-incubator, Grant Instruments (Cambridge) Ltd, Shepreth, UK) and incubated for 24 h at an orbital agitation of 170 rpm at 25 °C (Strain 837A was incubated for 48 h). Cell count was performed using a Thoma Hemocytometer with a depth of 0.1 mm (Blaubrand, Sigma-Aldrich, St. Louis, MO, USA).

2.4.2. Fermentation

Fermentation wort was prepared by dissolving 75 g/L spray-dried malt extract (Munton Spraymalt light) in 1 L of brewing water and sterilizing at 121 °C for 15 min, followed by filtration through a sterile grade 1V Whatman filter (Whatman plc, Maidstone, UK) to remove hot trub formed during sterilization. The analytical attributes of the fermentation wort for the yeast screening trial and RSM trial is shown in Table 1.

Table 1. Attributes of screening wort from wort extract.

Attribute	Unit	Value
Real Extract	°P	6.97 ± 0.00
pH	–	5.20 ± 0.01
Free amino nitrogen (FAN)	mg/L	115 ± 1
Maltotriose	g/L	8.12 ± 0.15
Maltose	g/L	32.37 ± 0.57
Sucrose	g/L	0.83 ± 0.04
Glucose	g/L	5.68 ± 0.91
Fructose	g/L	1.45 ± 0.10

Fermentation trials were carried out in 1 L sterile Duran glass bottles, equipped with an air lock. Per yeast strain, triplicate bottles were filled with 400 mL of wort and left untouched throughout the fermentation. Yeast cells for pitching were washed by centrifugation at 900 *g* for 5 min and resuspended in sterile water to ensure no carryover of sugars from the propagation wort into the fermentation wort. Pitching rate was 3×10^7 cells/mL. Fermentation temperature was 25 °C. Fermentation was performed until no change in extract could be measured for two consecutive days.

2.5. Scanning Electron Microscopy (SEM)

Yeast cultures for scanning electron microscopy (SEM) were prepared following the protocol for cultured microorganisms by Das Murtey and Ramasamy [28]. Single colonies were taken from a YPD agar plate and grown in YPD broth for 24 h at 25 °C. One milliliter of sample was centrifuged at 900 g for 2 min for pellet formation and resuspended in 5% glutaraldehyde solution prepared in 0.1 M phosphate buffer (pH 7.2) for fixation. After 30 min, the sample was centrifuged, the supernatant was discarded, and the pellet was washed twice in 0.1 M phosphate buffer. Consequently, the pellet was resuspended in 1% osmium tetroxide prepared in 0.1 M phosphate buffer. After 1 h, cells were again washed twice in 0.1 M phosphate buffer. The sample was then dehydrated through an ethanol series of 35%, 50%, 75%, 95%, absolute ethanol, and hexamethyldisilazane (HDMS), with 30 min per step (last two ethanol steps twice), centrifuging and discarding the supernatant at each change. Lastly, the second HDMS was discarded and the sample left drying overnight in a desiccator.

The dehydrated yeast sample was mounted onto plain aluminum stubs using carbon double surface adhesive and coated with a 5 nm gold-palladium (80:20) layer using a Gold Sputter Coater (BIO-RAD Polaron Division, SEM coating system, England), then observed under a constant accelerating voltage of 5 kV under a JEOL scanning electron microscope type 5510 (JEOL, Tokyo, Japan).

2.6. Response Surface Modeling (RSM)

To investigate optimal fermentation conditions for C6.1 to produce a fruity, non-alcoholic beer, response surface methodology (RSM) was performed using DesignExpert 9 software (StatEase, Minneapolis, MN, USA). A two-factorial, face-centered, central composite design with single factorial points and 5 replications of the center point was chosen. The predictor factors were temperature (17, 22, 27 °C), and pitching rate (10, 35, 60 × 10^6 cells/mL).

Spray-dried malt extract (Spraymalt light, Muntons plc, Suffolk, UK) served as the substrate. Wort preparation, propagation and inoculation were carried out as outlined in 2.4.1. The wort used was the same as in the screening (Table 1). Fermentation volume was 150 mL in 250 mL Duran glass bottles equipped with an air lock. Fermentation was performed until no change in extract could be measured for two consecutive days. Table 2 shows the experimental design.

Table 2. Response surface methodology (RSM) experimental design: Two-factorial, face-centered, central composite design with five repetitions of the center point. Factor 1, A: temperature, range 17, 22, 27 °C. Factor 2, B: pitching rate, range 10, 35, 60 × 10^6 cells/mL.

Run	Factor 1	Factor 2
	A: Temperature (°C)	B: Pitching Rate (×10^6 cells/mL)
1	22	60
2	22	10
3	17	35
4	27	35
5 *	22	35
6 *	22	35
7	17	60
8 *	22	35
9 *	22	35
10 *	22	35
11	17	10
12	27	10
13	27	60

* Center point.

Models were produced applying backward elimination regression of insignificant model terms with α to exit of 0.1 (detailed report in supplementary Data Sheet S1). For significant models with insignificant lack of fit (LOF), 3D response surface plots were produced. Fermentations for model validation were performed in the same wort with propagation as outlined in 2.4.1 and fermentation as outlined above.

2.7. Pilot-Scale Brewing

2.7.1. Wort Production

Wort for the pilot brew was produced in a 60 L pilot-scale brewing plant consisting of a combined mash-boiling vessel, a lauter tun and whirlpool (FOODING Nahrungsmitteltechnik GmbH, Stuttgart, Germany). The grain bill comprised 6.65 kg Weyermann Pilsner Malt and 0.35 kg Weyermann Acidulated Malt (Malzfabrik Weyermann, Bamberg, Germany). Grains were milled with a two-roller mill ("Derby", Engl Maschinen, Schwebheim, Germany) at a 0.8 mm gap size. The crushed malt was mashed-in with 30 L of brewing water at 50 °C. The following mashing regime was employed: 20 min at 50 °C, 20 min at 62 °C, 10 min at 72 °C and mashing out at 78 °C. The mash was pumped into the lauter tun, and lautering was performed after a 15 min lauter rest, employing four sparging steps of 5 L hot brewing water each. Boil volume was 50 L at a gravity of 1.030 (7.0 °P), and total boiling time was 60 min. Thirty minutes into the boil, 15 g of Magnum hop pellets (14% iso-α-acids) were added for

a calculated IBU content of 9. After boiling, gravity was readjusted to 1.030 (7.0 °P) with hot brewing water, and hot trub precipitates and hop residue were removed in the whirlpool with a rest of 20 min. Clear wort was pumped through a heat exchanger and filled into 60 L cylindroconical fermentation vessels at a temperature of 17 °C.

2.7.2. Propagation, Fermentation and Aftercare

A first propagation step was employed as described in 2.4.1. A second propagation step was performed by transferring the small-scale propagated wort into a 5 L carboy filled with 2 L of sterile wort extract at 7 °P and closed with sterile cotton. The second propagation step was conducted for 24 h under constant agitation at ambient temperature (20 ± 2 °C).

Yeast was pitched into the fermenter at a pitching rate of 10^7 cells/mL. Fermentation was carried out in cylindroconical fermentation vessels with a capacity of 60 L, at ambient pressure and at a glycol-controlled fermentation temperature of 17 °C. Samples were withdrawn every day. Fermentation was carried out until no change in extract could be measured for two consecutive days. The beer was then filled into a 50 L keg and carbonated by repeated pressurization with CO_2 to 1 bar at 2 °C. After 5 days, the carbonated beer was filled into 330 mL brown glass bottles with a counter-pressure hand-filler (TOPINCN, Shenzen, China) and capped. Bottles were pasteurized in a pilot retort (APR-95; Surdry, Abadiano, Vizcaya, Spain) with spray water at 65 °C for 10 min resulting in approximately 23 pasteurization units (PU). The successful pasteurization was confirmed by plating the pasteurized NAB on agar plates. Beer bottles were stored at 2 °C in a dark place for further analysis and sensory evaluation.

2.8. Sensory Evaluation

The sensory evaluation of the samples produced during yeast screening and RSM trial were judged by a panel of 12–15 experienced tasters. Samples were given at ambient temperature (20 °C) with a three-digit code. Each panelist evaluated the samples in an individual booth at ambient temperature (20 °C). The tasters were asked to desribe the sample in their own words, followed by evaluation of the intensity of a fruity smell and the overall acceptance of the smell of the sample on a hedonic scale from 0 ("not fruity"/"dislike extremely") to 5 ("extremely fruity"/"like extremely") according to MEBAK Sensory Analysis 3.2.1 "Simple Descriptive Test" and 3.2.2 "Profile Test", respectively.

The non-alcoholic beer samples (C6.1 pilot scale and commercial samples) were tasted and judged by a sensory panel of ten experienced and certified (DLG International Certificate for Sensory Analysis—beer and beer-based mixed drinks; Deutsche Landwirtschafts-Gesellschaft e.V.) panelists. A "Simple Descriptive Test" and "Profile Test" were performed according to MEBAK Sensory Analysis 3.2.1 and 3.2.2, respectively. Attributes for the aroma were "wort-like", "floral", "fruity", "citrus-like" and "tropical". A taste attribute "sweet taste" was also included. Panelists were asked to evaluate the attributes in their intensity on a line-marking scale from 0, "not perceptible", to 5, "strongly perceptible". Before the evaluation of the intensity, a descriptive sensory was performed, where the panelists were asked to describe the aroma of the samples in their own words. Samples were provided in dark glasses with a three-digit code and evaluated at a temperature of 20 °C in order to evaluate the full flavor profile (following DLG guidelines). The commercial samples NAB A and NAB B were non-alcoholic beers produced by limited fermentation [29] and "dialysis technology" [30], respectively. Each panelist tasted the samples in an individual booth at ambient temperature (20 °C). The amount of sample tasted was 50 mL per sample.

2.9. Wort and Beer Analyses

2.9.1. HPLC Analyses

Sugars and ethanol were determined by HPLC Agilent 1260 Infinity (Agilent Technologies, Santa Clara CA, USA) equipped with a refractive index detector (RID) and a Sugar-Pak I 10 µm,

6.5 mm × 300 mm column (Waters, Milford MA, USA), with 50 mg/L Ca-EDTA as mobile phase and a flow rate of 0.5 mL/min at 80 °C. Differentiation of maltose and sucrose was achieved with a Nova-Pak 4 µm, 4.6 mm × 250 mm column (Waters, Milford MA, USA), with acetonitrile/water 78:22 (*v/v*) as mobile phase and a flow rate of 1.0 mL/min. Quantification was achieved by external standards in a calibration range of 0.5 to 30 mM.

2.9.2. GC Analyses

Free vicinal diketones were quantified by a Clarus 500 gas chromatograph (Perkin-Elmer, Waltham MA, USA) with a headspace unit and Elite-5 60 m × 0.25 mm, 0.5 µm column using a 2,3-hexandione internal standard. Fermentation by products (esters, higher alcohols) was quantified using a Clarus 580 (Perkin-Elmer, Waltham MA, USA) gas chromatograph with a headspace unit and INNOWAX cross-linked polyethylene-glycol 60 m × 0.32 mm, 0.5 µm column (Perkin-Elmer, Waltham MA, USA). Vials containing beer samples were equilibrated for 25 min at 60 °C. The samples were injected at 50 °C, rising to 85 °C after one minute by heating at 7 °C/min. A temperature of 85 °C was maintained for one minute and then elevated to 190 °C at a heating rate of 25 °C/min.

2.9.3. Other

Glycerol was determined via enzymatic assay kit (glucokinase method), following the recommended procedure (K-GCROLGK, Megazyme, Bray Co. Wicklow, Ireland). The method is based on the use of ADP-glucokinase and an increase in absorbance on conversion of NAD^+ to NADH, and is performed at ambient temperature at a sample volume of 2 mL.

Free amino nitrogen (FAN) was measured using a ninhydrin-based dying method, where absorbance is measured at 570 nm against a glycine standard (ASBC Method Wort-12 A). The method is performed at a total volume of 10 mL. Following the color reaction at 95 °C, the samples are measured at ambient temperature.

Extract (apparent and real) and ethanol (for fermentation monitoring) were analyzed via density meter DMA 4500M with Alcolyzer Beer ME (Anton-Paar GmbH, Graz, Austria) at 20 °C and a sample volume of 30 mL.

The pH was determined using a digital pH meter (Mettler Toledo LLC, Columbus, OH, USA).

2.10. Statistical Analyses

Screening fermentations and analyses were carried out in triplicate. Statistical analysis was performed using RStudio, Version 1.1.463 with R version 3.5.2 (RStudio Inc, Boston, MA, USA; R Core Team, r-project). One-way analysis of variance (ANOVA) was used to compare means, and Tukey's post hoc test with 95% confidence intervals was applied for the pairwise comparison of means. When available, values are given as the mean ± standard deviation. Statistical analyses during the RSM trials were performed using the DesignExpert 9 software (StatEase, Minneapolis, MN, USA).

3. Results and Discussion

3.1. Yeast Strain Characterization

To identify the species of the yeast strains, amplification of the D1/D2 domain via PCR was performed and sequenced. The obtained sequences were compared to publicly available sequences in the NCBI nucleotide database via BLAST. The results of the strain identification are shown in Table 3.

Table 3. Yeast strain designation, species and origin of yeast strains used in this study.

Strain Designation	Species	Origin	Yeast Bank
837A	*Cyberlindnera misumaiensis*	Brewery cellar	FZW BLQ [1], Weihenstephan, Germany
NT Cyb	*Cyberlindnera fabianii*	Dried yeast starter for rice wine	FZW BLQ [1], Weihenstephan, Germany
L1	*Cyberlindnera jadinii*	Fruit of *Solanum quitoense*, "Lulo"	UCC Culture Collection, Cork, Ireland
C6.1	*Cyberlindnera subsufficiens*	Coconut	UCC Culture Collection, Cork, Ireland
CBS 1707 [T]	*Cyberlindnera mrakii*	Soil	Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands
CBS 5763[T]	*Cyberlindnera subsufficiens*	Soil	Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands

[1] Research Centre Weihenstephan for Brewing and Food Quality, Technische Universität München; [T] Type strain.

The yeast strains were found to belong to the species *Cyberlindnera misumaiensis* (837A), *C. fabianii* (NT Cyb), *C. jadinii* (L1), and *C. subsufficiens* (C6.1). The *Cyberlindnera mrakii* type strain CBS 1707 (former *Williopsis saturnus* var. *mrakii*; synonym NCYC 500) was included in this study as a strain that has previously been investigated for the production of a low alcohol beer with high levels of esters [20]. The *Cyberlindnera subsufficiens* type strain CBS 5763 was included as an example to investigate potential intraspecific differences from C6.1.

3.2. API Substrate Utilization

Before considering non-conventional yeasts for NABLAB brewing, their behavior regarding utilization of important wort sugars like maltose and sucrose should be investigated. An API ID 32C test was performed to investigate the utilization of those sugars and to show general, interspecific differences between the strains. The results of the API test are shown in Table 4.

Table 4. Results of the API ID 32C substrate utilization test of the individual strains. Substrates without brewing relevance, which were negative for all strains, are not shown. "+" positive, "−" negative, "w" weak.

Substrate	837A	NT Cyb	L1	C6.1	CBS 1707	CBS 5763
Cycloheximide (Actidione)	+	−	−	−	−	−
D-Cellobiose	+	+	+	+	+	+
D-Galactose	−	−	w	−	−	−
D-Glucose	+	+	+	+	+	+
D-Maltose	−	+	+	−	+[1]	−[1]
D-Mannitol	+	+	w	w	w	w
D-Melibiose	−	−	−	−	−	−
D-Melezitose	−	+	+	−	+	−
D-Raffinose	−	+	+	+	+	+
D-Sorbitol	+	+	w	+	−	−
D-Sucrose	−	+	+	+	−	+
D-Trehalose	−	+	−	−	+	−
D-Xylose	−	+	+	+	+	+
Esculin Ferric Acid	+	+	+	+	+	+
Glucosamine	−	−	−	w	−	−
Glycerol	+	+	+	+	+	+
Lactic Acid	−	+	+	+	+	+
Levulinic Acid	−	w	w	w	w	+
L-Sorbose	−	−	−	−	−	+
Methyl-αD-Glucopyranoside	−	+	−	−	−	−
N-Acetyl-Glucosamine	−	−	w	−	w	−
Palatinose	−	+	+	−	+	−
Potassium Gluconate	w	w	−	+	w	+

[1] Growth "variable" according to Kurtzman et al. [31].

Maltose utilization was positive for NT Cyb, L1 and CBS 1707, in accordance with the reported literature, although assimilation of maltose by CBS 1707 is classified as "variable" [31]. Sucrose utilization was positive for four of the six strains and negative for 837A and CBS1707. The results suggest that in brewers' wort, where maltose is the most abundant fermentable sugar, only NT Cyb, L1 and CBS 1707 have the capability to achieve high attenuations. However, the API test investigates substrate utilization under aerobic conditions. Sugar consumption during fermentation, under anaerobic conditions, can differ significantly [31], which is also known as the Kluyver effect [32]. Due to the inability of 837A and CBS 1707 to utilize sucrose, lower attenuations in fermentations in wort could be expected.

