Norwegian University
of Life Sciences

Master's Thesis 2023 60 ECTS
Faculty of Chemistry, Biotechnology and Food Science

Use of oligosaccharides from food side streams towards sour beer production

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Acknowledgements

The research and experiments for this master's thesis were conducted at the Faculty of Chemistry, Biotechnology and Food Science at the Norwegian University of Life Sciences (NMBU). This study was funded by the project "Green technology for plant-based food".

I was lucky enough to get to work on the topic of brewing and fermentation, a subject that on top of being complex and interesting, is close to my heart and deeply rooted in Norwegian tradition. Throughout the thesis I've been introduced to a broad specter of methods and analysis techniques expanding my knowledge and giving me valuable experience that I will take with me into the next phase of my career.

I would like to extend my gratitude towards Bjørge Westereng, my main supervisor, and the BioRef group for finding a way to incorporate my passion for brewing into the work performed by the group. Thank you Bjørge for the guidance, help, and the pigs. A big thank you also goes out to Philipp Garbers, who close to everyday was there to help me out, guide, criticize and share my enthusiasm towards beer and brewing. You can not underestimate my appreciation.

For sharing the frustration, tears, and headache I would like to thank fellow master student Aksel Vardeberg Skeie, but also for the good discussions, joy, and fun times we had throughout the process. Thank you to Lars Fredrik Moen for all the practical support, Hilde Østlie for providing bacterial stocks, and Kari Olsen for running analysis.

Finally, thank you to my brother Jonas for proofreading and being my only connection to the outside world close to the deadline, and thank you to my parents for providing support and being patient with me on this long journey of mine through higher education.

Norwegian University of Life Sciences Faculty of Chemistry, Biotechnology and Food Science

Ås, May 2023

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Abstract

This master's thesis explores the potential of Raffinose family oligosaccharides (RFO) from food side streams as an adjunct in sour beer production. The study investigates the effects of RFO on mixed fermentation with different bacterial strains and their enzymatic ability to hydrolyze oligosaccharides, the effect on yeast metabolism, and the overall flavor profile. A series of experiments were conducted using RFO enhanced wort as the base substrate, co-fermenting with various lactic acid producing bacterial strains, and yeasts. The fermentation process was monitored for sugar utilization, attenuation, organic acid production, pH, and volatile compound production. Tetrad analysis was performed to see if there was any discernable difference when adding RFOs. The study evaluated the different metabolic activities of a total of 10 bacterial strains and 4 yeasts. Their ability to metabolize RFO was assessed by monitoring their growth curves, as well as by a variety of chemical analysis techniques, at time intervals of 7, 14, and 19 days with the aim of producing sour beer within the normal time frame of traditional non-sour beer. The results suggest that the use of RFO as substrate in the production of beer is contributing beneficially towards the overall flavor and fermentation profile. It led to an increase in ethanol concentration in beer fermented with *Saccharomyces cerevisiae*, demonstrating that at least a proportion of the RFO extract can be fermented by brewer's yeast. However, the impact of RFOs on mixed fermentation, including the utilization of the oligosaccharide constituents, showed mixed results. Multiple strains demonstrated the ability to ferment individual constituents of RFO, but in co-fermentations, the fermentation conditions appeared to primarily promote organic acid production, rapidly lowering the wort's pH, inducing stress to the yeast, and affecting the production of metabolites, especially aldehydes and esters. The sensory evaluation of the experimental beer fermented with RFOs displayed a discernible differentiation from the beer fermented without RFOs, without increasing unwanted off-flavor associated compounds. This demonstrates that incorporating RFOs as an adjunct in the production of sour beers has promising potential. In conclusion, the incorporation of RFOs as a substrate in sour beer fermentation holds promise, with a discernable difference in organic compounds produced, but a more comprehensive understanding of the process and optimization of fermentation conditions are crucial to achieving consistent and desirable outcomes, as well as to quantify the enzymatic ability to hydrolyze the constituents of RFO.

Sammendrag

Denne oppgaven utforsker potensialet til Raffinose-familie oligosakkarider (RFO) fra sidestrømmer av matproduksjon som en tilsetning i produksjonen av surøl. Studien undersøker effekten RFO har på blandede fermenteringer og mikrobenes enzymatiske evne til å bryte ned oligosakkarider, hvilke effekter det har på gjærens metabolisme, og hva det har å si for den endelige smaksprofilen. Eksperimenter ble utført ved å bruke vørter tilsatt RFO i ko-fermenteringer med ulike melkesyrebakterier og gjær. Fermenteringsprosessene ble overvåket med fokus på utnyttingsevne av sukker, grad av utgjæring, produksjon av organiske syrer, pH, og produksjon av flyktige stoffer.

Studien evaluerte de metabolske aktivitetene til totalt 10 bakteriestammer og 4 gjær. Evnen deres til å metabolisere RFO ble vurdert ved å overvåke vekstkurvene deres, samt ved hjelp av ulike kromatografiske analyser. Målet var å produsere surøl innenfor de samme tidsrammene som vanlig ølproduksjon, så en tidsramme var satt på 19 dager.

Resultatene antyder at tilsetning av RFO har potensiale til å bidra til karakteristikker som hever den overordnede smaken og fermenteringsprofilen til ølet. Tilsetting av RFO førte til en økning i etanolkonsentrasjon når ølet ble gjæret med *Saccharomyces cerevisiae*, som demonstrerer at en bestanddel av RFO kan fermenteres av bryggegjær. Derimot var påvirkningen av RFO på blandende fermenteringer mer tvetydig, inkludert utnyttelsen av de individuelle oligosakkaride bestanddelene.

Sensorisk analyse av øl gjæret med RFO viste en tydelig forskjell sammenlignet med øl gjæret uten RFO. Resultatet var en mer kompleks syreprofil uten å ta bort de velsmakende karakteristikkene ved surøl. Dette viser at å inkludere RFO som en tilsetning i produksjonen av surøl har lovende potensial.

Konklusjonen er at inkludering av RFO som en substrat i surølgjæring har et lovende potensiale, med en tydelig forskjell i organiske forbindelser produsert, men en mer omfattende forståelse av prosessen og optimalisering av gjæringsbetingelsene er avgjørende for å oppnå konsekvente resultater, samt å kvantifisere den enzymatiske evnen mikrobene har til å hydrolysere RFO-komponentene.

Abbreviations

AAB	-	Acetic Acid Bacteria
AAS	-	Alcolyzer Analyzer System
ABV	-	Alcohol By Volume
ADF	-	Apparent Degree of Fermentation
ADY	-	Active Dry Yeast
ATT	-	Alcohol Acetyltransferase
BBB	-	*Brettanomyces claussenii*, *Lactobacillus brevis* and *Lactobacillus buchneri*
BLB	-	*Brettanomyces claussenii*, *Lactococcus cremoris* and *Lactobacillus brevis*
CFU	-	Colony Forming Unit
DAD	-	Diode Array Detector
DB	-	Dry Basis
DHB	-	2,5-Dihydroxybenzoic Acid
DMS	-	Dimethyl Sulfide
DO	-	Dissolved Oxygen
DOA	-	Dissolved Oxygen Analyzer
DW	-	Dry Weight
EBC	-	European Brewing Convention
EPS	-	Exopolysaccharide
FAN	-	Free Amino Nitrogen
FG	-	Final Gravity
GH	-	Glycoside Hydrolase Family
GOPOD	-	Glucose Oxidase/Peroxidase
GRAS	-	Generally Regarded As Safe
HPAEC-PAD	-	High-Performance Anion-Exchange Chromatography Pulsed Amperometric Detection
HPLC	-	High-Performance Liquid Chromatography

HSGC	-	Headspace Gas Chromatography
IBU	-	International Bitterness Unit
KBM	-	Faculty of Chemistry, Biotechnology, and Food Science
KEGG	-	Kyoto Encyclopedia of Genes and Genomes
KLB	-	*Saccharomyces cerevisiae* Lutra Kveik, *Lactococcus cremoris* and *Lactobacillus brevis*
LAB	-	Lactic Acid Bacteria
MALDI-ToF MS	-	Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
MEBAK	-	Methodensammlung der Mitteleuropäischen Brautechnischen Analysekommission
MRS	-	De Man, Rogosa and Sharpe
NAD	-	Nicotinamide Adenine Dinucletide
NMBU	-	Norwegian University of Life Science
OD	-	Optical Density
OG	-	Original Gravity
PFD	-	Piercing and Filling Device
PMF	-	Proton Motive Force
RI	-	Reflective Index
SB	-	*Saccharomyces cerevisiae* US-05 and *Bifidobacterium animalis subsp. lactis*
SG	-	Specific Gravity
SLB	-	*Saccharomyces cerevisiae*, *Lachancea thermotolerans* and *Lactobacillus buchneri*
SMM	-	S-Methyl-Methionine
SPT	-	Spread Plate Technique
TB	-	*Lachancea thermotolerans* and *Lactobacillus buchneri*
UV	-	Ultraviolet
VDK	-	Vicinal Diketones
VLB	-	Research and Teaching Institute for Breweries
YEP	-	Yeast Extract Peptone

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1 Introduction

1.1 Background

Prior to the advent of modern sanitation practices, beer was often contaminated with wild yeast and bacteria that caused it to sour. While this was considered a flaw in beer for many centuries, it eventually became a popular style in certain regions. It has become a unique and increasingly popular style of beer that is characterized by its tart and tangy flavor profile. While sour beer is a modern term, the production of sour beers can be traced back to ancient times when beer was fermented spontaneously with wild yeast and bacteria.

Yeast, along with malt, water, and hops, are a requisite component in the production of beer. Its principal function is to convert the sugars derived from cereals into ethanol and carbon dioxide, but the yeast's metabolic by-products are also critical in creating the unique sensory characteristics that define the final product. Historical analyses of beer and brewing suggest that the precursor to modern beer can be traced back to cereal-based beverages created in ancient Egypt and Mesopotamia around 6.000 BCE (Hornsey, 2003; McGovern, 2009). It is not unlikely that these tasted something akin to what a spontaneously fermented sour beer would taste like today.

The fermentation process of most contemporary beers is predicated upon a monocultural technique that employs either *Saccharomyces cerevisiae* or *Saccharomyces pastorianus*. In contrast, the fermentation and/or maturation of sour beers is dictated by non-traditional yeasts belonging to the indigenous non-starter populace or uncharacterized mixed starter cultures that comprise lactic acid bacteria (LAB) (Martens et al., 1997; Verachtert and Derdelinckx, 2005). These beers represent culturally significant alcoholic beverages, and their production and quality are contingent upon the intricate microbial communities that inhabit them. Recently, with the advent of the craft beer movement, these fermented beverages with their unique flavors have gained widespread recognition and popularity across the globe (Snauwaert et al., 2016). According to data by Vinmonopolet A/S, there has been a notable upward trajectory in the sales of sour beer in Norway. In 2021, the company reported a 3% increase in sales, totaling 212,000 liters (Vinmonopolet, 2023) This observed growth aligns with the broader consumer trend towards beverages with lower alcohol content and reduced-calorie profiles, as sour beers often have lower alcohol percentages and are by their

nature dry due to the over-attenuation performed by non-traditional yeasts and bacteria. Some sour beer produces however back sweeten their beers to mellow out the acidity.

Acquiring a deeper understanding of the microbiota that drive the sour beer fermentation processes is not only crucial for facilitating better quality management, but also enables the isolation of microorganisms that can be utilized as starter cultures. It is notable that, in many cases, the same microorganisms are repeatedly involved in these processes. Specifically, yeasts such as *S. cerevisiae* and *S. pastorianus*, along with *Brettanomyces* spp., notably *Brettanomyces bruxellensis*, as well as LAB and/or acetic acid bacteria (AAB), are commonly found to be key components of the microbiota involved in the sour beer fermentation process.

Side streams from the food industry make up renewable feedstocks that have the potential for utilization in a circular economy, particularly to produce bio-based chemicals and polymers (Ladakis, 2020). The industrial processing of plant-derived raw materials yields substantial quantities of by-products. These byproducts, while presenting a substantial disposal challenge due to seasonal growth and susceptibility to microbial decay, represent a potential abundant source of valuable compounds (Schieber, 2017).

These by-products are especially rich in secondary plant metabolites and cell wall materials, which can be extracted and used to enhance the functionality of food products and serve as natural alternatives to synthetic additives (Schieber, 2017). This means that the effective recovery and utilization of these valuable compounds not only can possibly contribute to a more sustainable food production, but also attribute to minimize environmental impact and the accumulation of waste associated with traditional food industry. With the integration of food industry side streams in sour beer production, it can potentially lead to the development of innovative, environmentally friendly, and resource efficient brewing techniques that align with the principles of the circular economy.

1.2 The brewing process

1.2.1 A quick overview of the brewing process

The characteristics, potency, and ingredients used in beer production have evolved significantly throughout history and vary across different cultures and communities worldwide. In contemporary times, most of the beer produced globally is crafted using malted barley (*Hordeum vulgare*), hops (*Humulus lupulus*), a cultivated strain of yeast (*S. cerevisiae* or *S. pastorianus*), and water.

In the production of beer, the process begins with the milling of malt, typically derived from barley. The roughly milled malt is called the grist, which is subsequently combined with heated water during a phase known as mashing. Throughout the mashing process, enzymes (predominantly α-amylase and β-amylase) catalyze the breakdown of starches into fermentable sugars. Both enzymes belong to the glycoside hydrolase family and target the α-1,4-glucosidic linkages found in starch molecules. However, they exhibit distinct modes of action and generate different end products (Briggs et al., 2004).

α-amylase (GH13): This enzyme is an endo-acting enzyme, meaning it cleaves the α-1,4-glucosidic linkages within the starch molecule at random locations. This amylase is highly active at elevated temperatures and functions optimally within the temperature range of 70-75°C (Briggs et al., 2004).

β-amylase (GH14): This enzyme is an exo-acting enzyme that specifically cleaves α-1,4-glucosidic bonds at the non-reducing end of the starch molecule, releasing maltose units in a stepwise manner. β-amylase has a lower optimal temperature range compared to α-amylase, being most efficient at temperatures between 60-65°C (Briggs et al., 2004).

Their combined action ensures the efficient conversion of starch into the necessary substrates for subsequent fermentation by yeast.

After the mashing phase, the insoluble fraction is isolated from the sacchariferous solution, denoted as wort, through a technique known as lautering. Concurrently, the spent grains undergo a process called sparging, which entails the rinsing of the grain bed with warm water to extract additional sugars, flavors and color following the mashing and lautering.

Subsequently, the wort undergoes boiling in the presence of hops, which isomerizes the α-acids (mainly humulone), after which it is cooled and inoculated with yeast. Both *S. pastorianus* and *S. cerevisiae* are referred to as brewer's yeast, and monoculture strains are predominantly used in beer fermentations. During the fermentation process, yeast consumes sugars, amino acids, and other nutrients in the wort, producing ethanol, carbon dioxide, higher alcohols, esters, and other metabolites in the resulting beer (Pires and Brányik, 2015).

The introduction of yeast cells, also known as the pitching rate, is one of the three crucial control variables in the fermentation process utilized in brewing. The other two variables being the provision of oxygen and the regulation of temperature. The efficacy of the

fermentation process, in terms of the production of ethanol relative to the growth of new biomass, can be optimized through the maintenance of a high pitching rate and the sufficient availability of oxygen, as demonstrated by Quain (2017). Several studies have investigated the effects of increasing the pitching rate on the fermentation process, including the works of Edelen et al. (1996), Erten et al. (2007), and Verbelen et al. (2009). As expected, an increase in the pitching rate leads to a faster fermentation process, with a higher and more rapid peak yeast cell count.

Underpitching can lead to a suboptimal fermentation outcome, the extent of which will vary based on the magnitude of the issue. This phenomenon is often the result of a lack of understanding regarding the role of dead yeast in pitching, leading to an increase in cell autolysis and undesirable flavor and aroma profiles (Quain, 2017).

Adaption to the acidity encountered in beer is critical for microorganisms, as low pH can impede enzymatic reactions, protein folding, and other intracellular processes in non-pH tolerant organisms. LAB and other pH-resilient microorganisms possess the ability to regulate their intracellular pH in response to acidic conditions. Mechanisms for such regulation include proton transport across the cellular membrane or the utilization of proton-translocating ATP synthase (de Angelis and Gobetti, 2011).

1.2.1 Brewing water

1.2.1.1 Ions

The mineral composition of brewing water plays a crucial role in determining the final characteristics of beer produced. Six primary ions influence the brewing process: calcium (Ca^{2+}), magnesium (Mg^{2+}), total alkalinity (as $CaCO_3$), sulphate (SO_4^{2-}), chloride (Cl^-), and sodium (Na^+). The mineral profile of the water is determined by its source (Palmer and Kaminski, 2013).

The effects of ions in the brewing water, and their effect on the final beer is described by Palmer (2017) and Palmer and Kaminski (2013). Calcium is the most important ion in the brewing process, acting as a cofactor in numerous biochemical reactions occurring in both the mash and fermentation stages. This ion stabilizes α-amylase under high temperatures and pH levels during the mash stage, enhances beer clarity through trub[1] coagulation and yeast flocculation, and interacts with malt phosphates to reduce mash

[1] The layer of sediment that appears at the bottom of the fermenter after yeast has completed the bulk of the fermentation.

pH. Although calcium itself does not have a distinct flavor, its absence can result in a watery-tasting beer. A minimum of 50 ppm of Ca^{2+} ions is recommended for ales. Magnesium ions function as a complementary element to calcium ions, participating in many of the same reactions. These ions are crucial for yeast nutrition, with a minimum required level of 5 ppm. However, malt typically provides sufficient magnesium to meet yeast requirements. Alkalinity, predominantly in the form of carbonate activity, is undesirable in most pale beers but can be beneficial in some selected darker beers. As the primary buffering system in water, alkalinity has a profound impact on the buffering capacity of the mash and wort, generally leading to increased mash pH. Low carbonate alkalinity levels prevent the beer from having a watery taste. Sulphate ions emphasize the hop character in beer, contributing to a drier and crispier taste. However, their efficacy varies among different hop families and beer styles. SO_4^{2-} ions do not influence mash or wort pH. Both chloride and sodium ions enhance the malt character of the beer, imparting a fuller and sweeter taste. Neither of these ions affects the mash or wort pH.

1.2.1.2 Mash and sparge pH

Mash pH is the second most critical factor, following temperature, that influences enzyme activity during the mashing process. Enzymes can become denatured due to excessively high temperatures, extreme pH levels, or a combination of both factors. Consequently, mash pH clearly impacts starch conversion, soluble and total nitrogen levels, lautering, fermentability, and yield (Palmer, 2017).

The optimum pH level can be affected by temperature in two distinct ways. First, the pH of any aqueous solution will change with temperature fluctuations; higher temperatures lead to increased molecular dissociation, resulting in greater ionization and subsequent pH changes. Second, the optimum pH for a particular process may vary depending on the temperature at which the process occurs. Generally, when discussing optimum mash pH, the focus is on achieving the maximum yield. Several sources concur that the ideal mash pH for yield ranges from 5.5 to 5.8 (Fix, 1999; Bamforth, 2002). However, these sources also agree that improved flavor, clarity, and flavor stability are obtained at lower mash pH levels. Therefore, the widely accepted target for mash pH lies between 5.2 and 5.6, as measured at room temperature (20-25°C). It has been demonstrated that pale beers tend to exhibit better flavor profiles at lower mash pH levels (5.2-5.4), whereas darker bees often taste better at slightly higher mash pH levels (Palmer, 2017).

The mash pH represents the balance achieved between the residual alkalinity of the water and the chemical composition of the malt. Each malt variety contains phosphates, proteins, and acids that influence the malt's chemical composition. Upon mashing in distilled water, every malt variety causes a decrease in pH from the initial water pH to a nominal baseline value. Notably, there is variation in baseline pH values (±0,2) among different barley cultivars. For base malts, the average pH range averages around 5.8, while specialty malts exhibit lower baseline pH values, falling within the range of 4.0 to 5.4. Moreover, it is important to note that malts with similar baseline pH values may possess distinct buffering capacities (Palmer, 2017).

Lowering the pH of sparge water is typically unnecessary, provided that the water contains adequate calcium levels. The malt's phosphates will react with calcium and buffer the pH until the gravity falls below 1.012. The critical factor is maintaining sufficient calcium levels in the water (Palmer, 2017). Taylor (1990) suggests that 50 ppm is enough to prevent excessive pH rise. Sparge water is generally neutralized to a pH range of 5.5 to 6.0 (Palmer and Kaminski, 2013).

1.3 Traditional sour beers

Sour beers represent a highly heterogenous category of beer, not confined to a singular definition based on production method, raw ingredients, or geographical provenance. A unifying attribute for sour beer is the elevated concentrations of organic acids and a lowered pH (ranging from 3.0 to 3.9) in comparison to commercial beers, resulting in enhanced sensory characteristics, such as a pronounced acidic flavor (Tonsmeire, 2014). In general, the increased levels of organic acids found in sour beer can be attributed to the participation of acidogenic bacteria during the fermentation process. Contrasting with the standard beer fermentation process, which typically involves single-strain yeast fermentations, sour beer is predominantly a product of a mixed fermentation process involving both yeast and bacteria.

Presently, only four classic sour beer styles have endured the test of time, persisting to this day. While a handful of other sour styles have been restored to prominence, such as Gose and Lichtenhainer, there is a lack of unanimity experts and practitioners regarding their precise production methodologies and recipes. Of the few surviving sour beer styles, three can be traced back to their origins in Belgium (Tonsmeire, 2014), which are described in detail below.

1.3.1 Lambic

Belgian sour beer, particularly the Lambic variety, has gained recognition for its distinct taste and unique production process. Lambic beer is traditionally brewed using 66% malted barley and 33% unmalted wheat. According to Belgian regulations, the inclusion of a minimum of 30% unmalted wheat in the mash is mandatory. The production of Lambic primary involves a turbid mash technique, which incorporates elements from both the English infusion and German decoction processes (Briggs et al., 2004). During the English infusion process, hot water is introduced to elevate the mash temperature. Meanwhile, in the German decoction process, a portion of the mash is separately boiled to break down starch granules and subsequently reintegrated into the mash tun, effectively raising the overall mash temperature, and ensuring optimal enzyme activity.

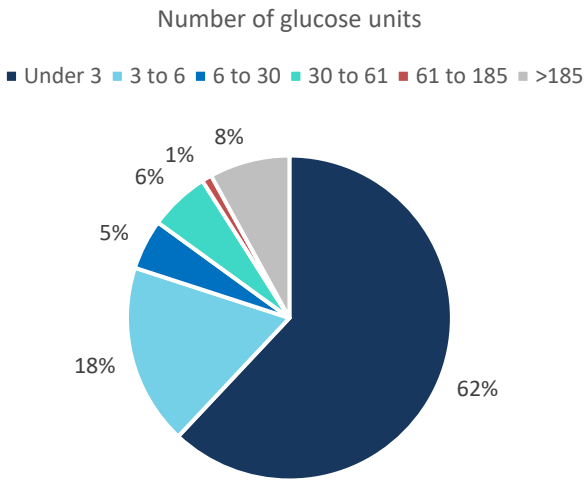


***Figure 1**: The carbohydrate composition of lambic wort. Adapted from (Kumara and Verachtert, 1991)*

However, in the turbid mashing method, the separately boiled wort, referred to as slime, is not introduced to the mash tun, resulting in the wort not passing through all temperature rests (Kumara and Verachtert, 1991). The employment of unmalted wheat and the turbid mashing process, yields a wort rich in malto-oligosaccharides or dextrins[2] (figure 1). Conventional *Saccharomyces* brewing yeast are unable to ferment these dextrins Kumara and Verachtert, 1991). Nonetheless, *Brettanomyces* yeasts and LAB, which are present during the maturation of Belgian sour beers, can ferment these longer chain carbohydrates (Martens et al., 1997).

The utilization of the turbid mashing technique not only facilitates starch conversion in the brewing process, but also leads to the breakdown of lengthy protein molecules into their constituent free amino acids. Consequently, the wort generated through this method possesses a reduced protein content, thereby providing a less hospitable environment for microorganisms that operate primarily during the initial phases of fermentation. The

[2] *Dextrins are sugar chains consisting of 3-10 glucose molecules linked by α-1,4 glucosidic bonds that are too long for standard brewing yeast to ferment, but too short to be considered starch (Bláhová et al., 2023)*

resulting wort offers an elevated abundance of dextrins and starches, which serve as a more favorable source of nourishment for microorganisms that are active during the later stages of the fermentation process (Sparrow, 2005) The original gravity (OG) of Lambics usually falls on the range of 1.048 to 1.057 specific gravity (SG) units (Tonsmeire, 2014)

The Lambic beer is characterized by a natural fermentation process driven by indigenous microorganisms in the brewery and its environment, involving a sequence of diverse bacteria and yeast (Spitaels et al., 2014). Traditionally, inoculation with these microorganisms takes place in a wide, open fermenter called a coolship. The following fermentation can be divided into four stages. The first stage of fermentation is dominated by Enterobacteriaceae and is reported to start after 3-7 days and lasting 30-40 days, followed by the second phase, being the main fermentation stage, starting with the emergence of *Saccharomyces* spp. in the second month and lasting 1-4 months. The disappearance of Enterobacteriaceae is explained by the depletion of glucose, the increase in ethanol and the decreased pH. The third phase is the acidification stage, dominated by *P. damnosus*, starting to appear after 2 to 3 months after the depletion of mono- and disaccharides. Subsequently, *B. bruxellensis* supplants *Saccharomyces* spp. after six months of maturation (Spitaels et al., 2014), signaling the start of the last phase, maturation. The complex fermentation process is essential to the development of the beer's unique sour and tart flavor profile, which is highly sought after by beer enthusiasts. This mixed fermentation can last up to 3 years and are traditionally performed in wooden casks or foeders after the initial inoculation in coolships.

Brettanomyces and LAB play an important role in the attenuation of sour beers. Previous studies have demonstrated that the combination of *Brettanomyces* and LAB results in a synergistic effect on beer (Andrews and Gilliland, 1952; Kumara and Verachtert, 1991). The residual dextrins that are not fermented by *Saccharomyces* spp. are degraded by *Brettanomyces* spp. working in tandem with LAB (Kumara and Verachtert, 1991). This degradation of residual dextrins is a crucial process in the production of sour beers, as it leads to super-attenuation, which is a hallmark characteristic of this beer style.

Kumara and Verachtert (1991) found that *Brettanomyces* is the primary contributor to super-attenuation in Lambic beer, with its effect being most pronounced in a mixed culture with *Pediococcus*. The production of α-glucosidase by *Brettanomyces* is another key factor in the degradation of residual dextrins. α-glucosidase is an enzyme capable of breaking down dextrins and shows both intracellular and extracellular activities (De Cort

et al., 1994). This enzyme removes a single glucose molecule from the oligosaccharide, and under optimal conditions, malto-oligosaccharides shorter than maltotetraose are not found in the presence of the enzyme (Kumara et al., 1993). This enzyme is fast acting, making it an essential factor in the super-attenuation of sour beers. However, Kumara et al. (1993) noted that while the α-glucosidase enzyme is fast-acting, the low pH of Lambic beers may slow down the over-attenuation process.

Brettanomyces yeast also plays a crucial role in the formation of various esters, as highlighted by Spaepen and Verachtert (1982). The synergistic interaction between LAB and *Brettanomyces* yeasts results in the production of numerous metabolites (Kumara and Verachtert, 1991; Van Oevelen et al.,1976). Among these metabolites are esters such as ethyl acetate and ethyl lactate, as well as long-chain fatty acids and their corresponding esters, including ethyl caprylate and ethyl caprate (Spaepen et al., 1978).

The presence of ethyl caprate in Lambic beers is particularly notable, as it is found in just trace concentrations in most other types of beer, thus establishing it as a distinctive aroma compound of Lambic beers when present at a relative high concentration (Spaepen et al., 1978). The authors also state that interestingly, a beer produced through a mixed fermentation process involving LAB carrying pitching yeast, followed by a secondary cask fermentation, exhibit similar concentrations of long-chain fatty acids and their esters as those found in coolship Lambics.

In an industrial setting, the production of Lambic wort may be achieved through the implementation of an infusion mashing technique as opposed to the traditional turbid mashing method. Upon completion of the wort boiling process, the wort is acidified to a pH level of 4.0 utilizing lactic acid. Subsequently, the wort undergoes pre-chilling prior to being transferred to a cooling tun. This approach allows industrial brewers to maintain a consistent production schedule, ensuring availability throughout the entire year. However, in order to attain a comparable level of dextrinous[3] wort, the incorporation of alternative adjuncts is necessary. Examples of such adjunct include the addition of flaked grains during the mashing process or maltodextrin powder during the boiling stage (Spitaels et al., 2017).

Belonging to the Lambic beer family, the Gueuze emerges from a blending process that combines young Lambic, typically aged one year, with more mature Lambics aged for

[3] Containing dextrins.

two to three years. This union initiates a secondary fermentation, yielding an effervescent beer that boasts both fruity and dry characteristics. Due to its similarity to the prestigious sparkling wine, gueuze has been dubbed “the champagne of beers” (Guinard, 1990).

1.3.2 Flemish red and brown (Oud Bruin)

In contrast to the Lambic beers, red-brown acidic beers originating from Flanders, Belgium exhibit a distinct composition in their grist, consisting primarily of malted barley, and cooked unmalted maize (Martens et al., 1997). Flemish reds and browns typically have a darker color compared to Lambics, which can be ascribed to the incorporation of Vienna and Munich malts[4]. The utilization of aroma and caramel malts is not uncommon. The original gravity for these beers typically falls within the range of 1.048-1.057 SG, the same as for Lambics. These beers were traditionally inoculated with an in-house starter culture, containing yeasts and LAB. This starter culture was harvested and reused from batch to batch. The fermentation starts with a main fermentation stage performed by the yeasts, after which an acidification stage follows, lasting 4-5 weeks. The final stage is maturation, performed in wooden casks, where *Pediococcus* spp., *Brettanomyces* spp., and AAB finish the beer. The AAB are supported by microoxygenation by the diffusion of O_2 through the pores of the wood in the casks (Martens et al., 1997). Furthermore, these beers exhibit heightened sweetness due to sugar often being added to the beer before bottling, and a more pronounced acetic quality in comparison to Lambics (Tonsmeire, 2014).

1.3.3 Berliner Weisse

The final traditional sour beer style, Berliner Weisse, diverges from its Belgian counterparts as it is exclusively produced within the city limits of Berlin, adhering to European Union regulation that protect the trademark (Burberg and Zarnkow, 2009). This beer is characterized by a blend of barley and wheat malts, typically possessing a low original gravity (1.028-1.032 SG) (Tonsmeire, 2014). Among the classic sour beer styles, Berliner Weisse is unique in that it always relies solely on *Lactobacillus* to produce lactic acid. The yeast to LAB ratio is generally between 4:1 to 6:1 (Burberg and Zarnkow, 2009). Traditionally Berliner Weisse had *B. bruxellensis* as the secondary fermentation yeast (Hieronymus, 2010).

[4] Vienna and Munich are kilned base malts, produced by increasing the moisture content and curing at a higher temperature. Munich malts are cured at a higher temperature than Vienna malts. The use of kilned base malts in the beer provides malt flavor to the beer (Palmer, 2017)

1.4 Lactic acid bacteria

LAB form a diverse group of Gram-positive, catalase-negative, non-sporulating, non-motile, and acid-tolerant microorganisms, which possess the ability to metabolize sugars into lactic acid as their primary fermentation product. Despite their shared characteristic of lactic acid production, these bacteria exhibit remarkable heterogeneity in terms of physiological attributes, metabolic and fermentation capabilities, and adaptability to various ecological niches (Pfeiler and Klaenhammer, 2007).

Moreover, specific LAB species that naturally inhabit food and beverage environments have been conferred with the designation “generally regarded as safe” (GRAS) due to their long-standing history of safe use in food production and preservation (Klaenhammer et al., 2005).

Ethanol concentrations and pH values exhibit considerable variation among global beer styles. When talking about sour beer, ethanol typically ranges from 2-9% (v/v) and pH values spanning 3.0-3.5 (Suzuki et al., 2008). Consequently, LAB isolated from these diverse environments are often well-adapted to one or both factors (Suzuki, 2011). Studies have demonstrated that LAB growth is not clearly impacted by decreased pH and ethanol levels in beer, and no correlation between these factors and contamination has been observed. However, pH values approaching 4.0 and lower exhibit a mildly inhibitory effect on LAB growth (Menz et al., 2010)

1.4.1 *Pediococcus*

Pediococcus is a genus of Gram-positive, non-motile, cocci-shaped LAB (Cai et al., 1999). Phylogenetically, *Pediococcus* belongs to the phylum Firmicutes, class Bacilli, order Lactobacillales, and family Lactobacillaceae (Ludwig et al., 2009)

Pediococcus isolates demonstrate the ability to grow in a range from facultative aerobic to microaerophilic conditions. These microorganisms exhibit a homofermentative metabolic pathway, whereby they exclusively produce lactic acid during glucose fermentation without generating carbon dioxide as a byproduct (Holzapfel et al., 2015).

The use of *Pediococcus* spp. in the food industry is largely attributed to their ability to produce antimicrobial substances, such as bacteriocins, organic acids, and hydrogen peroxide, which can inhibit the growth of spoilage and pathogenic microorganisms (Parvez et al., 2006). Consequently, *Pediococcus* has been employed in various food

fermentation processes, including the production of fermented vegetables, sausages, and beverages (Cai et al., 1999).

1.4.2 *Lactobacillus*

Recently, advancements in whole-genome sequencing have resulted in the reclassification of the formerly recognized *Lactobacillus* genus into 25 distinct new genera (Zheng et al., 2020). It should be noted that, for the purposes of this study, the former nomenclature will be utilized. For an overview of the taxonomical changes pertaining to relevant strains, refer to table 1.

***Table 1**: Taxonomical changes in the former genus Lactobacillus.*

Old nomenclature	New nomenclature
Lactobacillus pentosus	*Lactiplantibacillus pentosus*
Lactobacillus plantarum	*Lactiplantibacillus plantarum subsp. plantarum*
Lactobacillus brevis	*Levilactobacillus brevis*
Lactobacillus buchneri	*Lentilactobacillus buchneri*
Lactobacillus delbrueckii	Unchanged

Lactobacillus spp. predominantly exhibit anaerobic growth; however, some strains display aerotolerance. As described by Holzapfel and Wood (2014), they can be classified into three distinctive metabolic or fermentative groups based on their fermentation capabilities. The first group encompasses obligate homofermentative species, which exclusively ferment hexoses through the Embden-Meyerhof-Parnas pathway, with lactic acid as the primary by-product (Hammes and Vogel, 1995).

The second group comprises facultative heterofermentative species, which possess the ability to perform homofermentation but can also metabolize pentoses and gluconate through the pentose phosphate pathway under conditions of glucose limitation or starvation. As a result, these species generate by-products such as acetic acid, ethanol, and formic acid.

The third and final group is characterized by obligate heterofermentative species that metabolize both pentoses and hexoses exclusively via the initial portion of the pentose phosphate pathway, known as the phosphogluconate pathway. This process yields lactic acid, carbon dioxide, and either ethanol or acetic acid as by-products (Holzapfel and Wood, 2014; Sun et al., 2014; Zheng et al., 2015).

Phylogenetically they belong to the phylum Firmicutes, class Bacilli, order Lactobacillales and family Lactobacillaceae (Ludwig et al., 2009)

Professor Burghard Meyer of Versuchs- und Lehranstalt für Brauerei in Berlin (VLB) has highlighted a clear drawback of utilizing *Lactobacillus* for primary fermentation in beer production. When this bacterium is introduced during the brewing process, it produces a proteolytic enzyme that has the capacity to degrade all the protein present in the beer. As a result, the head retention of the beer can be severely compromised, leading to a suboptimal drinking experience (Meyer, 2012).

1.4.3 *Lactobacilli* as a beverage spoiling bacterium

In the context of beverage spoilage, *lactobacilli* are often regarded as undesirable contaminants due to their association with multiple adverse effects, including the formation of haze and sedimentation, production of off-flavors and acids, as well as the introduction of ropiness (Suzuki, 2011). The phenomenon of ropiness is attributed to the presence of exopolysaccharide (EPS) producing bacteria, which lead to an increased viscosity and a slimy texture in the affected products.

Exopolysaccharides are high molecular weight biopolymers that can be categorized into two primary types: homopolysaccharides, which consists of a single, repeating monosaccharide unit, and heteropolysaccharides, which are composed of distinct repeating units of multiple monosaccharides (Fraunhofer et al., 2017). The production of these EPS by *lactobacilli* and other spoilage microorganisms can compromise the quality and sensory properties of beer.

Brettanomyces strains have been shown to have the ability to break down EPS when pitched in a co-fermentation with LAB. In a study from Ovelen and Verachtert (1979) two strains of *B. bruxellensis* reduced the EPS produced by *Pediococcus cerevisiae*. However, when pitching it with a two-week delay, they recorded no reduction in EPS. This was attributed to the decrease in pH at the inoculation time. The precise enzymatic activity involved in the breakdown of EPS by *Brettanomyces* remains poorly characterized. However, it has been theorized that the α-glucosidase activity present in these yeasts play a role in this process.