3.3. Stress Tests

When considering non-*Saccharomyces* yeast strains for brewing purposes, several brewing-relevant parameters such as flocculation behavior, POF production and stress responses should be investigated [33]. The flocculation behavior can give initial indications regarding yeast handling in terms of potential bottom cropping. POF behavior is important because in most beer styles, POF is not desired. Substances like hop-derived iso-α-acids, ethanol content, or the pH value of the wort can have significant influences on yeast activity, manifesting mainly in a prolonged lag time, and even complete growth inhibition [33–35]. With the investigated yeast strains, iso-α-acid concentrations of up to 100 IBU had no significant effect on the yeast growth (data not shown), which is in accordance with previous reports on seven different non-*Saccharomyces* species [34,35]. However, Michel et al. [33] reported a minor prolongation in the lag time of *Torulaspora delbrueckii* strains in concentrations of up to 90 IBU. The results of the investigated characterization attributes are shown in Table 5.

Table 5. Characterization of yeast strains for flocculation behavior, phenolic off-flavor (POF) production and lag time in wort with and without a stressor at different concentrations. "—" no growth.

Characterization Attributes		Unit	837A	NT Cyb	L1	C6.1	CBS 1707	CBS 5763
Flocculation		%	78 ± 3	22 ± 2	35 ± 4	32 ± 1	85 ± 2	51 ± 4
POF		-	negative	negative	negative	negative	negative	negative
Ethanol	0% ABV	h	18	6	9	6	9	9
	2.5% ABV	h	120	12	18	18	12	18
	5% ABV	h	—	24	36	24	48	—
	7.5% ABV	h	—	42	—	—	126	—
pH	5.5	h	18	6	9	6	9	9
	5	h	18	6	9	6	9	9
	4	h	66	6	9	6	9	9
	3	h	—	12	24	18	78	42

CBS 1707 exhibited the strongest flocculation behavior, at 85%, followed by 837A and CBS 5763, at 78% and 51%, respectively. NT Cyb, L1 and C6.1 exhibited very low flocculation of below 35%. All strains were negative for POF behavior. NT Cyb and C6.1 exhibited the fastest growth in wort (without a stress factor), overcoming the lag time after only 6 hours, followed by L1 and the CBS strains after 9 hours. Strain 837A exhibited a long lag phase of 18 hours (Figure 1). Concentrations of 2.5% ABV ethanol in the wort affected the lag time of all investigated strains. 837A was especially susceptible, with a prolonged lag phase of 120 hours. The remainder of the strains showed an extension of the lag phase of 3 to 12 hours. At 5% ABV, growth was fully inhibited for 837A and CBS 5763, while the other strains again exhibited an extension of the lag phase, of up to a maximum of 48 hours in CBS 1707. Complete growth inhibition was observed for L1 and C6.1 at 7.5% ABV, while the lag phase of NT Cyb and CBS 1707 was prolonged to 42 and 126 hours, respectively. All strains except 837A, which showed a significant extension of the lag phase to 66 hours, remained unaffected by a lower pH of 4. Only at pH 3 were lag times affected, while 837A was fully inhibited. Growth at low pH is important when

considering the yeast for sour beer production, where the yeast must withstand pH values of below 4 [36]. However, it has been shown that organic acids like lactic acid can have a stronger inhibitory effect on yeasts and other microorganisms than HCl, which is caused by its chemical properties as a weak acid [35,37]. Inhibition by lactic acid could therefore be more pronounced than the HCl inhibition observed in this study. Figure 1 shows the growth of the investigated yeast strains in wort without the addition of a stressor.

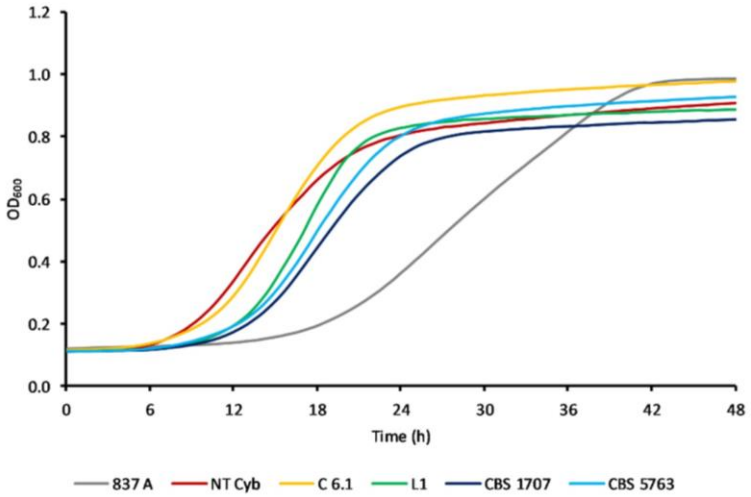


Figure 1. Growth of yeast strains in 7 °P wort extract at 25 °C without a stressor. Growth curves shown are the mean of a triplicate.

3.4. Screening

To investigate interspecific differences in the fermentation of wort, fermentation trials were performed in a diluted wort extract of 7 °P. Previous studies have shown that extract contents of around 7 °P will yield ethanol concentrations of around 0.5% ABV, a popular legal limit for NAB [7], in fermentations with maltose-negative yeast strains [1,14,34,38]. After aerobic propagation for 24 hours, NT Cyb exhibited the highest number of cells, at 2×10^9 cells/mL, more than four-fold the amount of cells compared to L1, C6.1, and the CBS strains with counts between 3.4 and 4.9×10^8 cells/mL (Table 6). Due to a delayed growth (compare Figure 1), 837A had to be propagated for 48 hours, reaching a cell count of 6.1×10^8 cells/mL. For the screening in wort, yeast cells were added at a concentration of 3×10^7 cells/mL, after a gentle washing step in water to prevent carry-over of propagation wort sugars. The results from the yeast screening are shown in Table 6. The fermentations were carried out until no change in extract could be measured for two consecutive days.

Table 6. Results of the screening of the investigated *Cyberlindnera* strains in wort extract.

Non-Alcoholic Beer (NAB) Attributes		Unit	837A	NT Cyb	L1	C6.1	CBS 1707	CBS 5763
Propagation	Cell count (24 h)[1]	$\times 10^6$ cells/mL	611 ± 34 [1]	2055 ± 21	486 ± 27	445 ± 4	338 ± 25	386 ± 48
Fermented wort	Real Extract	°P	6.53 ± 0.03 ^{b}	6.40 ± 0.04 ^{ab}	6.45 ± 0.05 ^{ab}	6.36 ± 0.03 ^{a}	6.57 ± 0.10 ^{b}	6.35 ± 0.10 ^{a}
	Attenuation	%	18 ± 1 ^{ab}	23 ± 1 ^{bc}	21 ± 2 ^{abc}	24 ± 1 ^{c}	17 ± 3 ^{a}	24 ± 3 ^{c}
	Ethanol	% ABV	0.55 ± 0.01 ^{a}	0.63 ± 0.01 ^{b}	0.66 ± 0.00 ^{cd}	0.63 ± 0.00 ^{bc}	0.54 ± 0.01 ^{a}	0.67 ± 0.02 ^{d}
	pH	-	4.41 ± 0.02 ^{ab}	4.51 ± 0.02 ^{c}	4.44 ± 0.01 ^{bc}	4.38 ± 0.03 ^{b}	4.37 ± 0.06 ^{ab}	4.33 ± 0.01 ^{a}
	FAN	mg/L	88 ± 1 ^{b}	83 ± 2 ^{ab}	80 ± 1 ^{a}	81 ± 4 ^{a}	78 ± 1 ^{a}	84 ± 5 ^{ab}
Sugar consumption	Maltotriose	%	3 ± 2	6 ± 1	4 ± 1	5 ± 1	3 ± 1	5 ± 3
	Maltose	%	4 ± 2	4 ± 0	3 ± 1	4 ± 0	4 ± 1	4 ± 4
	Sucrose	%	2 ± 10	100	100	100	2 ± 2	100
	Glucose	%	100	100	100	100	100	100
	Fructose	%	81 ± 1	75 ± 1	100	80 ± 1	73 ± 8	83 ± 2
Fermentation by-products	Glycerol	g/L	0.25 ± 0.05 ^{ab}	0.23 ± 0.04 ^{a}	0.36 ± 0.03 ^{b}	0.30 ± 0.04 ^{ab}	0.18 ± 0.05 ^{a}	0.21 ± 0.05 ^{a}
	Acetaldehyde	mg/L	9.70 ± 2.83 ^{b}	8.05 ± 1.48 ^{b}	2.60 ± 0.14 ^{a}	3.37 ± 0.71 ^{a}	3.83 ± 0.45 ^{a}	2.57 ± 0.21 ^{a}
	Ethyl acetate	mg/L	65.70 ± 14.57 ^{b}	22.55 ± 2.90 ^{a}	9.27 ± 3.23 ^{a}	4.90 ± 0.85 ^{a}	8.10 ± 0.28 ^{a}	5.17 ± 0.29 ^{a}
	Isoamyl acetate	mg/L	0.90 ± 0.14 ^{ab}	<LOD	0.15 ± 0.07 ^{a}	1.60 ± 0.62 ^{b}	1.67 ± 0.12 ^{b}	1.03 ± 0.23 ^{ab}
	Ethyl formate	mg/L	0.53 ± 0.04 ^{a}	0.31 ± 0.06 ^{a}	0.57 ± 0.09 ^{a}	0.25 ± 0.03 ^{a}	2.70 ± 0.57 ^{c}	1.45 ± 0.07 ^{b}
	Ethyl propionate	mg/L	0.13 ± 0.04 ^{a}	0.13 ± 0.01 ^{a}	<LOD	<LOD	0.16 ± 0.01 ^{a}	0.17 ± 0.03 ^{a}
	Isoamyl alcohols	mg/L	11.20 ± 0.14 ^{a}	16.40 ± 0.57 ^{b}	23.15 ± 0.92 ^{c}	11.67 ± 1.74 ^{a}	11.93 ± 0.93 ^{a}	10.50 ± 0.14 ^{a}
	n-Propanol	mg/L	4.03 ± 0.84 ^{ab}	3.73 ± 0.21 ^{ab}	4.40 ± 0.62 ^{b}	3.27 ± 0.15 ^{ab}	2.93 ± 0.29 ^{a}	3.33 ± 0.15 ^{ab}
	Isobutanol	mg/L	7.57 ± 1.24 ^{ab}	7.70 ± 0.36 ^{b}	8.27 ± 1.38 ^{b}	8.03 ± 0.40 ^{b}	5.33 ± 0.55 ^{a}	7.20 ± 0.20 ^{ab}
	Σ Esters	mg/L	67.26 ± 14.79 ^{b}	22.99 ± 2.87 ^{a}	9.99 ± 3.31 ^{a}	6.75 ± 0.61 ^{a}	12.62 ± 1.48 ^{a}	7.82 ± 0.30 ^{a}
	Σ Alcohols	mg/L	22.80 ± 0.14 ^{a}	27.83 ± 0.64 ^{b}	35.82 ± 1.48 ^{c}	22.97 ± 1.97 ^{a}	20.20 ± 0.17 ^{a}	21.03 ± 0.21 ^{a}
Sensory	Aroma	-	Solvent-like, unpleasant	Cabbage-like, unpleasant	Fruity, pleasant	Fruity, pleasant	Fruity, pleasant	Fruity, pleasant

[1] Cell count after 48 h due to delayed growth compared to other strains (compare Figure 1). LOD 'limit of detection'. Different superscripts of values within a row indicate a significant difference ($p \leq 0.05$).

Strains 837A and CBS 1707 exhibited the lowest attenuation of only 18% and 17%, respectively, owing to their inability to utilize sucrose (Table 4), which was confirmed by the lack of sucrose consumption. Liu and Quek [20] also reported the absence of sucrose utilization by CBS 1707. The other strains, which depleted sucrose completely, reached attenuations of 21% to 24%. Consequently, 837A and CBS 1707 also produced, at 0.55% and 0.56% ABV, the lowest amounts of ethanol ($p \leq 0.05$) compared to the remaining strains, where ethanol concentrations ranged from 0.63% to 0.67% ABV. The final pH of the fermented samples ranged from 4.33 (CBS 5763) to 4.51 (NT Cyb). Residual FAN ranged from 78 (CBS 1707) to 88 mg/L (837A). As expected, none of the strains consumed maltotriose. Maltose consumption was also neglectable in all strains, although the species *Cyberlindnera fabianii* (like NT Cyb) has been reported to be able to ferment maltose [31,39]. The observations also underlined that results from the API substrate utilization test (where NT Cyb, L1 and CBS 1707 were positive for maltose) are not necessarily reflected in practice, especially since sugar utilization during respiration and fermentation can differ [31,32,40]. While glucose was depleted by all strains, fructose was only fully depleted by L1. The remaining strains exhibited glucophilic behavior and consumed only 73% to 83% of fructose during fermentation. Regarding fermentation by-products, glycerol concentrations were low, ranging from 0.18 to 0.36 g/L. The strains 837A and NT Cyb accumulated significantly higher amounts of acetaldehyde, at 9.7 and 8.1 mg/L, respectively, compared to 2.6 to 3.8 mg/L in the remaining samples. The sample fermented with *Cyberlindnera misumaiensis* 837A exhibited extremely high values of ethyl acetate, at 65.7 mg/L, twice the flavor threshold concentration in beer [2,41]. Ethyl acetate is described to have a fruity, estery character but also solvent-like, especially in high concentrations. The remaining strains exhibited ethyl acetate production between 4.9 (C6.1) and 22.6 mg/L (NT Cyb). Isoamyl acetate, which is predominantly described as having a fruity, banana-like aroma, has a much lower flavor threshold of only 1.4–1.6 mg/L [2,41]. The strains C6.1 and CBS 1707 produced the highest amounts of isoamyl acetate, at 1.67 and 1.60 mg/L, followed by CBS 5763, 837A and L1, at 1.03, 0.90 and 0.15 mg/L, respectively. NT Cyb did not produce detectable amounts of isoamyl acetate. Concentrations of ethyl formate and ethyl propionate in the fermented samples were low, ranging from undetectable to 2.7 mg/L. Ethyl butyrate and ethyl caproate were not detected in either of the samples (data not shown). The strain L1 produced a significantly higher amount of higher alcohols, at 35.8 mg/L, followed by NT Cyb, at 27.8 mg/L, and the remaining strains at 20–23 mg/L. During sensory evaluation, the high ethyl acetate concentration in the sample fermented with 837A was indeed perceptible and described as an unpleasant, solvent-like aroma. The sample fermented with NT Cyb was described as having an unpleasant, cabbage-like aroma. The remaining samples were characterized by a pleasant, fruity aroma.

The unpleasant, solvent-like aroma in the sample fermented with 837A was attributed to the very high ethyl acetate concentration, well above the flavor threshold. However, the cabbage-like aroma, which is generally associated with sulfides or thiol compounds [41], that was detected in the sample fermented with NT Cyb could not be linked to the volatile by-products that were measured. Interestingly, ethyl acetate concentrations in the remaining samples, characterized by a pleasant, fruity aroma, were low, at only 2.6–3.8 mg/L. However, C6.1, CBS 1707 and CBS 5763 exhibited higher amounts of isoamyl acetate, a desired ester in beer (particularly ales) [42], when compared to the samples with unpleasant aroma. The concentrations of 1.0–1.6 mg/L are within the reported flavor threshold in beer of 0.5–2.0 mg/L [43]. Additionally, it is well known that synergistic effects between esters occur that can push the concentration of perception below their individual flavor thresholds [42,44,45]. Isoamyl acetate could therefore have been a cause of the fruity aroma in the samples fermented with C6.1, CBS 1707 and CBS 5763. However, the sample fermented with L1, which was also characterized by a fruity aroma, only contained a very low isoamyl acetate concentration of 0.15 mg/L. It is noteworthy, however, that the L1 sample contained a significantly higher amount of isoamyl alcohol, at 23.2 mg/L, which is described as having an alcoholic, fruity and banana-like flavor [2]. The results have confirmed that not a high amount of esters, but rather a balanced profile will lead to a pleasant, fruity aroma [5].

Based on the results from the screening, *Cyberlindnera subsufficiens* C6.1 was chosen for optimization of fermentation conditions by means of response surface methodology, followed by an up-scaled brewing trial at 60 L to create a fruity, non-alcoholic beer (≤0.5% ABV). Strains 837A and NT Cyb were eliminated because of their poor flavor characteristics. CBS 1707 was eliminated due to its inability to ferment sucrose, which apart from the lower attenuation, would remain in the wort after fermentation, acting as an additional sweetening agent and potential contamination risk. *Cyberlindnera jadinii* strain L1 was eliminated due to its very low isoamyl acetate production (Table 6) and due to its maltose utilization when oxygen was present (Table 4). The decision between the two similarly performing *Cyberlindnera subsufficiens* strains C6.1 and CBS 5763 was made in favor of C6.1 due to a more pleasant fruitiness. In addition, C6.1 showed increased tolerance towards stress caused by ethanol or low pH (Table 5).

3.5. Response Surface Methodology (RSM)

To find the optimal fermentation conditions for C6.1 for an up-scaled application to produce a fruity, non-alcoholic beer, RSM was performed. Michel et al. [46] applied RSM to optimize the fermentation conditions of a *Torulaspora delbrueckii* strain for brewing purposes. They found that the pitching rate and fermentation temperature were crucial parameters, which influenced the flavor character of the final beer. The optimal fermentation conditions were shown to be at 21 °C with a high pitching rate of 60×10^6 cells/mL. Especially for non-*Saccharomyces* yeasts, the pitching rate can be crucial since most non-*Saccharomyces* species have comparably smaller cell sizes [46]. Figure 2 shows an example of the differing cell size between *Cyberlindnera subsufficiens* strain C6.1 (A) and the brewers' yeast strain *Saccharomyces cerevisiae* WLP001 (B) at identical magnification.

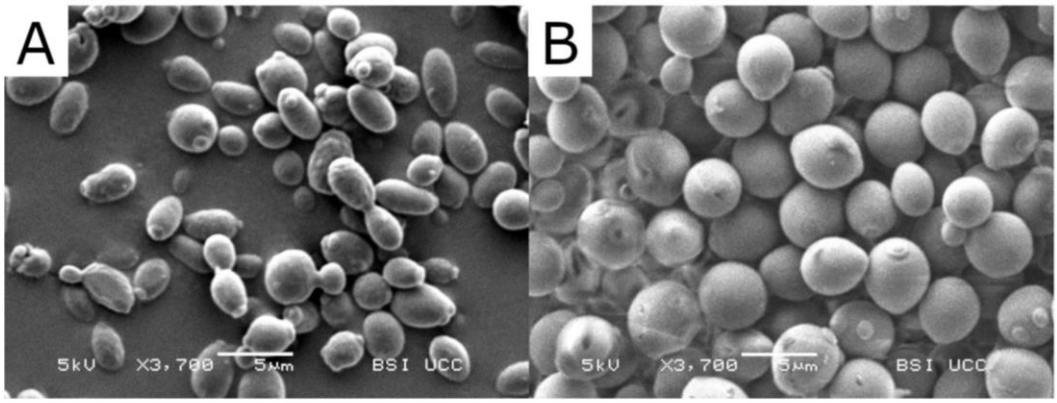


Figure 2. Scanning electron microscopy (SEM) picture of *Cyberlindnera subsufficiens* strain C6.1 (**A**) and the brewers' yeast strain *Saccharomyces cerevisiae* WLP001 (**B**) at a magnification of × 3700. Size of bar: 5 µm.

It is also known that temperature and pitching rate have an influence on ester production, though strain-specific differences also play a role [4,6]. Previously reported fermentation temperatures of *Cyberlindnera subsufficiens* and other *Cyberlindnera* spp. range from 20 to 25 °C [12,17,19,20,47]. Consequently, a two-factorial, face-centered central composite design was chosen with Factor A: fermentation temperature (17, 22, 27 °C), and Factor B: pitching rate (10, 35, 60×10^6 cells/mL). The individual experiment runs are listed in Table 2. The wort extract applied in the RSM trial was the same as that used for the screening, at an extract content of 7 °P (Table 1). Fermentation was conducted until no change in extract could be measured for two consecutive days. With the measured response values, significant models could be produced. The significant response models, with their respective minima and maxima and a summary of the model statistics, are shown in Table 7. Insignificant response models are not shown, and response models with a significant lack of fit will not be discussed in this

study but are included in the visualized data for the sake of a complete picture. For a full report on model statistics and response values, refer to the supplementary Data Sheet S1.

Table 7. Analysis of variance (ANOVA) results for response models of the response surface methodology (RSM) trial.

Response	Unit	Minimum	Maximum	Model	*P*-Value	LOF *P*-Value
Ethanol	% ABV	0.41	0.60	RQuadratic	2.80×10^{-3} **	0.648
Ethyl acetate	mg/L	3.4	9.3	2FI	3.12×10^{-2} *	0.007 **
Isoamyl acetate	mg/L	0.8	2.2	RQuadratic	1.42×10^{-2} *	0.046 *
Acetaldehyde	mg/L	1.9	3.4	RLinear	1.35×10^{-3} **	0.337
n-Propanol	mg/L	3.2	4.5	2FI	9.03×10^{-3} **	0.029 *
Isobutanol	mg/L	3.2	6.7	RQuadratic	4.30×10^{-9} ***	0.145
Isoamyl alcohols	mg/L	7.3	13.3	Quadratic	2.67×10^{-5} ***	0.270
Σ Esters	mg/L	4.2	11.1	RQuadratic	1.48×10^{-2} *	0.018 *
Σ Alcohols	mg/L	13.7	22.9	RQuadratic	3.28×10^{-8} ***	0.339
Glycerol	g/L	0.17	0.37	RQuadratic	4.85×10^{-5} ***	0.034 *
Acceptance	-	1.08	3.38	Linear	1.31×10^{-2} *	0.377
Fruitiness	-	1.13	3.38	Linear	7.31×10^{-3} **	0.484

Model terminology: "RQuadratic" Reduced Quadratic; "2FI" Two-Factor Interaction; "RLinear" Reduced Linear. "LOF" Lack of Fit. ANOVA significance codes: *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

It was possible to create significant models for 12 responses (Table 7). However, five also exhibited significant lack of fit (LOF), rendering them unusable for predictions. The aim of the RSM was to investigate the optimal fermentation conditions to create a fruity, non-alcoholic beer. The three-dimensional response surface plots of the interactive effects of temperature and pitching rate on the final ethanol content and the fruitiness of the produced NAB are shown in Figures 3 and 4.

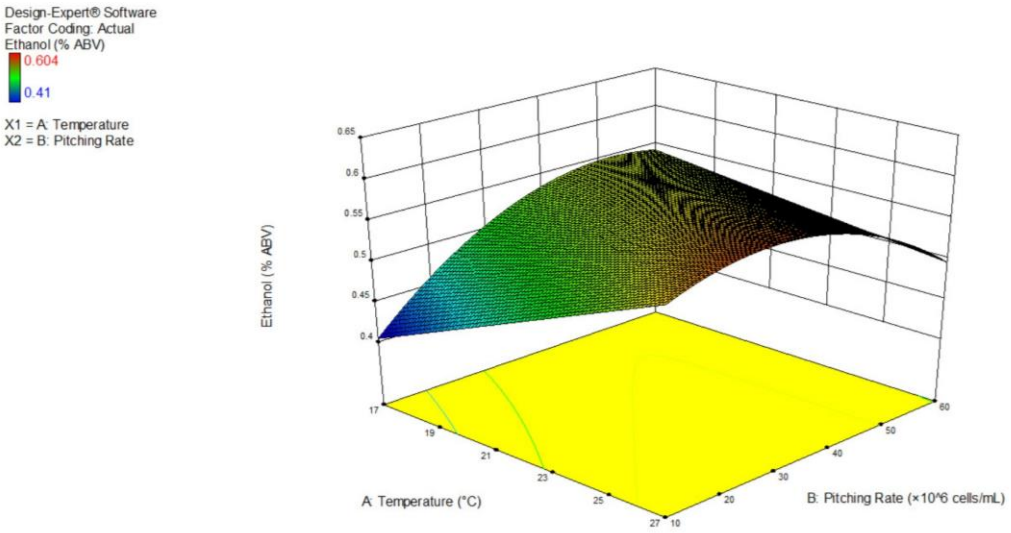


Figure 3. Three-dimensional response surface plot of the interactive effects of temperature and pitching rate on the ethanol content of the produced non-alcoholic beer ($p < 0.01$).

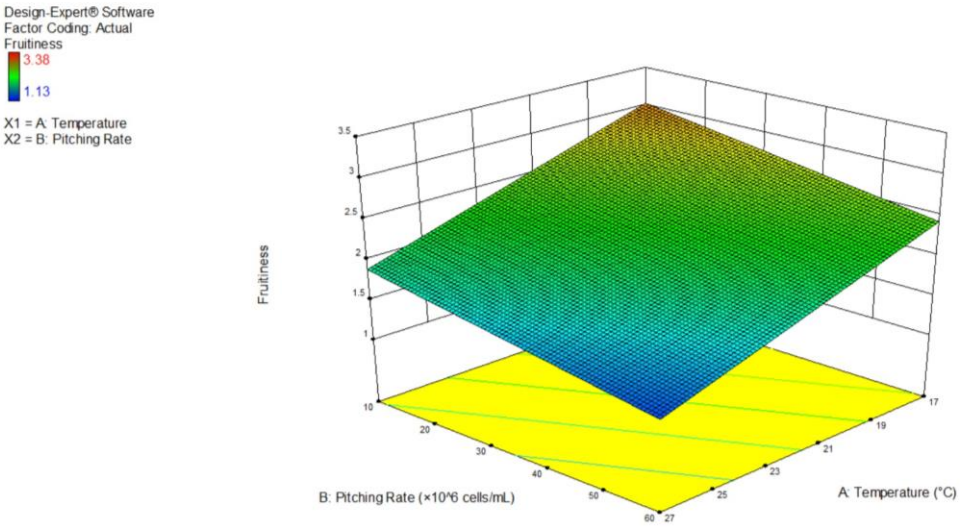


Figure 4. Three-dimensional response surface plot of the effects of temperature and pitching rate on the fruitiness of the produced non-alcoholic beer ($p < 0.01$).

Ethanol content was lowest at a low temperature of 17 °C and low pitching rate (10^7 cells/mL), and it went up with increasing temperature and pitching rate, but lowered again at a high pitching rate combined with a high fermentation temperature (Figure 3). The minium and maximum values were 0.41% and 0.60% ABV. Sugar analysis revealed that at 17 °C and 10^7 cells/mL, about 0.5 g/L of glucose was remaining after fermentation, while it was fully depleted in worts fermented at higher pitching rates and higher temperatures (data not shown). The residual sugar explained the lower final ethanol concentration. Fructose was only fully depleted in the samples that were fermented at 27 °C. At 22 °C, fermented samples exhibited residual fructose concentrations between 0.2 and 0.5 g/L, and at 17 °C, fermented samples showed remaining fructose concentrations between 0.2 and 0.7 g/L. Acetaldehyde concentrations were only dependent on the pitching rate, with increasing amounts of acetaldehyde found at lower pitching rates (Figure A1). This result correlates with other studies that found a decrease in acetaldehyde with increasing pitching rate in wort fermentations with brewers' yeasts [48,49]. However, overdosing yeast (>5 × 10^7 cells/mL) can lead to an increase in acetaldehyde again, as observed by Erten et al. [50]. The temperature did not have a significant effect on the acetaldehyde concentration and was therefore excluded from the model ($p = 0.39$; supplementary Data Sheet S1). However, regarding higher alcohols, the fermentation temperature had a stronger effect, with increasing amounts of higher alcohols found at higher temperatures (Figures 5 and A2), which is consistent with the literature [4,5]. Isoamyl acetate concentrations were generally high and ranged from 0.8 to 2.2 mg/L. Although the model was significant ($p < 0.05$), it was unsuitable for value prediction due to a significant lack of fit ($p = 0.046$).

Interestingly, the production of the esters ethyl acetate and isoamyl acetate did not show a clear correlation to temperature, which underlines that the general rule of thumb, that higher fermentation temperatures lead to increased ester production, is not valid for all yeast strains (Figure 5) [4]. Furthermore, the amount of esters that were quantified in this study did not correlate with the perceived fruitiness of the NAB, which tentatively suggests that the fruity flavor profile was caused by yet unidentified compounds (Figure 5).

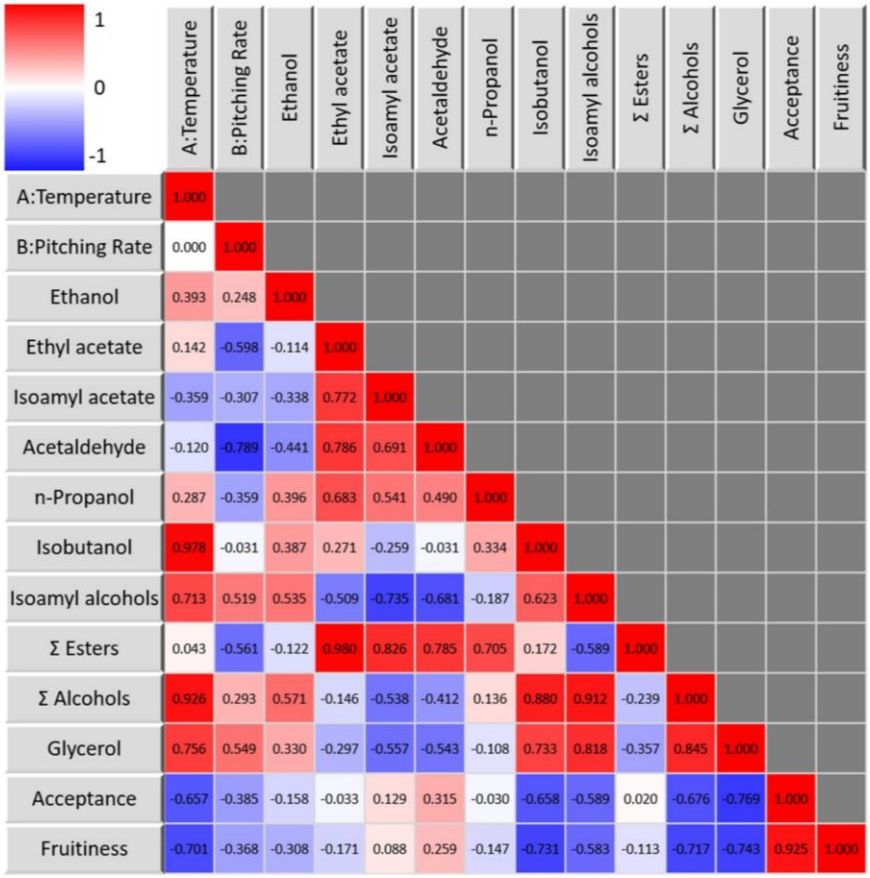


Figure 5. Map visualizing correlations of response surface methodology (RSM) factors and responses based on the Pearson Correlation Coefficient. 1 signifies strong positive correlation, 0 signifies no correlation, and −1 signifies a strong negative correlation.

In terms of fruitiness, a low fermentation temperature paired with a low pitching rate led to the highest perceived fruitiness. Indeed, the highest fruitiness was recorded at 17 °C and 1×10^7 cells/mL and the lowest at 27 °C and 6×10^7 cells/mL, following a linear model. General acceptance showed a strong positive correlation with the fruitiness, indicating that the panel preferred fruity samples (Figures 5 and A3).

Due to the ideal combination of lowest ethanol content and highest fruitiness and acceptance, the fermentation temperature of 17 °C and pitching rate of 1×10^7 cells/mL were chosen as the optimal fermentation conditions for application to produce a fruity, non-alcoholic beer.

A small-scale fermentation at the optimal conditions (17 °C, 10^7 cells/mL) was conducted to validate the RSM model. Table 8 shows the predicted mean including 95% prediction intervals (PI) and the measured ("observed") mean with standard deviation.

Although predicted by a significant model, the observed means for ethanol, acetaldehyde and isobutanol values were not within the 95% prediction interval. Sugar analysis revealed the complete depletion of glucose in the experimental fermentation trial at optimal conditions compared to the RSM model prediction, which explained the increased ethanol production (data not shown). The moderate success in model validation demonstrates the limitations in the application of RSM to optimize fermentations, where small differences in substrate and process conditions can have significant influences on the outcome. Because wort is a very complex substrate, comprising a complex mixture of different sugars, nitrogen sources, minerals and vitamins, among others, any interpretation or the

transfer of the RSM results to other substrates (even different wort substrates) should be made with caution. In particular, a different sugar composition will have a significant effect on the responses when applying maltose-negative yeasts. However, the improved fruitiness and therefore higher acceptance of the NAB produced at low temperature and low pitching rate, the main goal from the optimization, was significant and reproducable (Table 8).

Table 8. Response surface methodology (RSM) model validation via predicted value vs. observed value.

Response	95% PI Low	Predicted Mean	95% PI High	Observed Mean	Std. Dev.
Ethanol *	0.33	0.40	0.48	0.53	0.01
Ethyl acetate	0.89	4.74	8.60	6.83	0.59
Isoamyl acetate	0.78	1.63	2.47	2.50	0.10
Acetaldehyde *	2.19	2.97	3.74	1.27	0.29
n-Propanol	2.68	3.28	3.88	3.57	0.06
Isobutanol *	2.91	3.23	3.54	2.80	0.10
Isoamyl alcohols	5.78	7.03	8.29	4.10	0.10
SUM Esters	3.01	7.10	11.19	9.33	0.68
SUM Alcohols *	12.84	13.74	14.64	10.47	0.31
Glycerol	0.13	0.18	0.22	0.27	0.01
Acceptance *	2.12	3.23	4.34	3.75	0.62
Fruitiness *	2.02	3.03	4.05	3.58	0.87

* Significant model with insignificant lack of fit. 'PI' Prediction interval.

3.6. Pilot-Scale Brewing

Despite the limited model validation, the fermentation parameters were successfully optimized to enhance the fruity character of the NAB. Therefore, the pilot-scale brewing trial was conducted with the optimized conditions of 17 °C fermentation temperature and a pitching rate of 10^7 cells/mL.

The grain bill of the wort for the pilot-scale brewing trial consisted of 95% pilsner malt and 5% acidulated malt to lower the starting pH of the wort, to account for the reduced pH drop during fermentations with non-*Saccharomyces* yeasts compared to brewers' yeast. A low beer pH is desired to prevent microbial spoilage and to ensure good liveliness of the beer [51,52]. The analytical attributes of the wort produced at pilot-scale are shown in Table 9.

Table 9. Attributes of the wort produced on pilot-scale.