1.4.4 *Bifidobacterium*

Bifidobacteria, often mistakenly grouped with LAB, have distinct characteristics that set them apart from the traditional LAB used in fermented food production. Despite sharing

some similarities with *lactobacilli*, *Bifidobacteria* are not closely related to any LAB and exhibit unique biochemical and physiological properties (Hoover, 2014).

Unlike *lactobacilli*, which display acid tolerance and can be considered facultative anaerobic, *Bifidobacteria* are less tolerant to acidic conditions and are strictly anaerobic (Hoover, 2014). Although both groups of microorganisms produce lactic acid through carbohydrate fermentation, *Bifidobacteria* generate acetic acid in equal or higher amounts than lactic acid. Moreover, the catabolic pathway employed by *Bifidobacteria* is distinct from the homo- and heterofermentative pathways utilized by LAB (Hoover, 2014).

Bifidobacteria belong to the family Bifidobacteriaceae and are commonly found in the gastrointestinal tract of humans and animals, where they play essential roles in gut health and immune function (Turroni et al., 2012). In the food industry, *Bifidobacteria* are primarily used in dairy products such as yoghurt, kefir, and cheese (Tamime et al., 2007).

1.4.5 *Weissella*

Weissella, a genus of Gram-positive, catalase negative, non-endospore forming bacteria, exhibits coccoid or rod-shaped morphology and is classified within the group of LAB (Collins er al., 1993). Phylogenetically, *Weissella* belongs to the phylum Firmicutes, class bacilli, order Lactobacillales, and family Leuconostocaceae (Collins et al., 1993). These microorganisms are obligately heterofermentative, generating CO_2 through carbohydrate metabolism, with lactic acid and acetic acid as the primary end products.

The potential role of *Weissella* in the food industry is primarily linked to its ability to produce a range of organic acids, antimicrobial substances, and EPS (Patel et al., 2012).

1.5 Metabolism in LAB

During fermentation, yeast and bacteria are presented with a mixture of assimilable sugars in the wort. Maltose is typically the most abundant, followed by smaller amounts of glucose, fructose, sucrose, and maltose oligomers such as maltotriose and maltotetraose. The utilization of these sugars follows an orderly process, where maltose is not utilized until the supplies of glucose, fructose, and sucrose have been depleted. This is because glucose (and its related fructose) is the preferred carbon source for the yeast *S. cerevisiae*, and its presence triggers a series of wide-ranging metabolic changes, including the suppression of respiratory functions and the utilization of other less preferred carbon sources. This response is not influenced by the presence of oxygen (Broach, 2012).

LAB possess the ability to metabolize macromolecular substances in beer, which include the degradation of indigestible polysaccharides and the transformation of undesirable flavor compounds. Simultaneously, during their metabolic processes, LAB can generate a variety of products such as short-chain fatty acids, amines, bacteriocins, vitamins, and exopolysaccharides (Wang et al., 2021).

Oligosaccharides, comprising 3-10 monosaccharide residues, represent important carbohydrate sources within environments inhabited by *lactobacilli* (Gänzle and Follador, 2012).

1.5.1 Metabolism of starch

In the past, LAB were believed to play a more important role in the fermentation of dairy products, with a limited ability to hydrolyze sugars and proteins in grains. However, later analysis of LAB's (Kyoto Encyclopedia of Genes and Genomes) KEGG metabolic pathways revealed the presence of a corresponding starch metabolism pathway. Additionally, research has demonstrated that starch can be hydrolyzed by extracellular enzymes secreted by LAB (Gänzle and Follador, 2012).

Starch is composed of amylose, which consists of α-(1→4) linked glucose chains, and amylopectin, which features α-(1→4) linked glucose main chains and α-(1→6) linked glucose side chains (van der Maarel et al., 2002). Amylolytic degradation of amylose by α- and β-amylase and amyloglucosidase yields α-(1→4) linked maltodextrins, maltose, and glucose, respectively. The hydrolysis of amylopectin necessitates amylopullulanase or pullulanase to cleave the α-(1→6) linked branching points, resulting in the production of isomaltose and oligosaccharides with mixed α-(1→4) and α-(1→6) linkages (Gänzle and Follador, 2012). Due to starch hydrolysis by amylases derived from cereal grains, maltose and maltodextrins are the predominant oligosaccharides in cereal fermentations. Notably, almost all *lactobacilli* can metabolize α-glucans, and amylopullulanase is the sole extracellular polysaccharide hydrolyzing enzyme present in *lactobacilli* (Gänzle and Follador, 2012).

1.5.2 Metabolism of α-Glucans (Maltodextrins, isomalto-oligosaccharides and maltose)

Three intracellular α-glucosidases, namely MalL, MalN, and DexB, are responsible for the hydrolysis of maltodextrins or isomalto-oligosaccharides. These enzymes all belong to the glycoside hydrolase family 13 (GH13). Maltose phosphorylase, an enzyme with

high specificity for maltose, catalyzes the phosphorolysis of maltose into glucose and β-D-glucose-1-phosphate. This reaction is consistently associated with the activity of β-phosphoglucomutase, which converts β-D-glucose-1-phosphate into glucose-6-phosphate (Stolz et al., 1996). It is important to note that maltose phosphorylase does not act upon isomaltose or maltodextrins (Ehrmann and Vogel, 1998).

In the majority of obligate heterofermentative lactobacilli, including *L. brevis* and *L. buchneri*, maltose phosphorylase represents the sole enzyme that is active on maltose. In other *lactobacilli* species, maltose phosphorylase is an integral component of the MalEFG/MsmK maltodextrin operon, which also includes the α-glucosidases MalL and MalN (Gänzle and Follador, 2012).

1.5.3 Metabolism of sucrose

In *lactobacilli*, three distinct pathways for sucrose metabolism have been identified: (1) extracellular hydrolysis mediated by glucansucrases (GH70) or fructansucrases (GH68) enzymes; (2) transport and simultaneous phosphorylation of sucrose through the Pts1BCA phosphotransferase system, followed by hydrolysis via the invertase (GH32) enzymes SacA/ScrB; (3) transport of sucrose, succeeded by either phosphorolysis facilitated by sucrose phosphorylase or hydrolysis conducted by the invertase BrfA or SacA/ScrB (Gänzle and Follador, 2012).

1.5.4 Metabolism of Raffinose family oligosaccharides

The raffinose family oligosaccharides (RFOs), which include raffinose, stachyose, and verbascose, are composed of one, two, and three α-(1→6) D-galactose units, respectively, attached to sucrose as shown in figure 2. RFOs are widely distributed in plants, and relevant to this research, the seeds of grain legumes contain 2-10% RFOs (Gänzle and Follador, 2012). RFOs were initially considered as anti-nutritive factors, causing dose-dependent flatulence and gastrointestinal discomfort (Oku and Nakamura, 2002). However, recent research has revealed that raffinose holds potential as a prebiotic, specifically stimulating the growth of beneficial microbes in the gastrointestinal tract of humans and animals (Anggraeni, 2022). Studies on RFO metabolism in *lactobacilli* primarily focus on enabling their fermentative removal.

For the hydrolysis of RFOs into their individual monosaccharide components, the presence of two critical enzymes is required: α-galactosidase and invertase. These enzymes are members of the glycoside hydrolase family, specifically belonging to the GH36 and GH32 subfamilies, respectively. The α-galactosidase enzyme facilitates the hydrolytic cleavage of α-1,6-galactosific linkages within oligosaccharides, including RFOs, as depicted in figure 2.

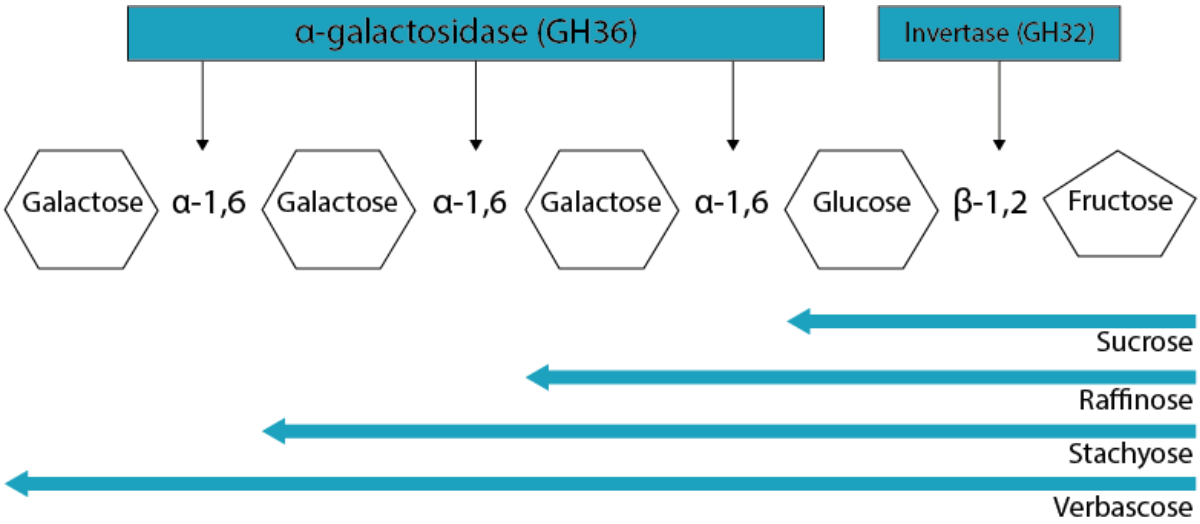


***Figure 2**: Different RFOs with their constituent sugars and the enzymes needed for their hydrolysis.*

The α-galactosidase activity in *lactobacilli* was initially described by Mital et al. (1973). In many *lactobacilli*, α-galactosidase is encoded by the melA gene. MelA is widely distributed in *lactobacilli*, including *L. brevis* and *L. buchneri*, highlighting the importance of α-galactosidases in plant ecosystems. The enzyme functions as a homotetramer and recognizes unbranched oligosaccharides, such as raffinose and stachyose, as substrates (Silvestroni et al., 2002). The hydrolysis of RFOs by α-galactosidase releases sucrose, and the complete degradation of RFOs depends on the presence of invertase (Gänzle and Follador, 2012).

Invertase, also known as β-fructofuranosidase, catalyze the hydrolysis of the β-1,2-glycosidic linkage in the disaccharide sucrose, converting it to its constituent monosaccharides, glucose, and fructose (figure 2), as previously mentions above.

1.6 Non-traditional yeasts

The craft beer industry has experienced growth and diversification in recent years, leading to an increased interest in utilizing non-traditional yeasts to create unique flavor profiles and styles. Among the most notable are *Brettanomyces*, Kveik, and *Lachancea*.

1.6.1 *Brettanomyces*

Brettanomyces represents a genus of unconventional, slow-fermenting yeast species within the Ascomycota fungal phylum. Historically, this genus has been perceived as a spoilage organism in the wine and beer industries, attributed to its capacity for generating off-flavors and aromas (Steensels et al., 2015). However, in recent years, *Brettanomyces* has experienced a surge in popularity among craft brewers and niche winemakers who purposefully employ it to enhance their products.

Several *Brettanomyces* species exist, with *B. bruxellensis*, *Brettanomyces anomalus*, and *Brettanomyces claussenii* being the most prevalent in brewing applications. These yeasts are recognized for their ability to metabolize a wide range of carbohydrates, including complex oligosaccharides and polysaccharides that conventional *S. cerevisiae* strains cannot ferment (Steensels et al., 2015). This attribute enables *Brettanomyces* to persist in fermenting carbohydrates in beer even after *Saccharomyces* strains have concluded fermentation, yielding a drier and more attenuated end-product.

Brettanomyces can generate a variety of flavor and aroma compounds, such as esters and phenols, which contribute to the distinctive sensory profile of the fermented products (Crauwels et al., 2017). These flavors are often characterized as funky, barnyard, horsey, leathery, or fruity, contingent on the strain, fermentation conditions, and substrate. The presence of these compounds primarily results from the yeast's metabolism of hydroxycinnamic acids into volatile phenolic compounds, including 4-ethylphenol and 4-ethylguaiacol (Steensels et al., 2015).

The utilization of *Brettanomyces* in beer production is typically linked to traditional Belgian styles, such as Lambics, Gueuze, and Flemish red/brown ales, as well as American wild ales. In these styles, the complex flavors generated are considered desirable and contribute to the beer's overall character (Steensels et al., 2015).

1.6.2 Kveik

Kveik, a traditional Norwegian farmhouse yeast, has garnered attention within the global brewing community due to its unique fermentation properties and versatility. Contrary to

being a single yeast strain, Kveik represents a diverse collection of *S. cerevisiae* strains that have been utilized in Norwegian farmhouse brewing for centuries (Preiss et al., 2018).

A key characteristic of Kveik yeasts is their remarkable ability to ferment at elevated temperatures, typically ranging between 30°C and 40°C, without generating off-flavors typically associated with other yeast strains under such conditions (Preiss et al., 2018). This high-temperature tolerance enables rapid fermentations, with certain Kveik strains capable of completing the process within a mere two to three days (Garshol, 2014).

Kveik strains display a broad spectrum of flavor profiles, from fruity and ester-driven to clean and neutral, contingent on the specific strain and fermentation conditions employed. This extensive flavor profile renders Kveik strains suitable for an array of beer styles, encompassing traditional Norwegian ales and contemporary craft beer styles such as IPAs, stouts, and sours (Garshol, 2014).

Additionally, Kveik yeasts exhibit a notable degree of ethanol tolerance, with some strains capable of fermenting up to 13% ABV or even higher (Preiss et al., 2018). This attribute, combined with their temperature tolerance and distinctive flavor profiles, positions Kveik as an appealing option for brewers seeking to explore innovative brewing techniques.

The adoption of Kveik in commercial brewing has increased in recent years, as numerous breweries worldwide have integrated these traditional Norwegian yeasts into their beer production processes, thus contributing to the diversification of flavors and styles within the global beer market (Garshol, 2014).

1.6.3 *Lachancea*

Lachancea is a genus of yeast within the Saccharomycetaceae family, and its species have gained interest in the brewing and fermentation industries due to their unique metabolic properties. One of the most notable species within this genus is *Lachancea thermotolerans,* which has been studied for its potential applications in winemaking and brewing (Banilas et al., 2016)

L. thermotolerans is known for its capacity to produce lactic acid during fermentation, which results in a pH reduction in the fermented product (Vilela., 2018). This characteristic has led to interest in its use for brewing sour beers and mixed fermentation processes, where the production of lactic acid contributes to the desired acidity and tartness of the final product. The utilization of *L. thermotolerans* in sour beer production

can offer an alternative to traditional methods that involve the use of LAB, such as *Lactobacillus* and *Pediococcus*, for acidification (Banilas et al., 2016).

Moreover, *L. thermotolerans* has demonstrated temperature tolerance, which enables it to ferment at a broad range of temperatures. This characteristic can be advantageous for brewers seeking flexibility in controlling fermentation temperature and adapting to various brewing conditions (Vilela, 2018).

In addition to its unique metabolic properties, *L. thermotolerans* can also contribute to the flavor profile of the fermented product, generating fruity and spicy esters and phenols that are desirable in certain beer styles (Banilas et al., 2016).

1.7 Intrinsic and extrinsic antimicrobial hurdles

Beer presents a seemingly inhospitable environment for microbial growth due to the presence of multiple physiological hurdles that bacteria must simultaneously overcome. These obstacles include the antimicrobial action of ethanol and hop-derived α-acids, low pH, limited availability of nutrients, and low oxygen (O_2) levels, along with high concentrations of carbon dioxide (Sakamoto and Konings, 2003). Despite these challenges, certain microorganisms can survive and proliferate in beer, potentially affecting its quality and flavor. Table 2 and 3 summarizes all extrinsic and intrinsic antimicrobial hurdles placed on the LAB and other potential contaminants.

***Table 2**: Extrinsic antimicrobial hurdles of beer (* not applicable for all beers). Adapted from Vriesekoop et al., 2012.*

Antimicrobial hurdles	Primary targets	Effect on cells
Mashing	Gram-negative bacteria	Thermal destruction
Kettle boil	All contaminants	Thermal destruction
Pasteurization*	All contaminants	Thermal destruction
Filtration*	All contaminants	Physical size exclusion
Bottle conditioning*	Aerobe bacteria	Anaerobic conditions created

Mashing typically occurs at temperatures ranging from 62 to 72°C. This elevated temperature effectively inactivates Gram-negative bacteria, yeasts, and molds. However, LAB and spore-forming bacilli can withstand the high temperatures encountered during mashing (Sakamoto and Konings, 2003). In the subsequent boiling phase, which

generally lasts around 60 minutes, both vegetative cells and spores are destroyed, ensuring the elimination of potential contaminants.

***Table 3**:Intrinsic antimicrobial hurdles of beer (* not applicable for all beers). Adapted from Vriesekoop et al., 2012.*

Antimicrobial hurdles	Primary targets	Effects on cells
Ethanol	All contaminants	Cell membrane functions inhibited
Low pH	All contaminants	Enzymes affected; inhibitory effect of hops enhanced
Iso-α-acids	Gram-positive bacteria	Cell membrane functions inhibited
Carbon Dioxide	Aerobe bacteria	Anaerobic conditions created, enzymes inhibited, pH lowered, and cell membrane functions inhibited
Low oxygen levels	Aerobe bacteria, Gram-negative bacteria	Anaerobic conditions created
Lack of nutrients	All contaminants	Cell starvation
Sulphur Dioxide*	Gram-negative bacteria	Metabolic systems affected

When it comes to sour beer, bottle conditioning is common. This is a secondary fermentation technique, where carbonation is being introduced to the beverage, by the introduction of extra sugar or unfermented young beer to an active microbial culture in the bottle, which triggers the yeast or bacteria to ferment and produce CO_2. As the active fermenting yeast reduces the O_2 content in the headspace, it diminishes the susceptibility of beer to microbial attack by approximately one-third (Simpson, 1993). However, in larger breweries, it is more common to expose the beer to post-fermentation treatment to ensure the microbial stability of the product before it is placed in its container. These treatments may include filtration, pasteurization, or the use of antimicrobial agents to inhibit growth of spoilage microorganisms.

CO_2 plays a crucial role in shaping the sensory experience of beverages, particularly in relation to three key aspects. First analogous to the function of acidity and tannins in

wine, CO_2 in beer serves to cleanse the palate and mouth of residual fat and oil. Second, the removal of fat and oil from the palate can result in the perception of heightened sweetness. Finally, CO_2 contributes a fundamental aspect to beer tasting, as the released gas facilitates the transport of aroma compounds, thereby enhancing the beverage's olfactory profile and potentially its appeal (Horne et al., 2014).

Hops are considered the defining stressor for microorganisms in the brewing process. When hops are introduced to wort and subsequently boiled, α-acids (mainly humulones) are extracted and converted into iso-α-acids, which constitute the primary bitter component in beer (Steenackers et al., 2015). The extent of α-acid isomerization is influenced by several factors, including boiling time, temperature (above 79°C), and the pH of the wort. A longer boiling time and higher temperatures generally result in a higher degree of isomerization and, consequently, increased bitterness in the beer (De keukeleire, 2000). These bitter compounds have been shown to exhibit antimicrobial effects on Gram-positive bacteria.

Bitter compounds functions as ionophores, sequestering protons within the cell and dissipating the pH gradient across the cellular membrane. This process reduces the proton motive force (PMF) and all PMF-dependent cellular activities, such as nutrient uptake (Sakomoto and Konings, 2003). Additionally, the inhibitory effect of hops on bacteria has been found to be dependent on pH and mediated by cation (K^+, Mn^{2+}) exchange across the bacterial membrane (Simpson, 1993). Further research revealed that a transmembrane redox reaction of hop compounds occurs at low pH in the presence of Mn^{2+}, leading to cellular oxidative damage (Behr and Vogel, 2010).

Thus, hop resistance in microorganisms is a multifactorial process, involving at least two distinct mechanisms that help bacteria withstand the stress induced by hops: proton ionophore-induced stress and oxidative stress mechanisms (Behr and Vogel, 2010).

One of the primary intrinsic factors contributing to microbial inhibition in beer is the ethanol concentration, which typically ranges between 3.5-5.0% (w/w) but can vary from as low as 0.5% to as high as >20% (w/w)[5] (Palmer, 2017). Ethanol is known to cause damage to bacterial cellular membranes, lead to protein denaturation, interfere with metabolic processes, and potentially induce bacterial lysis (Ashtavinayak and Elizabeth, 2016). These effects can alter bacterial morphology and impact a wide range of cellular

[5] Samuel Adams Utopias holds the record for the world's strongest naturally fermented beer at 28 % alcohol (w/w). (Samuel Adams, 2023).

functions (Kalathenos and Russel, 2003). However, at the concentrations commonly found in beer, ethanol exerts a limited effect on enzyme activity (Vriesekoop et al., 2012)

The pH of the final beer product typically ranges between 3.4 and 4.8 (Preedy, 2011), which creates an unfavorable environment for microbial growth. This low pH leads to an influx of weak organic acids into bacterial cells, causing intracellular acidification, disruption of enzyme systems, and a reduction in nutrient uptake, ultimately resulting in metabolic exhaustion (Vriesekoop et al., 2012). Furthermore, the low pH has a synergistic relationship with the bittering compounds derived from hops, as the antimicrobial effect of iso-α-acids increases with decreasing pH (Vriesekoop et al., 2012).

Finished beer is characterized by a low concentration of oxygen (0.1-0.3 ppm) and a high concentration of carbon dioxide (w/v) (Vriesekoop et al., 2012) This combination creates a predominantly anaerobic environment, which, along with the limited availability of nutrients, contributes to unfavorable growth for most bacteria. In the unfermented wort however, the extent of yeast growth is determined by the availability of oxygen, which facilitates the synthesis of sterols and mono-unsaturated fatty acids. An insufficiency of oxygen can lead to a reduction in lipid synthesis, resulting in insufficient yeast growth and incomplete fermentation. Conversely, if an excessive amount of oxygen remains accessible to the cells during the division phase, it can result in an excessive lipid synthesis, leading to unnecessary yeast growth (Quain, 2017). Contingent on the specific strains, yeast typically needs 8-12 ppm of oxygen to facilitate optimal fermentation. In the absence of adequate aeration, fermentations are inclined to exhibit under-attenuation (Palmer, 2017).

Beer's low oxygen content imposes selective pressure for microorganisms capable of anaerobic respiration. LAB, particularly *Lactobacillus* and *Pediococcus* isolates, can generate energy in oxygen-deprived environments by utilizing alternative electron acceptors to restore NAD^+ or through substrate-level phosphorylation during fermentation to regenerate NAD^+ (Coleman and Smith, 2014). Although the fermentation capabilities under anaerobic conditions differ among subgroups and even genera of LAB, their inherent anaerobic nature contributes to their resilience in the face of low oxygen stress.

1.8 Esters and phenols

Esterification occurs when yeast provide enzymes, called esterases, to act as catalysts in the presence of acids and alcohol. In sour beer, two esters in particular, ethyl lactate and ethyl acetate, contribute clearly to the aroma of the final product. These esters are derived from their respective acids, and each play a distinct role in shaping the character of the beer. Ethyl acetate becomes less fruity and more solvent-like as its concentration increases. In contrast, ethyl lactate provides a softer, balancing, and tart fruity character that is an essential hallmark of sour beer.

The enzyme group responsible for catalyzing ester formation in alcoholic beverages is referred to as alcohol acetyltransferases (ATTs). These enzymes facilitate the esterification process by reacting acetyl-CoA with various alcohols, resulting in the formation of diverse esters (Swiegers et al., 2005). ATTs can be classified into two primary categories based on substrate specificity: alcohol O-acetyltransferases (AATases) and alcohol O-acyltransferases (AATFases). AATases are responsible for catalyzing the transfer of an acetyl group from acetyl-CoA to an alcohol molecule, typically ethanol, thereby forming an ester, with ethyl acetate and isoamyl acetate being the most prevalent. Conversely, AATFases demonstrate a wider substrate specificity, catalyzing the transfer of acyl groups, not restricted to acetyl groups, from acyl-CoA molecules to alcohols (Saerens et al., 2010). Various factors impact the activity of AATs during the fermentation process, including yeast strains, fermentation conditions, and the presence of other microorganisms. By manipulating these factors, brewers can influence the flavor and aroma profiles of the resulting beer (Saerens et al., 2010).

In addition to ethyl lactate and ethyl acetate, several other esters can derive from alcohol and the corresponding acid, each with the potential to contribute a distinctive, overpowering fruity character to the beer when present in quantities that can be perceived by humans. These esters include ethyl caproate, ethyl caprate, ethyl caprylate, and ethyl butyrate. These esters are important in contributing to the overall complexity of the beer's aroma and flavor profile. However, care must be taken to ensure that their presence does not overwhelm the other flavor components in the beer, leading to an unbalanced or unpleasant end product (Sparrow, 2005). An overview of the most common esters and their characteristics can be seen in table 4.

Table 4: *Overview of the most common esters in sour beer, and their sources. Adapted from Sparrow, 2005.*

Ester	Alcohol	Acid	Enzyme group	Characteristic	Acid Source
Ethyl Acetate	Ethanol	Acetic acid	AATases	Sharp, musty, fruity, pineapple, black currant, apple, solvent, nail polish	Aerobic *Brettanomyces* spp. fermentation
Ethyl lactate	Ethanol	Lactic acid	AATases	Soft, tart, fruity, buttery, butterscotch	LAB
Isoamyl acetate	Isopentanol	Acetic acid	AATases	Banana	Yeast
Ethyl caproate	Ethanol	Caproic acid	AATFases	Waxy, fatty, fruity, pineapple, green banana	Fatty acids produced by *Saccharomyces* spp. and *Brettanomyces* spp. during cell growth phases.
Ethyl caprate	Ethanol	Capric acid	AATFases	Waxy, oily, fruity, apple, grape, brandy	
Ethyl caprylate	Ethanol	Caprylic acid	AATFases	Waxy, wine, floral, fruity, pineapple, apricot, banana, pear, brandy	
Ethyl butyrate	Ethanol	Butyric acid	AATFases	Fruity, bubble gum, pineapple, cognac.	*Clostridium* and other anaerobic bacteria. Common at low levels in spontaneous fermented beer.

The presence of polyphenolic compounds within hops, such as anthocyanogens, catechins, and tannins, offers an additional beneficial attribute to beer. Although polyphenols are commonly perceived as an undesirable constituent by many brewers due to their propensity to introduce color, haze, and astringency, aged hops exhibit a reduction in polyphenolic content, which moderates the sensation of mouth-puckering astringency. In addition to their sensory impact, polyphenols serve as natural antioxidants, conferring a safeguard against the deleterious effects of oxidation on beer, thus promoting greater longevity and stability of the product. This quality assumes heightened significance when considering beers that are intended for protracted maturation periods (Sparrow, 2005). It has been demonstrated that several strains of

LAB within genera *Lactobacillus* and *Pediococcus*, in addition to wild strains of *S. cerevisiae*, can produce volatile phenols (Lentz, 2018).

1.9 Objective and challenges

The objective of this study is to investigate yeast and LAB strains for their ability to utilize RFOs. This requires having α-galactosidase and invertase activity used for the hydrolysis of RFOs. The RFOs are sourced from pea protein concentrate, aiming to brew a beer comparable to other commercial sour beer without any off-flavors or unwanted aromas. Peas have a distinct taste and smell, with the flavor being described as beany (Trindler, 2022), with several molecules being responsible, among them being 3-methyl-1-butanol and 1-pentanol (Suwonsichon et al., 2004). In the general interest of the plant protein industry, there is compelling interest to remove these compounds from both the pea protein and RFOs, and the hope being that this could lead to a sustainable way to utilize food side streams derived from pulses suitable for the Norwegian climate in beer production.

When selecting a bacterial strain for fermentation, various characteristics beyond the capability to metabolize RFOs are taken into consideration, including attenuation, organic acid profile, stress tolerance, and the levels of volatiles produced. The choice of a bacterial strain must therefore be based on a comprehensive evaluation of all these factors, as each of them can clearly impact the final product quality. While *S. cerevisiae* strains do not possess the necessary enzymes to hydrolyze RFOs, the inclusion of LAB or non-traditional yeasts in the wort, such as *Brettanomyces* spp. or Kveik could yield results. The use of LAB would only be applicable in the production of sour beer.

In previous work by Dysvik (2019) it was shown that utilizing sugars derived from wood showed promise as a source for carbohydrates in the production of sour beer. The author further emphasized that traditional sour beer production faces numerous challenges, such as inconsistent product quality, wastage resulting from unsuccessful fermentations, and it is a time-consuming process. Many of these difficulties can be attributed to the use of spontaneous fermentations in open-air coolships. In order to try and circumvent these challenges, the present study will employ pure cultures in controlled mixed fermentations. All fermentations will be conducted under stringent temperature control and with regulated inoculation numbers. Alternative souring methods, such as sour mashing and kettle souring, will not be investigated in the context of this research.

2 Method

2.1. Media and substrate

2.1.1 Media

Microbiological analysis of different LAB was performed using De Man, Rogosa and Sharpe media (MRS) excluding polysorbate 80 and glucose (refer to Appendix 6.1). For the studies on the different yeast strains Yeast Extract Peptone (YEP) media (refer to Appendix 6.2) was used. For the pre-cultures 20 g/L glucose were added to the media. Inoculation from -80°C stock was performed, followed by incubation at strain-specific optimal temperatures for a period of 24 to 72 hours.

2.1.2 Substrate

Three distinct fractions of RFO were employed during the fermentation experiments. A summary of these various fractions is presented in table 5.

***Table 5**: List of RFO fractions used in fermentation experiments. *New and improved process from RFO3.The pea concentrates were provided by AM nutrition.*

Fraction	Source	Date
RFO1	Pea starch concentrate	January 2022
RFO3	Pea protein concentrate	April 2022
RFO4*	Fine pea protein concentrate	October 2022

2.1.3 Biorefining RFO4 from fine pea protein concentrate

In order to extract the RFOs from pea protein concentrate, the initial step involved the solubilization of oligosaccharides. A 10 kg sample of the pea protein concentrate was combined with 99 L of Milli-Q® water and 1 L of 2 M citric acid solution, yielding a pH of 5.1. The mixture was agitated at a speed of 200 rpm for 1 h at a temperature of 40°C.

Subsequently, the liquid and solid components were separated using a GEA Westfalia (Oelde, Germany) Easyscale 10.S 2-phase centrifugal separator operating at 12,000 rpm and 5 bar pressure. The flow rate and discharge intervals were adjusted according to the solids load. At a flow rate of 75 L/h, the separator was emptied every 60 s to prevent accumulation and achieve efficient separation.

The final purification step consisted of three consecutive filtration stages with progressively finer membranes, commencing with microfiltration. The microfiltration was conducted using a 0.2-0.4 µm DANMIL (Greve, Denmark) module connected to the pump in a GEA/Alfa Laval (Lund, Sweden) pilot-scale ultrafiltration/nanofiltration system. The filter was primed with approximately 20 L of Milli-Q® water, and the flow rate over the filter was about 3.58 kg/min.

The subsequent ultrafiltration stage employed an Alfa Laval ETNA01PP membrane (1 kDa) primed with tap water and circulated with 1.67 nM NaOH (ph 10.5-11.0) for more than 30 minutes, followed my flushing with Milli-Q® water until a conductivity of 0 µS/cm was attained. Filtration was performed at approximately 20°C and 2 bar, with a retentate flow rate of 500 l/h and conductivity of over 3000 µS/cm. the process was continued with repeated additions and retentate recirculation until a conductivity of approximately 268 µS/cm was reached. The system was then rinsed with water, washed with 1.67 mM NaOH for 30 min, and finally flushed with Milli-Q® water until again a conductivity of 0 µS/cm was achieved.

The final nanofiltration stage utilized an Alfa Laval NF membrane (300 Da) and was primed in the same manner as the ultrafiltration stage. The filtration conditions were set at over 30°C, 25 bar, and flow rates of 1400 L/h for retentate and over 40 L/h for permeate. At the end, the retentate was collected and freeze-dried before storage.

2.2 Fermentation studies

2.2.1 Fermentation studies 50 ml

The fermentation experiments were conducted using a novel fermenter system developed at the Bioprocess technology and biorefining group at NMBU (Norwegian University of Life Science). The fermenters are designed with a double-walled jacket that facilitates water circulation for temperature adjustment during the fermentation process. This is controlled by the Thermo Scientific Accel 500 LC (Waltham, USA), which can regulate water between 15°C and 100°C. Automatic pH control is achieved via a pH electrode submerged in the fermenter, which communicates data to a controller connected to peristaltic pumps that regulate the flow of NaOH and HCl into the fermenters.

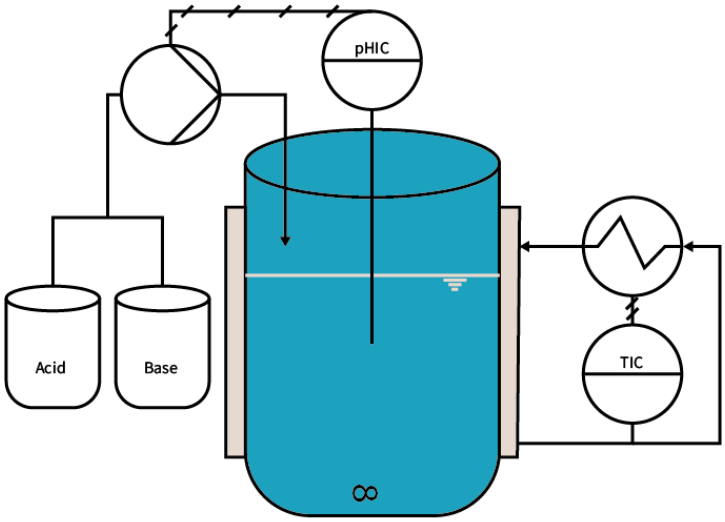


Figure 3: Schematic fermenter setup. Adapted from Garbers et al., 2022.

The fermenters have a total volume of 100 mL, with a functional volume of approximately 50 mL. The lid encompasses a pH electrode, openings for silicone tubing to accommodate acid and base inputs, a sampling port, and a VWR (Radnor, USA) 0,2 µm PTFE filter fitting to enable the release of CO_2. The temperature-controlled water flow within the outer jacket of the fermenters can be connected in series, allowing for simultaneous utilization of up to six fermenters. These fermenters are placed on a magnetic stirrer plate, controlled by a 2mag (München, Germany) Motion Mixdrive 15 magnetic controller. A schematic representation of the setup can be found in figure 3.

To explore the metabolic capabilities of the studied strains in relation to RFO utilization, a RFO mixture extracted from pea starch concentrate (RFO1 comprising 77% dry basis (db), as described by Garbers et al., 2022) was incorporated into the MRS media at a concentration of 20 g/L prior to inoculation.

To generate growth curves, samples were taken from the fermenter using a syringe and needle and turbidity was assessed through a spectrophotometer, measuring optical density at a wavelength of 600 nm (OD_{600}). To validate the data, dry weight samples and samples for high-performance liquid chromatography (HPLC) analysis were collected at regular intervals. All fermentations were executed in duplicate, with strains inoculated from the pre-cultures at 1% volume.

An overview of the experimental setup, detailing the various strains and their respective fermentation conditions, is provided in table 6.

***Table 6**: Setup for strains used in 50 mL fermentation for the generation of growth curves in MRS media containing RFOs.*

Experiment 1			
Strain	Time [h]	Temperature [°C]	pH
L. pentosus KW1	52	20	6±0.1
L. pentosus KW2	52	20	6±0.1
Experiment 2			
L. plantarum WildBrew^{TM}	72	30	6±0.1
L. brevis BSO464	72	30	6±0.1
L. buchneri CD034	72	30	6±0.1
Experiment 3			
W. confusa TM120	141	22	6±0.1
B. animalis subsp. Lactis BL04	141	22	6±0.1
P. damnosus WLP661	119	22	6±0.1

2.2.2 Fermentation studies 5 ml

A series of small-scale fermentations were conducted to investigate various characteristics of the different, microbial strains, including their metabolism of RFO constituents, growth in beer media, and tolerance to ethanol and IBU concentrations.

2.2.2.1 RFO Utilization

Ten bacterial strains, in addition to four yeast strains commonly utilized in beer production were examined throughout these small-scale fermentations. The bacterial strains included *L. pentosus* KW1, *L. pentosus* KW2, *W. confusa* TM120, *W. confusa* TM76, *L. plantarum* (Fermentis (Marquette-lez-lille, France) WildBrew^{TM} Sour pitch), *L. brevis* BSO464, *L. buchneri* CD034, *L. delbrueckii* (White Labs (San Diego, USA) WLP667), *P. damnosus* (White Labs WLP661), and *B. animalis subsp. Lactis* BL04. The yeast strains were *S. cerevisiae* (Fermentis US-05TM), *S. cerevisiae* (Omega Yeast (Chicago, USA) Lutra Kveik), *B. claussenii* (White Labs WLP645), and *L. thermotolerans* (Lallemand (Montreal, Canada) PhillySourTM).