Wort attributes	Unit	Value
Extract	°P	7.00 ± 0.01
pH		4.86 ± 0.01
FAN	mg/L	107 ± 3
Glucose	g/L	6.01 ± 0.08
Fructose	g/L	0.80 ± 0.01
Sucrose	g/L	2.13 ± 0.03
Maltose	g/L	31.59 ± 0.44
Maltotriose	g/L	9.32 ± 0.13

To assess the suitability of *Cyberlindnera subsufficiens* C6.1 to produce a fruity NAB, it was compared to two commercial NABs. NAB A was a commercial non-alcoholic beer produced by limited fermentation [29], and NAB B was a non-alcoholic beer produced by "dialysis technology" [30]. The NABs were analyzed for their extract, ethanol, FAN and glycerol content as well as their sugar composition and concentration of volatile fermentation by-products. The results are shown in Table 10.

Table 10. Attributes of the non-alcoholic beer (NAB) produced with C6.1 compared to two commercial NABs, NAB A and NAB B.

NAB Attributes	Unit	C6.1 NAB	NAB A	NAB B
Extract (real)	°P	6.60 ± 0.01	6.76 ± 0.07	7.05 ± 0.03
Extract (apparent)	°P	6.46 ± 0.02	6.57 ± 0.06	6.86 ± 0.01
Ethanol	% ABV	0.36 ± 0.00	0.50 ± 0.03	0.49 ± 0.04
pH		4.45 ± 0.01	4.29 ± 0.02	4.29 ± 0.04
FAN	mg/L	96 ± 2	86 ± 6	24 ± 0
Glycerol	g/L	0.30 ± 0.02	0.33 ± 0.01	1.40 ± 0.03
Glucose	g/L	2.77 ± 0.05	2.74 ± 0.04	5.61 ± 0.04
Fructose	g/L	1.65 ± 0.03	1.96 ± 0.03	0.19 ± 0.00
Sucrose	g/L	<LOD	<LOD	<LOD
Maltose	g/L	30.27 ± 0.62	30.11 ± 0.50	17.69 ± 0.24
Maltotriose	g/L	8.67 ± 0.24	8.31 ± 0.21	1.84 ± 0.03
Acetaldehyde	mg/L	10.55	2.40	0.70
Ethyl acetate	mg/L	12.00	<0.10	2.70
Isoamyl acetate	mg/L	0.80	<0.1	0.70
Isoamyl alcohols	mg/L	4.00	4.80	17.40
n-Propanol	mg/L	2.20	<0.5	2.50
Isobutanol	mg/L	3.60	1.00	4.90
Diacetyl	mg/L	<0.01	0.02	0.04
2,3-Pentandione	mg/L	<0.01	<0.01	<0.01
Σ Esters	mg/L	12.8	<0.1	3.4
Σ Alcohols	mg/L	9.8	5.8	24.8

The C6.1 NAB reached final attenuation after 13 days of fermentation at 17 °C, at an ethanol content of 0.36% ABV. At the end of fermentation, 2.77 g/L glucose was remaining in the wort and sucrose was fully depleted. Compared to the initial sugar concentration of the wort (Table 9), fructose concentrations in the final beer were significantly higher, at 1.65 g/L, twice as high as the starting concentration in the wort. Since sucrose was fully depleted, it can be assumed that it was converted to glucose and fructose by the yeast's invertase. The high residual fructose could therefore be attributed to the previously observed glucophilic character of the C6.1 strain in the screening and RSM trial. As a result, fructose was not consumed by the yeast due to the permanent presence of glucose until fermentation came to a halt. As expected, maltose and maltotriose consumption was negligible. Despite the limited fermentation, C6.1 produced a relatively high amount of esters, at 12.8 mg/L, the majority of which was ethyl acetate (12 mg/L). NAB A had an ethanol content of 0.50% ABV. Interestingly, the sugar composition was very similar to that of the C6.1 NAB. Regarding fermentation by-products, however, NAB A exhibited very low concentrations, at about half the amount of higher alcohols and a total lack of the esters ethyl acetate and isoamyl acetate. NAB B had an ethanol content of 0.49% ABV. Owing to its fundamentally different production method, the analyzed attributes were very different from those of the two NABs produced solely by limited fermentation. The low FAN content together with a high glycerol content compared to the other NABs were indicators of a more extensive fermentation, with subsequent removal of ethanol. However, NAB B still exhibited high amounts of monosaccharides, which suggested that the production of the NAB either also entailed a limited fermentation, or the dealcoholized beer was blended with wort (or other means of sugar addition). The increased amounts of higher alcohols in NAB B, at 24.8 mg/L, are uncommon for beers dealcoholized via dialysis, since the process commonly reduces their content in the final NAB by 90%–95% [7]. Despite the addition of acid malt during the wort production for the C6.1 NAB, the final pH after fermentation was, at 4.45, higher compared to 4.29 in the commercial NABs.

Due to the high amounts of residual sugars, proper pasteurization is essential for non-alcoholic beers produced by limited fermentation to avoid microbial spoilage [1,38,53]. After bottling, C6.1 NAB was therefore pasteurized with approximately 23 PU, and the successful pasteurization was confirmed

by plating the pasteurized NAB on agar to check for microorganism growth, which was found to be negative.

3.7. Sensory Evaluation

For a holistic evaluation of the C6.1 NAB compared to the two commercial NABs, a sensory trial was conducted with 10 trained and experienced panelists. The panel was asked to describe the flavor of the beer in their own words, followed by an assessment of several intensity attributes. The mean score values of the parameters wort-like, floral, fruity, citrus-like and tropical aroma, as well as sweet taste, of the NABs are shown in Figure 6.

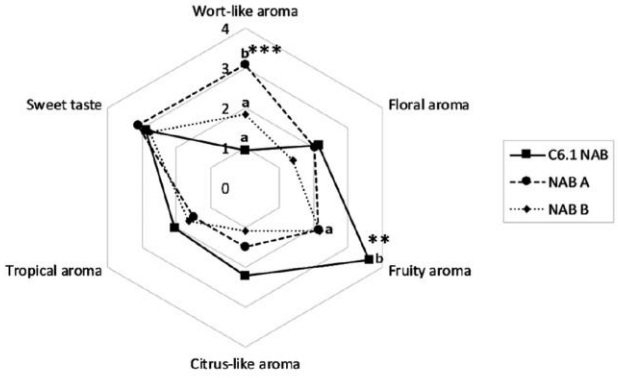


Figure 6. Spider web with the means of the descriptors from the sensory trial of the NAB produced with *Cyberlindnera subsufficiens* C6.1 and the two commercial NABs. Different letters next to data points indicate a significant difference as per Tukey's *post hoc* test. Significance codes: *** $p \leq 0.001$, ** $p \leq 0.01$.

The NAB produced with C6.1 was described as very fruity with aromas of pear, banana, mango and maracuja together with a slightly wort-like character. NAB A was described as malty, wort-like and hoppy, while NAB B was described as wort-like and caramel-like. The C6.1 NAB was indeed evaluated as being significantly more fruity than the commercial NABs ($p \leq 0.01$), at an average of 3.6 out of 5 compared to 2.1 and 2.2 out of 5, scoring also higher in citrus-like and tropical aromas. Consequently, the wort-like aroma, one of the most criticized flaws of NABs produced by limited fermentation [1,2,52], was least pronounced in the NAB produced with C6.1 with an average of 1 out of 5, followed by NAB B with 1.8 out of 5. NAB A exhibited, at an average of 3.2, a significantly more pronounced wort-like aroma ($p \leq 0.001$). A sweet taste, caused by a high amount of residual sugars, is another major point of criticism for NABs produced by limited fermentation [1,2,52]. All NABs scored similarly in sweet taste without significant differences. NAB B scored lower for "floral" compared to the other NABs. However, the difference was not statistically significant. When the panelists were asked for their favorite sample, 40% chose C6.1 NAB, 40% chose NAB A, and 20% chose NAB B. Similarly, Strejc et al. [3] investigated the production of a non-alcoholic beer (0.5% ABV) by a cold contact process (characterized by a low temperature and high pitching rate) with a mutated lager yeast strain (*Saccharomyces pastorianus*). The strain's targeted mutation resulted in an overproduction of isoamyl acetate and isoamyl alcohols. The authors reported that the fruity flavour of the NAB produced with the mutated strain was "partially able to disguise" the typical wort-like off-flavor [21]. However, the isoamyl acetate concentration of the resulting NAB was, at 0.5 mg/L, lower than the concentration in the C6.1 NAB in this study (Table 10). Furthermore, the complex mutation and isolation procedure paired with a potentially limited stability of the mutation limits its applicability in practice. Saerens and Swiegers [22] reported the successful production of a NAB at 1000 L scale with a *Pichia kluyveri* strain, owing to its high production of isoamyl acetate (2–5 mg/L), which reportedly gave the NAB a fruity flavor that was more like that of a regular beer than commercial NABs. In accordance, the results of the sensory indicated that a strong fruity aroma can mask the wort-like off flavor, and that

the non-*Saccharomyces* yeasts, which produce a pronounced fruity character, can therefore be a means to produce NAB with improved flavor characteristics.

4. Conclusions

The *Cyberlindnera* genus was found to be a promising non-*Saccharomyces* genus for application in the production of a fruity, non-alcoholic beer. Four of the six investigated species produced a fruity character, despite the limited fermentative capacity, which resulted in a low ethanol concentration. It was shown that through optimization of the fermentation parameters of temperature and pitching rate, the fruity character could be enhanced. Process up-scaling with *Cyberlindnera subsufficiens* strain C6.1 produced a NAB that was significantly more fruity compared to two commercial NABs. Owing to the strong fruity aroma, the often-criticized wort-like aroma could successfully be masked. Yeast handling throughout the process (i.e., propagation, yeast pitching, fermentation) proved to be suitable for pilot-scale brewing, with potential for application at industrial scale. Further studies should investigate if the masking effect was enhanced by a reduction of wort aldehydes via yeast metabolism.

This study demonstrated the suitability of the non-*Saccharomyces* species *Cyberlindnera subsufficiens* for the production of non-alcoholic beer (<0.5% ABV) with novel flavor characteristics that can compete with commercial NABs. The successful pilot-scale (60 L) brewing trial gives prospect to future studies with diverse non-*Saccharomyces* yeasts and strengthens their position as a serious and applicable alternative to established methods in non-alcoholic and low alcohol beer brewing.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-5637/5/4/103/s1, Data Sheet S1: RSM response values and model statistics.

Author Contributions: Conceptualization, K.B., M.M., M.H., M.Z. and E.K.A.; methodology, K.B. and M.M.; investigation, K.B., J.J.A., and A.H.; resources, M.H., F.J. and E.K.A.; formal analysis, K.B.; writing—original draft preparation, K.B.; writing—review and editing, K.B., M.M., M.Z., M.H., K.M.L. and E.K.A.; visualization, K.B.; supervision, E.K.A.; project administration, E.K.A.; funding acquisition, E.K.A.

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

Appendix A

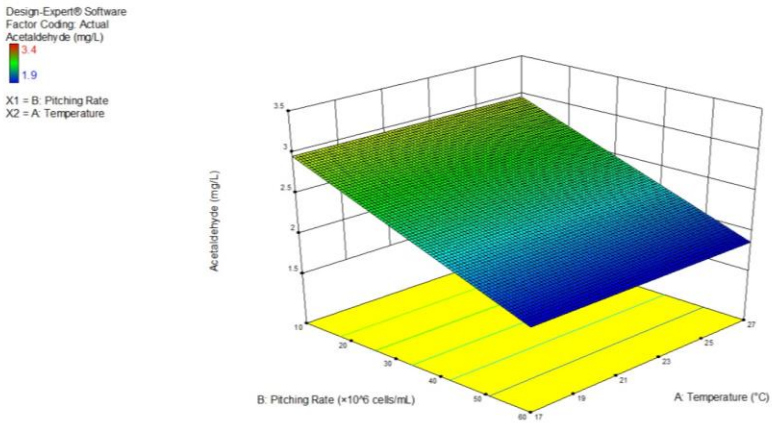


Figure A1. Three-dimensional response surface plot of the effect of pitching rate on the acetaldehyde content of the produced NAB ($p < 0.01$). The factor temperature was excluded from the model due to insignificance ($p = 0.39$; supplementary Data Sheet 1).

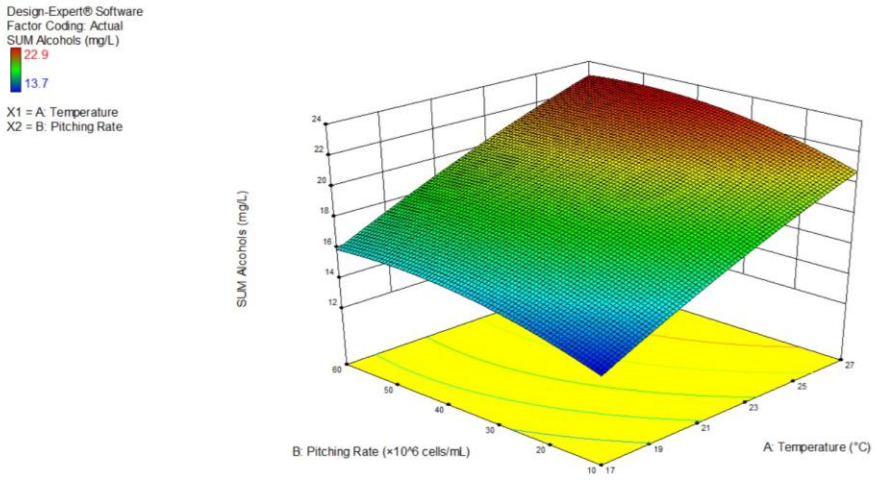


Figure A2. Three-dimensional response surface plot of the interactive effects of temperature and pitching rate on the sum of higher alcohols of the produced NAB ($p < 0.001$).

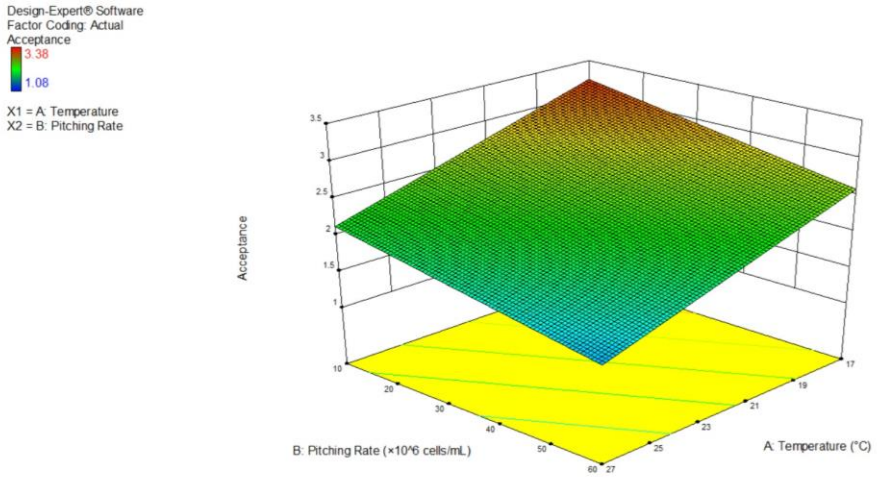


Figure A3. Three-dimensional response surface plot of the effects of temperature and pitching rate on the overall acceptance of the produced NAB ($p < 0.05$).

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ORIGINAL PAPER

On the suitability of alternative cereals, pseudocereals and pulses in the production of alcohol-reduced beers by non-conventional yeasts

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Abstract
The growing interest in non-alcoholic and low alcohol beers (NABLAB) has fuelled research into innovative production methods. One means to produce NABLAB is through limited fermentation by non-*Saccharomyces* yeasts which have a naturally low fermentative capacity in cereal-based wort substrates. At the same time, adjunct brewing, the partial replacement of barley malt on the grain bill, enjoys growing popularity. In this study, 13 cereals, pseudocereals, and pulses were investigated for their suitability to produce a wort with limited amounts of fermentable sugars. Subsequently, the fermentation performance of two non-*Saccharomyces* yeast strains, namely *Cyberlindnera subsufficiens* C6.1 and *Lachancea fermentati* KBI 12.1, in the produced worts was investigated and compared to that of a brewers' yeast strain. The worts were produced by harnessing endogenous amylolytic enzyme activity or the addition of an external amylase and analysed for their sugar composition and free amino acids (FAA) profile. All alternative substrates without endogenous β-amylase activity were found to be suitable for producing worts with a high proportion of unfermentable sugars. However, the extract yield was low for the pulses and most worts exhibited a low and/or unbalanced FAA profile. The ethanol production was limited and mostly dependent on the sugar spectrum of the worts and the sugar utilization characteristics of the applied yeast strains. The (partial) substitution of barley with alternative substrates when producing NABLAB by non-*Saccharomyces* yeast can be a means to alter the sugar and FAA profile of the wort, but must be considered in concert with the yeast strains' characteristics.