In the initial small-scale fermentations, each strain was inoculated at 1% into 5 mL of growth media. Four separate culture tubes were prepared for each strain, containing a negative control, 15 g/L raffinose, 15 g/L stachyose, and 15 g/L RFO1 mixture,

respectively. The bacterial strains were cultured in MRS media, with most strains incubated at 37°C, except for the two *Weissella* strains and the *Pediococcus* strain, which were incubated at 30°C. For the yeast stains, the experimental setup was similar, with YEP used as the growth medium, and all strains incubated at room temperature (21°C ±2°C).

Samples were collected from the culture tubes every 24 hours, starting at 0 hours, and the optical density was measured (OD_{600}) and plotted into a growth curve. Additionally, samples were collected for the analysis on high-performance liquid chromatography (HPLC).

2.2.2.2 Growth in beer media

In the second small-scale fermentation experiment, a simple beer was brewed to serve as the beer media for investigating the influence of stressors, primarily ethanol and IBU, on microbial growth. A summary of the critical parameters of the beer media is provided in table 7. For all parameters and brewing data, please refer to Appendix 6.3.

***Table 7**: Key parameters Beer media 1*

Beer media 1	
EtOH	3.27% (v/v)
Final gravity (FG)	1.010
pH	4,22
EBC	4.57
IBU	6.9

The malt bill for media 1 consisted of 59% pilsener and 59% wheat malt. In addition, 6% (w/w) of rice hulls were added and mixed well with the grist.

The gravity of the wort was 1.039, and the mash pH was adjusted to 5.2 to optimize the enzymatic conversion of starches to sugars. The grist was mashed in at a striking temperature of 2°C above the temperature-controlled single saccharification step at 68°C (60 min) to favor α-amylase activity and create a more dextrinous wort.

The wort was fermented using *S. cerevisiae* (Fermentis US-05) for a duration of 14 days. Following the completion of the primary fermentation, the beer was separated into two portions, with one of them mixed with 15 g/L of the RFO1 mixture. Both fractions were

then sterile filtered (0.22 µm) and subdivided into culture tubes. Each microbial strain was inoculated into 5 mL of beer media, both with and without the added RFO mixture, in duplicates. In contrast to the previous small-scale fermentation setup, the inoculation volume was increased to 2% to reduce lag phase duration. To simplify the experimental setup, all bacterial strains were incubated at 30°C, while all yeast stains were incubated at room temperature as mentions before.

Similar to the initial setup, samples were collected from the culture tubes every 24 hours, starting at 0 hours, and the optical density was measured (OD_{600}) and plotted into a growth curve. Additionally, samples were collected HPLC analysis.

2.2.2.3 Stressors: Ethanol and IBU

In the third and fourth small-scale fermentation experiments, the various microbial stains were subjected to increasing concentrations of ethanol and IBU in the beer growth media. The objective was to identify a threshold at which these stressors impeded growth.

A new beer media was brewed, and a summary of the most critical parameters is presented in Table 8. For a comprehensive recipe, refer to appendix 6.4.

Table 8: *Key parameters beer media 2.*

Beer media 2	
EtOH	4.49% (v/v)
Final gravity (FG)	1.008
pH	10.52
EBC	4.57
IBU	0

The recipe for the second beer media was slightly modified from the first one, with an increase in fermentable ingredients and a lower mash temperature (63°C) to produce a less dextrinous wort. This beer was brewed without hops, enabling precise adjustment of the IBU before inoculation by adding hops extract. The hops extract was prepared using Archer hops (1,54% α-acid), resulting in a solution containing 185 IBU, calculated using the MEBAK® convention (refer to section 2.2 Calculating IBU and α-acid concentration). To manipulate the ethanol concentration, the beer media was either diluted with Milli-Q® water or fortified with pure ethanol (96% containing 12 ppm emetic

compound). RFO1 mixture (15 g/L) was dissolved into the beer media prior to sterile filtration (0.22 µm).

The inoculation, incubation, and sampling procedures were conducted similarly to the previous setup. Table 9 displays the experiment design for the various IBU concentrations at each ethanol level for each strain. All samples were fermented in duplicates.

***Table 9**: Overview of all IBU concentrations at different ethanol levels in beer media 2.*

Ethanol	2.25%	3.5%	4.5%	6%
IBU	0	-	0	0
	2.5	-	2.5	2.5
	-	3.75	-	-
	5	-	5	5

2.2.3 Fermentation studies 0.5 and 3 liter

Due to the collaborative nature of some experiments conducted with fellow master's student Aksel Skeie, the data presented will include a strain of *Lactococcus cremoris* M121. However, this strain is not directly relevant to the research focus of this study.

All the yeast pitching rates for *S. cerevisiae* in this study will be based on the work of Fix and Fix (1997), where he states that a pitching rate of 0.75 million cells/mL/°Plato (°P) is an appropriate rate for most ales.

2.2.3.1 Pea Beer V1

Scaling up the fermentation experiments enabled the collection of larger sample sizes required for subsequent analyses. A new batch of beer media was brewed, retaining many of the same parameters as Beer Media 2, but reducing the amount of fermentables and the IBU slightly. In all subsequent beer media brews, the RFOs (RFO1) were directly added to the brew kettle at the end of the boil, at a concentration of 15 g/L. For a detailed recipe, refer to Appendix 6.5.

After cooling, the beer was divided into 3 L fractions and transferred to 5 L Brouwland Brewferm Royal Bubbler (Beverlo, Belgium) glass fermenters. The selection of strains for this experiment included the LAB strains *L. brevis*, *L. buchneri*, *L. pentosus* KW1, *P. damnosus*, and the yeast strain *L. thermotolerans*.

Except for the batch containing the *Lachancea* strain, all batches were inoculated with *S. cerevisiae* (Fermentis US-05) at a pitch rate of 0.75 million cells/ml/°Plato, followed by the immediate addition of 2% starter culture containing the separate LAB strains. The *Lachancea* batch was also inoculated with a 2% starter culture. All the batches were incubated at 24°C.

Samples were collected at 7, 14, and 19 days by pipetting 50 mL directly from the fermenters into Falcon tubes. The samples were then centrifuged, sterile filtered (0.22 µm), and stored at low temperatures for later HPLC, HSGC and HPAEC-PAD analysis.

After 19 days, following the collection of sampled for chemical analysis and Anton Paar Alcolyzer, the remaining liquid was bottled with 4 g/L of priming sugar for subsequent informal sensory analysis.

2.2.3.2 Pea Beer V2

In order to expand the results to further strains and co-fermentations, a second Pea Beer was brewed. This beer media, referred to as Pea Beer V2, adhered to the same recipe as Pea Beer V1, except for a higher saccharification temperature (65°C). For a comprehensive recipe, consult Appendix 6.5. The various strain combinations examined in this stage of the study are summarized in Table 10. *B. claussenii* was also added back into the study.

***Table 10**: Co-fermentations in 3 L pea beer media 2.*

Strains:	**Abbreviation:**
S. cerevisiae (Fermentis US-05) *B. animalis subsp. lactis*	SB
B. claussenii *L. cremoris* M121 *L. brevis*	BLB
S. cerevisiae (Lutra Kveik) *L. cremoris* M121 *L. brevis*	KLB
S. cerevisiae (Fermentis US-05) *L. cremoris* M121 *L. buchneri*	SLB
L. thermotolerans *L. buchneri*	TB

Samples were collected at 7, 14, and 19 days by pipetting 50 mL directly from the fermenters into Falcon tubes. The samples were then centrifuged and stored at low temperatures for later HPLC, HSGC and HPAEC-PAD analysis.

After 19 days, following the collection of sampled for chemical analysis and Anton Paar Alcolyzer, the remaining liquid was bottled with 7 g/L of priming sugar.

2.2.3.3 Pea Beer V3

In an effort to obtain more robust data, all fermentations from Pea Beer V1 and V2 were replicated in duplicates. The beer media employed adhered to the same recipe as Pea Beer V2, and the inoculation, incubation, and sampling procedures remained unchanged. For a detailed recipe, refer to Appendix 6.5.

However, modifications were made to the size and type of fermenter, utilizing 0.5 L borosilicate glass bottles with screw-on lids equipped with two barbed hose fittings. One of the fittings was sealed by attaching a silicone hose and tying the other end closed while the other fitting was connected to a VWR 0.2 µm PTFE filter. Due to a limited number of specialized lids, four regular blue screw-on lids were adapted using a drill press to create a hole large enough to accommodate a traditional S-shaped airlock with a rubber gasket. The RFO fraction used in these experiments were RFO3.

2.2.3.4 Pea Beer V4

The brewing recipe included 2.41 kg of Bestmalz (Heidelberg, Germany) Pilsen malt (49% w/w) and 2.41 kg of Bestmalz Wheat malt (49% w/w). To help with lautering, 2% (w/w) of rice hulls were added. The pH of the brewing water was determined to be 7.2 at room temperature.

Drawing upon the accumulated knowledge from all the previous experiments, a final brewing study was designed and implemented. In this experiment, a 30-liter batch of beer was divided into four 10 L plastic fermentation buckets, each containing 7.5 L of wort. Bucket 1 comprised wort without the addition of RFO and was fermented solely using *S. cerevisiae*. Bucket 2, which also lacked RFO, was co-fermented by *B. claussenii, L. buchneri*, and *L. brevis*. Bucket 3 contained wort with the addition of 15 g/L of RFO4 and was fermented using *S. cerevisiae*. Lastly, bucket 4 was co-fermented by *B. claussenii*, *L. buchneri*, and *L. brevis*, with the inclusion of 15 g/L of RFO4 in the wort.

Key ions in the brewing water are summarized in table 11. For a comprehensive water report (provided by ALS Laboratory Group (Oslo, Norway) and details on the calculation

of Na+ concentration, please refer to Appendix 6.13 and 6.14. A complete overview of the recipe can be found in Appendix 6.6. The targeted water profile aimed to achieve a crisp pale beer with a full body, with a sulphate-to-chloride ratio of 0.6.

Table 11: *Key ions in the brewing water before and after water adjustment*

The ion:	Source water profile:	Target water profile:	Final water profile:
Ca^{2+}	21 ppm	50 ppm	50 ppm
Mg^{2+}	3 ppm	5 ppm	3 ppm
Na^{+}	33 ppm	5 ppm	33 ppm
Cl^{-}	36 ppm	70 ppm	82 ppm
SO_4^{2-}	46 ppm	55 ppm	52 ppm
HCO_3^{-}	45 ppm	0 ppm	45 ppm

Salt additions to the brewing water were divided between the mash and the sparge water to reach the final concentrations as displayed in table 11. The pH of the mash, following the addition of the grist, was adjusted to 5.33, and the sparge water pH was adjusted to 5.7, both using lactic acid (80%).

The mashing profile was selected to maximize fermentability and yield, incorporating two saccharification steps: 63°C for 30 minutes and 70°C for 30 minutes. A mash-out step at 78°C for 10 minutes was included to help facilitate lautering.

After sparging, the wort was boiled for 60 minutes with the addition of 21.4 g of Archer hops (1.54% α-acid), resulting in an estimated 3 IBU. Prior to cooling, the wort fractions without RFO were transferred into two fermentation buckets and separately cooled using an ice water bath. In the remaining wort, 15 g/L of RFO (RFO4 fraction) was added before cooling to approximately 20°C.

Starter cultures were individually stepped up from frozen glycerol stocks, with 6 mL from the pre-cultures (2%) inoculated into 300 mL of media, out of which 150 mL (2%) was pitched into each fermenter. The growth media for LAB strains consisted of MRS with 20 g/L of glucose (35°C), while the growth media for the *Brettanomyces* strain was YEP with 20 g/L of glucose (30°C). The inoculation ratio between yeast and the two LAB strains were 1:1:1.

For the two buckets containing only *S. cerevisiae*, the pitching rate was 0.75 million cells/mL/°P of ADY. All four buckets were fermented at 24°C in an incubator.

2.2.3.5 Bottling Pea Beer V4

To remove most of the biomass, the beer was centrifuged at 8000 rpm for 30 minutes at 4°C (Beckman Coulter Avanti JXN-26, (Krefeld, Germany)). Sucrose was added for priming at a concentration of 7 g/L, aiming for a carbonation level of 2.7 volumes of CO_2 when stored at >16°C. Lallemand CBC-1 carbonation yeast was added before bottling at a concentration of 0.1 g/L.

2.3 Chemical Analysis

2.3.1 Calculating IBU and α-acid concentration.

2.3.1.1 Photometric determination of IBU according to the MEBAK® convention

An International Bitterness Unit (IBU) corresponds to 1 mg/L of isomerized α-acid in the liquid. According to the MEBAK® guidelines, beer's bitter substances are dissolved in a non-polar solvent following acidification through liquid-liquid extraction, after which the absorbance of the non-polar phase is measured. Other compounds also contribute to beer bitterness to a lesser extent; these are measured as part of the total photometric measurement.

A 10 mL beer sample was placed in a Falcon tube, acidified with 1 mL of 3 M hydrochloric acid, and treated with 20 mL of isooctane. The mixture was shaken for 15 minutes to ensure optimal mass transport between the phases.

Following centrifugation, 350 µL (equivalent to a pathlength of 1 cm) of the non-polar isooctane phase, containing the dissolved bitter substances, was extracted, and measured using a Genesys (Menlo Park, USA) 10S series spectrophotometer at 275 nm wavelength. To determine the final IBU value, the sample's absorbance was multiplied by a factor of 50. The samples were consistently run in quadruplicates, using a 96-well UV-compatible plate, and the average value of blank wells were subtracted from the measured sample values.

2.3.1.2 The effect of oxidation on the α-acid content in hops

To assess the impact of oxidation on hops, aged and unopened packets of Archer hops were investigated. The hops' age was indeterminate as no information was printed on the label. However, the initial α-acid contend was indicated at 3.7%.

To examine potential further oxidation, some Archer hops packets were opened and disperse in a tray with a loose-fitting lid. After six months, both the unopened and opened packets of hops were boiled at a concentration of 60 g/L for one hour. 50 mL duplicates of each concentrate were place in a Flacon tube and centrifuged, followed by IBU determination using the MEBAK® convention following 1:10 dilution.

Based on these finding, the novel combined α-acid and oxidized β-acid content, after an unknown period of aging was calculated (for detailed calculations, see Appendix 6.7).

2.3.2 Raffinose and Starch assay

In order to determine the complete starch content present within the RFO fractions, the Rapid Total Starch (RTS) methodology was employed, as outlined in the K-TSTA-100A assay protocol from by Megazyme (Brey, Ireland). Furthermore, to ascertain the concentrations of raffinose and free D-galactose within these RFO fractions, the K-RAFGA assay procedure was followed, which also is supplied by Megazyme.

2.3.2.1 Megazyme Total Starch Assay

Upon acquiring the assay, it was necessary to prepare the reagent solutions and suspensions as per protocol. A comprehensive description of these preparations can be found in Appendix 6.8.

The experimental procedure commenced with the measurement of 100 mg of test sample in duplicate using Falcon tubes, with one serving as sample blank. Subsequently, 10 mL of sodium acetate buffer containing calcium chloride was added to the tubes with the sample, followed by vortexing for 5 seconds. Then, 0.1 mL of undiluted thermostable α-amylase was introduced to the sample tubes, while an equivalent volume of sodium acetate buffer containing calcium chloride was added to the sample blank tubes. After vortexing for 3 seconds, the tubes were placed in a boiling water bath.

At 2, 5, and 10-minute intervals, the samples were removed from the water bath, vortexed for 5 seconds, and returned to the bath. 15 minutes after the addition of the enzyme, the tubes were once again removed from the water bath, vortexed, and then placed in a

separate water bath maintained at 50°C, allowing them to reach equilibrium with the temperature.

Subsequently, 0.1 mL of amyloglucosidase solution was added to the sample tubes, while an equal amount of sodium acetate buffer containing calcium chloride was added to the sample blanks. All tubes were then incubated at 50°C for 30 minutes, after which they were removed from the water bath and allowed to cool down to room temperature.

Next, 1.5 mL of each solution, encompassing both samples and sample blanks, was transferred to Eppendorf tubes and centrifuged at 13.000 rpm for 5 minutes. One milliliter of the resulting supernatant was then transferred to a new set of Falcon tubes containing 4 mL of sodium acetate buffer, and the samples were thoroughly mixed.

In duplicate, 0.1 mL aliquots of each sample, including the sample blanks, were transferred to glass test tubes. 3 mL of GOPOD reagent were added to each glass tube, followed by incubation at 50°C for 20 minutes. Absorbance measurements were recorded against the reagent blank using a spectrophotometer at a wavelength of 510 mL, with glucose control and reagent blank also being incubated.

To generate the glucose control, 0.1 mL of glucose standard solution was combined with 3 mL of GOPOD reagent in quadruplicate and incubated under identical conditions as the samples. For the reagent blank, 0.1 mL of sodium acetate buffer containing calcium chloride was mixed with 3 mL of GOPOD reagent in duplicate and incubated under the same conditions as the samples.

Finally, the obtained values were entered into the accompanying Excel spreadsheet provided on Megazyme's website.

2.3.2.2 Megazyme Raffinose/D-galactose Assay

Upon obtaining the assay, it was necessary to prepare the reagent solutions and suspensions as detained in Appendix 6.9 Preparations for the Raffinose Assay. Each sample was analyzed for raffinose and free D-galactose content. For free D-galactose testing, an identical assay was conducted without the inclusion of α-galactosidase. Duplicate preparations were made for each sample, as well as for each sample blank, with a concentration of 5 g/L of RFO. In cuvette 1, 100 µL of sample solution was combined with 50 µL of α-galactosidase. In the sample blank (cuvette 2), an equivalent volume of Milli-Q® water was added. The experimental design for D-galactose testing mirrored this setup, with the sample blank (cuvette 4) containing only 150 µL of Milli-Q®

water and the free D-galactose sample (cuvette 3) consisting of 100 µL sample solution and 50 µL Milli-Q® water. All four samples were gently mixed before incubation at room temperature for 20 minutes.

In order to streamline the procedure and eliminate the need to transfer samples between different types of containers during incubation and absorbance measurement, a custom cuvette holder was designed to fit within the thermoblocks, allowing the entire process to be conducted within cuvettes.

Following the 20-minute incubation, a mixture of 100 µL buffer, 1 mL Milli-Q® water, and 50 µL NAD+ solution was added to all cuvettes. After mixing and waiting for 3 minutes, the initial absorbance value was recorded at a wavelength of 340 nm. Subsequently, 20 µL of D-galactose dehydrogenase and galactose mutarotase suspension was added, and the samples were incubated at 40°C for 30 minutes before measuring the second absorbance value at the same wavelength.

During the second iteration of the assay, the samples were clarified using Carrez solution (Appendix 6.9) and diluted based on their estimated raffinose content. For the clarification 5 mL sample was mixed with 25 mL of Milli-Q® water in a 50 mL Falcon tube. Carefully, 2.5 mL of Carrez I solution, 2.5 mL of Carrez II solution and 5 mL of NaOH solution was added, mixing between each addition. The Falcon tube was topped off with Milli-Q® water reaching the 50 mL mark, before mixing and filtering (0.22 µm).

To calculate the results, the values were plotted into the corresponding Excel spreadsheet available on Megazyme's website.

2.3.3 Chromatography

2.3.3.1 High performance Anion-Exchange chromatography with Pulsed Amperometric Detection (HPAEC-PAD) analysis of monosaccharides and short oligosaccharides

The chromatographic method employed for analyzing carbohydrate consumption in the samples is founded on anion exchange principles. Anion exchange is conducted at elevated pH levels to facilitate the deprotonation of hydroxyl and carboxyl groups in the analytes. For carbohydrates, this typically results in one negative charge per hydroxyl group. The stationary phase of the column is positively charged, thereby interacting with the negatively charged counter-ions (Dionex).

In general, a greater number of negative charges per molecule corresponds to stronger affinity to the stationary phase, leading to extended retention times. For monosaccharides specifically, the foundation for separation is based on minimal differences in their chemical properties. Consequently, the spatial configuration of hydroxyl groups also plays a critical role in the separation process. This necessitates relatively weak eluting conditions to discern the subtle differences between analytes. Potassium hydroxide (KOH) is consistently used as eluent for the HPAEC of monosaccharides, generated electrolytically (Dionex).

The analytical system utilized was the Dionex ICS-6000, equipped with pulsed amperometric detection (PAD). The column employed for the analysis was the Dionex CarboPac™ PA210-Fast-4 µm, measuring 150 x 2 mm. For more detailed information regarding the operation protocol, refer to Appendix 6.10.

Sample preparation involved centrifuging each analyte, followed by a 1:10 dilution with Milli-Q® water and sterile filtration (0.22 µm). 200 µL of sample was then pipetted into HPLC vials with pre-cut lids. Air bubbles at the bottom of each vial were inspected and removed if present before placing the vial in the analytical tray. The injection volume for the Dionex was set to 0.4 µl, and each sample was subjected to a 20-minute run time, employing 12 mM KOH elution.

During sample analysis, blanks were incorporated into the sequence at regular intervals. Additionally, each run included a combination of mono- and oligosaccharide analytical standards for comparative purposes.

2.3.3.2 High performance liquid chromatography (HPLS) of organic acids

The analytical method employed for organic acid analysis was adapted from Grønnevik et al. (2011), incorporating modifications from Dysvik (2019). To prepare the samples for high-performance liquid chromatography (HPLC) they were filtered with α-cellulose filters with a pore size of 4-7 µm.

Following filtration, 1.0 g of each sample was transferred to 10.0 mL glass tubes with screw-on lids. Subsequently, 2,5 mL of deionized water, 200 µL of 0.5 M H2SO4, and 8.0 mL of acetonitrile (CH3CN) were added to each tube. After securing the lids and checking for leaks, the tubes were placed in MultiRS-60 BIOSAN programmable rotator for 30 minutes (30 rpm), followed by a 15-minute centrifugation at 3.400 rpm at room

temperature. The supernatant was then removed, and the samples were filtered using a 0.22 µm PTFE membrane.

Organic acids were separated using an Aminex HPX-87H column, measuring 300 x 7.8 mm. For further information on the operating protocol, refer to Appendix 6.11. Calibration standard solutions were prepared in the same way as the samples, and the components within the samples were identified based on retention time comparisons with the standard solutions. The organic acids analyzed included pyruvic acid, lactic acid, acetic acid, citric acid, and DL-pyroglutamic acid. The actual sample analysis was carried out by Kari Olsen, senior engineer at KBM, NMBU.

2.3.3.3 Headspace gas chromatography (HSGC) of volatile aromatic compounds

The detection and quantification of volatile aromatic compounds were conducted using a method adapted from Dysvik (2019). To analyze beer samples, it was necessary to remove CO_2, which was accomplished by filtration with α-cellulose filters with a pore size of 4-7 µm.

10.0 g of filtrate were weighed and transferred into Macheray-Nagel (Düren, Germany) crimp neck headspace vials. The vials were then sealed with Teflon-coated septa with aluminum rings.

The column employed for analyte separation was a CP-SIL 5CB from Varian (Palo Alto, USA), measuring 25 m x 0.53 mm with a 5 µm film thickness. For further information on the operating protocol, refer to Appendix 6.12. Identification and quantification were performed according to calibration with standards. The standards utilized in this analysis are presented in table 12. The execution of sample analysis was carried out by Kari Olsen, senior engineer at KBM, NMBU.

***Table 12**: Standards used in the HSGC analysis of beer media including RFO.*

Volatile aromatic compounds		
Acetaldehyde	Dimethyl sulfide (DMS)	2-methyl-propanal
1-propanol	Diacetyl	2-butanol
Ethyl acetate	2-methyl-1-propanol	3-methyl-butanal
2-methyl-butanal	Acetoin	3-methyl-1-butanol
2-methyl-1-butanol	Isobutyl acetate	Hexanal
2-hexanol	Isoamyl acetate	

2.3.4 Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Spectrometry (MALDI-TOF MS)

All four RFO fractions (RFO1-RFO4) were subjected to MALDI-ToF to verify the presence of constituent oligosaccharides and to obtain an estimation of their relative abundance. The analysis was conducted using an Ultraflextreme MALDI-ToF MS instrument (Bruker (Billerica, USA)) set up with a 337 nm nitrogen laser. Sample solutions were combined in a 1:2 ratio with a matrix composed of 2,5-dihydroxybenzoic acid (DHB). First 2 µL of DHB were added to the plate, after which 1 µL of sample was mixed in. The samples were then dried under a stream of warm air using a hairdryer.

The matrix helps with the ionization process and is made of a UV-absorbing compound that combines with the target substance. When exposed to a UV-laser, the matrix absorbs energy, causing it to be removed from the sample surface and pushing the target molecules into the gas phase. As they transition, the molecules become ionized. Time-of-flight mass spectrometry (ToF MS) is used to analyze these ionized molecules by accelerating them in an electric field and separating them based on their mass-to-charge ratio. A detector then measures the time it takes for the ions to travel a set distance (Croxatto et al., 2012).

2.3.5 Dissolved oxygen (DO)

The sensor of a Dissolved Oxygen Analyzer (DOA) DO9100 was carefully submerged into 150 mL of beer sample in a glass bottle. Following this, the opening of the glass bottle was sealed with parafilm. To ensure accuracy and reliability of the measurements, prior to each individual reading, the DOA probe underwent a single-point calibration process using atmospheric air as a reference standard. All the samples were measured at a temperature between 21-22°C.

2.3.6 Titratable acidity

In order to determine the concentration of acidic components within the beer samples and compare with the organic acid results from the HPLC analysis, the titratable acidity was measured. This analysis follows the directions laid out by the American Society of Brewing Chemists and was described by Lallemand Brewing (2023). To perform this test, a 0.1 M NaOH solution was incrementally introduced to the beer via a precision pipette, until reaching a predetermined pH. Rather than employing a pH-sensitive indicator, a

Sentron (Leek, The Netherlands) SI400 pH meter was used to monitor changes in the sample's pH value throughout the titration process.

2.3.7 Anton Paar Alcolyzer Analyzing System

To get accurate and repeatable data from the different beer fermentations, the modular Alcolyzer Analyzing System (AAS) from Anton Paar (Graz, Austria) was used. The included modules in this specific setup were DMA 4500 M Density Meter, pH 3200 for measuring pH, PFD piercing and filling device, Alcolyzer ME for measuring alcohol percentage, CarboQC ME measuring dissolved CO_2, and HazeQC ME turbidity meter.

Before and in-between each sample, the machine was rinsed with deionized water, using the machines rinsing program. Rinsing water and samples must be provided in a suitable container that can withstand pressure, as it is pressurized when pierced and added to the PFD module. The minimum amount of beer needed to perform the analysis was 125 mL.

After a container with the beer to be analyzed was placed in the piercing and filling (PFD) module and pressurized, the Alcolyzer Beer method was selected. Key parameters from the analysis include alcohol (%, v/v), SG extract, color value in EBC, and pH value.

When finished analyzing all the samples from one session, following another rinse, the check method was performed, to confirm the status of all the probes and sensors in the machine. If not satisfactory, further rounds of rinsing were performed, or the instrument was washed with sodium hydroxide.

2.4 Microbiological methods

2.4.1 Spread Plate Technique and total colony-forming units (CFUs)

In addition to serving as a positive control, spread plate technique (SPT) was used to quantify the bacteria in the starter cultures. 15 g/L of agar and 20 g/L of glucose was added to the MRS media, before being autoclaved and pipetted into petri dishes at a volume of 25 ml, whist still liquid, and then allowed to form a gel.

From each starter culture, 10-6 and 10-7 dilutions were spread on an agar plate using a glass Drigalski spatula. In-between each culture, the spatula was dipped in 96% ethanol and sterilized in the flame of a Bunsen burner.

Following an incubation period of 48 hours, at 30°C or 35°C depending on the strain, the colonies on the agar plates were counted. The total colony count was multiplied by the

corresponding dilution factor and divided by the volume of the sample. The result was expressed as colony-forming units per milliliter (CFU/mL).

2.4.2 Microscopy and Bürker hemocytometer

The Bürker hemocytometer was used in conjunction with a microscope to determine the concentration of yeast cells in the starter cultures.

The hemocytometer features a series of squares with known areas etched onto its surface. In this study, yeast cell counting was performed using a square with an area of 1/160 mm3. The placement of a glass cover slide on top of the counting plate creates a cell suspension chamber with a known height of 0.1 mm, enabling the calculation of cell number in a mL of liquid.

To determine the number of yeast cells in 1 g (dry weight) of *S. cerevisiae* (Fermentis US-05), 1 g of yeast was dissolved in 40 mL of Milli-Q® water. A 1:100 dilution was then prepared, and 10 µL of this dilution was pipetted beneath the glass cover slide. For each strain, the total number of cells was counted in 10 different squares, and the average value was employed to calculate the cell density.

This approach was also applied to the other three yeast strains, with the exception that the cells were derived from frozen glycerol stocks rather than from active dry yeast (ADY). Samples were collected from 60 mL starter cultures after 72 hours, having previously been inoculated at 2% from a pre-culture. Additionally, the microscope was utilized to examine the morphology of the yeast and bacteria strains before and after inoculation.

2.5 Sensory analysis

In the brewing industry, sensory analysis serves as a critical element in the production of beer, combining human sensory perception with scientific methodologies to assess the beer's characteristics and quality. This evaluation process not only ensures the consistency and quality of the final product, but also identifies potential areas for improvement and innovation, thereby enhancing the overall beer production process (Lawless and Heymann, 2010).

A variety of methodological approaches are employed in sensory analysis, encompassing descriptive analysis, discrimination testing, and consumer preference testing. The focus of this thesis is discrimination testing, used to determine if perceivable differences exist between two or more beer samples (Lawless and Heymann, 2010).

To evaluate the statistical significance of the results obtained from discrimination testing, hypothesis testing is conducted using the p-value. The p-value, a measure that ranges from 0 to 1, indicates the validity of the null hypothesis (no perceivable differences between the samples). A lower p-value signifies a higher likelihood of rejecting the null hypothesis, whereas a higher p-value suggests that the null hypothesis may be valid. In order for the p-value to be considered statistically significant, it must be equal to or less than 0.05.

The sensory evaluation of beer samples was conducted in a quiet, monochromatic room containing six individual sensory booths illuminated by neutral light. All samples were presented simultaneously on a tray through designated serving hatches. Access to the data entry system was granted via mobile devices by scanning a QR code. Each sample, measuring 25 mL, was served at room temperature in sealed plastic cups. Water was provided as a plate cleanser to mitigate potential interference between samples.

A tetrad test, a discriminative sensory analysis technique, was employed in this evaluation. Four samples were presented together, comprising two identical pairs. The panelist's objective was to group similar samples in pairs, aiming to determine if any discernable differences existed between the samples. The tree-digit codes assigned to the samples were randomly generated. In total, 25 individuals participated in the sensory analysis.

The tasting was divided into four distinct tests. The first test compared two batches fermented with *S. cerevisiae* US-05, with one containing added RFO and the other without. The second test paired a batch fermented with *B. claussenii*, *L. brevis* and *L. buchneri* with added RFO against a batch fermented with *S. cerevisiae* US-05, also enhanced with RFO. In the third test, a batch fermented with *B. claussenii*, *L. brevis* and *L. buchneri* was paired with a batch fermented with *S. cerevisiae* US-05, both devoid of RFO. The final test pitted two batches fermented with *B. claussenii*, *L. brevis and L. buchneri* against each other, one enriched with RFO and the other without.

3 Results

3.1 Substrate analysis

3.1.1 MALDI-ToF analysis of RFO fractions

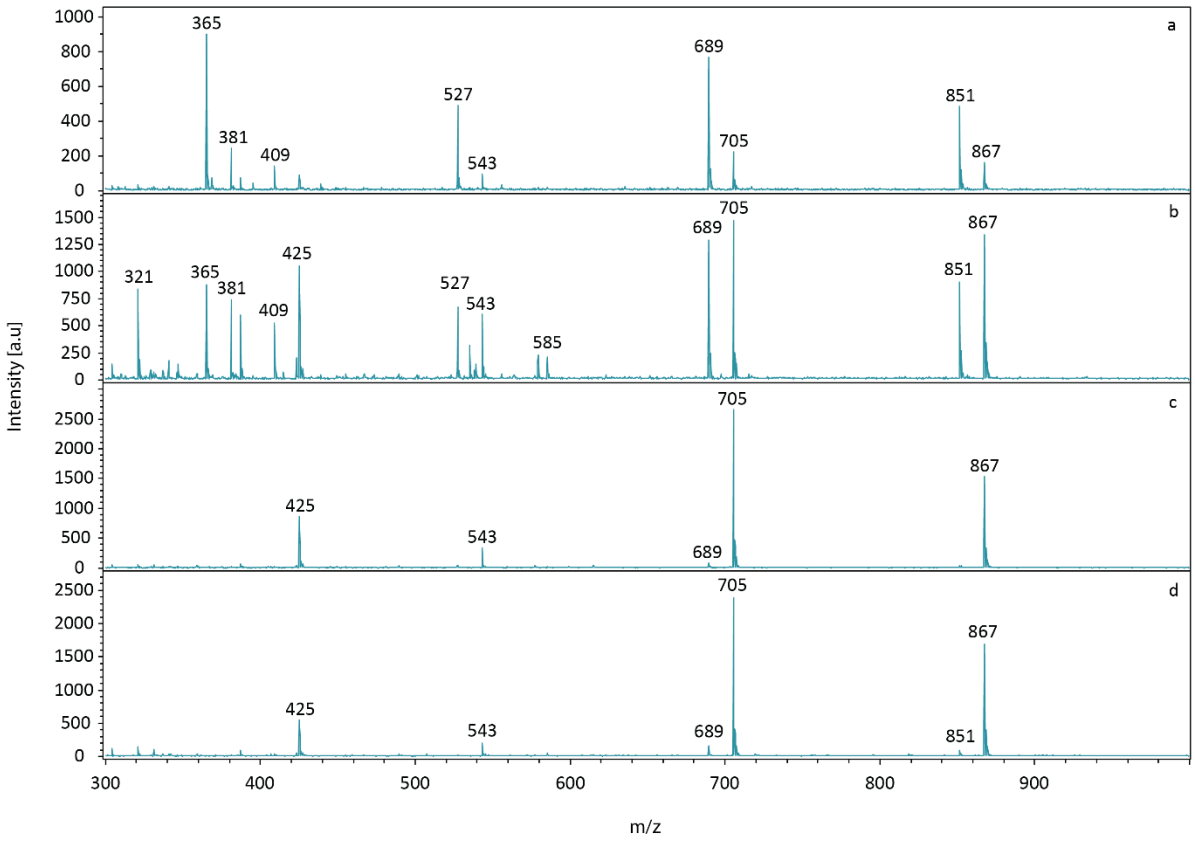


Figure 4: *Relative abundance of sucrose, raffinose, stachyose and verbascose in RFO fractions. All samples diluted 1:10 measured at 75% power.* ***(a)*** *RFO1* ***(b)*** *RFO2* ***(c)*** *RFO3* ***(d)*** *RFO4.*

Table 13*: Reference values for sugars with and without ions for MALDI-ToF.*

Sucrose	342	Raffinose	504	Stachyose	689	Verbascose	851
Sucrose + Na	365	Raffinose + Na	527	Stachyose + Na	705	Verbascose + Na	867
Sucrose + K	381	Raffinose + K	543	Stachyose + K	666	Verbascose + K	828

Figure 4 shows the relative abundance of the constituents of RFO as referenced in table 13. In In RFO3 and RFO4 no sucrose was recorded and the relative abundance in descending order was Stachyose, Verbascose, and Raffinose. In RFO1 and RFO2, each oligosaccharide is recording two peaks, on top of also measuring a relatively large abundance of sucrose.

3.1.2 Raffinose and starch assay

***Table 14**: Raffinose and starch content in the different RFO fractions. Calculated using Megazyme assays.*

	RFO1	RFO2	RFO3	RFO4
Raffinose (g/100g)	79.7	39.35	46.65	71.55
Starch (g/100g)	-	-	-	0.535

Table 14 shows the summarized results from the raffinose and starch assays from Megazyme.

3.2 Fermentation studies

3.2.1 50 ml fermentation studies

The outcomes derived from the 50 ml fermentation trials, as depicted in the growth curves on the left-hand side of figure 5, demonstrate that all eight distinct strains were capable of growing in MRS media supplemented with RFO, as measured at OD_{600}. Additionally, the associated graphs on the right-hand side of figure 5, show a corresponding growth in biomass concentration (g/L) during the exponential phase, as determined by the DW samples. The initial DW measurement for *P. damnosus* was obtained 12 hours into the fermentation process, accounting for the absence of a datapoint at the onset.

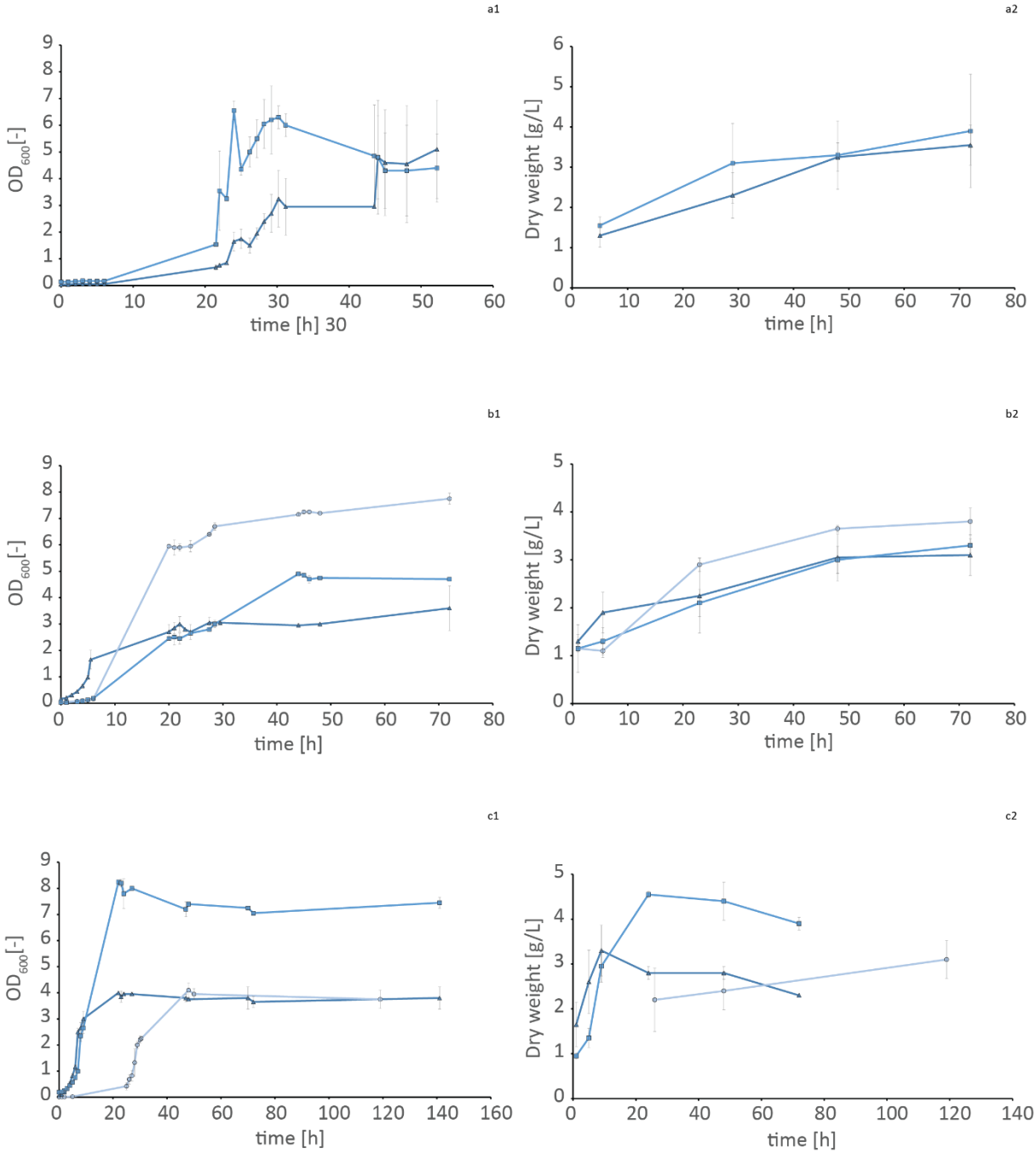


Figure 5: *To the left: Growth curves for strains grown in MRS media containing RFO1. To the right: Biomass concentration in g/l corresponding to their growth curves on the left. All curves are shown as averages of the duplicates grown.* ***(a)*** *▲ L. pentosus KW1, ■ L. pentosus KW2.* ***(b)*** *▲ L. plantarum, ■ L. brevis, ● L. buchneri.* ***(c)*** *▲W. confuse TM120, ■ B. animalis subsp. Lactis, ● P. damnosus.*

3.2.2 5 ml fermentation studies

3.2.2.1 RFO metabolism

Table 15 illustrates that the majority of the bacterial strains exhibited robust growth in media containing raffinose, with the exception of *W. confusa* TM76. *L. pentosus* KW1, *W. confusa* TM120, and *P. damnosus* demonstrated the most growth, all with an OD above 7. However, when exposed to media containing stachyose, all strains displayed more restricted growth patterns. Furthermore, all investigated strains demonstrated growth in the RFO1-containing media measuring an OD between 4 and 6. Upon comparing the yeast strains with the bacterial strains, a marked preference for the RFO media was discerned among the yeast strain. The observation suggest that the yeast strains exhibit a distinct metabolic preference compared to their bacterial counterparts.

***Table 15**: A color coded representation of bacteria and yeast quantification, a comparison between samples. Presented as optical density at a wavelength of 600 nm. All samples with growth above 1 was diluted 1:10.*

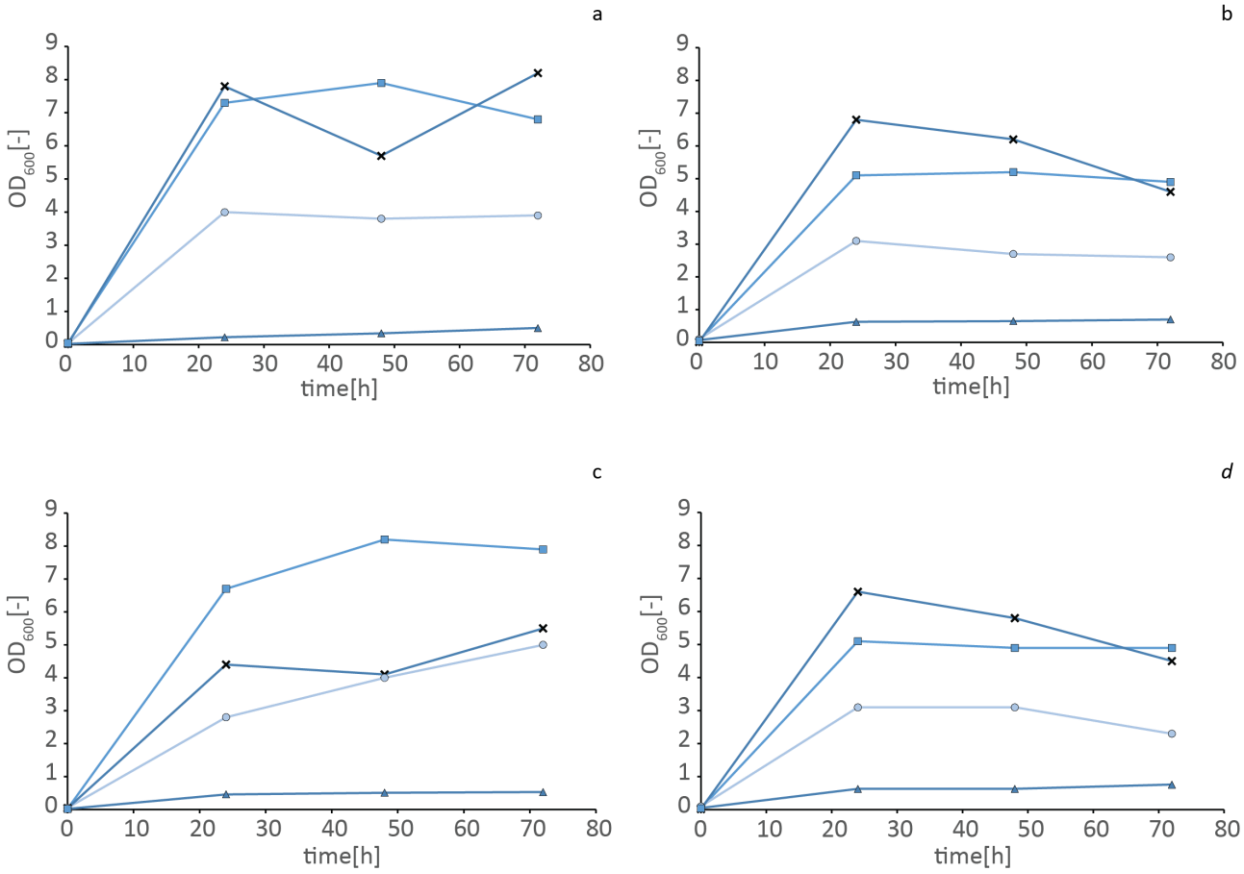
Strain	Temperature	MRS-US	MRS Raf 15 g/L	MRS Sta 15 g/L	MRS RFO 15 g/L
L. pentosus KW1	37 °C				
L. pentosus KW2	37 °C				
Weissella TM120	30 °C				
Weissella TM76	30 °C				
L. plantarum	37 °C				
L. brevis	37 °C				
L. buchneri	37 °C				
L. delbrueckii	37 °C				
P. damnosus	30 °C				
B. animalis	37 °C				

Strain	Temperature	YEP-US	YEP Raf 15 g/L	YEP Sta 15 g/L	YEP RFO 15 g/L
S. cerevisiae US-05	21°C ±2				
S. cerevisiae Kveik	21°C ±2				
B. claussenii	21°C ±2				
Lachancea spp.	21°C ±2				

> 7	>6	>5	>4	>3	>2	>1	~0	OD

In figures 6 and 7, the outcomes of the small-scale fermentation trial examining various strains' capability to metabolize distinct oligosaccharides within the RFO family are depicted. Owing to constraints in cost and accessibility, verbascose was excluded from the trial. *L. buchneri* and *B. claussenii* demonstrated no growth in the starter culture.

In this trial, it was evident that the growth conditions provided by Beer Media 1 was inhospitable for the growth of the bacterial strains under examination. The intrinsic hurdles presented by the media, did not exert an equivalent impact on the yeast strains, as indicated by the OD measurements displayed in figure 7. The recorded OD values for the yeast strains ranged from 3.2 for *L. thermotolerans* to 9.6 for *S. cerevisiae* US-05. In contrast, all bacterial strains exhibited a markedly reduced growth, as evidenced by their OD values remaining below 1.



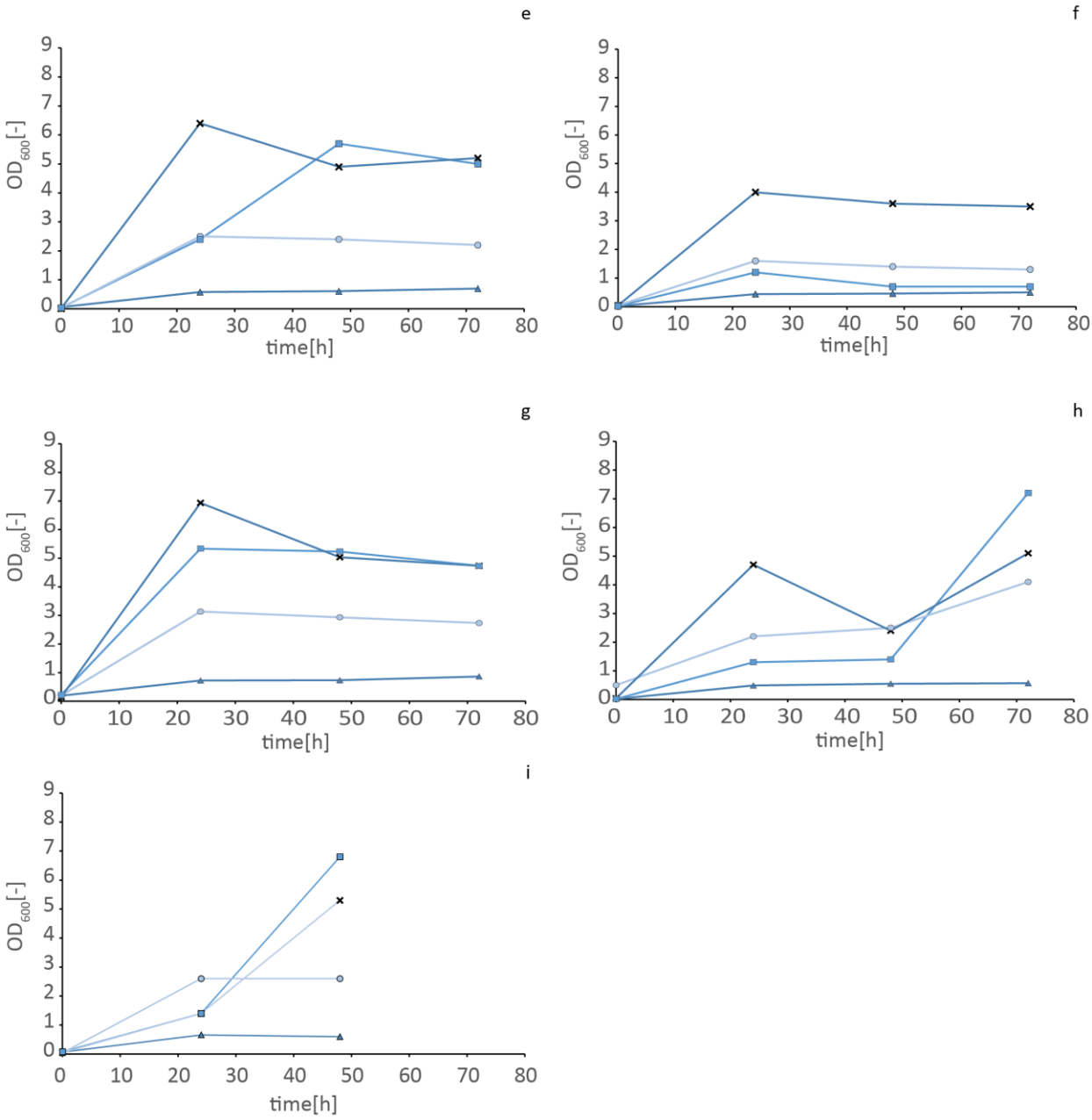


***Figure 6**: Growth in MRS media with different carbon sources. ▲MRS without any carbon source added, ■ MRS with 15 g/L raffinose, ● MRS with 15 g/L stachyose, ✕ MRS with 15 g/L RFO1. **(a)**L. pentosus KW1. **(b)** L. pentosus KW2. **(c)** W. confuse TM120. **(d)** L. brevis. **(e)** L. plantarum. **(f)** W. confuse TM76. **(g)** L. delbrueckii **(h)** P. damnosus. **(i)** B. animalis subsp. Lactis.*

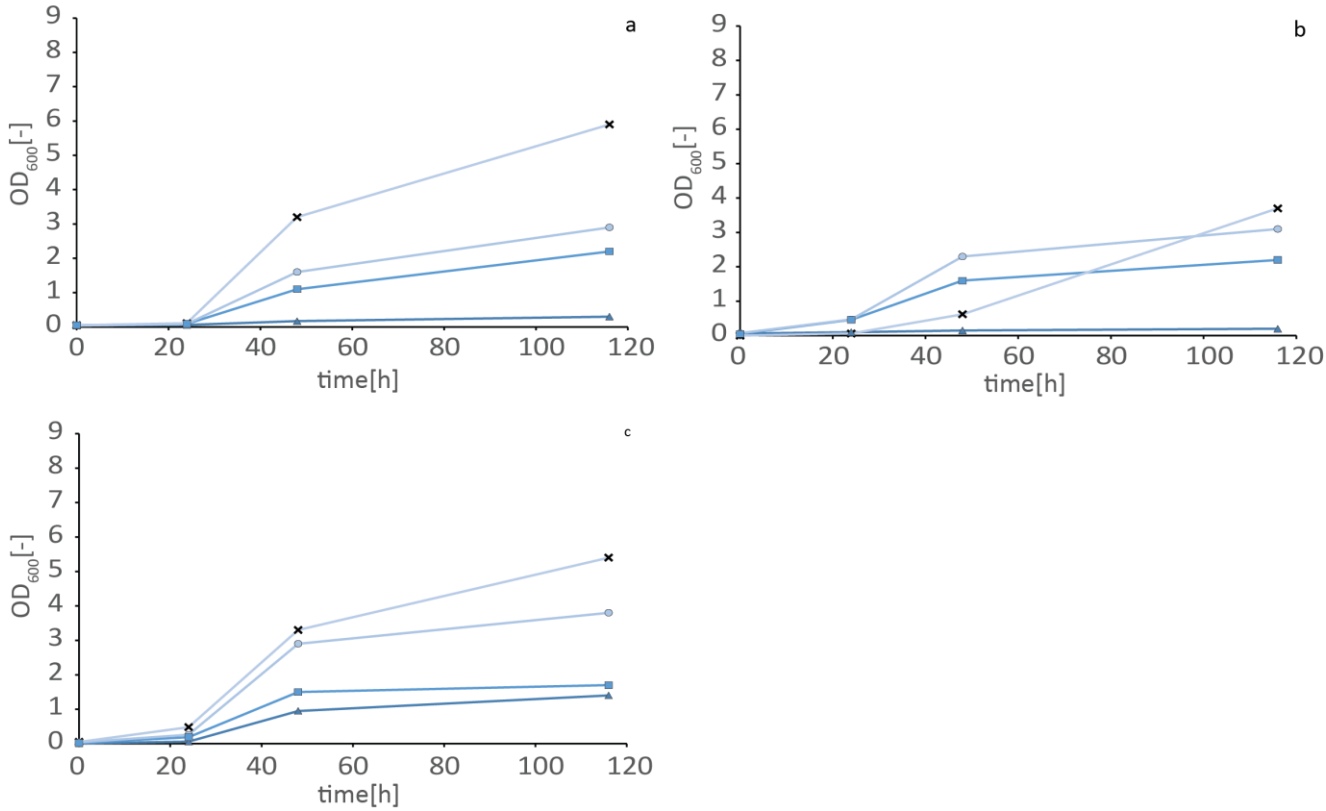


***Figure 7**: Growth in YEP media with different carbon sources. ▲YEP without any carbon source added, ■ YEP with 15 g/L raffinose, ● YEP with 15 g/L stachyose, ✕ YEP with 15 g/L RFO1. **(a)** S. cerevisiae (Fermentis US-05). **(b)** S. cerevisiae (Lutra Kveik). **(c)** L. thermotolerans (Lallemand Philly Sour).*

Figure 8 shows the reduction in area from the HPAEC-PAD analysis represented as a bar graph. *L. brevis* showed a reduction in verbascose, stachyose, and raffinose of 44.41%, 29.04%, and 58.54% respectively. For *L. buchneri* the reduction was 41.87%, 39.11%, and 26.84%, and for *L. plantarum* the reduction was 46.85%, 39.98%, and 76,80%. In all three fermentations, the sucrose was completely metabolized.

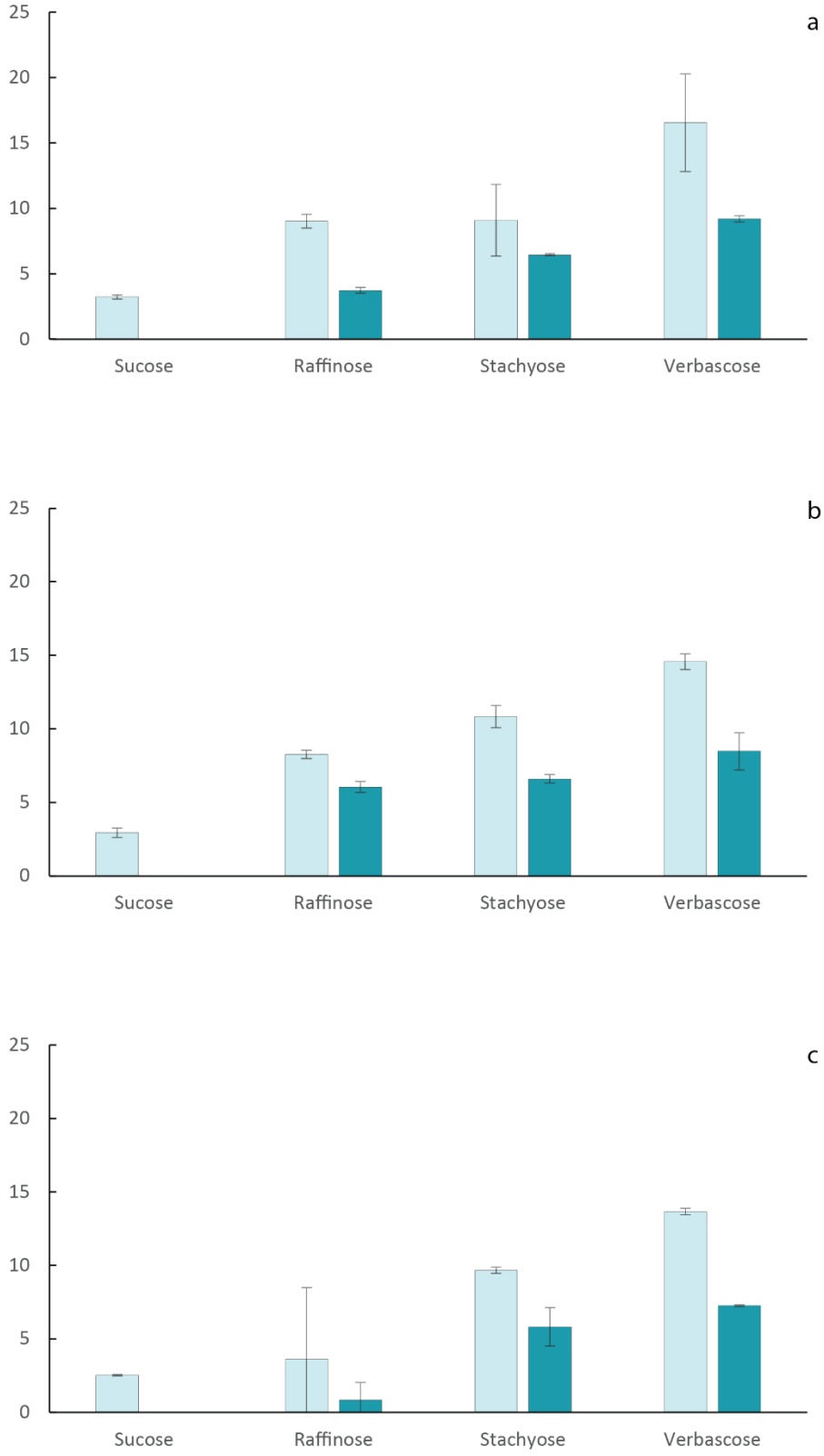


***Figure 8**: HPAEC-PAD results of 50 mL fermentations in MRS media containing 15 g/L RFO1. Measured in area (nC*min). Light bars represent area at 0 hours, and dark bars at 72 hours. **(a)** L brevis **(b)** L. buchneri **(c)** L. plantarum.*

3.2.2.2 Growth in beer media

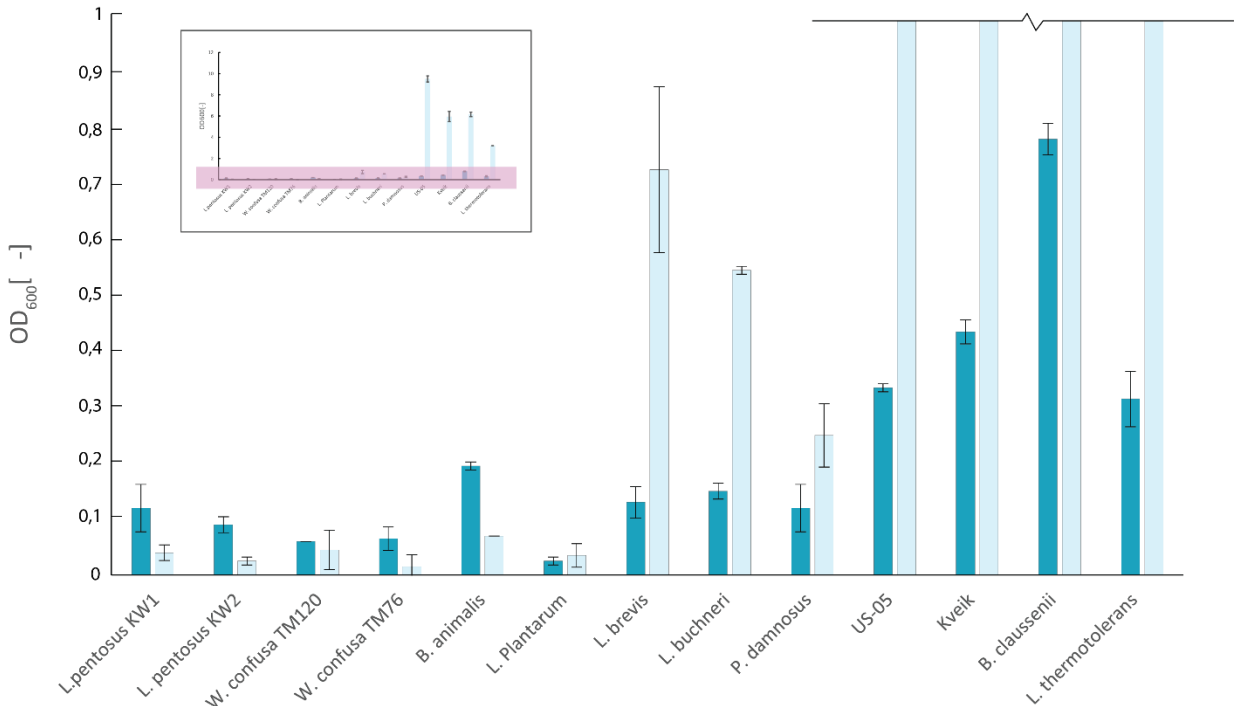


Figure 9: *Growth in beer media with 3.27% EtOH and 6.9 IBU. Dark bars are beer media without any RFOs added, light bars are with 15 g/L added RFO. Yeast strains measured after 99 hours; bacterial strains measured after 116 hours. Zoomed in on growth below 1 OD. US-05 with RFO (9.5 OD), Kveik with RFO (5.95 OD), B. claussenii (6.15 OD), and L. thermotolerans (3.2 OD), are all outside of focus area.*

When grown in beer media 1, containing 3.27% EtOH and with an IBU content of 6.9, none of the bacterial strains measured an OD above 1, with *L. brevis* coming the closest with an OD of 0.73 with the addition of RFO. Without RFO, none of the bacterial strains measured an OD above 0.2, as seen in figure 9. The growth was also limited for the yeasts when not utilizing RFO as a substrate, with *B. claussenii* performing the best with an OD of 0.78. However, with the addition of RFO, the yeasts grew better with *S. cerevisiae* performing the best with an OD of 9.5.

3.2.2.3 Stressors: Ethanol and IBU

In summary, an examination of the growth curves depicted in figure 10 demonstrates that the yeast strains exhibited a lower sensibility to variations in ethanol and IBU concentration within the medium, as opposed to their bacterial counterparts (figure 11). Disregarding the outlier in figure 10c, the average growth of the yeast strains, as quantified by OD at the culmination of the fermentation experiment, was 2.87. The best performing strain was *B. claussenii* with an average recorded OD of 4.43 (disregarding the outlier) after 166 h, as can be seen in figure 10c.

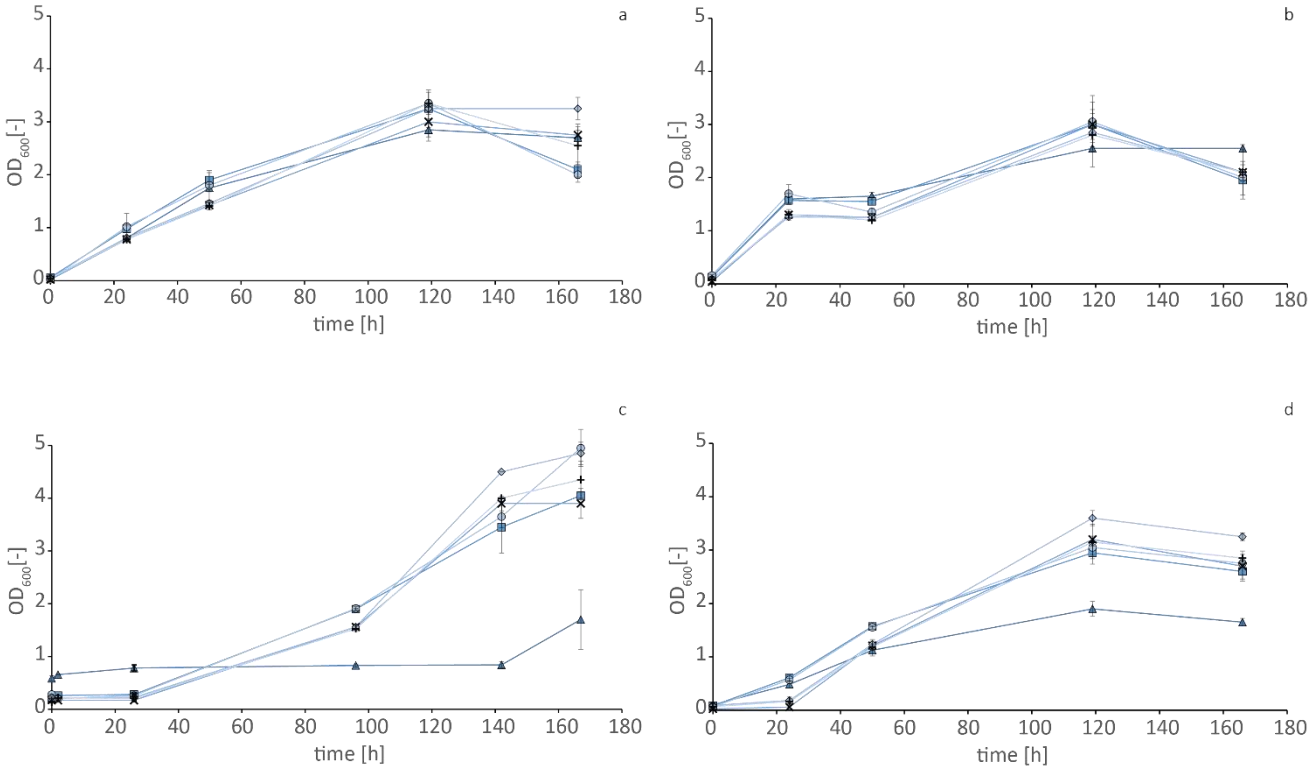


***Figure 10**: Growth of yeast strains in Beer Media 2 with different ethanol concentration and IBU. **(a)** S. cerevisiae US-05. **(b)** S. cerevisiae Lutra Kveik **(c)** B. claussenii **(d)** L. thermotolerans. ▲ 2.5% EtOH, 0 IBU, ■ 2.25% EtOH, 2.5 IBU, ● 2.25% EtOH, 5 IBU ✕ 4.5% EtOH, 0 IBU. ◆ 4.5% EtOH, 2.5 IBU. ✛ 4.5% EtOH, 5 IBU.*

Conversely, the bacterial stains displayed heightened susceptibility to the stressors, with the IBU values exerting a pronounced effect, as seen in figure 11 and 12. Examining the average growth curves of the bacterial strains in figure 12, gives an understanding of the influence of ethanol and IBU value in on the growth of Gram-positive bacteria. Although IBU serves as the principal inhibitor to growth, the concentration of ethanol also contributes to a lesser extent. On average, the growth of the bacteria measured in OD at the end of fermentation are summarized in table 16.

***Table 16:** Average OD_{600} values as presented in figure 12.*

2.25% EtOH, 0 IBU	2.25 EtOH, 2.5 IBU	2.5% EtOH, 5 IBU	4.5% EtOH, 0 IBU	4.5% EtOH, 2.5 IBU	4.5% EtOH, 5 IBU
1.38	0.64	0.37	1.2	0.77	0.43

W. confusa TM76, *L. brevis*, and *L. delbrueckii* results all showed a maximum growth of <=1 with 2.5% EtOH and 0 IBU being on top, with growth curves mirroring *W. confusa* TM120 in

figure 11c closely. or in the case of *L. plantarum* just above 1. They are excluded from figure 11, but can be seen in Appendix 6.15

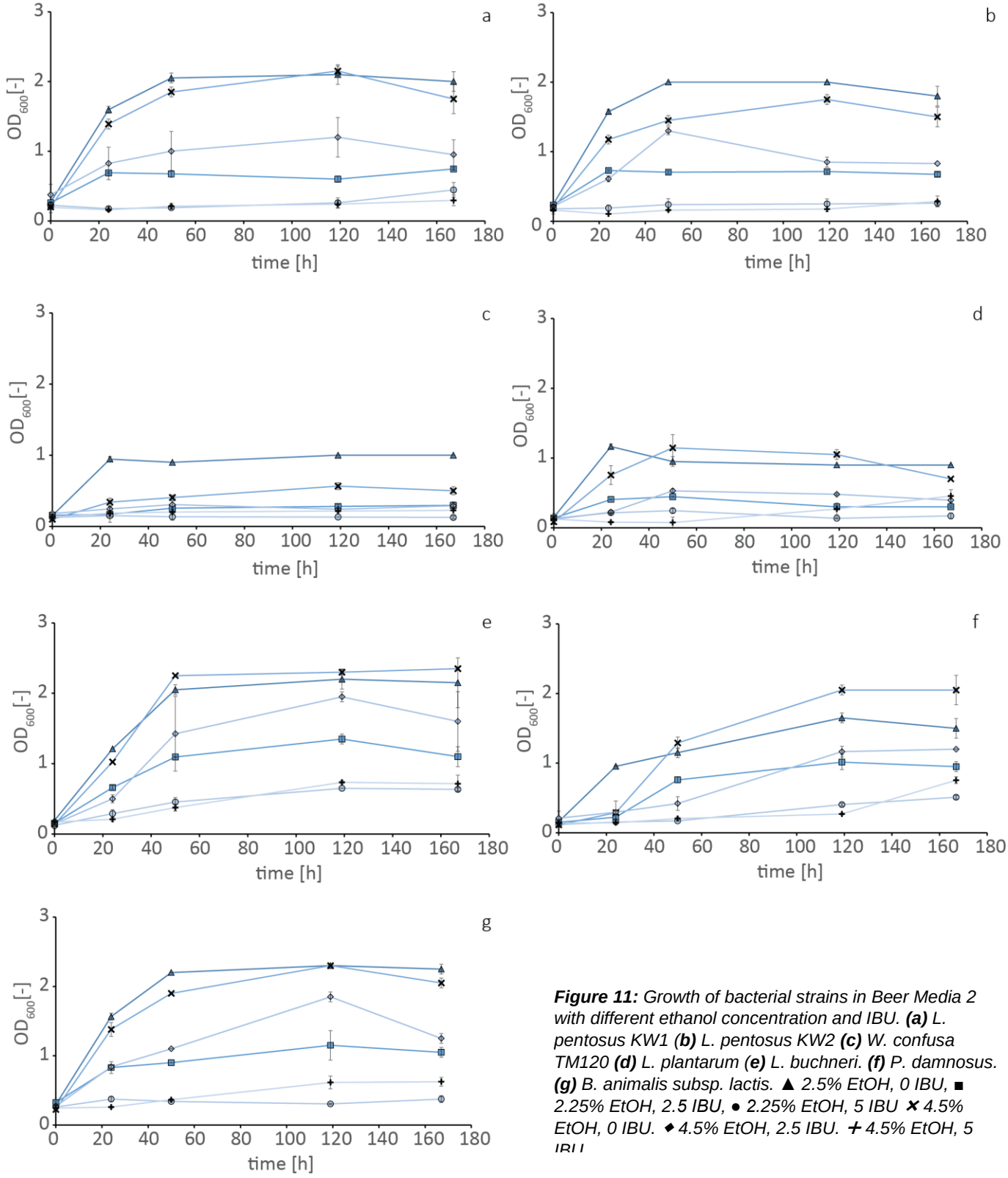


Figure 11: *Growth of bacterial strains in Beer Media 2 with different ethanol concentration and IBU.* ***(a)*** *L. pentosus KW1 (**b**) L. pentosus KW2 **(c)** W. confusa TM120 **(d)** L. plantarum (**e**) L. buchneri. **(f)** P. damnosus. **(g)** B. animalis subsp. lactis. ▲ 2.5% EtOH, 0 IBU, ■ 2.25% EtOH, 2.5 IBU, ● 2.25% EtOH, 5 IBU ✕ 4.5% EtOH, 0 IBU. ◆ 4.5% EtOH, 2.5 IBU. ✛ 4.5% EtOH, 5 IBU*

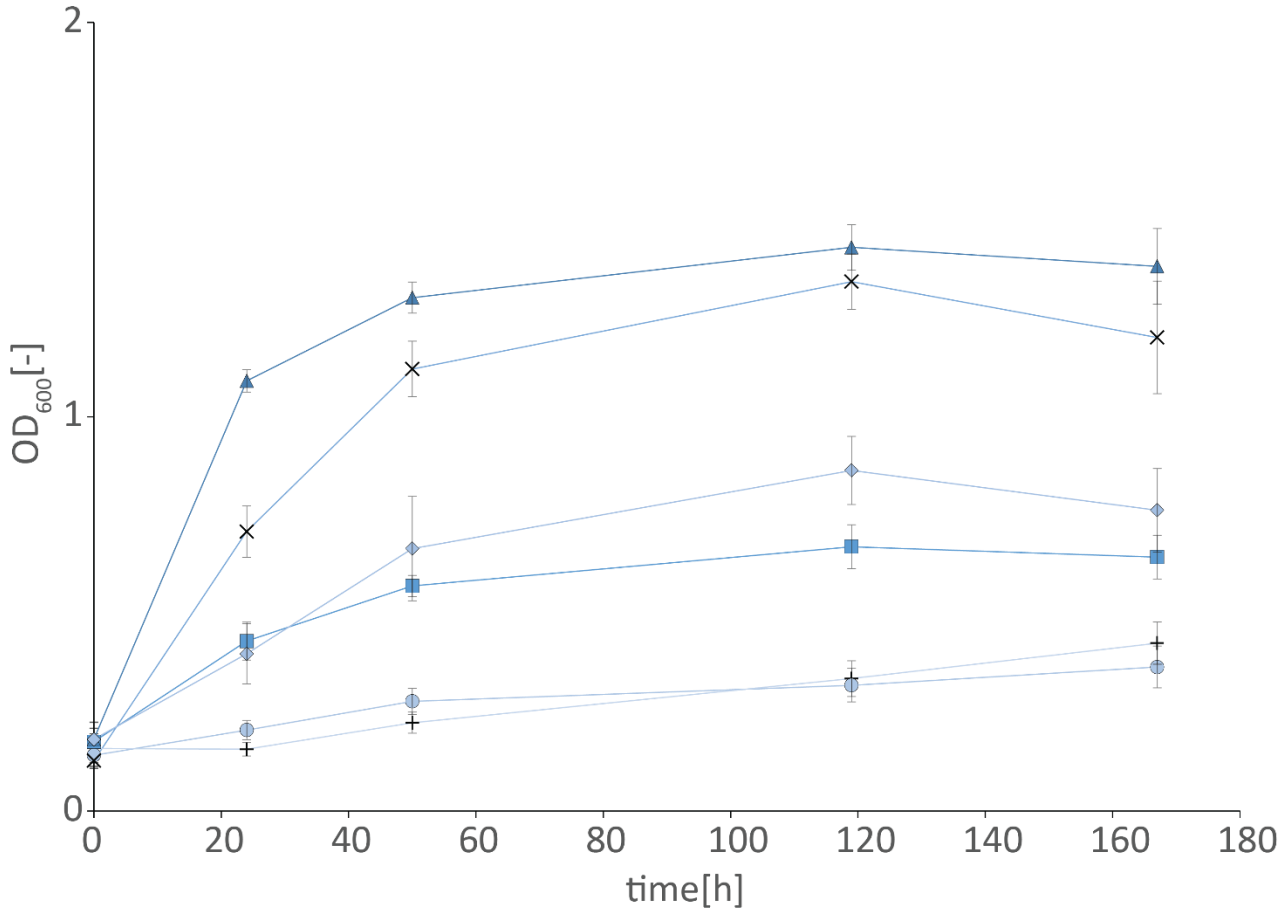


***Figure 12**: Average growth curves for all bacterial strains exposed to intrinsic ethanol and IBU stressors. ▲ 2.5% EtOH, 0 IBU, ■ 2.25% EtOH, 2.5 IBU, ● 2.25% EtOH, 5 IBU ✕ 4.5% EtOH, 0 IBU. ◆ 4.5% EtOH, 2.5 IBU. ✛ 4.5% EtOH, 5 IBU.*

In the case of the specific strains used in the second trial testing for stressors, elevating the ethanol concentration to 6% did not notably impact the growth of either yeast or bacterial strains, as evidenced by figures 13 and 14.