Keywords Cereals · Pseudocereals · Pulses · Non-conventional yeast · Non-*Saccharomyces* yeast · Low alcohol beer

Introduction

Increasing customer demands fuelled by recent lifestyle trends have led to an increase in the production of non-alcoholic and low alcohol beers (NABLAB) [1]. The production methods of NABLAB can generally be divided into two categories: physical methods and biological methods (Fig. 1). Physical methods focus on the removal of ethanol from a finished beer, while biological methods focus on a limited ethanol formation by the yeast. Investigations into the use of "special yeast", especially non-*Saccharomyces* species, have picked up in the recent years, which have the potential to lead to the introduction of NABLAB with innovative flavours into the market [1]. The principle behind the application of non-conventional yeasts in NABLAB production is their sugar utilization capabilities. Most non-*Saccharomyces* yeasts are unable to consume maltose and maltotriose, the most abundant sugars in cereal-based wort substrates. Thus, fermentations with these yeasts yield less ethanol due to their reduced fermentative capacity. However, research into the use of non-*Saccharomyces* yeasts is predominantly in the early stages and far from application. Furthermore, most studies investigate the performance of non-conventional yeasts in barley malt-based substrates. Barley has a justified traditional pole position in brewing

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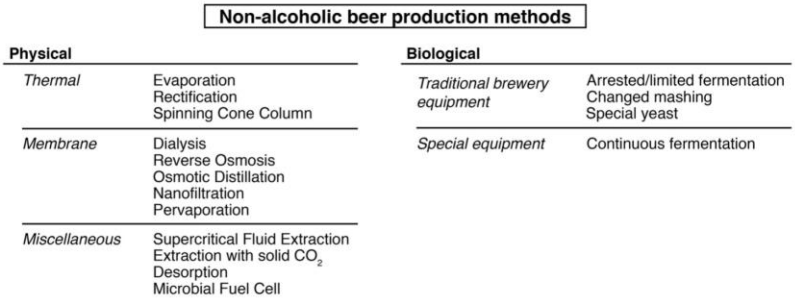


Fig. 1 Non-alcoholic beer production methods [1]

due to its sensorial, physical, and technological characteristics which make it a perfect grain for brewing purposes. However, other cereals and pseudocereals have made their way into the brewing sector. Wheat has long been a popular choice amongst Belgian and German brewers, while oat and rye are a means to enhance the mouthfeel of beer, especially in the craft beer sector. Furthermore, the use of pseudocereals such as quinoa, amaranth, and buckwheat has been investigated in the context of gluten-free beer brewing [2]. In fact, adjunct brewing, the partial replacement of barley malt by unmalted grains, alternative malted grains, or sugar syrup, is a common practice amongst breweries around the globe. It is believed that worldwide up to 85–90% of beer is produced with adjuncts, with the reasons for their use being cost reduction, to support local agriculture, or to alter the sensory and physical properties of the beer [3, 4]. Usually, only a small part of the grain bill is substituted by adjuncts, since the high amylolytic power of barley malt can make up for low or non-existent amylolytic activity of the respective adjuncts. Higher adjunct percentages require the addition of exogenous enzymes during the mash to fully saccharify the starch and to yield a high amount of fermentable sugars [5]. The choice of enzymes added can significantly influence the yield of fermentable sugars and the overall composition of sugars in the wort.

In this study, the wort production from selected cereals, pseudocereals, and pulses and their subsequent fermentation with selected non-*Saccharomyces* yeast strains was investigated. In the first part of the study, 13 cereals (barley, corn, millet, oat, rye, spelt, wheat), pseudocereals (amaranth, buckwheat, quinoa), and pulses (lentil, lupine, pea) were analysed for their chemical composition and amylolytic enzyme activity. The investigated substrates were sourced as malted or sprouted seeds since the germination, also called sprouting, of a seed leads to the activation and de novo synthesis of a range of hydrolytic enzymes. Additionally, sprouting is a means to reduce antinutritional compounds and increase the nutritional value of the grain or pulse [6–8]. Worts from the aforementioned cereals, pseudocereals, and pulses were produced and analysed in detail for their sugar, free amino acid (FAA), and free amino nitrogen (FAN) composition. A thermostable α-amylase was added to saccharify the starch when required, and an external protease was applied to optimize the FAN yield when required.

Subsequently, the worts were fermented with two non-*Saccharomyces* yeast strains, namely *Cyberlindnera subsufficiens* C6.1 and *Lachancea fermentati* KBI 12.1, and one *Saccharomyces cerevisiae* WLP001 brewers' yeast strain, in small-scale fermentations. Yeast strains of the *Cyberlindnera* genus have been reported to produce high concentrations of acetate esters, in particular isoamyl acetate, and have the potential to create fruity beers with reduced ethanol content due to their inability to consume the most abundant wort sugars [9–11]. *Lachancea fermentati* strain KBI 12.1 has recently been investigated by Bellut et al. [12] for its potential for low alcohol beer brewing due to its trait of producing significant amounts of lactic acid during alcoholic fermentation, which is uncommon for yeasts. *Saccharomyces cerevisiae* WLP001 is a popular commercial ale yeast. It has been applied as a control brewers' yeast in many studies, especially when used in comparison to non-*Saccharomyces* yeasts or wild-type *Saccharomyces* strains [12–16]. The final fermented substrates were analysed for ethanol production and extract reduction, pH drop, FAN consumption, and lactic acid production (where applicable).

Materials and methods

Enzymes, yeast strains, and substrates

Hitempase STXL, a heat-stable α-amylase (*Bacillus lichenformis*) was sourced from Kerry Group (Tralee, Ireland). It is an endo-amylase which randomly hydrolyses the α-1,4-glycosidic linkages in amylose and amylopectin, resulting in the production of dextrins. Bioprotease P1, a proteolytic enzyme, was also sourced from Kerry Group (Tralee, Ireland). It is a complex enzyme system derived from selected microbial strain and plant species and is

used in sorghum brewing to ensure adequate levels of free α-amino nitrogen in the wort.

The *Saccharomyces cerevisiae* brewers' yeast strain WLP001 (California Ale Yeast©) was sourced from Whitelabs (San Diego, CA, USA). *Lachancea fermentati* strain KBI 12.1 was isolated from a kombucha culture as described by Bellut et al. [12]. *Cyberlindnera subsufficiens* strain C6.1 was sourced from the in-house culture collection and had been isolated from a coconut. Yeast strains were kept as stock cultures in 50% (v/v) glycerol at − 80 °C. Strains were grown on peptone dextrose agar (PDA) plates for 48–72 h at 25 °C and stored at 4 °C. The substrates used in this study are shown in Table 1.

Compositional analysis

The moisture content was determined by the air-oven method according to the AACC method 44-15.02. Ash content was determined by the AACC method 08-01.01. Fat content was determined by Soxhlet solid–liquid extraction following AACC method 30-25.01. Total nitrogen content was determined by Kjeldahl method (AACC 46-12.01). Total starch content was determined colorimetrically by Megazyme enzyme assay kit K-TSHK following the recommended procedure (Megazyme, Bray Co. Wicklow, Ireland).

Alpha-amylase activity was determined via Megazyme enzyme assay kits. The Ceralpha kit K-CERA (Megazyme, Bray Co. Wicklow, Ireland) was applied for barley, oat, rye, wheat malt, and sprouted corn. For the remaining grains and pulses with lower α-amylase activity, the Amylase SD kit (Megazyme, Bray Co. Wicklow, Ireland) was applied due to its high sensitivity. Beta-amylase was determined using the Beta-Amyl-3 K-BETA (Megazyme, Bray Co. Wicklow, Ireland) enzyme assay kit.

Wort production

The grains were milled with a Bühler disc mill (Bühler Group, Uzwil, Switzerland) at a gap size of 2 mm. Fifty grams was placed in a beaker of the Lochner Congress mashing device (Lochner Labor and Labortechnik GmbH, Berching, Germany) and 350 g of brewing water was added. After mixing, enzymes were added where applicable (Fig. 2). A detailed scheme of the wort production is illustrated in Fig. 2. Hitempase STXL was added at concentrations of 1 µL/g grist (220–286 U/g grist). In the corn mash, the concentration was 4 µL/g grist (880–1144 U/g grist). Bioprotease P1 was added at 0.5 mg/g grist (125–163 U/g grist), where applicable (Fig. 2). If enzymes were applied, 100 ppm calcium chloride was added to stabilize the enzymes. The mashing regimes for the respective substrates are outlined in Fig. 2. The mashing regime without the addition of enzymes was 30 min at 50 °C, followed by 60 min at 62°, 60 min at 72 °C, and mashing out after 10 min at 78 °C. The mashing regime for the mashing with enzymes was 30 min at 50 °C, followed by 60 min at 90 °C (Fig. 2). Heating rate was 2 °C/min and stirring speed was 100 rpm. At the end of mashing, starch negativity was checked by iodine test. At the end of mashing, 100 g of brewing water was added, and the mashes were filtered through a Whatman folded filter grade 1 V paper (Whatman plc, Maidstone, UK). Filtration was stopped when no liquid was standing above the filter cake. Worts were boiled vigorously for 5 min before filling into sterile bottles and storing at − 20 °C.

Fermentation

For the preparation of the inoculum, a single colony was picked from plate stocks, streaked on PDA agar, and incubated for 48 h at 25 °C. Subsequently, a single colony was transferred into a 250 mL Schott bottle filled with 150 mL

Table 1 List of substrates used in this study

Designation	Class	Supplier designation	Supplier
Barley	Cereal	Best Pilsen malt	Bestmalz, Heidelberg, Germany
Wheat	Cereal	Wheat malt	Muntons, Suffolk, UK
Rye	Cereal	Rye malt	Bestmalz, Heidelberg, Germany
Oat	Cereal	Oat malt	Muntons, Suffolk, UK
Spelt	Cereal	Spelt sprouts flour	Ziegler, Wunsiedel, Germany
Millet	Cereal	Brown millet sprouts flour	Ziegler, Wunsiedel, Germany
Corn	Cereal	Corn sprouts	Keimkraft, Pöttelsdorf, Austria
Amaranth	Pseudocereal	Amaranth sprouts	Ziegler, Wunsiedel, Germany
Buckwheat	Pseudocereal	Buckwheat malt	Mälzerei Steinbach, Zirndorf, Germany
Quinoa	Pseudocereal	Quinoa sprouts	Ziegler, Wunsiedel, Germany
Lentil	Pulse	Lentil sprouts	Keimkraft, Pöttelsdorf, Austria
Lupine	Pulse	Lupine sprouts	Keimkraft, Pöttelsdorf, Austria
Pea	Pulse	Pea sprouts	Keimkraft, Pöttelsdorf, Austria

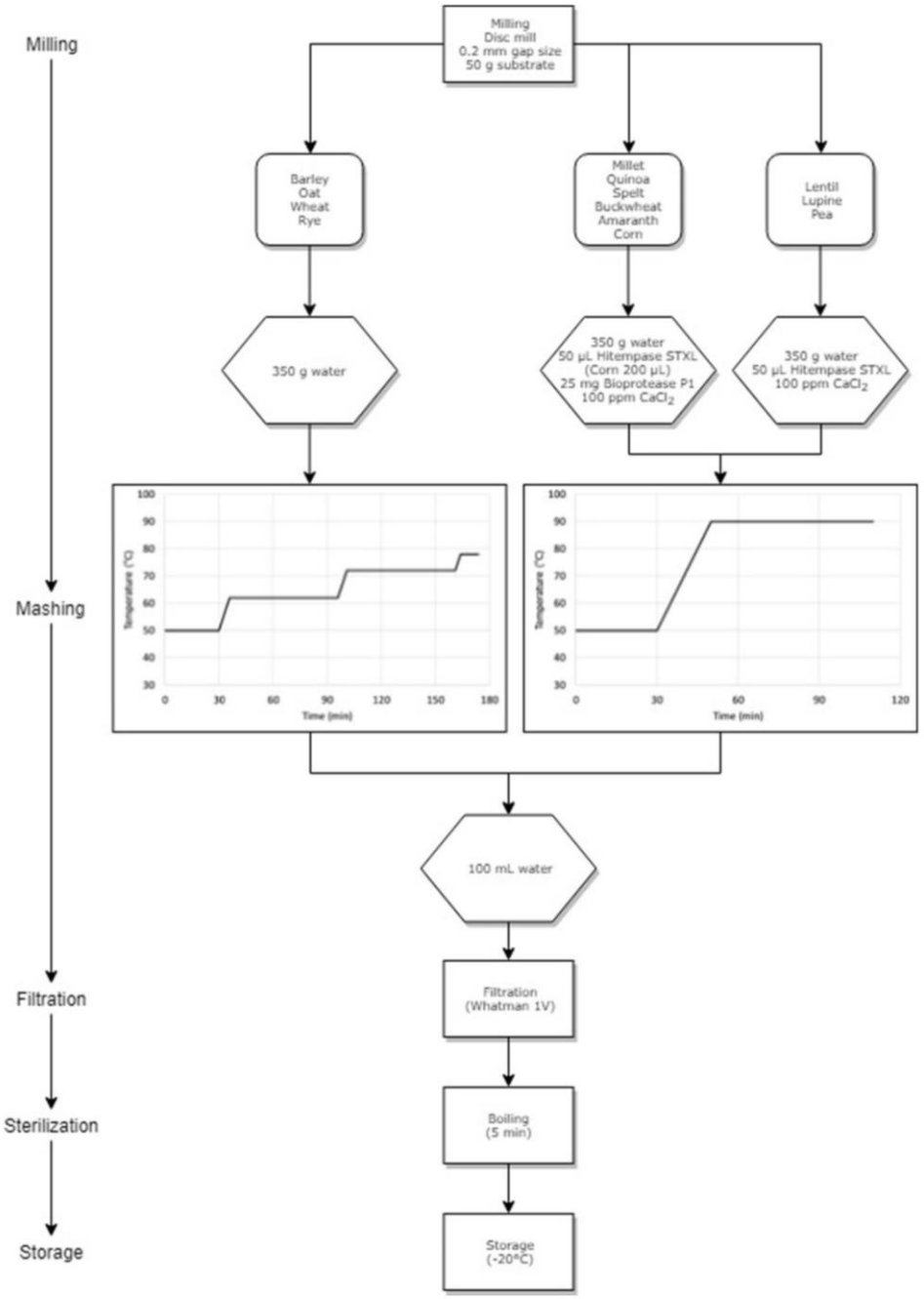


Fig. 2 Wort production scheme

sterilized propagation wort comprising 7.5% (w/v) malt extract, Spraymalt light (Muntons plc, Suffolk, UK), and 3% (w/v) glucose (Gem Pack Foods, Dublin, Ireland). The bottles were closed with sterilized cotton to allow aerobic conditions and incubated at 25 °C for 48 h in an incubation chamber (ES-80 shaker-incubator, Grant Instruments (Cambridge) Ltd, Shepreth, UK) with orbital shaking at 180 rpm.

Cell count was determined using a Thoma counting chamber (0.1 mm depth). Before pitching, the propagation wort containing the yeast cells was centrifuged at 3000*g* for 5 min (Rotina 380 R, Andreas Hettich GmbH & Co.KG, Tutlingen, Germany). The supernatant was discarded and the cells were resuspended in sterilized brewing water to eliminate carryover of sugars from the propagation wort into the wort samples for fermentation. Pitching rate was 10^7 cells/mL with a maximum pitching volume of 1 mL (max. 3.3% dilution of wort samples). Fermentations were carried out in 50 mL sterile Sarstedt tubes filled with 30 mL of the respective wort samples. The tubes were closed with an air lock and incubated at 25 °C. After 7 days of fermentation, the cell count was determined for each sample after homogenization. The fermented samples were then centrifuged (5000*g*, 10 min) and the supernatant frozen (− 20 °C) for further analysis.

Wort and fermented wort analyses

The supernatant from the fermentation trials was used for extract measurements with a density meter DMA 4500 M with Alcolyzer Beer ME (Anton-Paar GmbH, Graz, Austria). The pH value was determined using a digital pH meter (Mettler Toledo LLC, Columbus OH, USA). The fermented worts were analysed using the following methods. Sugars and ethanol were determined by high-performance liquid chromatography HPLC Agilent 1260 Infinity (Agilent Technologies, Santa Clara CA, USA) equipped with a refractive index detector (RID) and a Sugar-Pak I 10 µm, 6.5 mm × 300 mm column (Waters, Milford MA, USA) with 0.1 mM Ca-EDTA as mobile phase and a flow rate of 0.5 mL/min at 80 °C. Lactic acid was quantified via HPLC (Waters 2690 Separations Module, Waters, Milford MA, USA) with diode array detector (DAD) and a Hi-Plex H 8 µm, 7.7 mm × 300 mm column (Agilent Technologies, Santa Clara CA, USA) with 5 mM H_2SO_4 as mobile phase and a flow rate of 0.5 mL/min at 60 °C.

Amino acid content was quantified using the HPLC MEBAK II 2.8.4.1 method. FAN was measured using a ninhydrin-based dying method where absorbance is measured at 570 nm against a glycine standard (MEBAK 2.6.4.1).

Statistical analyses

Fermentations and analyses were carried out in triplicate, unless stated otherwise. Statistical analysis was performed using RStudio, Version 1.1.463 with r version 3.5.2 (RStudio Inc, Boston MA, USA; R Core Team, r-project). One-way ANOVA was used to compare means and Tukey's test with 95% confidence intervals applied for the pairwise comparison of means. Values are given as means (± standard deviation). Principal component analysis (PCA) was performed with the r package *FactoMineR* [17].

Results

Compositional analysis

To analyse the substrates, a compositional analysis was performed entailing analysis for moisture, ash, protein, fat, and total starch content. Alpha-amylase and β-amylase activity was determined to investigate the necessity for the addition of technical enzymes. The results of the compositional analysis and enzyme activity are shown in Table 2.