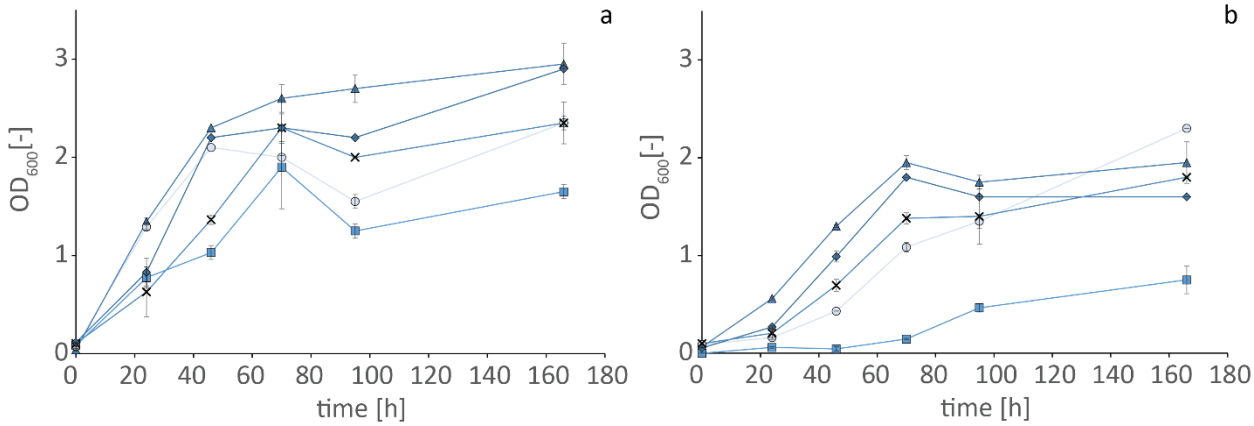


***Figure 13**: Second trial of yeast growth in media containing intrinsic stressors **(a)** S. cerevisiae US-05 **(b)** L. thermotolerans. ● 3.5% EtOH, 3.75 IBU. ▲ 4.5% EtOH, 0 IBU. ◆ 6% EtOH, 0 IBU. ✕ 6% EtOH, 2.5 IBU. ■ 6% EtOH, 5 IBU.*

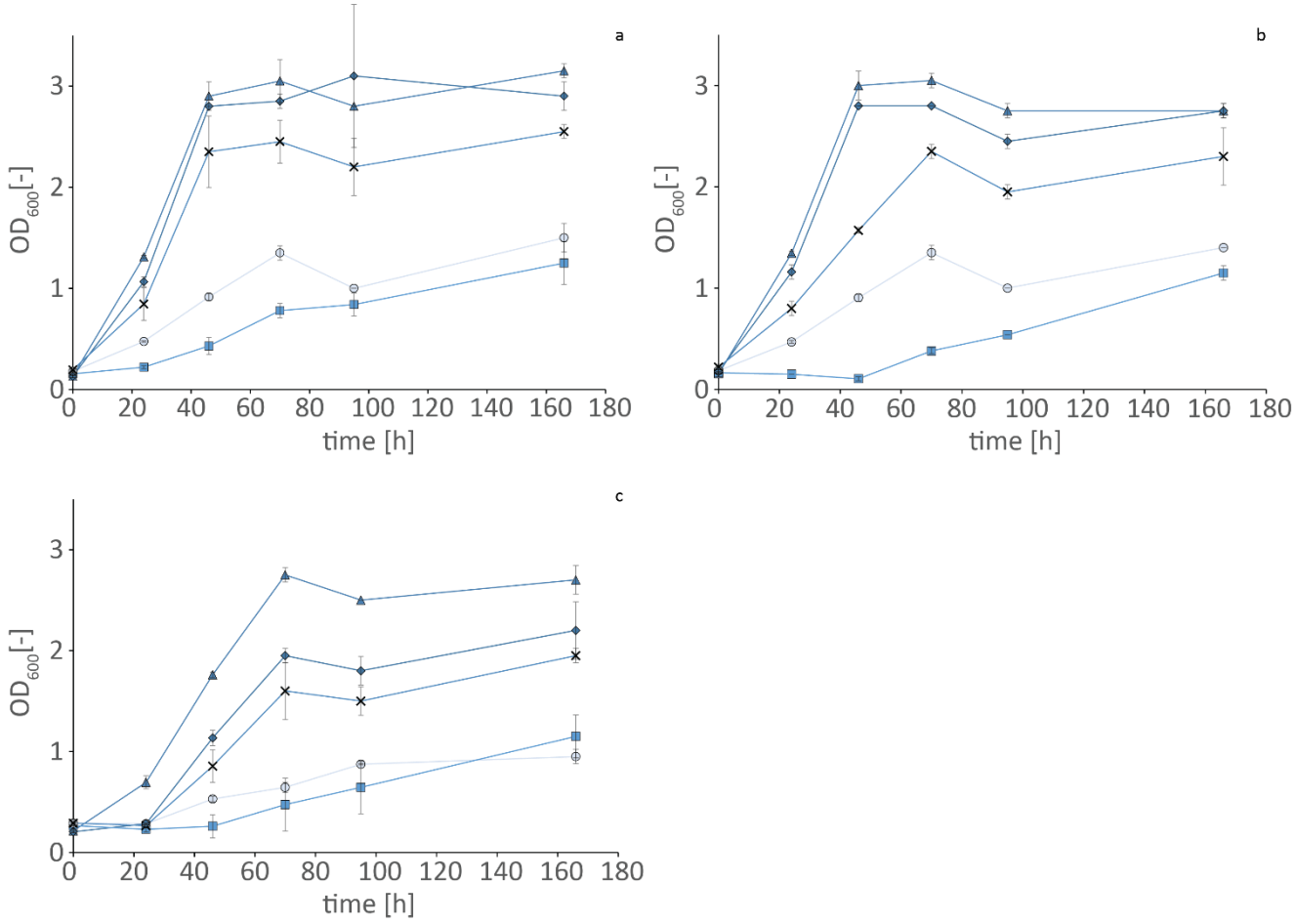


Figure 14: *Second trial of yeast growth in media containing intrinsic* ***(a)*** *L. brevis.* ***(b)*** *L. buchneri.* ***(c)*** *B. animalis subsp. lactis. ● 3.5% EtOH, 3.75 IBU. ▲ 4.5% EtOH, 0 IBU. ◆ 6% EtOH, 0 IBU. ✕ 6% EtOH, 2.5 IBU. ■ 6% EtOH, 5 IBU.*

3.2.3 Pea Beer

In brewing the beer media throughout all batches, a discrepancy between the estimated brewhouse efficiency and the actual brewhouse efficiency was recorded. The estimated efficiency in the Brewfather software was 72.8%, The actual brewhouse efficiency in the four pea beers, varied between 60.24-71.60%.

During certain bacterial fermentations, a pellicle may form on the surface of the beer. This was observed in all mixed fermentations in the 5-liter fermenters, except for the one fermented with *B. claussenii*, *L. cremoris* M121, and *L. brevis*. A similar observation was made in all fermentations in Pea Beer V3.

3.2.3.1 Pea Beer V1

Figure 15 displays the key parameters from the Anton Paar AAS analysis of Pea Beer V1. The one fermented with *S. cerevisiae* US-05 and *L. brevis* produced the least amount of EtOH at 3.31%. In comparison the other fermentations produced 3.5%±0.5. This is also reflected in the ADF and remaining sugar concentration, where US-05 and *L. brevis* ended with 73.57% attenuation and over 23 g/L of sugar at the end of fermentation. This was the lowest of all the samples tested. *L. thermotolerans* performed slightly better than US-05 and *L. Brevis* in regard to attenuation. However, these two batches were the once that produced the most acid, reflected in the pH, with *L. thermotolerans* lowering the pH to 3.64 and Us-05 with *L. brevis* lowering it to 3.76. All the other samples produced similar results to each other in all the parameters, except for US-05 co-fermented with *L. buchneri*, lowering the pH to 3.94 in comparison to 4.15±0.03 in the others.

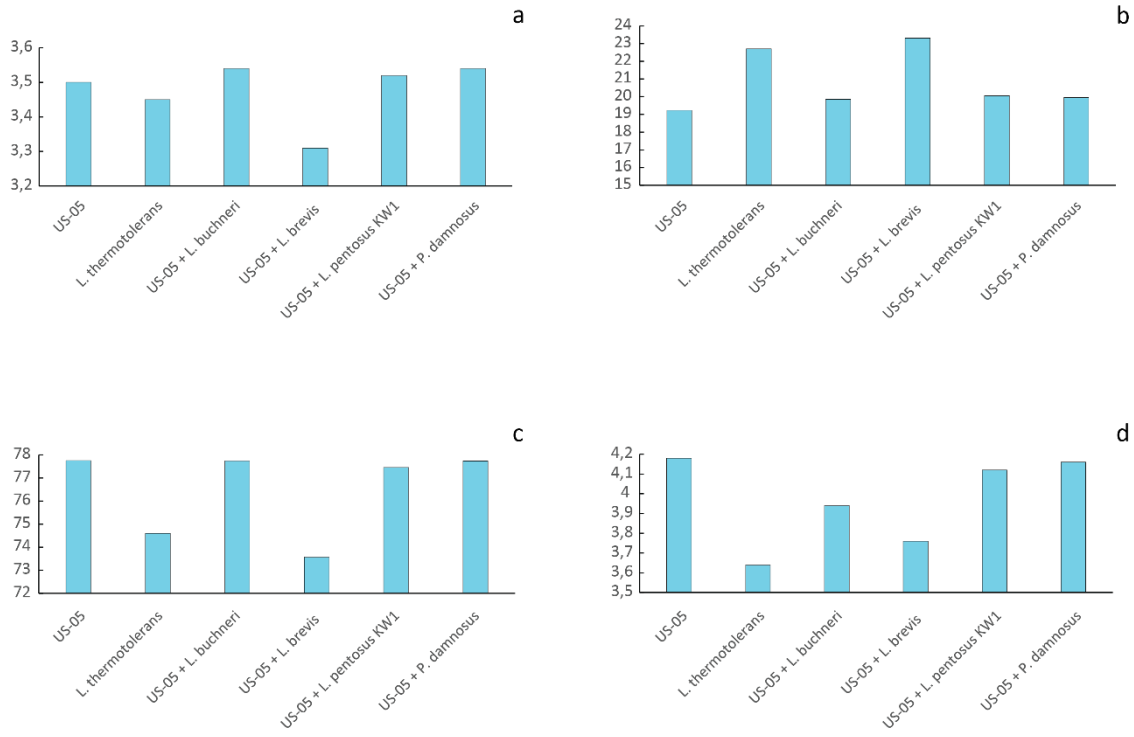
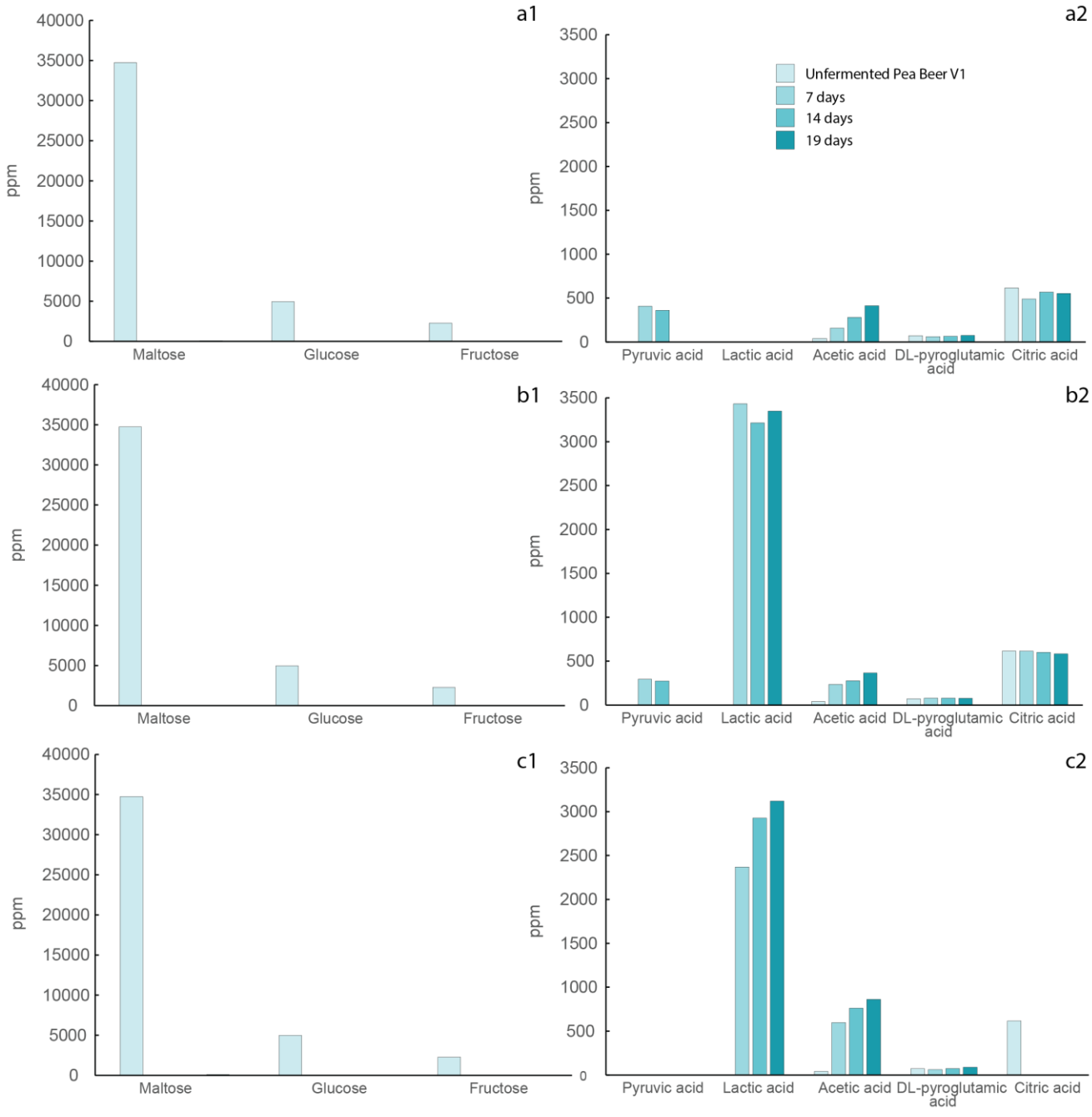


***Figure 15**: Data from Anton Paar Alcolyzer Pea Beer V1. **(a)** Ethanol (% v/v). **(b)** Sugar concentration (g/L). **(c)** ADF (% w/w). **(d)** pH value. Fermented with the addition of 15 g/L of RFO1 fraction.*

The analyses of sugars and organic acids using HPLC and volatile compounds using HSGC in Pea Beer V1 are presented in figures 16 and 17. Throughout all fermentations, simple sugars were metabolized by day 7, displaying indistinguishable results across the various strains. This pattern persisted in Pea Beer V2 and V3. *L. thermotolerans* and *L. brevis* were the most effective strains, producing over 3000 ppm of lactic acid. *L. buchneri* generated more than 1500 ppm, while *L. pentosus* KW1 and *P. damnosus* produced over 1000 ppm. *L. brevis* yielded the highest acidic acid concentration at 862 ppm and was the sole strain capable of metabolizing citric acid as can be seen in figure 16 c2.



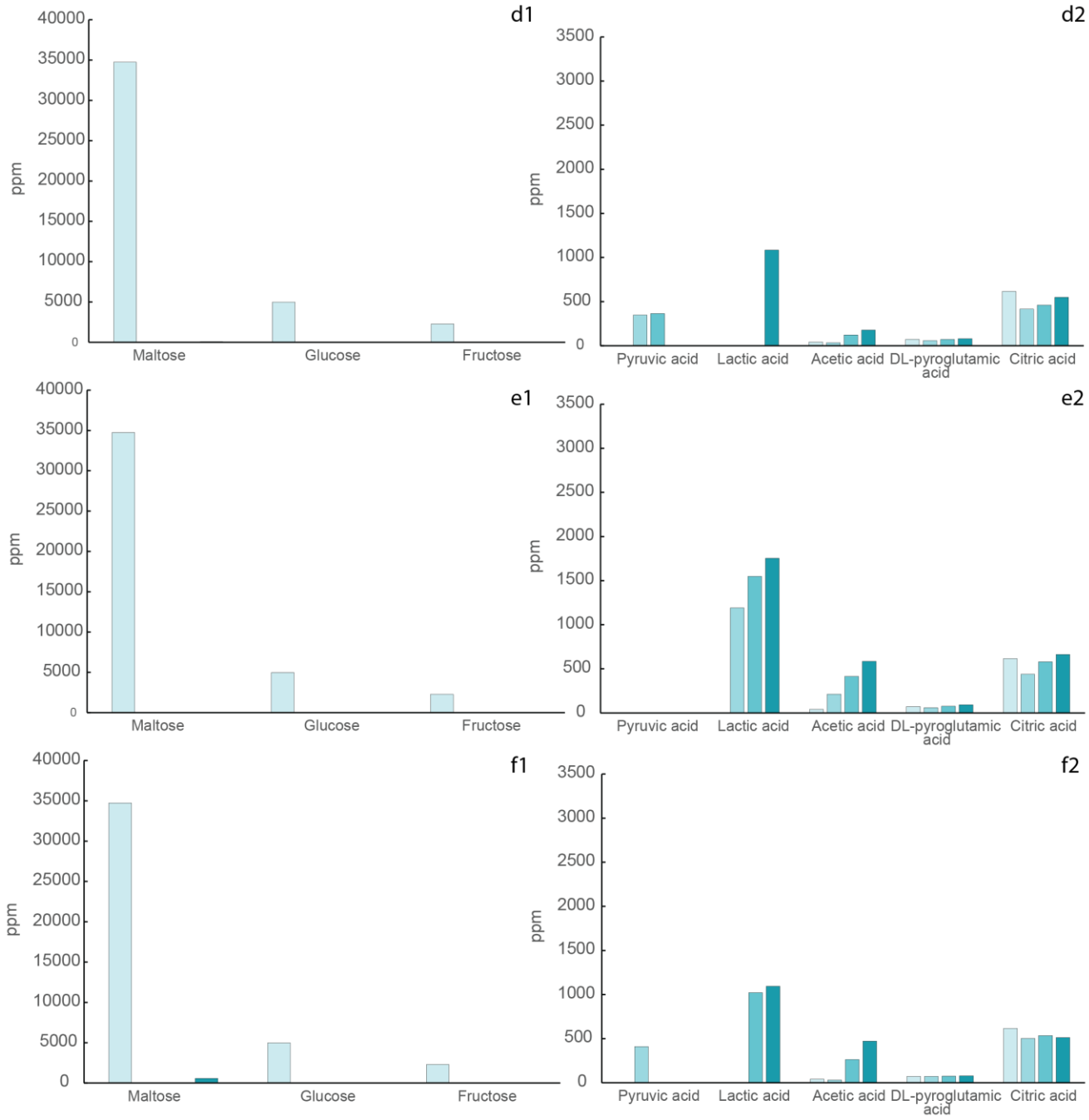
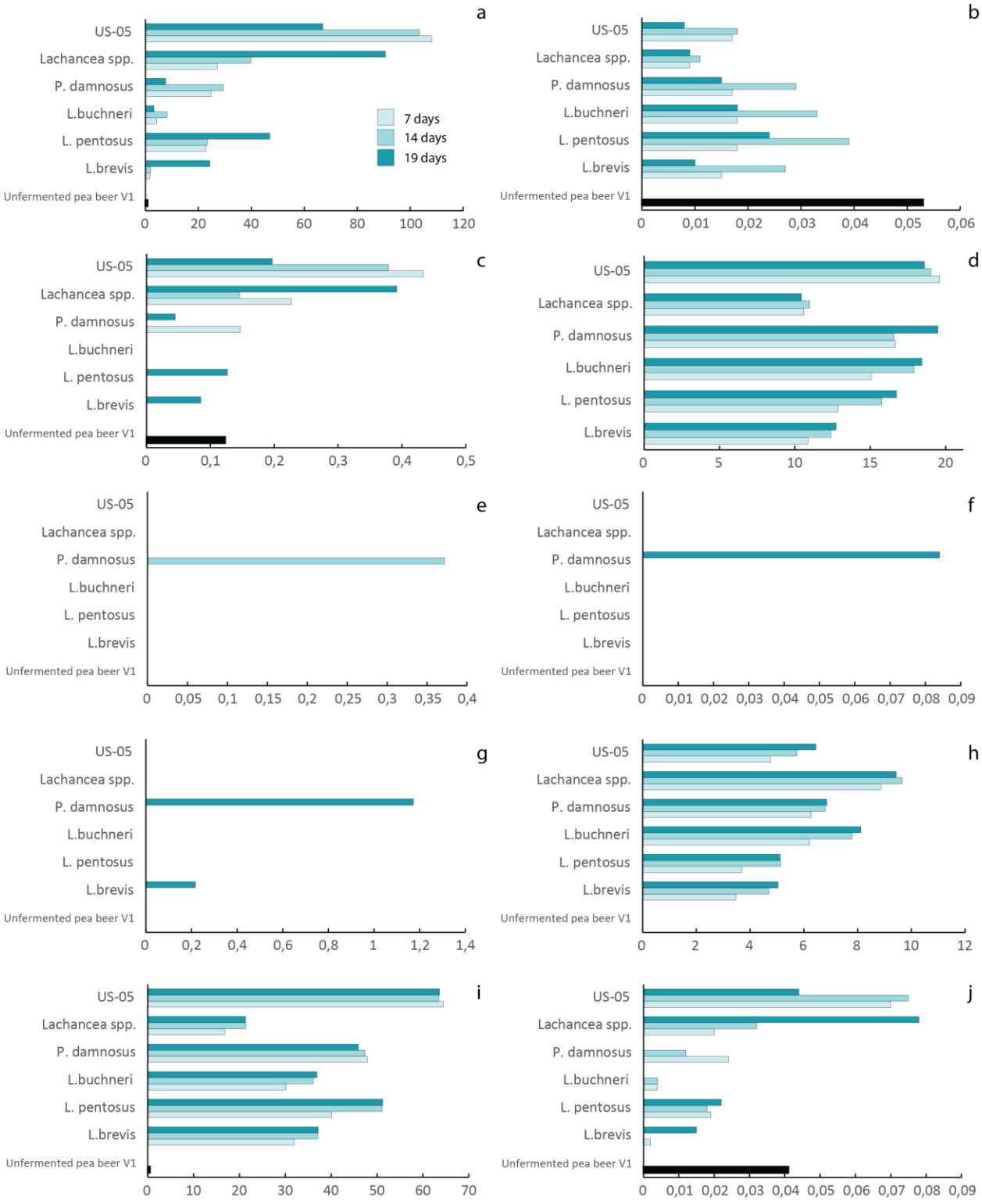


Figure 16: *HPLC analysis of sugars and organic acids in Pea Beer V1 presented in as a graphical representation. The left portion of the graph delineates the concentration of sugars during the fermentation process, at 0-, 7-, 14- and 19-days. Concurrently, the right segment of the graph illustrates the concentration of organic acids across the same time points.* ***(a)*** *S. cerevisiae US-05* ***(b)*** *L. thermotolerans* ***(c)*** *S. cerevisiae US-05 and L. brevis* ***(d)*** *S. cerevisiae US-05 and L. pentosus KW1* ***(e)*** *S. cerevisiae US-05 and L. buchneri* ***(f)*** *S. cerevisiae and P. damnosus.*

In terms of ester production, all strains exhibited relatively low levels. *L. thermotolerans* generated the highest acetaldehyde concentration at 90 ppm, while *L. buchneri* produced the most isoamyl acetate and isobutyl acetate at 0.176 ppm and 0.063 ppm, respectively. All strains yielded comparable amounts of higher alcohols.


a
US-05
Lachancea spp.
P. damnosus
L.buchneri
L. pentosus
L.brevis
Unfermented pea beer V1
7 days
14 days
19 days
0 20 40 60 80 100 120
b
US-05
Lachancea spp.
P. damnosus
L.buchneri
L. pentosus
L.brevis
Unfermented pea beer V1
0 0,01 0,02 0,03 0,04 0,05 0,06
c
US-05
Lachancea spp.
P. damnosus
L.buchneri
L. pentosus
L.brevis
Unfermented pea beer V1
0 0,1 0,2 0,3 0,4 0,5
d
US-05
Lachancea spp.
P. damnosus
L.buchneri
L. pentosus
L.brevis
0 5 10 15 20
e
US-05
Lachancea spp.
P. damnosus
L.buchneri
L. pentosus
L.brevis
Unfermented pea beer V1
0 0,05 0,1 0,15 0,2 0,25 0,3 0,35 0,4
f
US-05
Lachancea spp.
P. damnosus
L.buchneri
L. pentosus
L.brevis
Unfermented pea beer V1
0 0,01 0,02 0,03 0,04 0,05 0,06 0,07 0,08 0,09
g
US-05
Lachancea spp.
P. damnosus
L.buchneri
L. pentosus
L.brevis
Unfermented pea beer V1
0 0,2 0,4 0,6 0,8 1 1,2 1,4
h
US-05
Lachancea spp.
P. damnosus
L.buchneri
L. pentosus
L.brevis
Unfermented pea beer V1
0 2 4 6 8 10 12
i
US-05
Lachancea spp.
P. damnosus
L.buchneri
L. pentosus
L.brevis
Unfermented pea beer V1
0 10 20 30 40 50 60 70
j
US-05
Lachancea spp.
P. damnosus
L.buchneri
L. pentosus
L.brevis
Unfermented pea beer V1
0 0,01 0,02 0,03 0,04 0,05 0,06 0,07 0,08 0,09

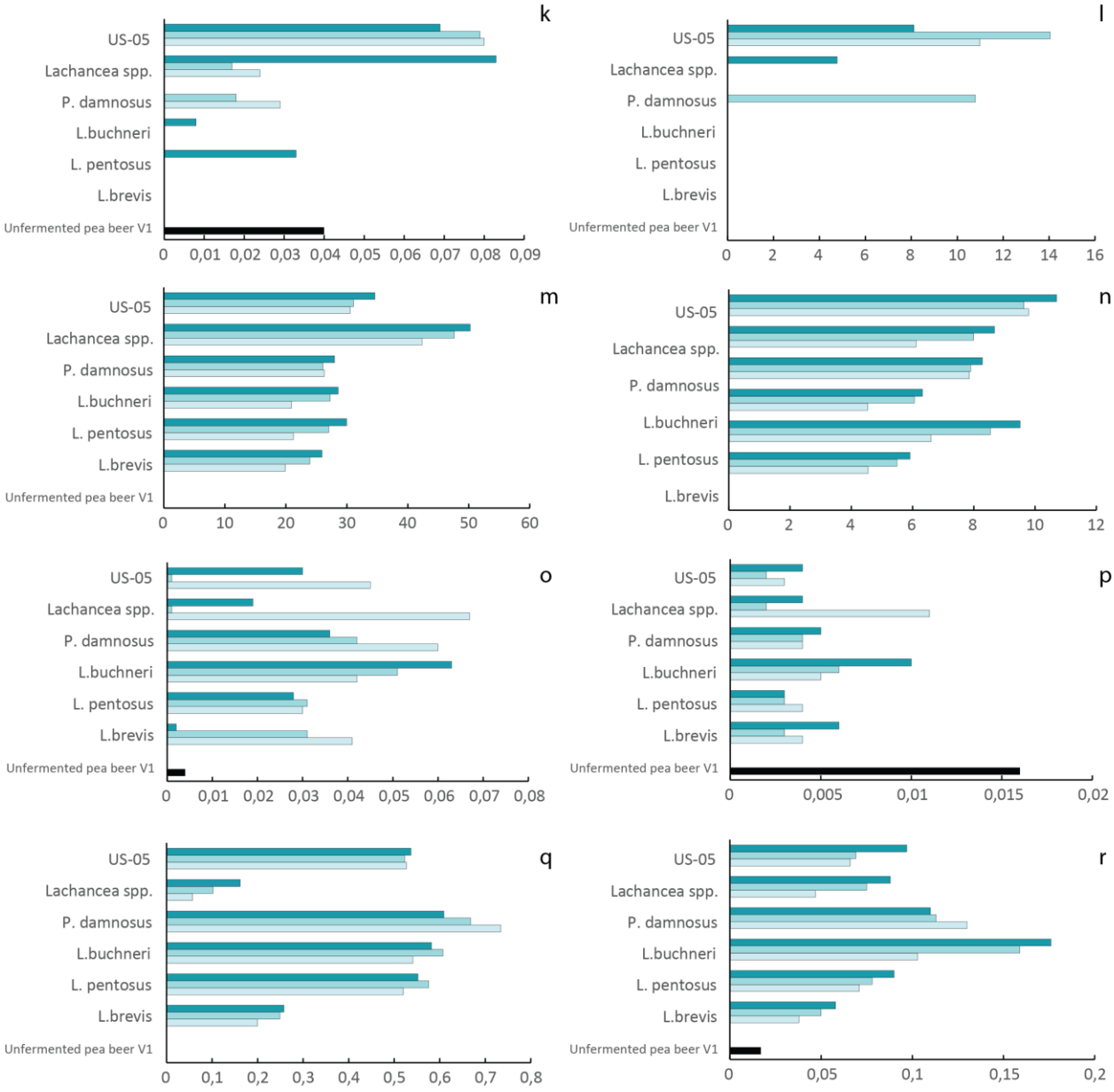


Figure 17: *Concentration of volatile compounds in Pea Beer V1 in ppm at 0-, 7-, 14-, and 19-days.* ***(a)*** *Acetaldehyde* ***(b)*** *Dimethyl sulfide* ***(c)*** *2-methyl-propanal* ***(d)*** *1-propanol* ***(e)*** *Diacetyl* ***(f)*** *2-butanon* ***(g)*** *2-butanol* ***(h)*** *Ethyl acetate* ***(i)*** *2-methyl-1-propanol* ***(j)*** *3-methyl-butanal* ***(k)*** *2-methyl-1-butanal* ***(l)*** *Acetoin* ***(m)*** *3-methyl-1-butanol* ***(n)*** *2-methyl-1-butanol* ***(o)*** *Isobutyl acetate* ***(p)*** *Hexanal* ***(q)*** *2-hexanol* ***(r)*** *Isoamyl acetate.*

Concerning aldehyde production, strains could be categorized into three groups. The first group, consisting of *S. cerevisiae* and *L. thermotolerans*, generated high aldehyde concentrations: 67-90 ppm acetaldehyde, 0.044-0.078 ppm 3-methyl-butanal, 0.069-0.083 ppm 2-methyl-butanal, and 0.197-0.392 ppm 2-methyl-propanal. The second group, including *L. pentosus* KW1 and *L. brevis*, produced moderate aldehyde amounts: 24-46 ppm

acetaldehyde, 0.015-0.022 ppm 3-methyl-butanal, 0-0.033 ppm 2-methyl-butanal, and 0.085-0.127 ppm 2-methyl-propanal. The third group, featuring *P. damnosus* and *L. buchneri*, generated minimal to no aldehydes. None of the strains produced sulfur compounds or diacetyl above sensory threshold levels (Table 18), except for the 14-day sample of *P. damnosus*.

3.2.3.2 Pea Beer V2

When running the Anton Paar analysis for the Pea Beer V2 (figure 18) sample fermented with *L. thermotolerans* the container exploded during testing, not yielding any results. Out of the four samples not exploding, the one fermented with *B. claussenii*, *L. cremoris* M121, and *L. brevis* was the outlier, only producing 2.92% EtOH in comparison to the others producing 3.8%±0.03, showing a poor attenuation at 59.87% in comparison to the rest at 75-76%. However, it did produce the most acid, resulting in a pH of 3.52, while none of the other batches produced a pH lower than 4.0.

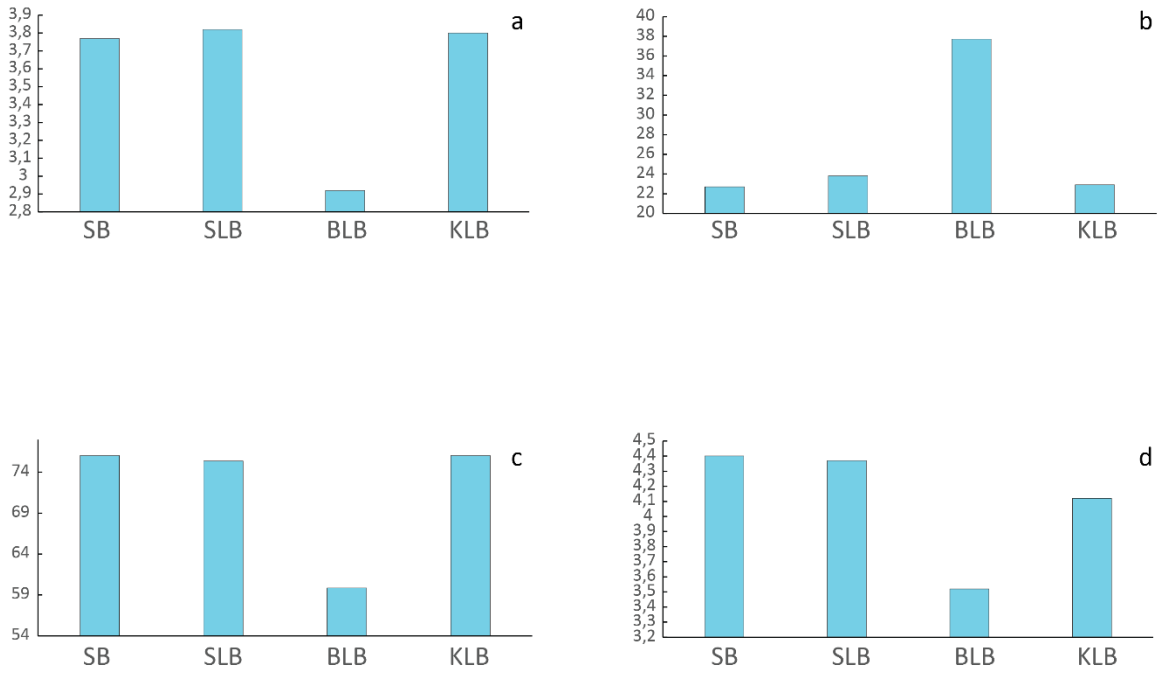
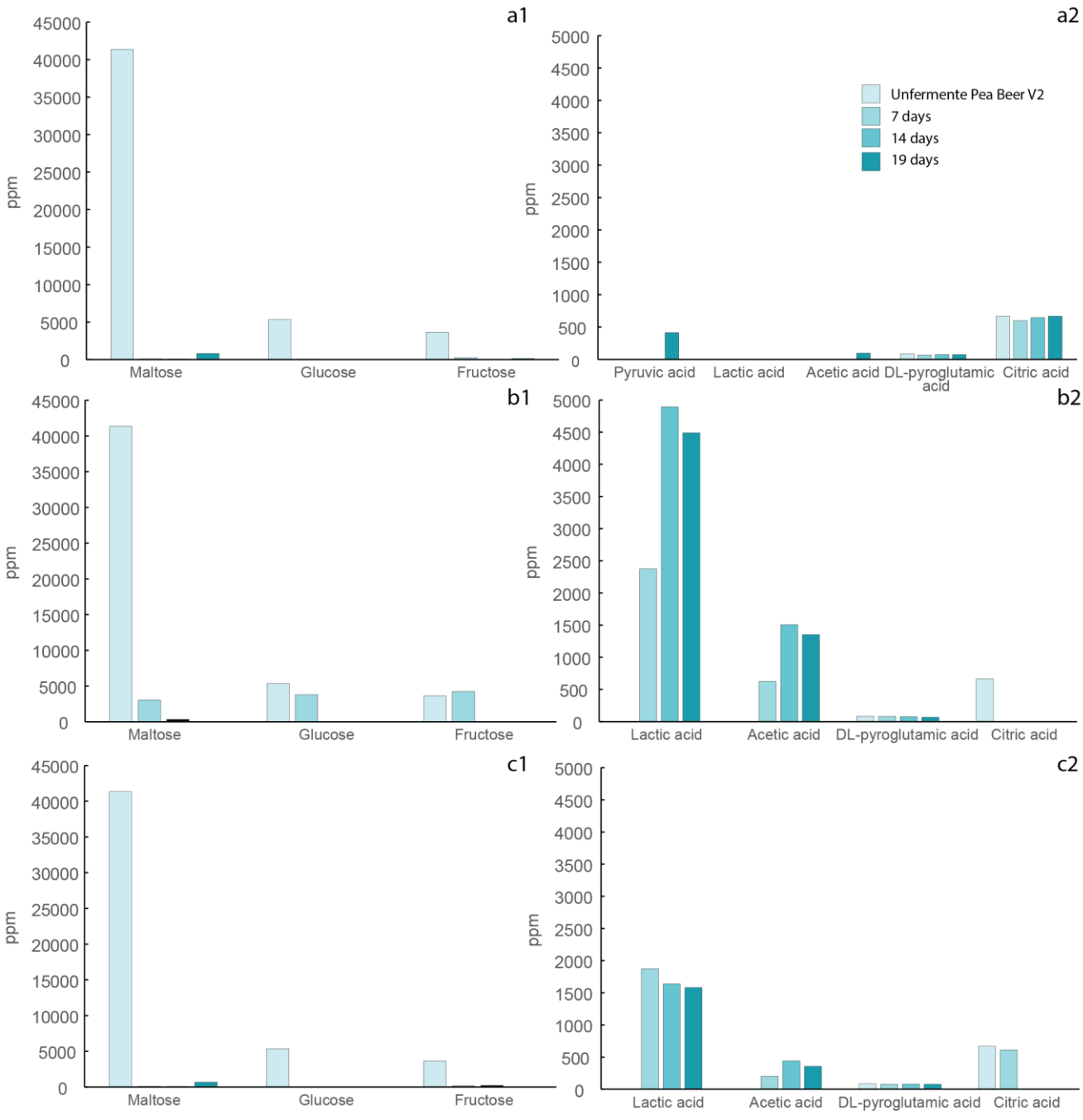


Figure 18: *Data from Anton Paar Alcolyzer Pea Beer V2.* ***(a)*** *Ethanol (% v/v).* ***(b)*** *Sugar concentration (g/L).* ***(c)*** *ADF (% w/w).* ***(d)*** *pH value. Fermented with the addition of 15 gIL of RFO1 fraction.* ***(SB)*** *S. cerevisiae US-05 with B. animalis subsp. Lactis* ***(SLB)*** *S. cerevisiae with L. cremoris M121 and L. buchneri* ***(BLB)*** *B. claussenii with L. cremoris M121 and L. brevis* ***(KLB)*** *Kveik with L. cremoris and L. brevis.*

The evaluation of sugars and organic acids using HPLC and volatile compounds using HSGC in Pea Beer V2 are illustrated in Figures 19 and 20. The most notable batch was the one fermented with BLB, generating a lactic acid concentration of 4489 ppm and an acetic acid concentration of 1352 ppm. Most of the other batches yielded similar results, with lactic acid concentrations exceeding 1500 ppm at the end of fermentation and low to moderate levels of acetic acid (275-609 ppm). The batch fermented with *B. animalis subsp. Lactis*, however, exhibited a near-complete failure to produce any acid.



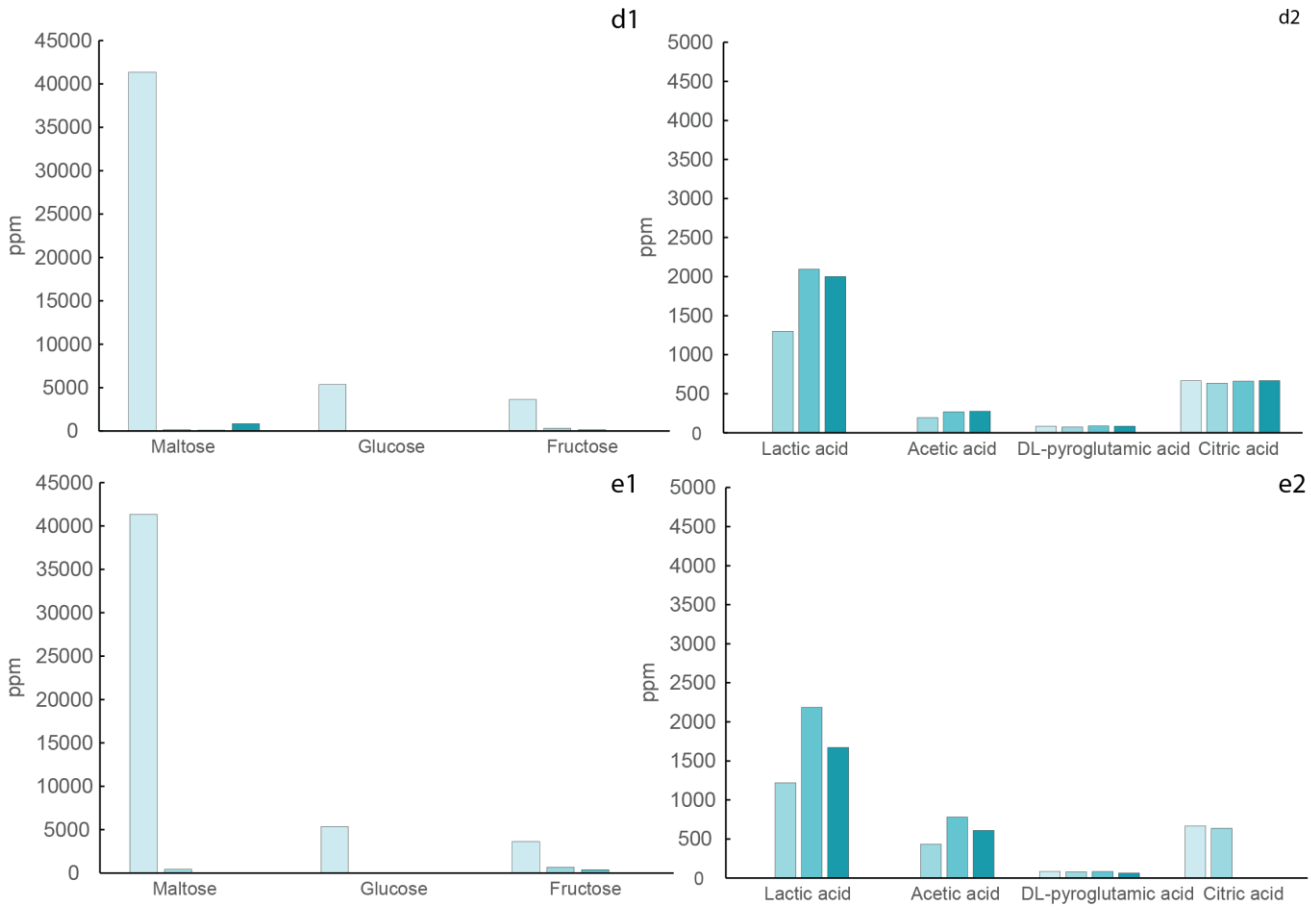
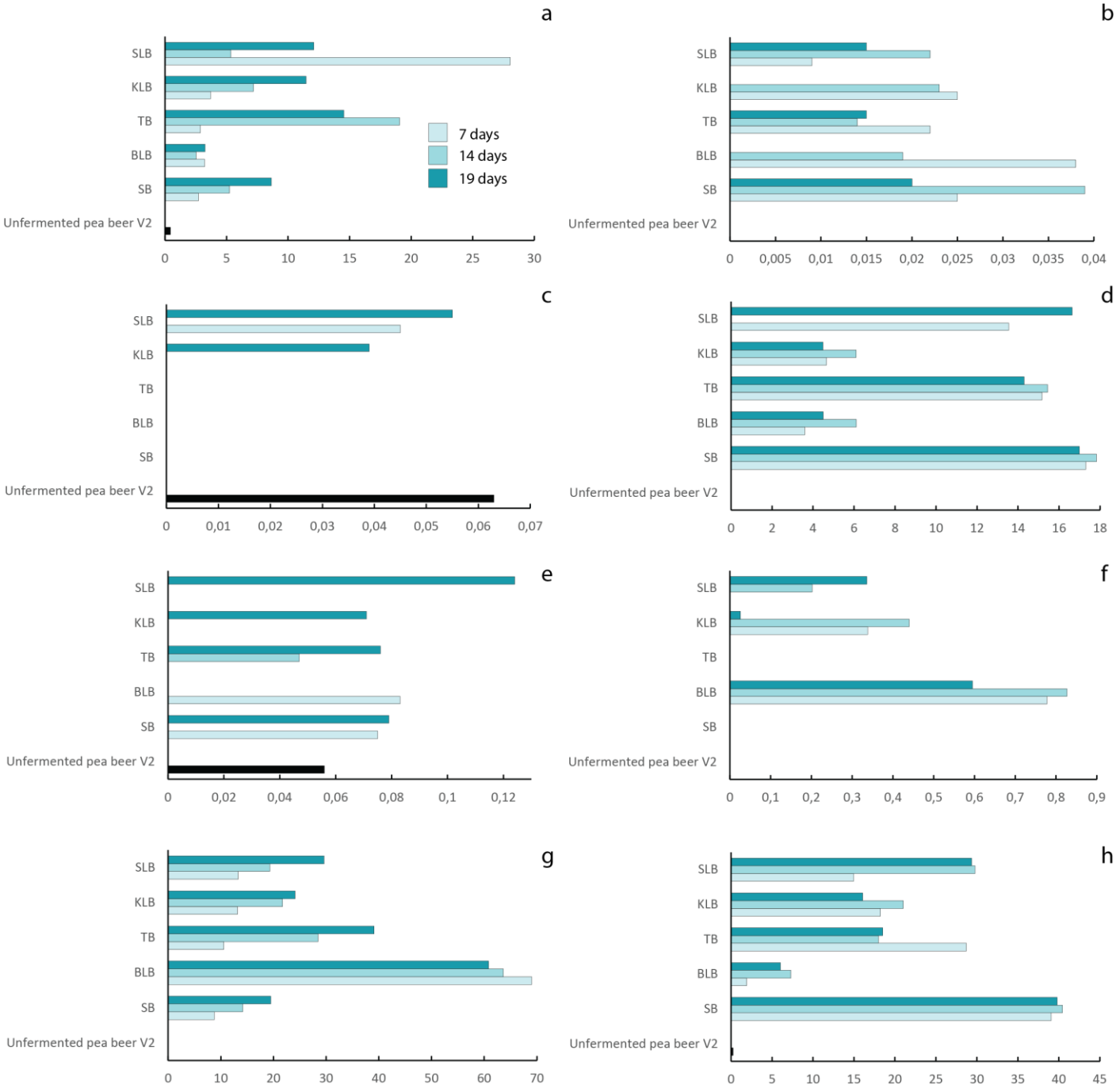


Figure 19: *HPLC analysis of sugars and organic acids in Pea Beer V2 presented in as a graphical representation. The left portion of the graph delineates the concentration of sugars during the fermentation process, at 0-, 7-, 14- and 19-days. Concurrently, the right segment of the graph illustrates the concentration of organic acids across the same time points* ***(a)*** *S. cerevisiae US-05 and B. animalis subsp. lactis* ***(b)*** *B. claussenii, L. cremoris M121, and L. brevis* ***(c)*** *S. cerevisiae US-05, L. cremoris M121, and L. buchneri* ***(d)*** *L. thermotolerans and l. buchneri* ***(e)*** *S. cerevisiae Lutra Kveik and L. brevis.*

In terms of ester production, isobutyl acetate and isoamyl acetate concentrations remained below the sensory threshold (Table 18). The batch fermented using BLB generated the highest ethyl acetate concentration at 60 ppm. All strains produced comparable amounts of higher alcohols, all of which remained below the sensory threshold.

Most of the batches exhibited acetaldehyde concentrations around the sensory threshold, ranging from 8.6 to 14.5 ppm. The exception was the BLB batch, which produced only 3.2 ppm. KLB, TB, and BLB generated concentrations of 2-methyl-butanal above the sensory threshold (0.081-0.108 ppm), while the two batches containing *S. cerevisiae* US-05 produced none. BLB was the outlier in the production of 3-methyl-butanal, yielding 0.08 ppm.

SLB, TB, and SB were unable to eliminate DMS from the fermented beer, resulting in trace amounts between 0.015 and 0.2 ppm. Diacetyl was not detected in any of the samples.


a
SLB
KLB
TB
BLB
SB
Unfermented pea beer V2
7 days
14 days
19 days
0 5 10 15 20 25 30
b
0 0,005 0,01 0,015 0,02 0,025 0,03 0,035 0,04
c
0 0,01 0,02 0,03 0,04 0,05 0,06 0,07
d
0 2 4 6 8 10 12 14 16 18
e
0 0,02 0,04 0,06 0,08 0,1 0,12
f
0 0,1 0,2 0,3 0,4 0,5 0,6 0,7 0,8 0,9
g
0 10 20 30 40 50 60 70
h
0 5 10 15 20 25 30 35 40 45

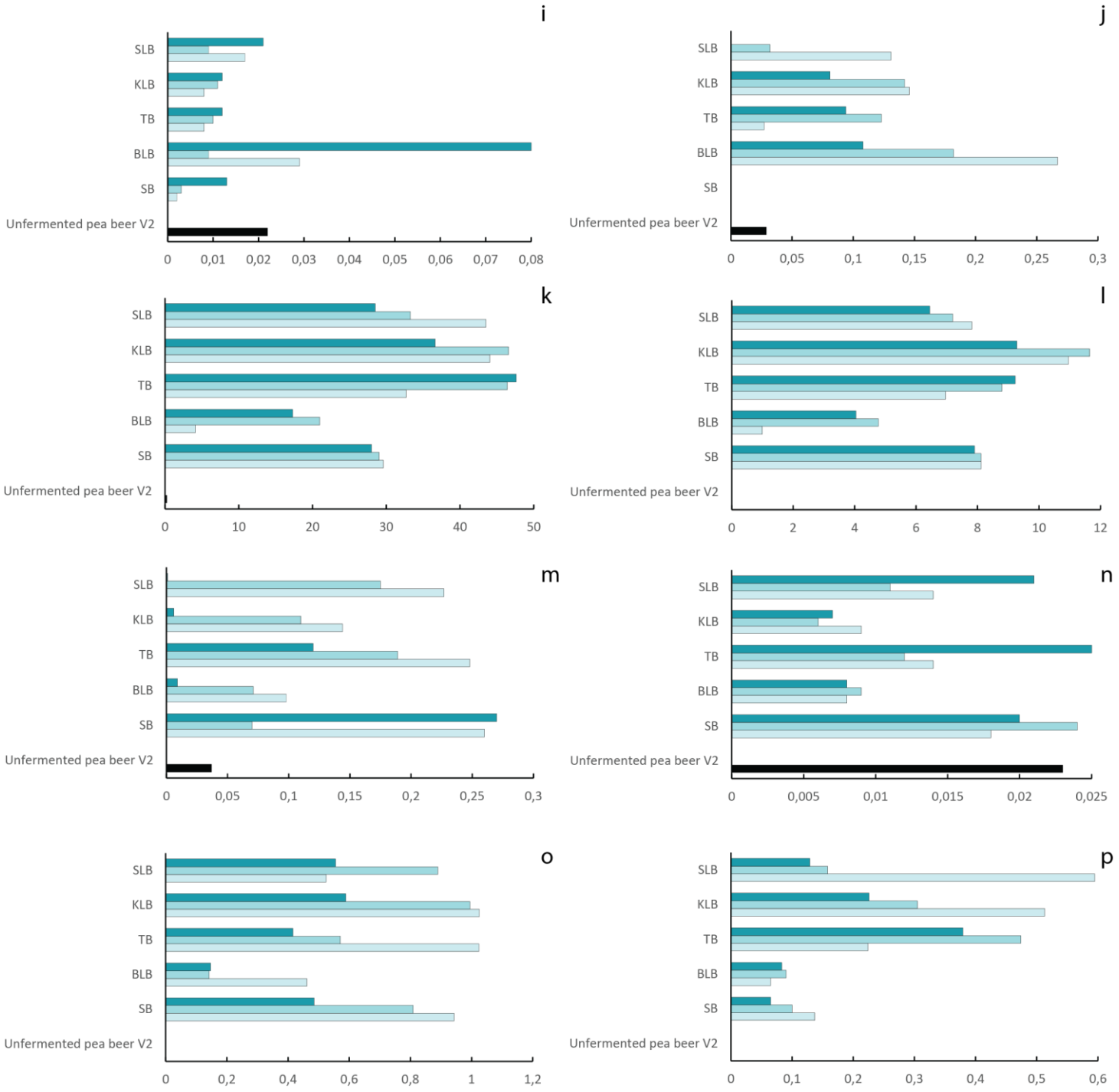


Figure 20: *Concentration of volatile compounds in Pea Beer V2 in ppm at 0-, 7-, 14-, and 19-days.* ***(a)*** *Acetaldehyde* ***(b)*** *Dimethyl sulfide* ***(c)*** *2-methyl-propanal* ***(d)*** *1-propanol* ***(e)*** *Diacetyl* ***(f)*** *2-butanon* ***(g)*** *2-butanol* ***(h)*** *Ethyl acetate* ***(i)*** *2-methyl-1-propanol* ***(j)*** *3-methyl-butanal* ***(k)*** *2-methyl-1-butanal* ***(l)*** *Acetoin* ***(m)*** *3-methyl-1-butanol* ***(n)*** *2-methyl-1-butanol* ***(o)*** *Isobutyl acetate* ***(p)*** *Hexanal* ***(q)*** *2-hexanol* ***(r)*** *Isoamyl acetate.*

3.2.3.3 Pea Beer V3

The same thing happened with the Pea Beer V3 samples fermented using *S. cerevisiae* US-05 with *L. pentosus* KW1, and *S. cerevisiae* Lutra Kveik with *L. cremoris* M121 and *L. brevis* as it did for the sample containing *L. thermotolerans* in Pea Beer V2, the bottle containing the sample exploded when applying pressure in the Anton Paar AAS yielding no results.

Again *B. claussenii*, *L. cremoris* M121, and *L. brevis* (BLB) performed the worst when it came to EtOH production, producing only 0.51% (figure 21). The two batches fermented with *L. thermotolerans* performed the second worst with an EtOH production between 1.17-1.42%. All the other samples produced less than the control beer at an average of 2.64% with the control producing 3.38%. These results were reflected in the attenuation. BTB also produced the most acid, lowering the pH to 3.91, while none of the others produced enough acid to lower the pH below 4.0.

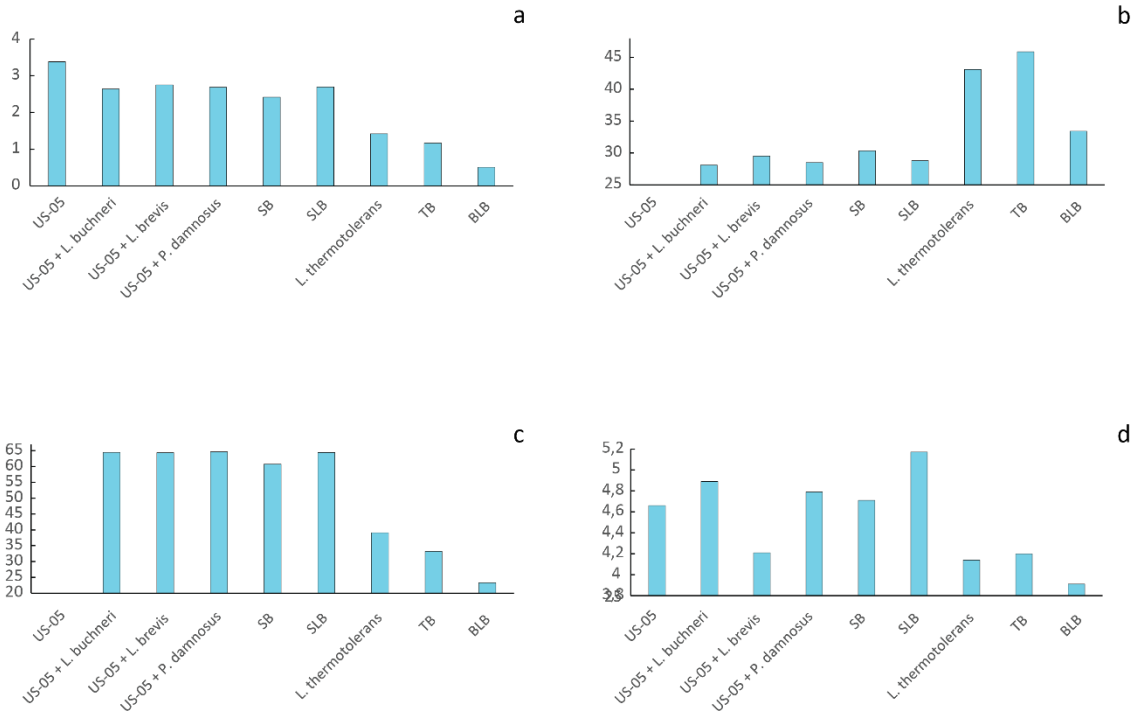


Figure 21: *Data from Anton Paar Alcolyzer Pea Beer V3.* ***(a)*** *Ethanol (% v/v).* ***(b)*** *Sugar concentration (g/L).* ***(c)*** *ADF (% w/w).* ***(d)*** *pH value. Fermented with the addition of 15 g/L of RFO3 fraction.*

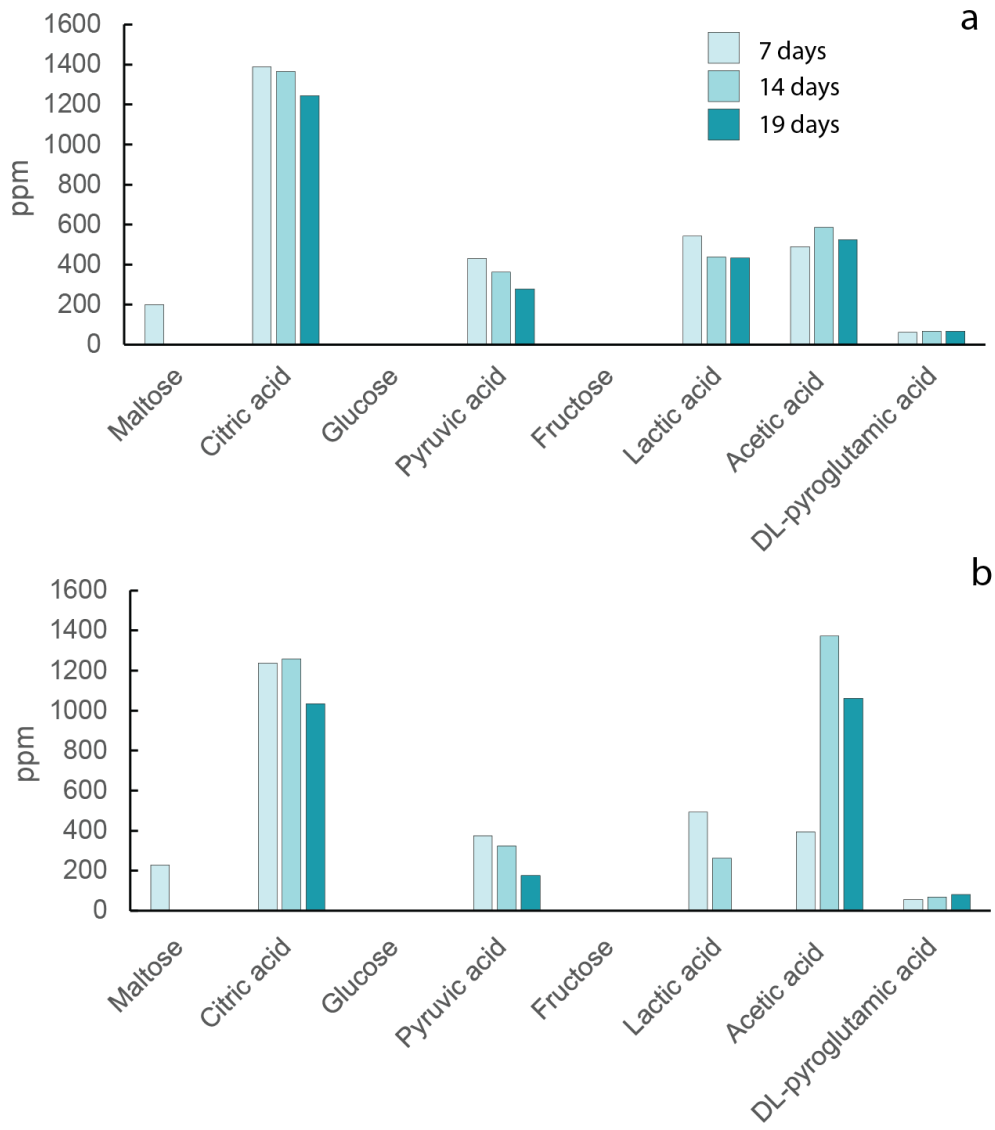


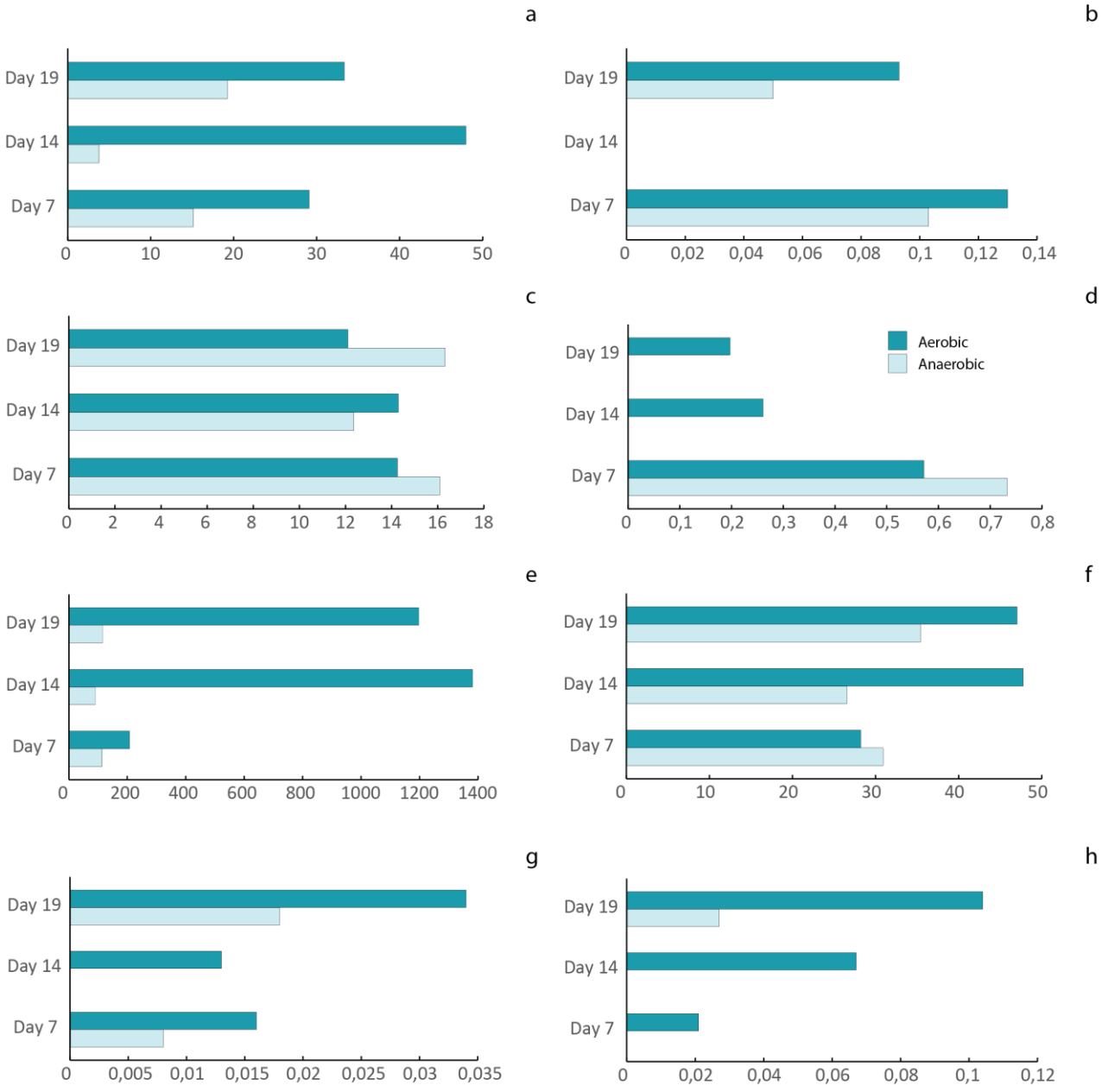
***Figure 22**: HPLC analysis of sugars and organic acids in Pea Beer V3 fermented with S. cerevisiae under **(a)** anaerobic and **(b)** aerobic conditions. The figure displays concentration changes at 7, 14, and 19 days of fermentation.*

Regarding Pea Beer V3, the main takeaway from figure 22, is the increased levels of acetic acid in the sample fermented under aerobe conditions, producing 1061 ppm at the end of fermentation. A notable decrease in lactic acid can also be observed, with it being completely gone after 19 days. When inspecting the fermenters when sampling, they contained an excessive amount of pellicle on top of the beer.

Looking at the volatiles in figure 23, the beer fermented under oxic conditions, produced notably more esters. It produced 0.133 ppm of isobutyl and 0.542 ppm of isoamyl acetate, 375% and 198% more than their respective anoxic counterparts. However, when looking at ethyl acetate, it produced 1197 ppm, a 941% increase as in comparison to the beer fermented with a regular air lock under anaerobic conditions.

Fermenting under aerobic conditions also led to an increase in aldehydes. The oxic sample recorded 33.332 ppm of acetaldehyde, 0.093 ppm of 2-methyl-propanal, 0.034 ppm of 3-methyl-butanal, and 0.104 ppm of 2-methyl-butanal, all values above the sensory threshold as described in table 18.

However, no diacetyl or DMS was measured in the beer fermented under oxic conditions after 19 days of fermentation.



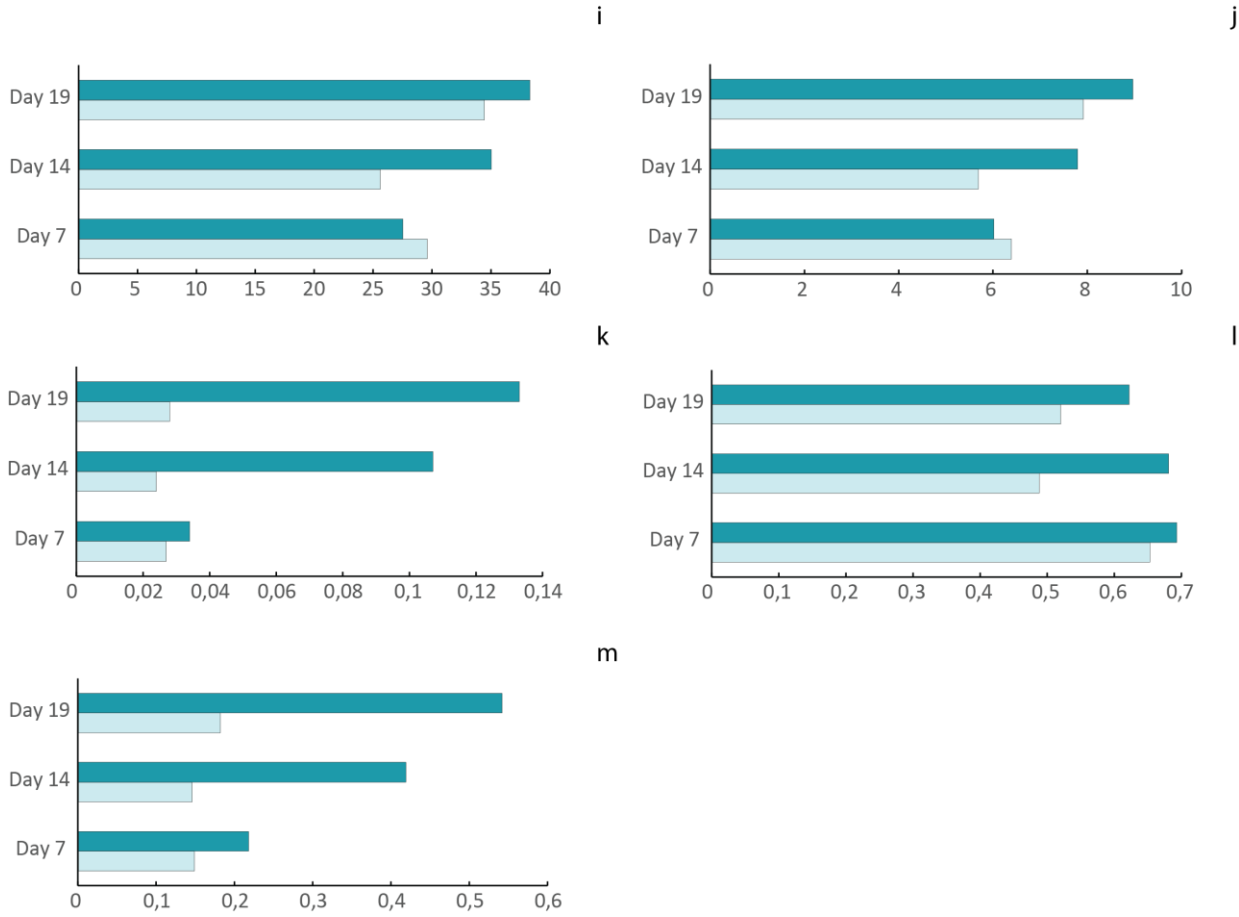


Figure 23: *Graphical depiction of the HSGC results of volatile compounds in Pea Beer V3 fermented with S. cerevisiae US-05 under aerobic and anaerobic conditions. Showing the concentration in ppm at 7-, 14-, and 19-day intervals.* ***(a)*** *Acetaldehyde* ***(b)*** *2-methyl-propanal* ***(c)*** *1-propanol* ***(d)*** *Diacetyl* ***(e)*** *Ethyl acetate* ***(f)*** *2-methyl-1-propanol* ***(g)*** *3-methyl-butanal* ***(h)*** *2-methyl-1-butanal* ***(i)*** *3-methyl-1-butanol* ***(j)*** *2-methyl-1-butanol* ***(k)*** *Isobutyl acetate* ***(l)*** *2-hexanol* ***(m)*** *Isoamyl acetate*

3.2.3.4 Pea Beer V4

The main takeaway from the HPLC results in figure 24, is the low attenuation and resulting high EtOH concentration in the beers co-fermented with *B. claussenii*, *L. brevis*, and *L. buchneri* after 19 days on average they only managed an attenuation of 20%, and with a resulting average EtOH concentration of 1.04%, with the beer fermented with RFO performing slightly better.

When looking at pH the two mixed fermentations, with and without RFO, produced analogue amounts of acid, finishing with a pH of 3.44±0,02. After 19 days, the sample fermented by *S. cerevisiae* US-05 without RFO recorded a pH 0.63 lower than the sample fermented with the addition of RFO.