The malted grains (barley, oat, wheat, rye, buckwheat) generally exhibited a lower moisture content compared to the sprouted grains and pulses (pea, lentil, lupine, corn, amaranth, quinoa). The moisture content of the substrates ranged from 5.2 to 12.5%. The protein content of the cereal grains ranged between 7.9 and 13.4%, with corn exhibiting the lowest value and spelt the highest (Table 2). Of the pseudocereals, amaranth exhibited the highest protein content, at 15.9%, significantly higher than that of quinoa and buckwheat, both at 11.1%. These findings are similar to reported values from previous studies [18, 19]. Pulses, especially lupine, have a high protein content. In literature, reported values range between 30 and 38% for lupine, 19–35% for pea, and 26–31% for lentil [20–22]. As expected, the pulses exhibited a significantly higher protein content compared to the pseudocereals and cereal grains. At 34.8%, lupine exhibited the highest protein content followed by lentil, at 26.7%, and pea, at 25.5%. The highest fat content amongst the cereals was found in oat, at 5.1%, which is known to exhibit high fat contents among the class of cereals [23]. Millet showed only traces of fat, although reported values range between 1.5 and 5% [24]. The remaining cereals contained fat in the range of 1.4–3.3%. It is known that buckwheat contains less fat than quinoa or amaranth [18]. However, compared to reported values between 2.1 and 2.9% fat, the measured value of 0.07% appeared unexpectedly low [18]. Quinoa contained, at 7.6%, a significantly higher fat content compared to amaranth, at 6.5%. Except for lupine, whose fat content can reach up to 20%, pulses are generally low in fat [21, 25]. The fat content of lentil and pea was low with 1.8%

Table 2 Compositional analysis and enzyme activity of the respective substrates

Substrate	Moisture (%)	Ash (% DM)	Protein (% DM)	Fat (% DM)	Total starch (% DM)	Alpha amylase (U/g grist)	Beta amylase (U/g grist)
Barley[m]	7.08 ± 0.11^{c}	2.14 ± 0.02^{c}	9.25 ± 0.43^{ab}	2.36 ± 0.09^{d}	59.53 ± 1.14^{ef}	166.42 ± 8.88^{d}	13.99 ± 0.39^{d}
Wheat[m]	5.87 ± 0.11^{b}	1.30 ± 0.06^{a}	11.04 ± 0.70^{ab}	2.05 ± 0.01^{cd}	48.96 ± 2.09^{cd}	172.17 ± 1.48^{d}	36.52 ± 0.94^{f}
Rye[m]	6.06 ± 0.03^{b}	1.73 ± 0.02^{b}	8.76 ± 0.44^{a}	1.65 ± 0.17^{bc}	48.13 ± 0.59^{bcd}	99.08 ± 3.22^{c}	9.01 ± 0.21^{c}
Oat[m]	9.25 ± 0.22^{e}	2.33 ± 0.01^{de}	10.51 ± 0.95^{ab}	5.07 ± 0.31^{f}	47.03 ± 5.18^{bcd}	38.87 ± 1.81^{b}	1.85 ± 0.08^{b}
Spelt[s]	11.70 ± 0.02^{g}	2.10 ± 0.03^{c}	13.42 ± 1.23^{bc}	3.25 ± 0.04^{e}	71.15 ± 1.75^{g}	3.87 ± 0.21^{a}	16.62 ± 0.37^{e}
Millet[s]	10.83 ± 0.03^{f}	3.33 ± 0.01^{h}	11.08 ± 0.79^{ab}	0.04 ± 0.05^{a}	61.18 ± 1.16^{f}	0.79 ± 0.01^{a}	0.07 ± 0.03^{a}
Corn[s]	12.45 ± 0.07^{h}	1.32 ± 0.01^{a}	7.90 ± 0.66^{a}	1.39 ± 0.11^{b}	78.06 ± 0.01^{g}	2.55 ± 0.49^{a}	0.22 ± 0.09^{a}
Amaranth[s]	11.08 ± 0.05^{f}	3.34 ± 0.10^{h}	15.86 ± 1.17^{c}	6.49 ± 0.32^{g}	51.85 ± 2.68^{de}	0.18 ± 0.02^{a}	ND
Buckwheat[m]	5.20 ± 0.06^{a}	2.22 ± 0.01^{cd}	11.12 ± 0.33^{ab}	0.07 ± 0.03^{a}	40.68 ± 1.98^{abc}	0.05 ± 0.01^{a}	0.07 ± 0.03^{a}
Quinoa[s]	8.19 ± 0.27^{d}	2.38 ± 0.07^{e}	11.12 ± 0.66^{ab}	7.59 ± 0.17^{h}	39.07 ± 2.70^{ab}	ND	ND
Lentil[s]	11.60 ± 0.04^{g}	2.87 ± 0.03^{f}	26.73 ± 2.12^{d}	1.78 ± 0.16^{bc}	31.56 ± 2.33^{a}	0.15 ± 0.01^{a}	ND
Lupine[s]	10.82 ± 0.01^{f}	2.89 ± 0.02^{f}	34.81 ± 3.52^{e}	7.31 ± 0.20^{h}	ND	ND	ND
Pea[s]	11.64 ± 0.02^{g}	3.13 ± 0.01^{g}	25.50 ± 2.31^{d}	1.86 ± 0.08^{bc}	42.75 ± 3.47^{bc}	ND	ND

% DM Percent dry matter. [m]Malt. [s]Sprouts. Protein conversion factor 6.25. Different superscripts of values within a column indicate a significant difference ($p \leq 0.05$)

ND not detected

and 1.9%, respectively. Lupine showed a high fat content of 7.3%. The total starch content of the cereals ranged between 48.1% for rye and 78.1% for corn. Spelt showed a total starch content of 71.1%, followed by millet and barley, at 61.2% and 59.5%, respectively. Wheat and oat exhibited values of 49.0% and 47.0%, respectively. Total starch values of the cereals were in line with previously reported values [26]. Of the pseudocereals, amaranth exhibited a significantly higher total starch content, at 51.9%, compared to buckwheat and quinoa, at 40.7% and 39.1%, respectively. Those values are lower compared to reported values of between 56 and 64% [18]; however, the previously reported values are of the unsprouted seeds. The buckwheat malt and amaranth and quinoa sprouts used in this study were expected to have lower starch contents due to the preceding germination which reduced the starch content and led to an apparent increase in the percentage of other components. The analysis of total starch content of lupine showed no result. However, reported values are very low (2.8–4.5%) and the preceding germination entailed starch degradation [21, 27]. The total starch contents of lentil and pea were relatively low, at 31.6% and 42.8%, respectively. The ash content of the substrates ranged from 1.3 to 3.3%.

Alpha-amylase activity varied widely amongst the group of cereal grains. Barley, wheat, and rye showed high activity, at 166.4, 172.2, and 99.1 U/g, respectively. Oat exhibited an α-amylase activity of 38.9 U/g. Spelt, corn, and millet showed low activity of between 0.8 and 3.9 U/g (Table 2). Beta-amylase activity was highest for wheat, at 36.5 U/g, followed by spelt and barley, at 16.6 and 14.0 U/g, respectively. Rye exhibited β-amylase activity of 9.0 U/g, while oat exhibited a low activity, at 1.9 U/g. Corn and millet showed extremely low β-amylase activity levels, at 0.1–0.2 U/g. Of the pseudocereals, only amaranth and buckwheat showed slight α-amylase activity, at 0.15 U/g and 0.05 U/g, respectively. Concerning β-amylase activity, buckwheat showed trace activity of 0.07 U/g, while no activity was detected for amaranth and quinoa. However, research has shown that with an optimized malting regime, amylase activity in buckwheat could be preserved [6, 28, 29]. For the pulses, neither α-amylase activity nor β-amylase activity was detected in lupine or pea. Lentil showed very low α-amylase activity, at 0.15 U, but no β-amylase activity.

Wort production and analysis

The aim of wort production was to saccharify the starch without yielding a high proportion of fermentable extract. For that reason, Hitempase STXL, a thermostable α-amylase which yields mostly dextrins, was added to the mashes which were lacking endogenous α-amylase activity (corn, millet, spelt, amaranth, buckwheat, quinoa, lentil, lupine, pea) (Fig. 2). The mashes with added Hitempase STXL yielded starch-negative worts with a high amount of unfermentable extract after 1 h at 90 °C, above their respective gelatinization temperature [26]. Bioprotease P1 is commonly applied in sorghum and high adjunct brewing to optimize FAN levels and was thus applied in mashes which exhibited a low FAN yield in pre-trials to ensure a sufficient amount of FAN for the subsequent fermentations (corn, millet, spelt, amaranth, buckwheat, quinoa) [5].

The properties of the produced worts are shown in Table 3. The real extract of the worts ranged between 6.0 and 9.0% (w/v). One outlier, lupine wort, only exhibited a real extract of 2.6% (w/v) owing to its low starch content. The rye, wheat, and barley worts showed the highest extract contents at 8.3%, 8.5%, and 9.0% (w/v), respectively. The percentage of fermentable sugars shown in Table 3 was calculated as the sum of maltotriose, maltose, sucrose, glucose, and fructose concentrations as measured via HPLC analysis. The unfermentable extract was calculated as the difference from real extract minus fermentable sugars and represents the entirety of soluble extract (dextrins of four glucose molecules and higher, proteins, soluble fibre, minerals, etc.) excluding fermentable sugars. It was found that the worts from barley, wheat, and oat exhibited a high proportion of fermentable sugars and thus comprised only one-quarter unfermentable extract. Conversely, the worts from millet, lentil, lupine, and pea comprised three-quarters unfermentable extract. The worts from the pseudocereals and corn exhibited around 65% unfermentable extract, while the rye and spelt worts showed lower values, at 35% and 44%, respectively.

Corresponding with the high raw protein content of the pulses, the FAN content in the worts was highest for the worts from pulses. For total FAN, the lentil wort exhibited the highest value of 205 mg/L, significantly higher compared to the lupine and pea worts, at 166 and 161 mg/L, respectively. In relation to the total extract content, lupine showed the highest FAN content at 63 mg/L per 1% extract, followed by lentil and pea, at 33 and 23 mg/L per 1% extract, respectively (Table 3). Barley showed, with 16 mg/L per 1% extract, the highest FAN yield of the cereal grains at a total of 139 mg/L. The spelt, millet, and corn worts, which were treated with Bioprotease, exhibited 64, 43, and 29 mg/L, respectively. The pseudocereals amaranth, buckwheat and quinoa, which were also treated with Bioprotease during mashing, exhibited total FAN values of 114, 81, and 69 mg/L, respectively. The oat wort exhibited the lowest pH value of the worts from cereal grains at pH 5.40. The remaining cereal worts showed similar pH values, ranging between 5.64 and 5.71. The amaranth wort exhibited the highest pH value of 6.15, and the only value above pH 6. The buckwheat and quinoa worts had pH values of 5.68 and 5.41, respectively. The pH values of the worts from pulses were 5.40, 5.77, and 5.91 for the lupine, pea, and lentil wort, respectively. The lentil, pea, and rye worts exhibited a very long filtration time, exceeding 3 h. Barley, wheat, spelt, and millet had a fast filtration, at approx. 30 min. Filtration of the remaining mashes took approx. 1 h (Table 3).

Figure 3 shows the sugar content of the respective worts. Amongst the worts from cereals, maltotriose values ranged from 7.8 to 12.9 g/L, with the millet wort exhibiting the lowest value and the corn wort exhibiting the highest. The worts from pseudocereals contained 7.1–7.7 g/L maltotriose. Concerning the worts from pulses, lupine showed a low concentration of only 1.8 g/L. The pea and lentil worts exhibited maltotriose concentrations of 9.6 and 10.0 g/L, respectively. Owing to the method of sugar determination, maltose concentrations include sucrose. However, sucrose concentrations in cereals, pseudocereals, and pulses are very low (ca. 0.5–0.9% dry weight) [30, 31]. The barley, wheat, and rye worts exhibited the highest maltose concentrations ranging from 42.8 to 44.8 g/L, followed by the spelt and oat worts, at 32.9 g/L and 29.9 g/L,

Table 3 Analysed parameters of worts from the respective substrates

Substrate	Real extract E_r (°P)	Fermentable extract (°P)	Unfermentable extract[1] (°P)	Unfermentable extract (% of E_r)	FAN (mg/L)	FAN per °P E_r (mg/L °P)	pH	Filtration time
Barley[m]	8.3 ± 0.1^{efg}	6.1 ± 0.2^{ef}	2.2	27	130 ± 10^{f}	15.7 ± 1.4^{f}	5.71^{abc}	30 min
Wheat[m]	8.5 ± 0.1^{fg}	6.3 ± 0.1^{f}	2.2	26	115 ± 2^{ef}	13.4 ± 0.3^{def}	5.63^{abc}	30 min
Rye[m]	9.0 ± 0.2^{g}	5.9 ± 0.1^{e}	3.1	35	94 ± 1^{de}	10.5 ± 0.4^{cd}	5.70^{abc}	> 3 h
Oat[m]	6.0 ± 0.1^{b}	4.5 ± 0.2^{d}	1.5	24	82 ± 1^{cd}	13.8 ± 0.4^{ef}	5.40^{a}	1 h
Spelt[s]	8.0 ± 0.1^{ef}	4.5 ± 0.1^{d}	3.5	44	64 ± 1^{bc}	7.8 ± 0.7^{bc}	5.64^{abc}	30 min
Millet[s]	7.0 ± 0.1^{cd}	1.9 ± 0.1^{b}	5.1	73	43 ± 1^{ab}	6.1 ± 0.1^{ab}	5.69^{abc}	30 min
Corn[s]	7.7 ± 0.1^{de}	2.8 ± 0.1^{c}	4.9	64	29 ± 1^{a}	3.8 ± 0.1^{a}	5.64^{abc}	1 h
Amaranth[s]	7.8 ± 0.4^{ef}	2.7 ± 0.2^{c}	5.1	66	114 ± 12^{ef}	14.5 ± 0.9^{ef}	6.15^{d}	1 h
Buckwheat[m]	6.6 ± 0.1^{bc}	2.5 ± 0.1^{c}	4.1	62	81 ± 1^{cd}	12.2 ± 0.2^{de}	5.68^{abc}	1 h
Quinoa[s]	8.0 ± 0.1^{ef}	2.7 ± 0.1^{c}	5.3	66	$69 \pm ^{2c}$	8.6 ± 0.2^{bc}	5.41^{ab}	45 min
Lentil[s]	6.3 ± 0.1^{bc}	1.8 ± 0.1^{b}	4.5	72	205 ± 4^{h}	32.9 ± 1.2^{h}	5.91^{cd}	>3 h
Lupine[s]	2.6 ± 0.2^{a}	0.7 ± 0.1^{a}	2.0	75	166 ± 9^{g}	63.0 ± 1.5^{i}	5.40^{a}	1 h
Pea[s]	6.9 ± 0.8^{c}	1.8 ± 0.3^{b}	5.0	73	161 ± 13^{g}	25.0 ± 2.7^{g}	5.77^{bc}	>3 h

[1]Calculated from real extract and fermentable extract. [m]Malt. [s]Sprouts. Different superscripts of values within a column indicate a significant difference ($p \leq 0.05$)

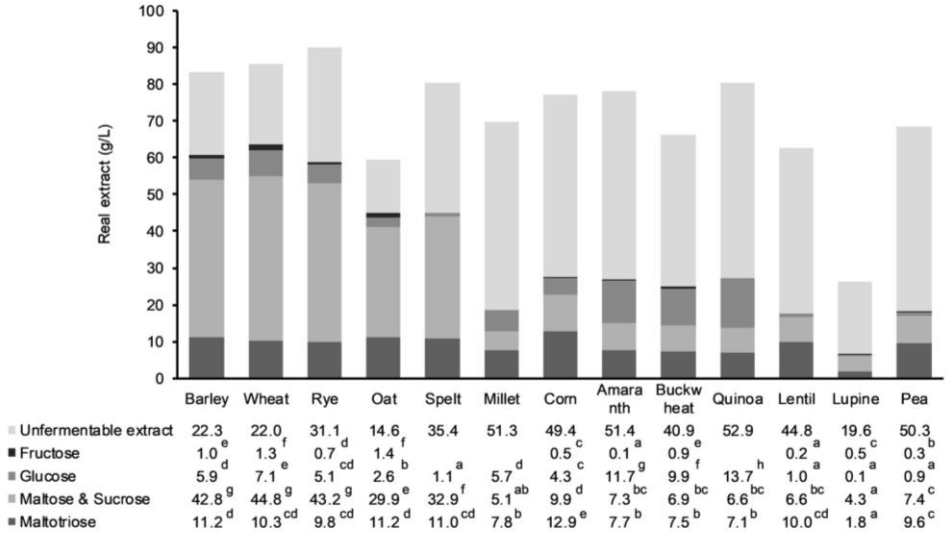


Fig. 3 Composition of real extract of the respective worts as means. Different superscripts of values within a row indicate a significant difference ($p \leq 0.05$)

respectively. The high maltose concentrations of the barley, wheat, rye, and spelt worts correlated ($r = 0.80$, $p \leq 0.01$) with the presence of β-amylase activity (Table 2). While α-amylase is mostly responsible for breaking down the starch to higher dextrins and maltotriose, β-amylase activity enables the production of maltose [32]. With no β-amylase activity present, the starch of the remaining grains and pulses was mostly broken down to maltotriose and higher dextrins by the added α-amylase. Consequently, the worts of the remaining grains and pulses contained little maltose, between 4.4% in lupine wort and 9.9% in corn wort (Fig. 3). The worts from the group of pseudocereals contained significantly more glucose than any other cereal or pulse, making glucose the most abundant fermentable sugar of the worts. Since other cereals and pulses were treated with the same technical α-amylase, the higher glucose content can be considered as a unique characteristic of those three pseudocereals. Previous studies have already reported high glucose levels of worts from buckwheat and quinoa when compared to barley wort [33–35]. The quinoa wort exhibited the highest glucose concentration of 13.7 g/L, followed by the amaranth and buckwheat worts, at 11.7 g/L and 9.9 g/L, respectively. Glucose concentrations in the cereal worts ranged from 1.1 g/L in the spelt wort, to 7.1 g/L in the wheat wort. Little glucose was found in the worts from pulses. The lupine wort contained only 0.1 g/L of glucose. The pea and lentil worts contained 0.9 and 1.0 g/L of glucose, respectively. Fructose concentrations were generally low with a maximum concentration of 1.4 g/L in the oat wort (Fig. 3). No fructose was found in the worts from millet, spelt, and quinoa.