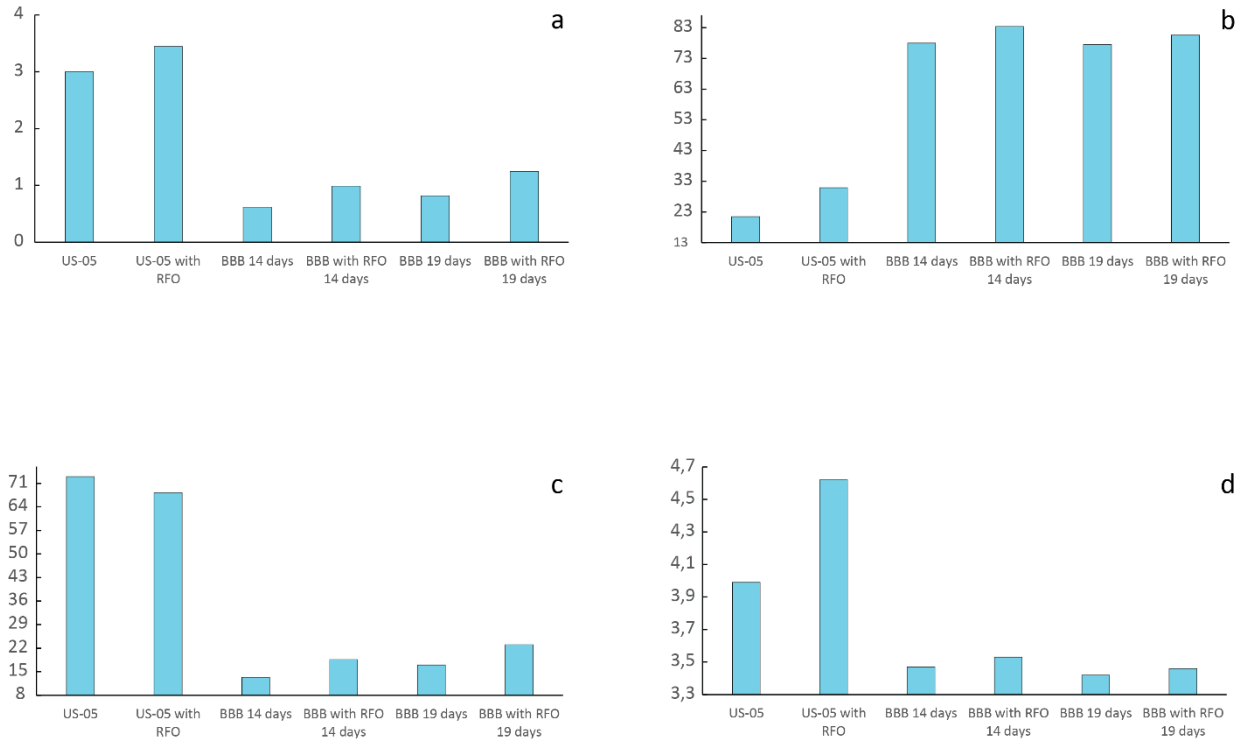


***Figure 24**: Data from Anton Paar Alcolyzer Pea Beer V4. **(a)** Ethanol (% v/v). **(b)** Sugar concentration (g/L). **(c)** ADF (% w/w). **(d)** pH value. Fermented with the addition of 15 g/L of RFO4 fraction. BBB: B. claussenii, L. buchneri and L. brevis.*

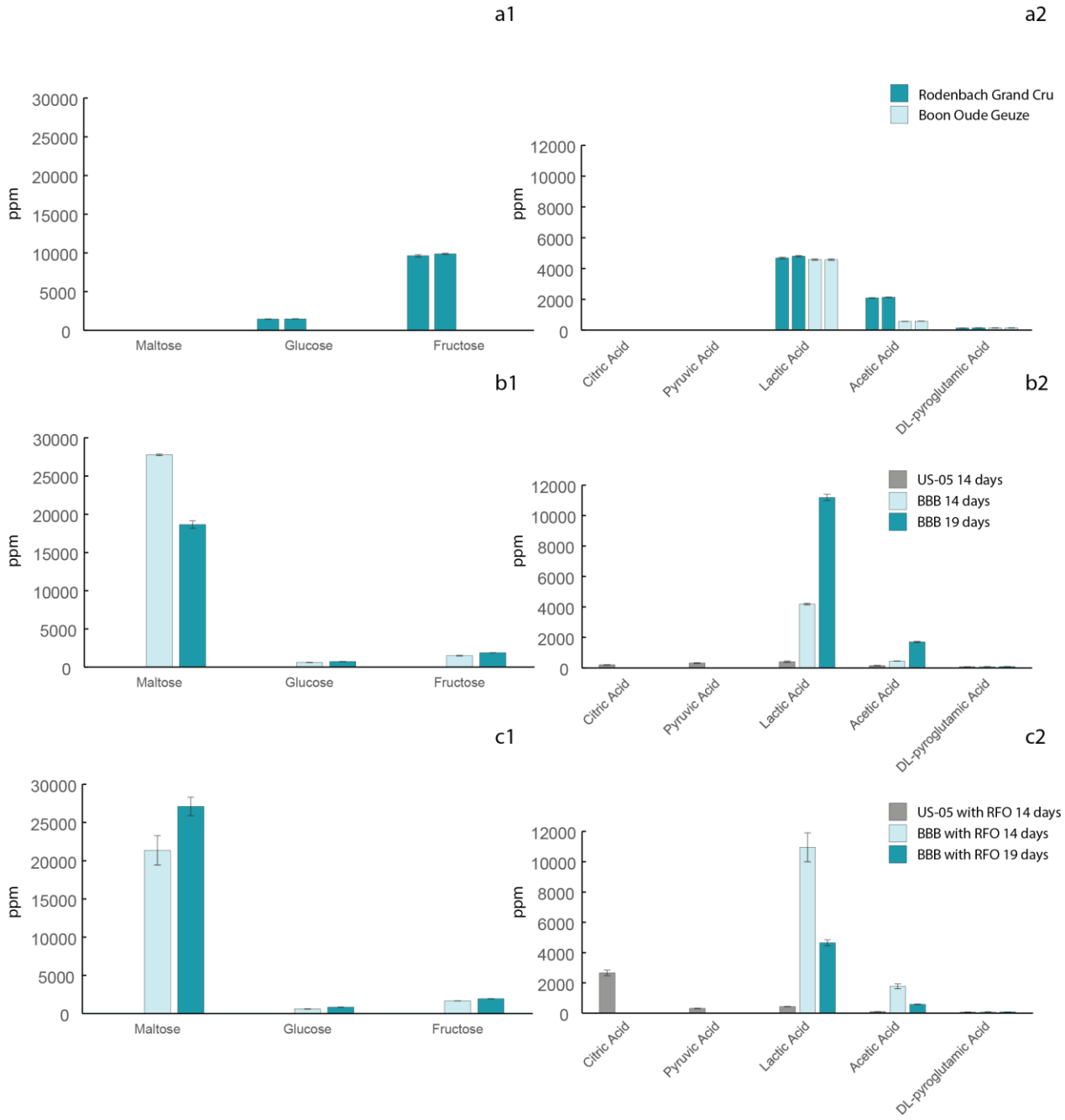


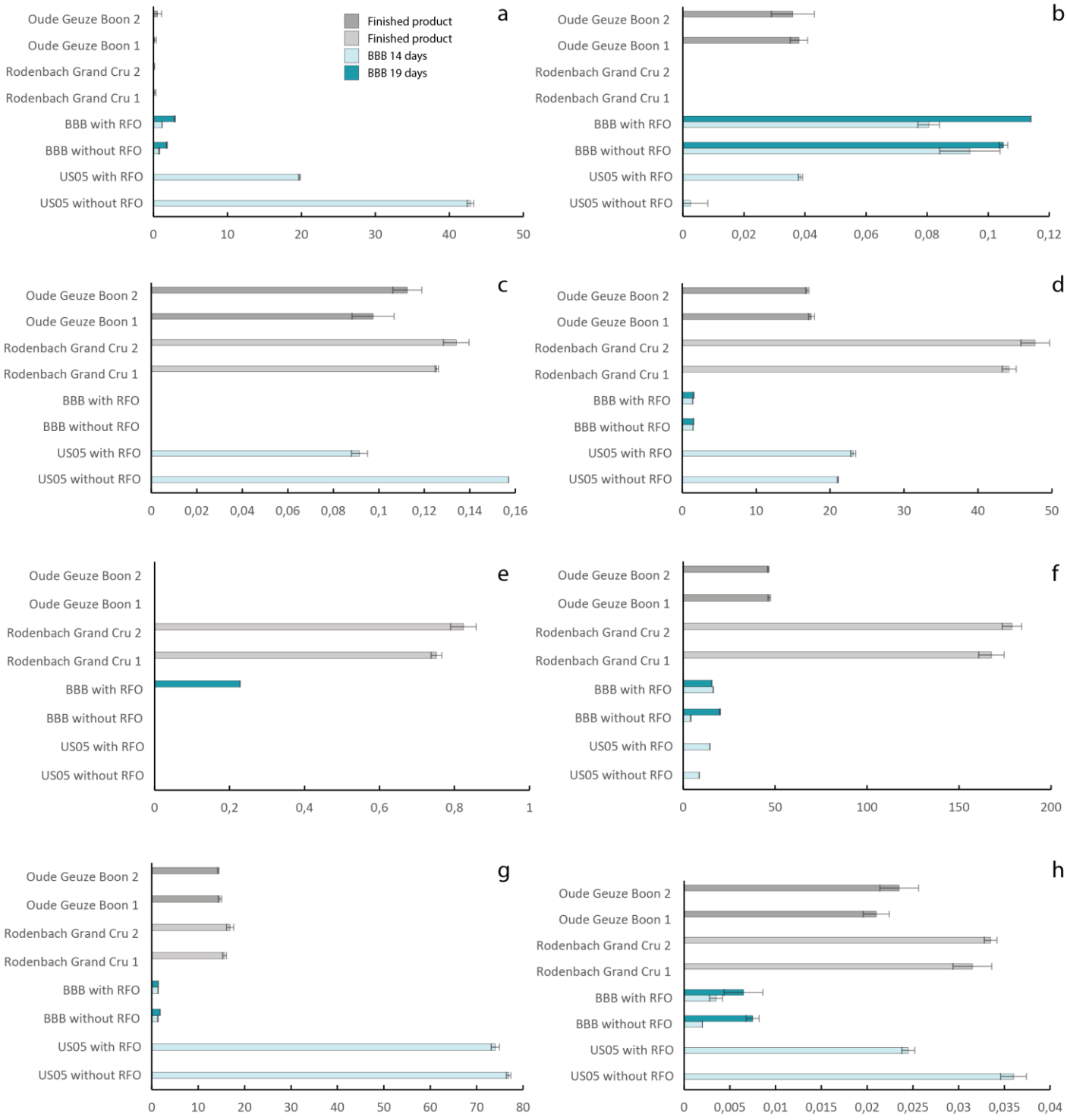
***Figure 25**: HPLC analysis of sugars and organic acids in Pea Beer V4 presented in as a graphical representation. The left portion of the graph delineates the concentration of sugars during the fermentation process, at 0-, 7-, 14- and 19-days. Concurrently, the right segment of the graph illustrates the concentration of organic acids across the same time points. Concentration values are denoted as ppm **(a)** Technical duplicates of commercial sour beers **(b)** B. claussenii, L. buchneri, and L. brevis without RFO added **(c)** B. claussenii, L. buchneri, and L. brevis with RFO added.*

Figure 25 presents a comparison of the concentrations of simple sugars and organic acids in the fermented beers, as analyzed by high-performance liquid chromatography (HPLC) and headspace gas chromatography (HSGC), with those found in commercially available sour beers Rodenbach Grand Cru and Boon Oude Geuze. Rodenbach adds sugar back into the beer after fermentation to sweeten it, resulting in a high concentration of fructose (9600-9800 ppm) in the final product, in addition to approximately 1450 ppm of glucose. In contrast, the fermented beers with and without raffinose family oligosaccharides (RFOs) exhibit large concentrations of maltose in the wort. The exception is the beers fermented using *S. cerevisiae* US-05, which completely metabolized the sugars. The increase in maltose observed in Figure 25 c1 is likely attributable to a mislabeling of the sample prior to analysis.

Limited conclusions can be drawn from the HSGC analysis of the mixed fermentations in figure 26, due to incomplete fermentation. Consequently, the remaining results primarily focus on the *S. cerevisiae* fermentations. The batch with RFO generated more ethyl acetate (14 ppm) than the one without RFO (8 ppm). This pattern was also observed for the other two esters, with the RFO-containing batch producing 0.0925 ppm of isobutyl acetate and 0.6425 ppm of isoamyl acetate, nearly double the amounts of the batch without RFO.

No differences were detected between the two batches in terms of higher alcohol production. The batch without RFO generated more acetaldehyde (42 ppm) compared to the one with RFO (19 ppm). This trend extended to other aldehydes as well, with the batch fermented with RFO producing almost double the amount of aldehydes.

None of the batches exhibited diacetyl, and all dimethyl sulfide (DMS) was eliminated by the yeast at the end of fermentation in both instances.


a
Finished product
Finished product
BBB 14 days
BBB 19 days
Oude Geuze Boon 2
Oude Geuze Boon 1
Rodenbach Grand Cru 2
Rodenbach Grand Cru 1
BBB with RFO
BBB without RFO
US05 with RFO
US05 without RFO
0 10 20 30 40 50
b
0 0,02 0,04 0,06 0,08 0,1 0,12
c
0 0,02 0,04 0,06 0,08 0,1 0,12 0,14 0,16
d
0 10 20 30 40 50
e
0 0,2 0,4 0,6 0,8 1
f
0 50 100 150 200
g
0 10 20 30 40 50 60 70 80
h
0 0,005 0,01 0,015 0,02 0,025 0,03 0,035 0,04

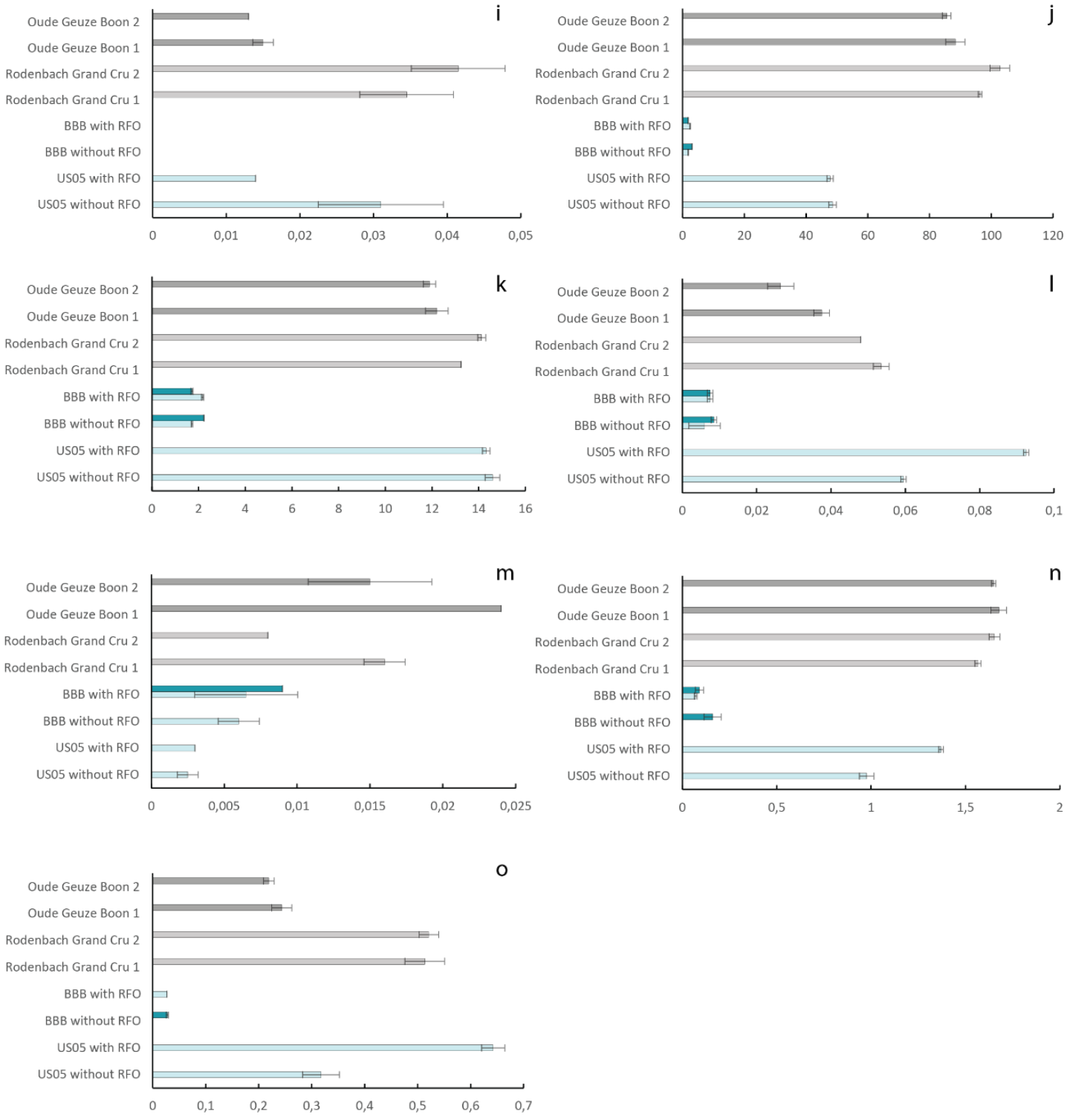
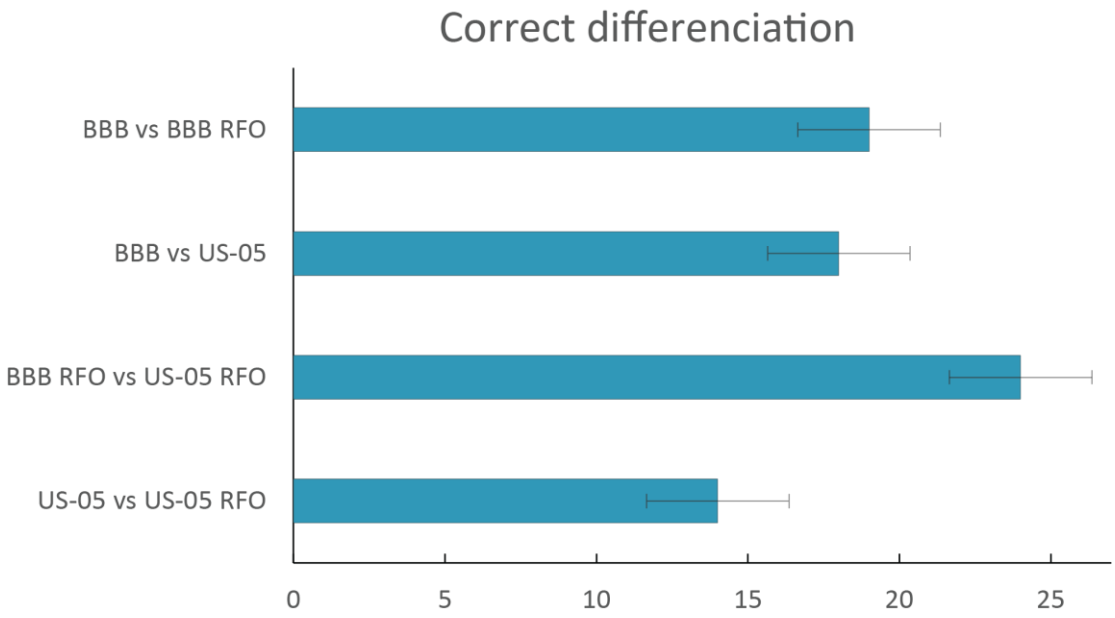


Figure 26: *Concentration of volatile compounds in Pea Beer V4 in ppm at 0-, 7-, 14-, and 19-days.* ***(a)*** *Acetaldehyde* ***(b)*** *Dimethyl sulfide* ***(c)*** *2-methyl-propanal* ***(d)*** *1-propanol* ***(e)*** *2-butanol* ***(f)*** *Ethyl acetate* ***(g)*** *2-methyl-1-propanol* ***(h)*** *3-methyl-butanal* ***(i)*** *2-methyl-butanal* ***(j)*** *3-methyl-1-butanol* ***(k)*** *2-methyl-1-butanol* ***(l)*** *Isobutyl acetate* ***(m)*** *Hexanal* ***(n)*** *2-hexanol* ***(o)*** *Isoamyl acetate.*

***Table 17**: Titratable acidity test Pea Beer V4*

Sample	Initial pH	Total Acidity (mol/L)
US-05	3,86	0,021
US-05 with RFO	4,54	0,03
BBB 14 days	3,36	0,052
BBB 14 days with RFO	3,5	0,125
BBB 19 days	3,46	0,052
BBB 19 days with RFO	3,51	0,132
Rodenbach Grand Cru	3,29	0,102



US-05 vs US-05 RFO

Number of sucesses in trials	14
Number of independent trials	25
Probability of successes in trials	33 %
Binomal distribution (p-value)	0,01
SD	2,351

BBB RFO vs US-05 RFO

Number of sucesses in trials	24
Number of independent trials	25
Probability of successes in trials	33 %
Binomal distribution (p-value)	4,66E-11
SD	2,351

BBB vs US-05

Number of sucesses in trials	18
Number of independent trials	25
Probability of successes in trials	33 %
Binomal distribution (p-value)	6,28E-05
SD	2,351

BBB vs BBB RFO

Number of sucesses in trials	19
Number of independent trials	25
Probability of successes in trials	33 %
Binomal distribution (p-value)	1,14E-05
SD	2,351

***Figure 27**: Results from sensory analysis (tetrad test) for Pea Beer V4.*

Pea Beer V4 was the sole trial that incorporated both titratable acidity testing and sensory analysis of the final product (table 17). The titratable acidity samples containing RFO compared to those without RFO was higher across all samples which is in line with the higher concentrations of organic acids in these samples (see figure 25). The initial pH in the samples fermented with BBB were all similar at 3.48±0.03. However, the beer with RFO recorded a much higher total acidity at 0.132 mol/L in comparison to the beer fermented without RFO at 0.052 after 19 days.

In the tetrad sensory analysis summarized in figure 27, a total of 25 participants were involved, comprising 8 females and 17 males. The age distribution was as follows: 16 individuals between 18-29 years old, 7 between 30-40, 1 between 45-59, and 1 above 60. Out of the participants, 16 had previous experience in sensory analysis, while 9 had not. In the first trial, the aim was to differentiate between two samples fermented with *S. cerevisiae*, one with the addition of RFO and the other without. Fourteen participants were able to correctly identify the difference between the samples. The second trial's objective was to distinguish between samples both containing RFO, one fermented in a mixed fermentation with *B. claussenii*, *L. brevis*, and *L. buchneri*, and the other fermented using only *S. cerevisiae*. In this trial, 24 participants correctly differentiated between the samples.

The third trial mirrored the second trial but with both samples lacking RFO. In this case, 18 participants managed to correctly identify the differences between the samples. The final trial focused on samples all fermented with *B. claussenii*, *L. brevis*, and *L. buchneri*, one containing RFO and one without. In this trial, 19 participants successfully differentiated between the samples.

4 Discussion

Sour beers have gained increasing popularity in recent years (Forbes, 2020; Time, 2017; Vinmonopolet, 2023), leading to a demand for efficient and sustainable production methods. Kettle souring and mash souring are currently the fastest approaches for producing sour beers. Despite their speed and simplicity, these methods often result in a one-dimensional sour profile, primarily due to the use of monocultures for separate-stage fermentation, which optimizes conditions for rapid lactic acid production (Palmer, 2017; Tonsmeire, 2014). In contrast, traditional Belgian sour beers require extended production times and specialized equipment, such as coolships and foeders (Tonsmeire, 2014). This master thesis aims to investigate the potential of LAB strains to utilize RFOs derived from pea protein concentrate in the production of high-quality and sustainable sour beers within the timeframe of standard non-sour beer production. RFOs would serve as a nutrient source for LAB strains, analogous to the role of unmalted grains in Belgian sour beers. The study seeks to develop an innovative approach that addresses some of the challenges faced in traditional sour beer brewing while promoting sustainability and efficiency in beer production. Additionally, this thesis examines the feasibility of using RFOs as substrates to support the growth of beneficial sour beer bacteria, both as single strains and in mixed fermentations.

4.1 Strain selection

The selection of strains for this study combined in-house strains and commercial strains known for their use in sour beer production. In-house strains included *L. brevis* BSO464 and *L. buchneri* CD034, which were previously investigated in the doctoral thesis of Anna Dysvik (2019) for their potential to use xylooligosaccharides from birch wood as substrates in sour beer production. These strains were interesting because they have α-galactosidase and invertase in their genomes, the enzymes necessary to break down RFOs. Other in-house strains, *L. pentosus* KW1 and KW2, and *W. confusa* TM76 and TM120, were used in research on exopolysaccharide production. Commercial strains *L. plantarum* Lallemand WildBrew™, *P. damnosus* WLP661, and *L. delbrueckii* WLP667 from White Labs were also included. The last strain, *B. animalis subsp. lactis*, was considered due to its potential in sour beer production but limited research available.

The baseline yeast selected for this study was *S. cerevisiae* US-05 from Fermentis. This strain was chosen for its convenience as an active dry yeast, low ester production, apparent attenuation of 78-82%, and good flocculation properties (Fermentis, 2023). *B. claussenii* was selected due to its prevalence in spontaneously fermented sour beers. To investigate the potential of using a monoculture for sour beer production, *L. thermotolerans* was included. Its heterofermentative qualities, which enables the production of both ethanol and lactic acid, and its classification as a yeast, make it particularly interesting from a production standpoint as this offers several advantages, including ease of maintaining a single culture, cost-effectiveness, and reduced risk of cross-contamination between batches, particularly when using the same equipment for the production of bacteria free beers. *S. cerevisiae* Omega Lutra Kveik, was chosen due to its rising popularity in the brewing community.

4.2 Substrate

The MALDI-ToF analysis of the different RFO fractions revealed a distinct difference in the distribution of peaks between RFO1/2 and RFO3/4, as illustrated in Figure 4.1. RFO fractions 1 and 2 displayed increased noise at the lower end of the spectrum, which may be attributed to an underdeveloped refinement process. The presence of two separate ionic peaks in RFO fractions 1 and 2 could be a result of not using deionized water during the RFO refinement process.

The subsequent refinement process, as described earlier, appeared to be more effective in separating oligosaccharides from disaccharides, including sucrose, as evidenced by the spectrum in Figure 4.1. Across all fractions, the relative abundance of RFOs followed a descending order of stachyose, verbascose, and raffinose. A peak of unknown origin at m/z 425 was observed in all samples.

Table 13 presents the raffinose assay results, demonstrating a clear trend of improved purity of the RFO extract as the refinement process evolved. This observation pertains to RFO2, RFO3, and RFO4, all derived from pea protein concentrates, and excludes RFO1, which was sourced from pea starch concentrate.

During the attempt to sterile filter samples fermented with RFO3, a filtration challenge was encountered without an evident cause. By gradually applying finer filtration gradients, the beer media could be filtered at 4-7 µm, but not at finer levels. Further investigation is required to understand the underlying factors impeding finer filtration

and to optimize the process for efficient sterile filtration. This problem was solved for RFO4 by acidifying the pea protein concentrate to remove the proteins during the biorefining process. This was important, not only for the experimental use of the substrate, but it also takes up a lot of time and resources to produce the RFOs. From this experience it is clear that small changes in the substrate could lead to large consequences as to the execution of the process.

4.3 50 mL fermentations in pH-controlled bioreactors

As a proof of concept, this study aimed to investigate whether LAB strains would grow in MRS media with RFO as a substrate. All strains exhibited a classic growth curve with a lag, exponential, and stationary phase. The corresponding DW graph demonstrated an increase in biomass in accordance with the increasing optical density.

However, certain aspects of the experimental design might not be directly applicable to beer fermentation.

One limitation of the experimental setup was that it was not entirely anaerobic, with a small tube (~1mm inner diameter) connected to a filter, allowing O_2 to enter the fermenter. The extent to which O_2 would affect the production and release of waste products remains speculative. It would be interesting to explore in future studies whether this factor influences biomass production, as some *Lactobacillus* strains have shown potential respiratory capabilities, potentially increasing biomass yield at the expense of organic product synthesis (Stevens et al., 2008). This consideration would be particularly relevant if yeast were introduced in these trials, with the Crabtree effect possibly occurring, depending on the glucose concentration in the media (Hagman and Piškur, 2015). The Crabtree effect, as described by Pfeiffer and Morley (2014), refers to a phenomenon where yeast, notably *S. cerevisiae*, exhibit a metabolic preference for fermentation over respiration in oxic conditions. Despite the greater yield of ATP provided by respiration, the yeast will ferment glucose to ethanol at high glucose concentrations.

Another aspect of the fermentation design that could potentially impact the fermentation profile is the continuous mechanical stirring. In a heterofermentative process with CO_2 as one of the products, the recirculation of CO_2 from the headspace could cause hydrodynamic stress-induced damage to yeast cells (Boswell et al., 2003).

Other studies have reported changes in yeast morphology due to mechanical stress (Wucherpfennig et al., 2012) and the potential effects of shear forces on microbial viability (Lange et al., 2001).

The final parameter in this trial, which may not be transferable to sour beer production, was the constant regulation of pH to maintain optimal bacterial growth conditions (*Lactobacillus*: 5.5-6.2 (Śliżewska and Chlebicz-Wójcik, 2020), *Pediococcus*: 6.0 (Papagianni and Anastasiadou, 2009), *Weissella*: 4.0-9.0 (Fusco et al., 2015), and *Bifidobacterium*: 6.5-7.0 (Hoover, 2014)) at pH 6. Such pH regulation would negate the souring effect in sour beer production. Future studies should address these limitations to ensure a more accurate translation of the proof of concept to sour beer fermentation processes.

The 50 mL fermentations of *L. brevis*, *L. buchneri*, and *L. plantarum* were the only fermentation in media where HPAEC-PAD results could be extracted and analyzed. Figure 8 shows the potential of LAB, *Lactobacillus* strains specifically, to metabolize RFOs under optimal pH conditions. A clear reduction in the quantity of the RFO constituents was recorded. However, the high degree of uncertainty of the HPAEC-PAD results due to multiple challenges further explained in section 4.6, makes it hard to draw any definite conclusion, as the methodology needs to be addressed in future experiments to gain more trustworthy results.

4.4 Recipe design

In an effort to establish an efficient brewing process with a turnaround time of approximately two weeks and to minimize the number of variables involved, this study opted for a simplified grain bill. Contrary to the Belgian tradition of incorporating unmalted wheat or similar adjuncts to supply nutrients for LAB and wild yeast over time, this research drew inspiration from the Berliner Weisse brewing technique. The malt bill was comprised of 50% Bestmalz pilsner malt and 50% Bestmalz malted wheat, deliberately excluding specialty malts to ensure an unobstructed assessment of the fermentation profile. This specific ratio was consistently maintained throughout all trials, with only the quantities adjusted for recipe fine-tuning.

Inherent challenges exist when determining the precise brewhouse efficiency while using new equipment. These challenges are reflected in the discrepancies between the expected OG calculated by the brewing software (Brewfather) and the measured

OG as determined by a refractometer at the final step of the brewing session. Brewhouse efficiency ranged between 60.24% and 71.60%, resulting in a difference in output of more than 5 gravity points and a potential 0.66% difference in ethanol concentration in the final product, contingent upon the attenuation. This difference could be reinforced by the small batch sizes and could lead to variations in OG measurements when trying to replicate the recipe. Several factors could contribute to this variance, including fluctuations in the grain mill's distance, water-to-grist ratio in the mash, mashing temperatures, and mash pH. Additionally, potential extract variations between malt batches might play a minor role.

When assessing the apparent degree of attenuation, final ethanol concentration, and FG using the Anton Paar Alcolyzer, it became evident that the filtration degree of the sample influenced the outcome. To facilitate sparging and lautering due to the high β-glucan content in wheat malt, the grist was combined with 6% rice hulls (w/w). As long-chain polysaccharides, β-glucans contribute to wort viscosity, potentially complicating the lautering process (Vis and Lorenz, 1997). The chosen percentage aimed to compensate for the absence of hulls in the wheat malt. No issues with stuck mash or sparging and lautering were encountered across the brewed batches. However, the rice hull percentage was reduced to 2% (w/w) for Pea Beer V4 due to an oversight in estimating the remaining storage quantity. This reduction did not appear to impact the lautering efficacy of the grist.

Breweries specializing in Lambic production typically utilize hops that have undergone an extended aging process. This aging is often facilitated by storing hops at elevated temperatures and exposing them to air, thereby promoting the gradual oxidation of α-acids and reducing the bitterness they impart to the beer. Although isomerized α-acids are crucial for the antimicrobial properties of hops, it is essential to acknowledge that other key compounds survive the oxidation process, allowing the hops to maintain good antimicrobial activity. This consideration is vital, as aged hops provide a higher hopping rate that effectively protects against detrimental microbial activity without adding excessive bitterness to the beer (Sparrow, 2005), which is especially important in sour beer fermentations given the low α-acid tolerance of the Gram-positive bacteria used in its production.

In the context of hops and their role in beer production, it is noteworthy that approximately one-third of the compounds contributing to hop bitterness, specifically

trans-iso-humulones, possess a half-life of about one year. Conversely, the remaining compounds responsible for bitterness, known as cis-iso-humulones, exhibit a substantially longer half-life of approximately five years (de Keukeleire, 2000). Hops also contain another group of compounds called β-acids, which demonstrate relatively lower solubility in wort compared to α-acids. Upon oxidation, β-acids are transformed into more soluble hulupones, which subsequently contribute to the overall bitterness of the beer. The α-acid to β-acid ratio in a particular hop variety determines the extent to which aged hops will impart bitterness to the beer (Tonsmeire, 2014). While no experiments were performed to quantify the ratio of α-acids in comparison oxidized β-acids, their combined concentration was measured using the MEBAK® convention and converted into % of α-acids for use in the Brewfather software for recipe design and estimation of final IBU content in the beer. In this study, the primary criterion for hop selection was a low α-acid content and, to an unknown extent, oxidized β-acids, maintaining low IBU levels relating to the inherent sensitivity of Gram-positive bacteria to hops.

The interplay of pronounced bitterness and sourness in beer may lead to unfavorable flavor outcomes. While low-level sourness can potentially enhance bitterness, the high acidity characteristic of sour beers tends to render robust hop bitterness as harsh and unpalatable (Tonsmeire, 2014). Therefore, it is generally recommended to limit the International Bittering Units (IBU) level in sour beers. It was also clear from the trials performed related to the IBU concentration when looking at the average growth of lactic acid bacteria (figure 12), that an IBU concentration of 5 clearly inhibited proliferation. This led to the choice of aiming for a final IBU in the pea beers between 3.0 and 3.5.

Archer hops, having the lowest α-acid content available in storage at NMBU, were selected as the best candidate. The hop packets had an unknown year of origin and were vacuum-packed in silver foil bags. When evaluating the impact of an additional six months of open-air storage, it was concluded that no further oxidation would occur, as the average IBU calculated for the open-air samples was 181, which was negligibly less than the samples taken directly from unopened packages which was 185.

Similar to the malt bill, discrepancies were observed between the estimated final IBU values and the results. The Brewfather software consistently underestimated the extraction of bitter compounds from the hops, necessitating adjustments to achieve the desired target. This discrepancy could be attributed to the software's formula for

calculating α-acid extraction compared to oxidized β-acids, which would be present in much higher concentrations in the utilized hops than in hops with a more recent production date. However, since the calculations are concealed behind the software's graphical user interface, no definitive conclusions could be drawn.

In all the trials except for Pea Beer V4, the sole water adjustment made involved modifying the pH to maximize the yield from the mash. However, for Pea Beer V4, the water profile was adjusted to enhance the perceived body of the beer and increase the low calcium values, as discussed in Chapter 1.2.1.1. To adjust the water profile, the missing sodium values from the provided water report (ALS laboratories) had to be calculated. Assuming that the concentrations of potassium, iron, nitrate, nitrite, and fluoride in the water were low enough not to impact the ion balance and taking into account that water is electrically neutral (anions = cations) (Palmer and Kaminski, 2013), an approximate concentration was calculated.

The fermentation temperatures for Pea Beer V1-V4 were set at 24°C, which is on the higher end of the recommended range for ale fermentation. This aimed to strike a balance between yeast and bacterial growth without compromising flavor. For non-sour fermentations, elevated fermentation temperatures may result in rapid growth and stalled fermentation, high concentrations of fusel alcohols, acetaldehyde, and other undesirable off-flavors, including selected esters and phenols, particularly concerning *Brettanomyces* (White and Zainasheff, 2010). However, the fermentation temperatures for Pea Beer V4 exceeded 26°C at some points. This could be attributed to fermentations being exothermic reactions (White and Zainasheff, 2010), and the conditions within the incubator (Binder (Neckarsulm, Germany) FED 400) were unable to dissipate the heat swiftly enough. Another possibility is that the incubator was influenced by the ambient room temperature, which may have increased for unknown reasons. According to the manufacturer's manual, the temperature setting must be 5°C above the room temperature. However, it maintained a stable temperature of 24°C for previous fermentations (Pea Beer V1-V3).