Table 4 shows the mean values of the free amino acids (FAA) concentrations of the respective worts. Concerning the sum of FAA in wort, the corn wort exhibited the lowest, at 23 mg/100 mL, lacking threonine, valine, and methionine. With a total of 32 mg/100 mL, the millet wort also exhibited a low FAA concentration, lacking aspartic acid and threonine. The highest sum of FAA was found in the worts from pulses with total concentrations of 136–150 mg/100 mL. The barley wort exhibited a balanced amino acid profile, also exhibiting the highest concentrations for nine amino acids, namely histidine, glycine, threonine, valine*, methionine*, isoleucine*, phenylalanine*, leucine*, and lysine* (*with statistical significance; $p \leq 0.05$). Contrary to the balanced amino acid profile of the barley, wheat, rye, and oat worts, the worts produced from pulses showed extremely high concentrations for selected FAA (i.e. arginine, glutamic acid, asparagine), but very low concentrations for other FAA such as glutamine and methionine. Arginine concentrations were extremely high for lupine and pea at 52.8 and 44.2 mg/100 mL, respectively. With the example of the pea wort, three amino acids (glutamic acid, asparagine, arginine) were responsible for nearly two-thirds (64%) of the total FAA concentration. Conversely, the glutamine concentration in the pea wort was, at 1.3 mg/100 mL, only a tenth of that of the wheat wort, which exhibited the highest glutamine concentration amongst the worts (13 mg/100 mL). The corn, millet and spelt worts, as well as the pseudocereal worts,

Table 4 Concentration of free amino acids (FAA) in the wort substrates

Substrate	Arg	Lys	Asn	Asp	Glu	Ser	Thr	Gln	His	Leu	Ile	Val	Met	Gly	Phe	Tyr	Ala	Trp	Σ
Barley [m]	8.96^{c}	6.30^{g}	6.42^{b}	3.42^{c}	4.53^{abc}	6.09^{bc}	3.13^{c}	10.72^{f}	3.71^{f}	11.11^{i}	5.28^{h}	8.84^{f}	2.50^{f}	2.80^{f}	10.03^{g}	7.08^{e}	7.34^{cd}	4.04^{b}	112.25^{f}
Wheat [m]	8.20^{bc}	2.88^{d}	6.88^{b}	1.45^{ab}	4.51^{ab}	5.76^{bc}	1.73^{abc}	13.02^{g}	2.67^{cde}	8.68^{h}	4.67^{g}	5.74^{g}	1.84^{e}	2.32^{def}	8.56^{f}	4.17^{d}	6.22^{bc}	4.97^{i}	94.21^{e}
Rye [m]	4.86^{abc}	3.21^{d}	14.42^{d}	5.93^{d}	7.40^{cd}	4.40^{ab}	1.28^{abc}	10.52^{f}	2.28^{bcde}	4.52^{f}	2.82^{f}	4.47^{de}	1.32^{cde}	2.09^{cde}	5.39^{e}	3.34^{cd}	5.88^{bc}	2.35^{cde}	86.42^{de}
Oat [m]	8.11^{bc}	5.21^{f}	9.81^{c}	1.12^{ab}	2.54^{a}	3.10^{ab}	1.31^{abc}	3.93^{cd}	2.95^{def}	5.85^{g}	2.47^{ef}	4.21^{d}	1.44^{de}	1.73^{abcd}	5.00^{e}	3.86^{d}	5.51^{b}	3.36^{fg}	71.48^{cd}
Spelt [s]	1.43^{a}	1.49^{a}	2.69^{a}	0.23^{a}	1.78^{a}	1.50^{a}	ND	1.07^{a}	1.18^{a}	1.05^{a}	0.53^{a}	ND	ND	1.23^{a}	1.02^{a}	1.08^{a}	2.97^{a}	1.29^{ab}	20.52^{a}
Millet [s]	3.34^{ab}	1.78^{ab}	2.78^{a}	ND	1.79^{a}	1.81^{a}	ND	1.18^{a}	1.45^{ab}	2.47^{bc}	0.91^{ab}	0.88^{a}	0.60^{ab}	1.22^{a}	1.90^{b}	1.51^{a}	3.12^{a}	1.80^{abc}	28.50^{b}
Corn [s]	3.47^{ab}	2.22^{bc}	3.12^{a}	ND	2.73^{a}	2.08^{a}	ND	1.68^{ab}	1.42^{ab}	3.32^{de}	1.51^{c}	1.65^{ab}	0.70^{abc}	1.71^{abcd}	2.31^{bc}	1.68^{ab}	3.95^{a}	3.56^{ab}	37.07^{b}
Amaranth [s]	15.68^{d}	2.56^{cd}	2.13^{a}	0.30^{a}	7.13^{bcd}	4.02^{ab}	0.34^{a}	4.81^{de}	3.11^{ef}	2.99^{cd}	1.69^{cd}	1.67^{ab}	0.99^{bcd}	1.96^{bcde}	2.63^{bcd}	3.38^{cd}	7.92^{d}	2.85^{ef}	66.12^{c}
Buckwheat [m]	4.59^{abc}	1.43^{a}	1.88^{a}	ND	5.95^{bcd}	2.63^{ab}	0.67^{ab}	1.93^{ab}	1.35^{ab}	2.49^{bc}	1.34^{bc}	1.14^{a}	0.14^{a}	1.70^{abcd}	2.33^{bc}	1.96^{ab}	2.80^{a}	1.82^{abc}	36.13^{b}
Quinoa [s]	6.10^{abc}	2.60^{cd}	1.46^{a}	0.92^{a}	7.49^{d}	1.87^{a}	ND	2.69^{bc}	2.72^{cde}	2.90^{cd}	1.49^{c}	1.51^{ab}	ND	1.65^{abc}	2.22^{bc}	2.00^{ab}	4.01^{a}	1.84^{bc}	43.43^{b}
Lentil [s]	52.78^{f}	4.21^{e}	5.77^{b}	1.04^{a}	6.85^{bcd}	3.07^{ab}	2.67^{bc}	1.81^{ab}	3.06^{cd}	3.76^{e}	2.21^{de}	2.61^{bc}	0.45^{ab}	2.55^{ef}	2.55^{bcd}	2.57^{bc}	12.77^{f}	2.47^{de}	113.15^{f}
Lupine [s]	44.24^{e}	3.06^{d}	20.00^{e}	0.58^{a}	31.90^{f}	8.43^{c}	0.90^{ab}	1.27^{a}	2.03^{abcd}	2.61^{bcd}	1.85^{cd}	2.82^{bc}	0.61^{ab}	2.50^{ef}	3.35^{d}	9.72^{g}	9.72^{e}	2.16^{cd}	147.71^{g}
Pea [s]	17.59^{d}	4.08^{e}	23.46^{f}	2.56^{bc}	23.60^{e}	4.34^{ab}	2.66^{bc}	5.26^{e}	1.86^{abc}	1.95^{b}	1.65^{c}	3.70^{cd}	0.25^{a}	1.39^{ab}	2.95^{cd}	8.35^{f}	27.33^{g}	1.28^{a}	134.21^{g}

Values are shown as means of three replicate measurements in mg/100 mL. [m] Malt. [s] Sprouts. Different superscripts of values within a row indicate a significant difference ($p \leq 0.05$)

exhibited a more balanced but low FAA profile, completely lacking several amino acids (Table 4).

Figure 4 shows the principal component analysis (PCA) performed for the wort analysis parameters and FAA. The class of pulses can be clearly discriminated from the classes of cereals and pseudocereals (Fig. 4b). The pulses were characterized by high concentrations of the FAA glutamic acid, arginine, alanine, and asparagine and a generally high FAN concentration, while having low real extract and low fermentable extract, especially low glucose concentrations. The class of pseudocereals also forms a cluster on the individuals factor map (Fig. 4b). Quinoa, buckwheat, and amaranth were characterized by a rather low FAA and FAN content, a high glucose content, and a high amount of unfermentable extract. Within the cluster, amaranth, which exhibited higher FAA and FAN contents, is situated slightly separated from buckwheat and quinoa which are very close to each other (Fig. 4b). The group of cereal grains is spread out over the plane due to the varying wort composition of the individual cereal grains. Millet, corn, and spelt form a separate cluster

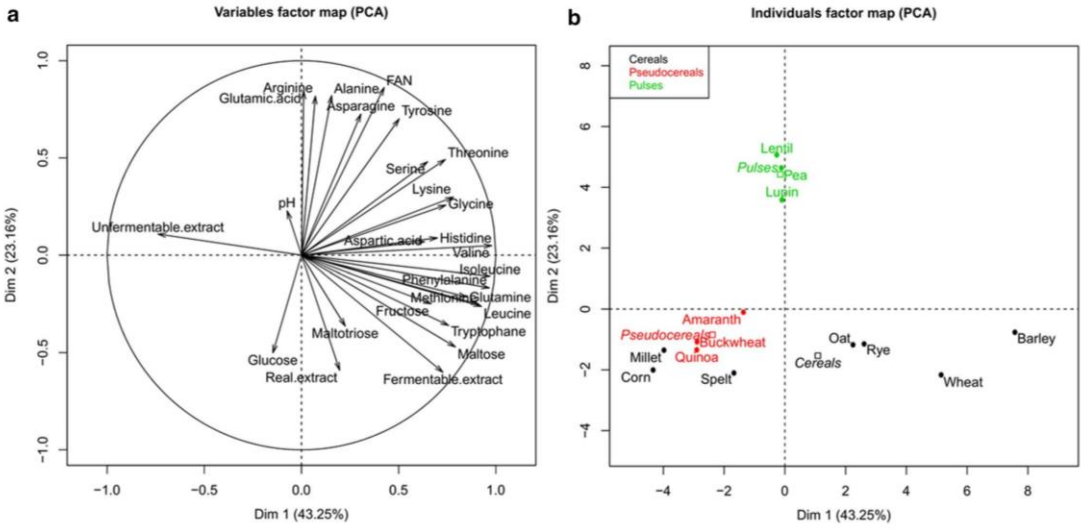


Fig. 4 Principal component analysis (PCA) of the wort attributes. **a** Variables factor map. **b** Individuals factor map

close to the pseudocereals, which was characterized by a low amount of FAN and FAA and high amounts of unfermentable sugars. The separation of millet, corn, and spelt from the remaining cereal grains became even more evident when they were treated as supplementary individuals during the PCA (data not shown). The cereals oat, rye, wheat, and barley were characterized by high fermentable extract, low unfermentable extract, high maltose concentrations, and a high concentration of individual FAA. In particular, the barley and wheat worts were characterized by a balanced and rich FAA profile.

Fermentation trials

To investigate the suitability of the alternative substrates as adjuncts for ethanol reduction, they were fermented with three different yeast strains. Two non-*Saccharomyces* yeast strains, namely *Cyberlindnera subsufficiens* C6.1 and *Lachancea fermentati* KBI 12.1, were compared to the commercial *Saccharomyces cerevisiae* ale yeast strain WLP001. The analysis results for the fermented worts are shown in Table 5. The general observation in terms of apparent degree of fermentation (ADF) was that, with few exceptions, the ADF was highest for *S. cerevisiae* WLP001, followed by *L. fermentati* KBI 12.1, and lowest for *C. subsufficiens* C6.1.

Cyberlindnera subsufficiens C6.1 showed the lowest ADF and ethanol values in spelt wort, at 6% and 0.11% ABV, respectively. In the residual cereal worts, ethanol concentrations were between 0.24 and 0.49% ABV. In barley wort, *C. subsufficiens* C6.1 exhibited an ADF of 16% and produced 0.49% ABV. The highest ADF and ethanol values were observed in quinoa wort at 22% and 0.66% ABV, respectively (Table 5). It was closely followed by buckwheat wort with 22% and 0.56%ABV, respectively. In the worts from the pulses, ethanol concentrations reached 0.12–0.22% ABV. The high cell counts of *C. subsufficiens* C6.1, observed in pea and lupin wort, seemed to correlate with the high FAN content of these worts. Consequently, C6.1 exhibited the highest cell concentrations in the pea and lupine worts, at 6.2 and 7.1×10^7 cells/mL, respectively. Differences in the cell counts in the remaining worts showed no statistical significance due to a high standard deviation. The final pH values of the worts fermented with *C. subsufficiens* C6.1 were relatively high, at 4.4–5.4. The highest pH drop, resulting in the lowest reported final pH of the worts fermented with *C. subsufficiens* C6.1, was observed for millet and buckwheat, with a final pH of 4.4. *C. subsufficiens* C6.1 showed an average FAN consumption of approximately 20% of the total FAN content of the respective worts.

In the worts fermented with *Lachancea fermentati* KBI 12.1, the highest ethanol concentration was found in the fermented barley wort at 3.0% ABV, followed by wheat, at 2.5% ABV, and oat, at 1.9% ABV. In contrast to *C. subsufficiens* C6.1, *L. fermentati* KBI 12.1 is able to utilize maltose [12]. However, maltotriose is not utilized by the yeast strain [12]. The ADF and ethanol concentration for wheat was lower compared to that of barley due to an incomplete maltose utilization (data not shown). The fermented spelt wort and rye wort showed ethanol concentrations of 1.6% ABV and 1.4% ABV, respectively. Additionally, *L. fermentati* KBI 12.1 exhibited the lowest cell concentration in rye wort, at 3.5×10^7 cells/mL. The worts from the pseudocereals amaranth, buckwheat, and quinoa exhibited ethanol concentrations between 0.7 and 0.8% ABV. The fermented corn and millet worts and the fermented worts from pulses exhibited ethanol concentrations between 0.2 and 0.6% ABV, with lupine exhibiting the lowest ethanol concentration, at 0.2% ABV, however, without statistical significance. Lactic acid production by *L. fermentati* KBI 12.1 varied greatly between the substrates (Fig. 5). The highest lactic acid concentration was found in the fermented barley wort, at 1.23 g/L. No lactic acid was found in the fermented worts from millet and lentil. Although the extract content and composition of fermentable sugars of the wheat wort was comparable to that of the barley wort, significantly less lactic acid was produced, at only 0.34 g/L. The same pattern of having similar composition of fermentable sugars and free amino acids while exhibiting low lactic acid production was observed for the fermented rye wort, at a concentration of only 0.20 g/L. However, the cell count in the rye wort was also significantly lower (Table 5). Lactic acid production in relation to the consumed fermentable sugars showed a comparably high performance of KBI 12.1 in the barley and amaranth worts (Fig. 5). Conversely, in the wheat, rye, spelt, buckwheat and quinoa worts, KBI 12.1 showed a significantly poorer performance. Differences in the performance in terms of lactic acid production in relation to extract reduction were not statistically significant for the corn, oat, lupine, and pea worts. Cell counts in the fermented wort showed no correlation with lactic acid or ethanol production. The highest pH drops were observed for barley, amaranth, and oat wort in correspondence with the lactic acid production in those worts. Consequently, the fermented barley wort exhibited the lowest pH, at 3.54, followed by oat, at 3.79. The high initial pH of the fermentation in amaranth wort (pH 6.61) led to a final pH of 4.40, despite the extensive pH drop. The worts from pea, lentil, and lupine showed only marginal pH drop and exhibited high final pH values of 5.22–5.33. The remaining worts showed final pH values between 4.04 and 4.54 (Table 5). The average FAN consumption of *L. fermentati* KBI 12.1 during the fermentation of the worts from cereals, pseudocereals, and pulses was 50%, 26%, and 11%, respectively.

The brewers' yeast strain *Saccharomyces cerevisiae* WLP001 was used in this study as a control strain to demonstrate the performance of a commercial brewers' yeast in

Table 5 Results of analyses of worts fermented with the respective yeast strains

Wort substrate	*Cyberlindnera subsufficiens* C6.1						*Lachancea fermentati* KBI 12.1						*Saccharomyces cerevisiae* WLP001					
	E_r (°P)	ADF (%)	Ethanol (% ABV)	Cell count (Mio. cells/mL)	pH	FAN (mg/L)	E_r (°P)	ADF (%)	Ethanol (% ABV)	Cell count (Mio. cells/mL)	pH	FAN (mg/L)	E_r (°P)	ADF (%)	Ethanol (% ABV)	Cell count (Mio. cells/mL)	pH	FAN (mg/L)
Barley[m]	7.14^{fg}	16^{ef}	0.49^{fg}	34^{ab}	4.71^{d}	104^{e}	3.22^{b}	74^{g}	3.02^{g}	150^{d}	3.54^{a}	58^{d}	2.98^{b}	78^{e}	3.33^{g}	67^{d}	4.17^{bc}	43^{f}
Wheat[m]	7.46^{gh}	15^{de}	0.39^{e}	33^{ab}	4.68^{d}	93^{de}	4.44^{c}	58^{ef}	2.46^{f}	105^{bcd}	4.05^{c}	47^{cd}	3.49^{e}	73^{e}	3.24^{g}	46^{bcd}	4.04^{b}	36^{def}
Rye[m]	7.80^{h}	14^{de}	0.39^{e}	38^{ab}	5.05^{f}	83^{cd}	6.14^{e}	37^{cd}	1.38^{d}	35^{a}	4.42^{de}	47^{cd}	4.86^{ef}	56^{d}	2.56^{f}	30^{abc}	4.36^{d}	34^{def}
Oat[m]	5.06^{b}	17^{ef}	0.24^{bc}	36^{ab}	5.06^{cd}	67^{bc}	2.68^{ab}	67^{fg}	1.94^{e}	140^{d}	3.79^{b}	40^{bcd}	1.79^{a}	86^{f}	2.53^{f}	53^{cd}	3.84^{a}	22^{bc}
Spelt[s]	6.82^{ef}	13^{cd}	0.27^{cd}	27^{a}	4.55^{bc}	19^{a}	6.14^{e}	24^{ab}	0.63^{abc}	91^{abcd}	4.09^{c}	14^{a}	5.78^{hi}	30^{b}	1.15^{de}	25^{ab}	4.09^{b}	8^{a}
Millet[s]	6.13^{d}	14^{de}	0.31^{d}	35^{ab}	4.42^{ab}	30^{a}	5.62^{e}	23^{ab}	0.45^{abc}	101^{abcd}	4.54^{e}	26^{ab}	5.27^{fg}	30^{b}	1.01^{cd}	42^{abcd}	4.16^{bc}	14^{ab}
Corn[s]	7.50^{gh}	6^{a}	0.11^{a}	27^{a}	5.19^{g}	59^{b}	4.71^{cd}	50^{de}	1.55^{de}	145^{d}	4.23^{cd}	34^{bc}	4.20^{d}	58^{d}	2.50^{f}	32^{abc}	4.09^{b}	27^{cd}
Amaranth[s]	6.14^{d}	19^{fg}	0.48^{f}	32^{ab}	4.89^{e}	91^{de}	5.39^{de}	32^{bc}	0.70^{bc}	57^{ab}	4.40^{de}	97^{e}	6.20^{i}	19^{a}	0.59^{ab}	20^{a}	5.21^{e}	107^{h}
Buckwheat[m]	5.37^{b}	22^{gh}	0.56^{g}	41^{ab}	4.40^{a}	64^{b}	4.78^{cd}	33^{bc}	0.73^{bc}	70^{ab}	4.41^{de}	56^{d}	4.56^{de}	38^{c}	1.15^{de}	33^{abc}	4.33^{cd}	41^{ef}
Quinoa[s]	6.53^{e}	22^{h}	0.66^{h}	40^{ab}	4.52^{abc}	57^{b}	5.68^{e}	34^{bc}	0.81^{c}	64^{ab}	4.32^{de}	42^{bcd}	5.50^{gh}	39^{c}	1.48^{e}	44^{abcd}	4.41^{d}	30^{cde}
Lentil[s]	2.17^{a}	21^{gh}	0.22^{bc}	71^{c}	5.34^{h}	143^{f}	2.08^{a}	25^{abc}	0.19^{a}	62^{ab}	5.33^{f}	140^{f}	2.11^{a}	26^{b}	0.31^{a}	27^{ab}	5.37^{e}	138^{i}
Lupine[s]	5.87^{cd}	10^{bc}	0.18^{ab}	62^{c}	5.42^{h}	85^{cd}	5.50^{de}	18^{a}	0.35^{abc}	75^{abc}	5.22^{f}	111^{e}	4.90^{ef}	30^{b}	0.96^{cd}	38^{abc}	5.37^{e}	94^{g}
Pea[s]	5.76^{c}	9^{ab}	0.12^{a}	52^{bc}	5.38^{h}	150^{f}	5.31^{de}	18^{a}	0.30^{ab}	78^{abc}	5.22^{f}	169^{g}	4.98^{ef}	25^{ab}	0.68^{bc}	29^{abc}	5.35^{e}	165^{j}

E_r real extract, *ADF* apparent degree of fermentation. [m]Malt. [s]Sprouts. Each value is the mean of three replicate measurements. Different superscripts of values within a column indicate a significant difference ($p \leq 0.05$)

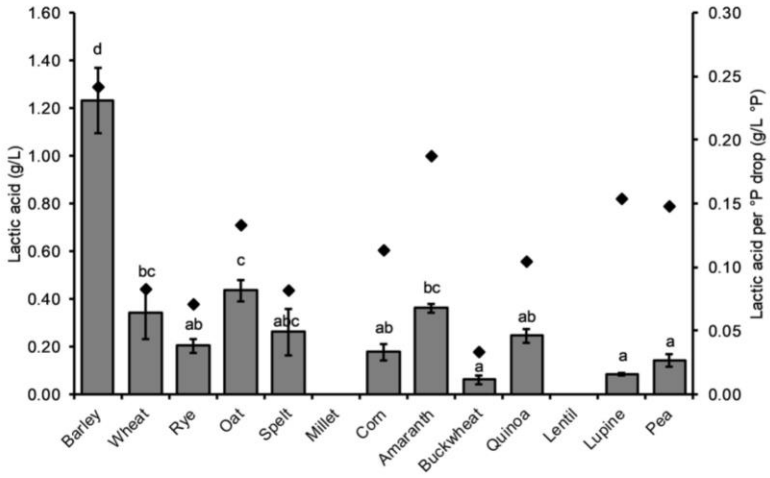


Fig. 5 Final lactic acid concentrations in worts fermented with *Lachancea fermentati* KBI 12.1 (bars) and lactic acid per °P extract drop (filled diamond). Different letters above bars indicate a significant difference in final lactic acid concentration ($p \leq 0.05$)

comparison to the two non-conventional yeasts. *S. cerevisiae* WLP001 generally exhibited the highest ADF and final ethanol concentrations amongst the yeast strains due to its ability to utilize maltotriose (Table 5). However, the amaranth wort exhibited a significantly low ADF of 19% and correspondingly low ethanol concentration of 0.6% ABV. In correlation to this, the cell count in the amaranth wort was, at 2×10^7 cells/mL, the lowest of all fermentations. Sugar analysis revealed a maltotriose and maltose consumption of about 20% (data not shown). The highest ADF (86%) was observed in oat wort due to the relatively high proportion of fermentable to unfermentable sugars (Fig. 3). The highest ethanol concentrations were found in the fermented barley and wheat worts, at 3.3% ABV and 3.2% ABV, respectively. The lowest ethanol concentration was found in the fermented lupine wort, at 0.3% ABV. Cell counts in the fermented worts were in the range of 2.0–6.7 × 10^7 cells/mL. The highest cell count was observed in barley wort at 6.7×10^7 cells/mL. In the worts from the cereals, WLP001 exhibited a pH drop of around 1.3–1.6 with oat showing the lowest final pH, at 3.8. The average FAN consumption of *S. cerevisiae* WLP001 during the fermentation of the worts from cereals, pseudocereals, and pulses was 67%, 35%, and 17%, respectively. The lowest pH drops and highest final pH values were observed in the worts from pulses, a pattern that could be observed amongst all strains due to the limited fermentation and potentially high amount of buffering substances in those worts.

Discussion

Alternative substrates have been investigated in brewing research for a long time in the search for a gluten-free alternative to barley-based and wheat-based beers, as well as to enable brewing with local ingredients for cost reduction or for the development of specialty beers [2]. In light of recent trends, research into innovative methods to produce non-alcoholic and low alcohol beers (NABLAB) has gained momentum [1]. Alteration of the sugar profile of the wort is a means to produce lower alcohol beers which is usually achieved by an alteration of the mashing regime [36]. The main principle is to shift the proportion of saccharification during mashing from fermentable sugars to unfermentable sugars (higher dextrins). In this study, it was possible to create worts with low amounts of fermentable sugars and high amounts of unfermentable extract due to the application of alternative substrates and the choice of external α-amylase. It can be assumed that the largest part of the unfermentable extract for the cereal, pseudocereal grains, and pulses treated with Hitempase STXL were dextrins, since the grains exhibited high starch contents which were liquified to dextrins during mashing (Table 2). Additionally, soluble fibre contents are usually low in cereals, pseudocereals, and pulses [22, 23, 27, 37–39].

Concerning FAA, the barley and wheat worts particularly showed a balanced profile. Many FAA such as lysine, glutamine and methionine were available in significantly high amounts in the barley and wheat worts as opposed to low concentrations in the worts from alternative substrates (Table 4). Procopio et al. [40] investigated amino acid uptake in lager and ale yeast strains and found that methionine, asparagine, glutamine, and lysine were taken up and depleted quickly during fermentation. However, they stated that the amino acid uptake by the lager and ale yeast strains did not follow a defined course, highlighting the existence of interspecific and intergeneric differences which limit the transferability of those findings to other yeast genera [40].

The use of pulses proved impractical owing to long filtration times and/or low extract yields (Table 3). Additionally, the worts produced exhibited an unpleasant taste which was still noticeable after fermentation. The use of high amounts of rye malt is impractical due to its high amount of pentosans that lead to long filtration times and a very viscous wort, which was also observed in this study (Table 3) [41]. The observed high viscosity of the rye wort was likely connected to the poor ADF and low cell counts in the subsequent fermentation trials (Table 5).

Endogenous β-amylase activity in barley, rye, oat, wheat, and spelt led to a high maltose production and thus a high amount of fermentable sugars. Consequently, high final ethanol concentrations were reached in those worts when fermented with *L. fermentati* KBI 12.1 or *S. cerevisiae* WLP001 (Table 5). The ethanol yield with *C. subsufficiens* C6.1 was not influenced by maltose availability due to its inability to utilize maltose. The worts from the substrates without endogenous β-amylase activity indeed showed low amounts of fermentable sugars, and thus exhibited low ethanol concentrations after fermentation with the respective yeasts.

In cereal-based substrates, the most abundant sugars are maltose and maltotriose. Most non-*Saccharomyces* yeasts are unable to ferment maltose and/or maltotriose, since they are not adapted to cereal environments. *C. subsufficiens* C6.1 is unable to ferment maltose and maltotriose which is common for the species [42]. *L. fermentati* KBI 12.1 is unable to ferment maltotriose, while the commercial brewers' yeast *Saccharomyces cerevisiae* WLP001 is able to ferment both maltose and maltotriose [12, 16]. These metabolic differences were the reason for the large variance in terms of ADF and final ethanol concentrations amongst the individual yeast strains.

Yeast strains of the *Cyberlindnera* genus have been reported to produce high concentrations of acetate esters, in particular isoamyl acetate [9, 10]. In screenings in wort extract, Van Rijswijck et al. [43] reported that 9 *Cyberlindnera fabianii* isolates produced a higher volatile ester to volatile alcohols ratio compared to 16 wild *Saccharomyces cerevisiae* isolates. Liu et al. [11] investigated the application of *Cyberlindnera mrakii* (formerly *Williopsis saturnus* var. *mrakii*) strain NCYC 500 to produce a fruity beer in a 13.8°P barley malt wort with added glucose. The concentrations of isoamyl acetate detected in the beer fermented with *C. mrakii* were approximately 20 times higher than in those fermented with Safale US-05. The final ethanol content was at 1.7% ABV lower compared to that of the *Saccharomyces cerevisiae* brewers' yeast (Safale US-05), at 6.9% ABV, due to the yeasts' inability to consume maltose. Strain *Cyberlindnera subsufficiens* C6.1 was included in this study as a maltose-negative strain that showed low ethanol production in pre-trials in wort, while developing a very fruity character. For *Cyberlindnera subsufficiens* C6.1, the degree of fermentation was dependent on the concentration of monosaccharides in the wort. Due to the low monosaccharide content of the spelt wort, C6.1 showed the lowest ADF and ethanol values. In the worts from the pulses, the low ethanol concentrations could also be explained by the low concentrations of monosaccharides (Fig. 3). Correspondingly, due to the high glucose content in the worts from the pseudocereals, the highest ADF and ethanol values were observed, in quinoa and buckwheat wort. These results show how crucial the sugar spectrum of the wort is regarding ethanol production when using a maltose-negative yeast. The sugar spectrum is very important when considering the use of alternative grains or adjuncts, since for most non-*Saccharomyces* yeasts only monosaccharides, and sometimes sucrose, contribute to the fermentable extract. The high final pH values of the worts and the low pH drops caused by the limited fermentation must be considered. A rapid pH drop and low final pH are important to avoid microbial spoilage, especially at the beginning of fermentation. The final pH values of the worts fermented with *C. subsufficiens* C6.1 barely crossed the hurdle of pH 4.5, known to be necessary to limit microbial spoilage [44]. A pH adjustment of the wort before fermentation, i.e. with lactic acid, the use of acid malt, or biological acidification, is therefore advisable. This is also particularly important since the low ADF leaves residual fermentable sugars which makes pasteurization of the finished beer essential. The low average FAN consumption of *Cyberlindnera subsufficiens* C6.1 of only approximately 20% was in accordance with previous studies where non-*Saccharomyces* yeasts consumed relatively low amounts of wort FAN due to limited fermentation [16, 45].

Lachancea fermentati strain KBI 12.1 has recently been investigated for its potential for low alcohol beer brewing due to its uncommon trait of producing significant amounts of lactic acid during alcoholic fermentation [12]. In the study by Bellut et al. [12], the strain produced 1.30 g/L lactic acid in a 6.6°P wort from barley wort extract. In the current study, *L. fermentati* KBI 12.1 was included to investigate its performance in terms of ethanol and lactic acid production in alternative substrates. In accordance with the previous reported lactic acid value, in this study, *L. fermentati* produced 1.23 g/L lactic acid in 8.3°P barley malt wort. However, the different results of lactic acid production in barley wort compared to the poor performance in wheat wort underline the, to date, poor understanding of the factors influencing lactic acid production by *Lachancea fermentati*. Although the worts were very similar in extract, sugar composition and, to an extent, amino acid profile, lactic acid concentrations were significantly different (over three times higher in barley wort) (Fig. 5). In addition, no lactic acid was found in the worts from millet and lentil, although those worts did not exhibit extreme values in terms

of fermentable sugars or nitrogen sources (Table 3; Figs. 3 and 4). Future research should investigate the factors influencing the production of lactic acid by *Lachancea fermentati* to further understand this metabolic trait. As reported by Bellut et al. [12], *Lachancea fermentati* KBI 12.1 is unable to ferment maltotriose. The lower ADF and correspondingly lower ethanol production compared to the brewers' yeast WLP001 was attributed to this characteristic. In their previous study, Bellut et al. [12] suggested the use of *Lachancea fermentati* KBI 12.1 for low alcohol beer brewing. As the results of this study indicate, due to a lower performance in ADF and ethanol production in all substrates other than barley, a partial substitution of the cereals used could benefit a lower ethanol production. However, in this particular case, a blending of the worts after mashing is advisable to retain the high amount of dextrins and low amount of fermentable sugars in the wort from an alternative substrate. The high β-amylase activity from malted barley would otherwise lead to the breakdown of the dextrins to fermentable maltose. However, lactic acid production was only high in barley and much lower in all other substrates. This fact has to be considered in the proposed scenario of the use of lactic acid to counteract the often criticized residual sweetness of NABLAB produced by limited fermentation [12, 46, 47]. Substituting parts of the barley malt on the grain bill could be a means to adjust lactic acid production and ethanol production by *L. fermentati* KBI 12.1 and would be worth investigating further. The lowest pH drops, and highest final pH values, were observed for the worts from pulses, a pattern that was observed amongst all strains. Peyer et al. [48] showed a linear correlation between the FAN content of wort and its buffering capacity which may have contributed to this finding aside the obvious reason of a limited fermentation.

Conclusion

Starch-negative worts from all the investigated cereals, pseudocereals, and pulses were successfully created by harnessing endogenous enzyme activity or through the addition of an external amylase. The worts from barley, oat, wheat, and rye exhibited high amounts of fermentable sugars, solely based on the activity of endogenous enzymes. The remaining grains and pulses were liquified by the addition of Hitempase STXL. Due to the characteristic of this enzyme to yield mostly dextrins, the resultant worts showed high proportions of unfermentable extract. The maltose concentration in the worts was solely dependent on endogenous β-amylase activity of the grains. Lupine proved unsuitable as an alternative substrate due to a significantly low extract yield, owing to the low starch content. The pea, lentil, and rye mashes proved to be impractical due to very long filtration times. ADF and ethanol concentrations in subsequent fermentation trials with non-*Saccharomyces* yeasts depended mostly on the sugar spectrum of the respective worts. Interestingly, the pseudocereals showed significantly high glucose concentrations, thus yielding the highest ethanol concentrations in fermentations with the maltose-negative yeast *Cyberlindnera subsufficiens* C6.1. The variation in lactic acid production by *Lachancea fermentati* KBI 12.1 could not be explained by the composition of the sugar or nitrogen sources of the respective worts and requires further research. Generally, the sugar spectrum of the wort proved to be the most important factor influencing the final ethanol concentration after fermentation with the respective yeasts. For the production of NABLAB by non-*Saccharomyces* yeast, the (partial) substitution of barley with alternative substrates can be a means to alter the sugar and amino acid profile of the wort, but must be considered in concert with the individual characteristics of the yeast strain.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Compliance with ethics requirements This article does not contain any studies with animal or human subjects.

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