An increase in temperature causes yeast cells to accelerate their metabolism and eventually express heat shock proteins. These proteins protect the membrane and prevent other proteins from unfolding, but they also hinder the cell's ability to express proteins necessary for cell division, fermentation, and other essential cell functions (White and Zainasheff, 2010). This issue is compounded by the increased production

of secondary metabolites and flavor-active compounds. The initial plan involved monitoring the temperature during fermentation within the beer. However, due to difficulties connecting the digital hydrometer (KegLand (Springvale, Australia) RAPT Pill) to the secure campus Wi-Fi and inconsistencies in receiving the signal through the incubator's walls, this idea was abandoned. It would have been intriguing to further investigate if the substrate clearly affected heat output, as there is little research done on the subject of temperatures in mixed culture fermentations.

As previously mentioned, the general pitching rate for *S. cerevisiae* across all trials was 0.75 million cells/ml/°P. A low pitching rate results in increased total cell growth and higher synthesis of amino acids, leading to more byproducts from the fermentation process. In contrast, a higher pitching rate leads to less total cell growth and, consequently, fewer byproducts (Palmer, 2017). Based on microscopy using a hemocytometer, the cell density of the *S. cerevisiae* US-05 ADY employed in all trials was calculated to be 2.18 x 1010 cells/g. For *Brettanomyces* spp., the maximum cell density per mL in wort is three to six times higher than that of S. cerevisiae. Employing the same pitching rate calculations for *S. cerevisiae* would result in excessively high cell counts. However, there appears to be a lack of data regarding optimal inoculation rates for *Brettanomyces* in primary fermentation, as well as clear variations in cell density among different strains (Yakobson, 2023). The cell density of *B. claussenii* was calculated from a sample of starter culture, yielding an estimated density of 6.4 x 1011 cells/mL. Nonetheless, these results should be interpreted with caution, since counting the smaller *Brettanomyces* was more challenging compared to *S. cerevisiae*.

Regarding Kveik, it is common practice to underpitch the yeast in typical beer fermentations to stress the yeast and promote greater flavor production, as it is considered a desirable trait (Preiss et al., 2018). However, in this thesis, the emphasis was on optimizing fermentation conditions. It is worth noting that all yeast strains used in this trial exhibit higher optimal growth temperatures (*S. cerevisiae* = 30°C-35°C (Walsh and Martin, 1977), *B. bruxellensis* = 32°C (Brandam et al, 2008), Kveik = >30°C (Foster et al., 2022), and *L. thermotolerans*=29°C (Kogan et al., 2023)). No specific data could be found for *B. claussenii*, with the assumption being that it would perform similarly to *B. bruxellensis*. Lutra Kveik, a strain of *S. cerevisiae*, exhibited indistinguishable morphology from *S. cerevisiae* US-05, with a starter culture cell density of 3.1 x 1010 cells/mL in the starter culture. The morphology of *L.*

thermotolerans was too small to count using a hemocytometer. The viability of individual cells was not considered, which could be assessed in future experiments using vitality staining, to assess the percentage of live cells in the samples. This could include both colorimetric and fluorescent dyes, with the mechanism of action depending on the properties of the cell membrane (Kwolek-Mirek and Zadrag-Tecza, 2014).

The LAB strains used in this thesis have optimal fermentation temperatures ranging between 30°C-37°C, which are not dissimilar to the yeast strains. The decision to adopt a fermentation temperature of 24°C was made early in the process, and no mixed fermentations were performed at other temperatures. Investigating higher fermentation temperatures in future experiments would be of interest, particularly with Kveik, which is known to produce cleaner fermentation profiles at higher temperatures when inoculated with appropriate pitching rates compared to traditional ale strains (Kits and Garshol, 2021).

As this beer was never intended for commercial release, limited attention was given to post-fermentation treatment, including potential filtration or other clarification techniques for increased stability and shelf-life, production costs, and the impact of maturation and conditioning. Another unexplored aspect was the proteolytic enzymes in LAB and their effect on reducing foam-promoting proteins in sour beer, which is often a challenge.

4.5 Intrinsic hurdles

In examining the various factors that influence the quality and characteristics of beer, it has been observed that the α-acid from hops constitutes the primary inhibitory factor affecting bacterial growth, while ethanol plays a secondary role (as seen in figures 10-14). The ethanol tolerance of different yeast strains used in this study, including *S. cerevisiae* US-05, *B. claussenii* WLP645, Omega Lutra Kveik, and *L. thermotolerans* Philly Sour, correspond well with the data obtained from trials investigating the impact of ethanol and IBU stressors on their growth. None of the beer media produced in these trials exceeded 6% ethanol content, and all yeast strains exhibited good growth. In Beer Media 1, which had an IBU value of 6.9, all yeast strains demonstrated substantial growth as seen in figure 9, while bacterial strains experienced inhibition with only limited growth observed in *L. brevis*, *L. buchneri*, and *P. damnosus*.

The findings from these trials prompted further investigation into the ethanol and IBU tolerance of different strains, with the aim of identifying a threshold or an optimal balance of stressors. *Lactobacillus* strains, in general, exhibit high ethanol tolerance, although there is profound variation depending on the specific strain. Most strains exhibit ethanol tolerance above 13% (Nojiro, 1983; Kleynmans et al., 1989), with certain *L. plantarum* strains ceasing growth at around 5-6% ethanol (Wibowo et al., 1985). However, Pittet et al. (2011) found no correlation between inherent ethanol tolerance and the ability to grow in beer, with all *Lactobacillus* strains in this study demonstrating growth in beer to a certain extent. *P. damnosus* has been observed to tolerate ethanol concentrations up to 10% (w/v) (Harrison, 2009).

Limited data is available on the ethanol tolerance of *W. confusa* and *Weissella* spp., with Chorianopoulos et al. (2015) reporting that a *Weissella* spp. did not grow at 10% ethanol. No prior information on the ethanol tolerance of *B. animalis subsp. lactis* was found in literature. The scarcity of data on *Weissella* and *Bifidobacterium* is not surprising, given their infrequent occurrence in beer or wine fermentations. Nonetheless, under the conditions of the first trial testing the effect of stressors, the average reduction in growth was 0.1 (measured in OD_{600}) when exposed to 4.5% ethanol compared to 2.25% ethanol, without any IBU present.

Hazelwood et al. (2010) demonstrated that the resistance of *S. cerevisiae* to α-acids involves three key processes: active proton pumping into the vacuole by an ATPase to enable sequestration of isomerized α-acids, alteration of the cell wall structure, and, to a lesser extent, the active export across the plasma membrane. These mechanisms ensure that *S. cerevisiae* can grow efficiently in beer with an IBU content of 6.9, which was the highest value tested in this study. Generally, research into α-acid tolerance and resistance has been traditionally focused on LAB strains, particularly in the context of LAB as spoiler bacteria in regular beer production.

Tonsmeire (2014) suggests aiming for less than 5 IBU in beer with *Lactobacillus* activity, while Palmer (2017) cites that Berliner Weisse usually has an IBU ranging between 5-8 IBU. *Pediococcus* is known to have a higher tolerance and can grow in beers with up to 30 IBU (Palmer, 2017). Schurr et al., (2015) concluded that the presence of isomerized α-acids appeared as the most stressful factor, especially in combination with other stress factors such as low pH and ethanol, acting as ionophores. In the first stressor trial, an average decrease in growth of 0.3, measured

in OD_{600}, was observed, and the graph in Figure 11 showed a clear separation between 0, 2.5, and 5 IBU. The same was true for trial two, with an average decrease of 1.08 measured in OD_{600} when comparing the 2.5 and 5 IBU in the beer media containing 6% ethanol. It should be noted that Beer Media 2, used in this experiment, measured a pH of 10.55 when analyzed using Anton Paar Alcolyzer. This likely indicates an error in the sampling side of the analysis, potentially due to not running the rinsing program on the Alcolyzer and leaving cleaning solution in the tubes before sampling.

Numerous studies have established the optimal pH range for *S. cerevisiae* to be between 4 and 6 (Narendranath and Power, 2005; Wu et al., 2022), with some strains demonstrating adaptability to environments with pH as low as 2.5 (Liu et al., 2015). Rozpedowska et al. (2011) compared the pH tolerance of different yeast species and found that *B. bruxellensis* was capable of propagating at pH 2.3, and *B. anomalus* at pH 2.6, whereas *S. cerevisiae* ceased growth at pH 3.2. Lallemand Brewing (2023) reported that the *L. thermotolerans* strain could produce beer with a pH as low as 3.2, a finding corroborated by research on wine fermentation (Hranilovic, 2022). However, there is limited literature available on the pH tolerance of Kveik yeast strains.

LAB leverage lactic acid production as a competitive advantage against other microorganisms in their environment, despite their own susceptibility to the acidic conditions they create (Dysvik, 2019). LAB generally prefer an initial pH of 6-7 for optimal growth, which is slightly higher than the pH range of 5.0-5.4 found in unfermented beer (Palmer, 2017). The pH tolerance of certain *Lactobacillus* species has been reported to be as low as 2.8, with *L. brevis* and *L. plantarum* exhibiting pH tolerances of 3.1 and 3.3, respectively (Shillinger et al., 2006). *Pediococcus* spp. have been demonstrated to reduce pH to 3.6 (Papagianni and Anastasiadou, 2009).

In the case of Pea Beer V1 and Pea Beer V3, in the batches which were fermented exclusively using *S. cerevisiae* US-05, the final pH values were 4.18 and 4.66, respectively. During fermentation, yeast cells assimilate amino acids and other buffering compounds as nutrients while excreting protons. As the wort typically contains a low amount of free amino nitrogen (FAN), it does not exhibit good buffering capacity, causing the pH to decrease by approximately 0.5 units during fermentation (Palmer, 2017). Intriguingly, the inclusion of RFOs in the beer media appeared to enhance the buffering capacity, as evidenced by measuring titratable acidity, which was higher in samples containing RFOs.

4.6 Experimental design errors

The experimental design for Pea Beer V3 aimed to generate additional data through duplicate fermentations, repeating those conducted in V1 and V2. However, the use of borosilicate glass bottles with filters instead of traditional air locks, led to excessive oxygen leakage into the fermenters, promoting biomass growth instead of the production of fermentation products. Consequently, all separate fermentations exhibited an ADF of approximately 30%, with *B. claussenii*, *L. cremoris* M121, and *L. brevis* performing the poorest at just 23.32%. Furthermore, these fermentations produced a higher proportion of acetic acid relative to lactic acid (figure 22), resulting in a potentially unpalatable sensory profile. Just one of the batches registered a pH below 4 (*B. claussenii*, *L. cremoris* M121, *L. brevis* at 3.91), with many of the batches ending up with a higher pH than the beer fermented with just *S. cerevisiae*. This outcome was also reflected in the volatile compounds detected. Ultimately, only the results from the two duplicates of *S. cerevisiae* fermentation, one with a regular airlock and the other with a filter setup, were included in the study as a demonstration of the presumably aerobe vs anaerobe fermentation conditions. The reduction in lactic acid over time in the sample fermented under oxic conditions (see figure 22) could indicate some malolactic fermentation activity, possibly due to contamination.

As illustrated in figure 22, the aerobic fermentation generated nearly 1400 ppm of ethyl acetate, which has a sensory threshold of around 25-30 ppm. Consequently, the fermented beers likely exhibited a nail polish remover-like sensory profile. Additionally, the RFO3 fraction utilized in this batch proved challenging to filter, adding to the issues with this experiment. Due to these issues, the majority of the results were excluded from this thesis.

The reduction in lactic acid with increased time in the sample fermented under oxic conditions could indicate some malolactic fermentation activity, possibly due to contamination.

The HPAEC-PAD method utilized for oligosaccharide analysis also requires improvement. Previously poor column management (the column had never been washed) resulted in suboptimal results from fermentations performed on MRS and YEP media. Although column maintenance and washing improved the quality of the results over time, the complexity of the beer media made interpreting the results from

the potential enzymatic hydrolysis of RFOs impossible. The presence of acids and ethanol in the beer media might be a reason for shifting retention times, further complicating the analysis. Additionally, the impurities in the RFO1 fraction, as seen in the MALDI-ToF spectrum, likely contributed to the issue.

In summary, the data obtained from the HPAEC-PAD analysis is inconclusive at best and misleading at worst. A new method is needed to eliminate background noise and generate definitive data. Due to the inconclusive HPAEC-PAD data, no definitive conclusion can be drawn regarding the strains' ability to hydrolyze RFOs.

4.7 Fermentation studies with RFO as the substrate

In Pea Beer V1 figure 15, the lowest pH value was observed in the batch fermented exclusively with *L. thermotolerans* (pH 3.64). In contrast, the beers fermented with US-05 in combination with *L. pentosus* KW1 *and P. damnosus* exhibited a pH decrease of only 0.4 and 0.2, respectively, compared to the US-05 base beer. Notably, *L. buchneri* (pH 3.94) and *L. brevis* (pH 3.76) imparted a subjective distinct fruity acidic taste profile when tasting the fermentation samples. All batches in Pea Beer V1 exhibited comparable performance concerning fermentation parameters, as shown in figure 15. The batch with US-05 and *L. buchneri* had the lowest ADF (73.57%) and a final ethanol concentration of 3.32% (v/v). A potential explanation for these observations could be that the production of lactic acid (3121 ppm) and acetic acid (862 ppm) and the subsequent decrease in pH affected the yeast's metabolism. *L. thermotolerans* rapidly produced lactic acid (>3000 ppm) within 7 days, with no change in the subsequent 12 days. The batches containing *L. brevis* and *L. buchneri* also performed comparatively well to *L. thermotolerans*, producing lactic acid well above the flavor threshold, at 3120 ppm and 1753 ppm, respectively at the end of fermentation.

Both *L. brevis* and *L. buchneri* are heterofermentative strains, so ethanol production is expected from them, and to a certain degree, *L. plantarum* KW1, being facultative heterofermentative. On the other hand, *P. damnosus*, being homofermentative, may exhibit a discrepancy between the ADF and the ethanol concentration, as some sugars/oligosaccharides are only metabolized to produce acid. None of the batches displayed higher attenuation than the base beer, leaving inconclusive evidence regarding the strains' ability to metabolize RFOs.

The analysis of sugars and organic acids via HPLC revealed that, as anticipated, maltose, glucose, and fructose were depleted after seven days in all beers. HPLC and HSGC analyses of fermentation samples were conducted simultaneously, with the 19-day samples analyzed a week later. This led to some discrepancies, as a few 19-day samples indicated residual sugars in the beer. This observation implies a margin of error in the sampling process, with presumably greater consistency achieved by analyzing all samples at once.

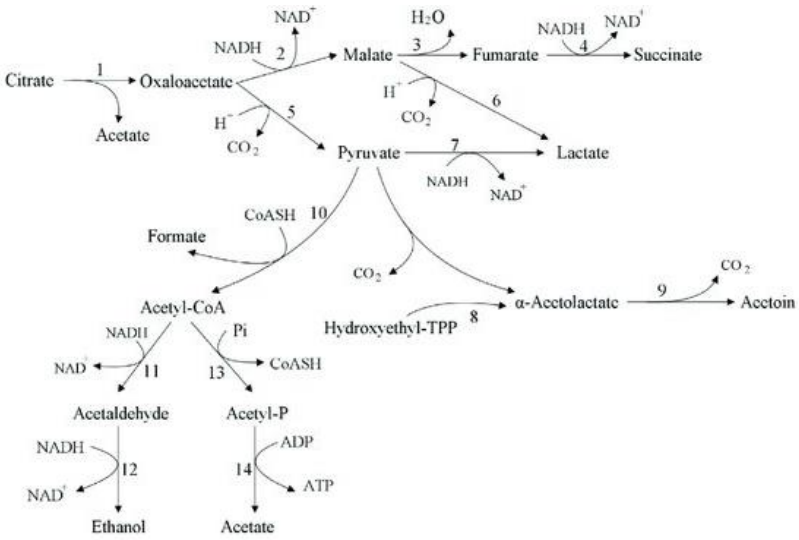


Figure 28: *Metabolism of citric acid in LAB (Gänzle, 2015): Citric acid, present in LAB, is converted into succinate, lactate, acetate, and ethanol or acetylacetone via the intermediate metabolite oxaloacetate.*

In the base beer, 616 ppm of citric acid was present, exceeding the typical range of 50-250 ppm. This result can be attributed to the yeast's metabolism and its role as a key component in the tricarboxylic acid cycle. Although citric acid contributes to the overall acidity of the beer, it has minimal impact on flavor (Klopper, 1986). Interestingly, *L. brevis* appears capable of metabolizing citric acid, as illustrated in figure 16.c2. In LAB, citric acid can be converted into succinate, lactate, acetate, and ethanol or acetylacetone via the intermediate oxaloacetate (figure 28) (Gänzle, 2015). This metabolic pathway might explain the elevated levels of acetic acid in the beer. In cases where citric acid levels slightly increase over time, cell autolysis could be a potential explanation (Wang, 2018), assuming the cells contain an intracellular concentration of citric acid. The flavor thresholds of organic acids in beer are 400 ppm for lactic acid and 200 ppm for acetic acid (Tan and Siebert, 2004).

Pyruvic acid was present in some of the fermentations (figure 16, 19, 22, and 25). It is a product of glycolysis and can be metabolized by yeast and bacteria through the intermediate acetaldehyde at the end of fermentation to produce ethanol and CO_2 (Malakar et al., 2020). In halted fermentations, this could lead to an increase in acetaldehyde in the beer.

DL-pyroglutamic acid, which is present in all wort, remains unaffected during fermentation (Coote, 1977), and little research has been conducted on its effect in beer. It was detected in small amounts in every sample analyzed.

Table 18: *Volatile aromatic compound in beer and their sensory thresholds. Adapted from Aasen (2020). (Briggs et al., 2004; Dong et al., 2015; Harrison, 1970; Holt et al., 2019; Humia et al., 2019; Olaniran et al., 2017; Preiss et al., 2018; Saison et al., 2009; Tan and Siebert, 2004; Gonzalez Viejo et al., 2019; Xu et al., 2017).*

Class	Compound	Threshold (ppm)	Sensory characteristics
Ester	Ethyl acetate	25-50[1]; 30[2,10]	Solvent[1, 2], butter[1], fruity[2], sweet[2]
	Isoamyl acetate	1-2.5[1]; 1.2[2,5,6,10]; 0.51[8]	Fruit[1,2,6], banana[1,2,6,8], apple[2,6], solvent[2,6], estery[2,6,8], tropical fruit[6], berry[6]
	Isobutyl acetate	0.67[7]; 1.6[10]	Fruit[7], solvent[7]
Higher alcohols	1-propanol	800[1,2]	Alcohol[2]
	2-methyl-1-propanol	100-175[1]; 200[2]	Alcohol[2], apple[6]
	3-methyl-1-butanol	70[2]	Tropical fruit[6], orange[6], alcohol[2]
	2-methyl-1-butanol	65[1,2]	Alcohol[2], banana[2], solvent[2], medicinal[2]
	2-hexanol	4[10]	
Aldehyde	Acetaldehyde	10-25[2]; 25[10]	Green apple[2], green leaves[2], fruity[2], sweat[4], pungent[4]
	2-methyl-propanal	0.0023[4]; 0.086[8]	Wine[4], solvent[4], malty[4]
	3-methyl-butanal	0.032[4]; 0.056[8]	Malt[4]
	2-methyl-butanal	0.032[4]; 0.045[8]; 0.157[8]	Cocoa[4], candy[8], flowery[8]
	Hexanal	0.030[4]; 0.088[8]; 0.35[10]	Grass[4], tallow[4], fat[4]
Sulphur components	Dimethyl sulfide	0.06[9]	Cooked sweet corn[11]
Ketone	Diacetyl	0.1-0.2[1]; 0.1-0.15[2]; 0.017[8]; 0.15[10]	Stale milk[1], butter[1,8], solvent[1], toffee[1, 2]

[1] Humia et al., 2019; [2] Olaniran et al., 2017; [3] ; Gonzalez Viejo et al., 2019; [4] Dong et al., 2014; [5] Preiss et al., 2018; [6] Holt et al., 2019; [7] Xu et al., 2017; [8] Saison et al., 2009; [9] Harrison, 1970; [10] Tan and Siebert, 2004; [11] Briggs et al., 2004.

Table 18 summarizes the threshold levels for volatile aromatic compounds as analyzed by HSGC. The lowest reported value is considered to be the threshold level for each compound. Upon examining the esters in the beer, none of the beers produced levels that would be expected to be perceptible in the beer when considering concentrations

after 19 days (Figure 17). Selecting a neutral yeast strain and avoiding excessive fermentation temperatures help maintain low ester production, particularly for isoamyl acetate and isobutyl acetate. Ethyl acetate, a characteristic ester in sour beer production, was also below or at the threshold for all tested strains.

In the raw data, two unidentified peaks were observed, which may represent some of the characteristic esters in sour beer, such as ethyl lactate, ethyl caproate, ethyl caprate, or ethyl caprylate (see further details in Section 1.8). To confirm this, further analysis with standards would need to be conducted. The findings from Pea Beer V4 suggest that RFOs might play a role in increasing the concentration of esters in the fermented beer, especially for *S. cerevisiae*. Despite the suboptimal fermentation observed in the beer containing *B. claussenii*, *L. brevis*, and *L. buchneri*, a considerable amount of ethyl acetate was produced, with the duplicates containing RFO exhibiting four times as much as the control brew. This highlights the potential influence of RFOs on ester production in beer fermentation and warrants further investigation to better understand the underlying mechanisms and impacts on the overall sensory profile of the finished product.

The production of higher alcohols in beer is predominantly attributed to elevated fermentation temperatures and the metabolism of amino acids through the Ehrlich pathway (Pires et al., 2014). None of the fermentation samples demonstrated higher alcohol concentrations above the sensory threshold, indicating that a fermentation temperature of 24°C could be suitable for mitigating higher alcohol or fusel off-flavors. The data from Pea Beer V4 does not suggest that RFOs play a substantial role in the production of higher alcohols, which in low concentrations can enhance the beer, but in larger concentration can be detrimental to the sensory characteristics of beer (Palmer, 2017). Out of the reported higher alcohols attributing to the beany character of peas, 3-methyl-1-butanol was tested for using HSGC. None of the fermented beers tested displayed values above the threshold (70 ppm as seen in table 18), with *L.* thermotolerans producing the most with 50 ppm and 47 ppm in Pea Beer V1 and V2 respectively.

Dimethyl sulfide (DMS) in beer results from the breakdown of the precursor S-methyl methionine (SMM), which forms during the malting process when kilning temperatures exceed 80°C. In malt production with higher kilning temperatures, a profound proportion of SMM is evaporated, making this issue primarily relevant for pilsner malt.

During mashing, SMM is released into the wort, and subsequently broken down into DMS during boiling. Since DMS is a volatile compound, a vigorous boil typically evaporates most of it, but some traces remain in the fermenting beer. Higher fermentation temperatures can aid removal of the remaining DMS, which is why it is typically a problem in lager beers (Palmer, 2017). As presented in the table above, the sensory threshold for DMS is 0.06 ppm, and almost all fermentations in this study had concentrations below this, except for the beers fermented with *B. claussenii*, *L. brevis*, and *L. buchneri* in the Pea Beer V4 trial. This is likely a result of the weak fermentation; a more vigorous fermentation might have reduced the DMS concentration to a greater extent. However, this beer was also fermented using a new batch of malts, and the concentration of SMM in this batch could have been higher than in the previous ones used. The inclusion of RFOs did not appear to affect the amount of DMS in the beer.

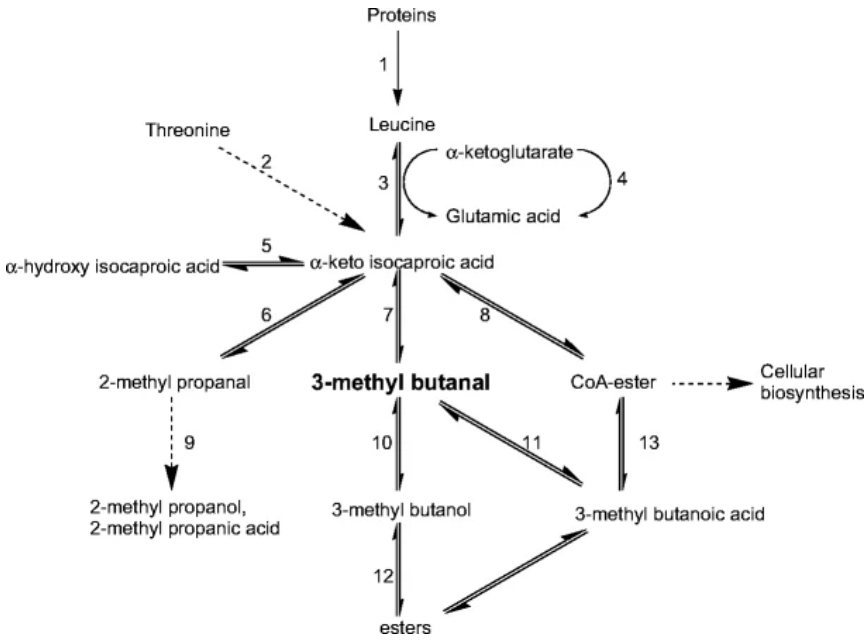


Figure 29: *Metabolic network around 3-methyl butanal in the catabolism of leucine. (Smit et al., 2009)*

The aldehydes 2-methyl-propanal, 2-methyl-1-propanol, 3-methyl butanal, and 3-methyl-1-butanol are all intermediates in the catabolism of leucine, in a pathway parallel to the Ehrlich pathway (figure 29). These compounds have been extensively studied in lactic acid fermentations (Smit et al., 2009). In the context of beer, 3-methyl butanal and 2-methyl butanal are considered the most odor-active compounds in malt (Cramer et al., 2005). Although aldehyde levels in beer are generally low, except for acetaldehyde, they tend to increase in aged beer and may sometimes be undesired

(Vanderhaegen et al., 2007). These aldehydes are present in varying degrees in most beers.

In Pea Beer V4, when comparing fermentations with and without RFOs (figure 26), a slight trend towards higher concentrations of these aldehydes in the beer fermented without RFOs can be observed, particularly in beers fermented using only *S. cerevisiae* US-05. However, more data is needed to draw any definitive conclusions from this observation. Due to the incomplete fermentation by *B. claussenii*, *L. brevis*, *and L. buchneri*, no trend can be identified regarding the impact of RFOs on aldehyde production in the beer.

Acetaldehyde is predominantly found as an intermediate in the formation of ethanol. The amount produced can be strain-dependent, but in general, its presence indicates a stressed or incomplete fermentation caused by underpitching or overpitching (as in Pea Beer V1), high dissolved oxygen levels (as the case being in Pea Beer V3), or by pitching at a high temperature and then allowing the fermentation temperature to decrease. A high amount of simple sugars available early in the fermentation also promotes acetaldehyde formation (Palmer, 2017). As previously mentioned, it can also arise from the metabolism of pyruvic acid. The fermenting microbe usually removes acetaldehyde toward the end of fermentation (Palmer, 2017).

In Pea Beer V1, excessive amounts of acetaldehyde were detected in the beer after 19 days, particularly in the single-fermentation samples. This is most likely attributed to either overpitching or underpitching as a result of inaccurate pitch rate calculations. The acetaldehyde levels in Pea Beer V2 were reduced, most likely due to the correction in inoculation rate. Similar to other esters mentioned above, a higher amount of acetaldehyde was recorded in the beer fermented with *S. cerevisiae* US-05 without RFO, compared to the beer with RFO. Further research is needed to confirm these findings, but it generally appears that RFOs influence the metabolism of aldehydes in beer fermented with *S. cerevisiae* US-05. The incomplete fermentation in the mixed-fermentation samples did not provide any data to clarify the effect of RFOs on aldehyde concentrations in the finished beer.

Vicinal diketones (VDKs), particularly diacetyl, are undesirable off flavors in beer, originating from both yeast and LAB. In yeast, diacetyl production is associated with valine metabolism. During valine anabolism, the intermediate α-acetolactate leaks out

of the cell and into the beer. In the wort, α-acetolactate is chemically converted to diacetyl through an oxidation reaction, which accelerates with increasing temperature. A lack of free amino nitrogen (FAN) also contributes to elevated diacetyl levels (Santos et al., 2019). To reduce diacetyl in beer, a diacetyl rest is recommended towards the end of fermentation. Diacetyl is typically a problem in colder fermentations. Certain LAB strains, such as *P. damnosus*, are notorious diacetyl producers. The most common pathway for LAB to produce diacetyl starts from pyruvate, with an alternative pathway originating from acetaldehyde (Escamilla-Hurtado et al., 1996). Diacetyl was detected only in Pea Beer V1 in the co-fermentation of *S. cerevisiae* US-05 and *P. damnosus through* HSGC analysis. The addition of RFOs in the wort does not seem to affect the production of VDKs in beer.

Throughout the fermentation studies, *L. buchneri* and *L. brevis* were the most consistent performers in terms of fermentation parameters and flavor. *L. delbrueckii*, *L. pentosus* KW2, and *W. confusa* TM76 were the first strains to be eliminated from contention, as they performed poorly under the given fermentation conditions. *L. pentosus* KW1, *W. confusa* TM120, *B. animalis subsp. lactis*, and *P. damnosus* did not produce desirable sensory profiles after 19 days of fermentation. *B. claussenii* was selected as the yeast for Pea Beer V4 based on its known inherent α-galactosidic activity, with the aim of optimizing the beer's success. However, B. claussenii exhibited inconsistent results throughout the trials, ultimately proving to be a questionary decision. The choice to include *L. brevis* and *L. buchneri* in the co-fermentation with *B. claussenii* for Pea Beer V4, was a combination of the fermentation characteristics recorded through the various trials, in combination with subjective taste notes as to their sensory profile.

The presence of a pellicle, a floating biofilm formed at the interface between air and liquid produced by yeast or bacteria (Kwak et al., 2020), observed in Pea beer V2 and V3 suggests that oxygen is reaching the beer, and the pellicle may serve as a protective mechanism. However, limited research exists on this subject, and professional sour beer brewers generally recommend leaving the pellicle undisturbed (Palmer, 2017). AAB are known to cause pellicles in beer, and their presence could indicate contamination (Van Vuuren, 1999). If this were the case, an increase in acetic acid in the beers would be expected. Nevertheless, due to other pathways in LAB that produce acetic acid, it remains uncertain whether the beers were contaminated. To

confirm this, additional microbiological analyses, such as growth in selective media or sequencing techniques, would be required.

In Pea Beer V4, there were indications of pellicles starting to form in both batches fermented with *B. claussenii*, *L. brevis*, and *L. Buchneri*, suggesting that the addition of RFOs did not affect pellicle formation in sour beer. The reduction in pellicle presence can most likely be attributed to the decreased headspace in the fermenters used in Pea Beer V4 compared to those in Pea Beer V2.

Throughout Pea Beer 1, 2, and 4, the average ADF in beers co-fermented with *S. cerevisiae* US-05 and one or more bacterial strains was 76.32%. In comparison, the beer fermented solely with *S. cerevisiae* US-05 exhibited an ADF of 77.76%, indicating that mixed-fermentations generally underperformed in terms of sugar metabolism in the wort compared to regular brewer's yeast. Insufficient attenuation could be attributed to low levels of dissolved oxygen (DO) in the wort. As previously mentioned in Section 1.9, yeast typically requires 8-12 ppm of DO for optimal fermentation. The measured DO values for the different beer media were Beer media 1 = 3.3 ppm, Pea Beer V1 = 3.1 ppm, Pea Beer V2 = 3.1 ppm, Pea Beer V3 = 2.4 ppm, and Pea Beer V4 = 2.5 ppm.

However, in the four batches of Pea Beer V4, US-05 had an ADF of 73% in the wort without RFO and 68.3% in the wort with RFOs. This difference can be explained by the increased OG and higher FG, as the addition of 15 g/L of RFOs led to an increase in ethanol concentration of 0.45%, demonstrating that a good proportion of the constituents in the RFO adjunct can be fermented by *S. cerevisiae*. However, the higher FG of 1.017 (21.48 g/L sugar concentration) compared with 1.013 (30.86 g/L sugar concentration) in the batch without RFO (figure 24), suggesting a proportion of oligosaccharides have been added that *S. cerevisiae* cannot ferment. In Pea Beer V4, the final ethanol concentration in mixed-fermentation batches was 0.82% without RFO and 1.25% with RFO, with a >6% higher ADF. This is equivalent to approximately 5 SG points, the same as the expected gravity increase from the addition of 15 g/L RFO, reflecting the results from the *S. cerevisiae* fermentation. However, when looking at the final sugar concentration and FG in the mixed fermentations, the difference is smaller compared to the two batches fermented with *S. cerevisiae*. The one batch fermented without RFO had a final sugar concentration of 77.55 g/L (1.031 FG) and the one fermented with RFO 80.63 g/L (1.033 FG). This gap would likely increase if

the fermentations would reach completion, but it could indicate better utilization of the RFO constituents in BBB.

The flocculation qualities of the strains could impact the final attenuation numbers, as a more flocculant strain quickly clumps together and sinks to the bottom of the beer, spending less time in contact with the wort and resulting in under-attenuated beers (Palmer, 2017). All beers showed a good degree of sedimentation in the fermenter at the end of fermentation, but overall little attention was directed towards the flocculation abilities of the strains.

The fermentation conditions in Pea Beer V4 appeared to primarily promote the production of organic acids, rapidly lowering the wort's pH. This is likely the main factor inhibiting the growth of *B. claussenii* and the low ethanol production. To address this issue, acid shock starter techniques could be employed to prepare the yeast for the acidic conditions in the wort. In this approach, the yeast is gradually introduced to wort with increasing acidity before being pitched into the wort. This technique, however, is generally used when introducing yeast in the bottle before carbonation (Rogers et al., 2016), and little research has been done on this technique to prepare the yeast for primary fermentation. A more cautious approach would be to ferment the beer in two stages, first a clean fermentation with yeast, and then pitching the lactic acid bacteria after the primary fermentation is done. The main drawback with this would be the increase in fermentation time, as this could take weeks to months to reach the desirable acidity due to less available nutrients and more intrinsic stressors.

In Pea Beer V4 the density of cells in the starter cultures was estimated using spread plate technique. The density of *L. buchneri* was calculated to be an estimated 1.7 x 108 cfu/mL and the density of *L. brevis* was calculated to be 1.25 x 1010 cfu/mL. This equals a pitching density of 1.7 x 107 cfu/mL/°P and 1.25 x 109 cfu/mL/°P. *B. claussenii* was estimated to have 6.4 x 1011 cells/ml in the starter culture, counted using hemocytometer, equaling 6.4 x 1010 cells/ml/°P. How many cells make out a CFU in this case can only be speculated about. On top of this, *B. claussenii* was also counted without using viability staining, increasing the likelihood of the viable cell count being lower. The high pitching rate of LAB in comparison to yeast likely accounts for the quick souring of the beer and the accompanying poor attenuation, and more emphasis on pitching ratios should be taken into account in future experiments on mixed fermentations.

In the sensory analysis performed on Pea Beer V4, all the p-values for the four trials, calculated using binomial distribution, were lower than the significance value of 0.05. This meant that the null hypothesis could be rejected, indicating a statistically significant difference between the samples. Further research could explore more of the sensory characteristics that contributed to the differentiation, including aroma, taste, and mouthfeel, for example through descriptive analysis by a trained sensory panel. With these results the potential is there to elucidate how RFO in mixed fermentations shape the sensory attributes.

5 Conclusion

Designing an experiment to determine the optimal fermentation conditions for mixed fermentations presents a considerable challenge, as demonstrated throughout this thesis. Achieving a delicate balance among several factors is crucial for a successful outcome. These factors include optimizing the ratio of yeast to bacteria being pitched, managing their fermentation temperatures to maximize extract yield and minimize the production of undesirable volatile components, and maintaining a balance between the production of organic acids and the acid tolerance of the strains involved, as well as the overall flavor profile they generate. Competition between strains in a mixed fermentation also adds to the complexity, as certain strains employ various tactics to outcompete others, such as the production of bacteriocins. This interplay between strains makes it hard to determine which microbe affects specific fermentation parameters and which metabolites they produce.

In conclusion, the use of RFO as a substrate in the manufacture of beer has revealed promising indications in contributing beneficially towards the overall flavor and fermentation profile of the beer. The integration of RFOs into the brewing process has resulted in a notable elevation in the concentration of ethanol, which serves to demonstrate that a good proportion of the RFO constituents can be fermented by *S. cerevisiae* and shows that it has potential as an adjunct also outside of sour beer production, maybe in a role mirroring maltodextrin, enhancing the body and mouthfeel of the beer, without contributing any noticeable sweetness (Rübsam et al., 2012). The results also suggest that RFOs affect the metabolism of aldehydes in beer fermented with *S. cerevisiae* US-05, although further research is required to confirm this observation.

However, the impact of RFOs on mixed fermentation, including the utilization of the oligosaccharide constituents, showed mixed results. The main objective to make sour beer using RFO as an adjunct was achieved, and multiple strains showed promising results as to their ability to metabolize the individual constituents of RFO. In co-fermentations however, the fermentation conditions appeared to primarily promote organic acid production, rapidly lowering the wort's pH, and especially when using *B. claussenii*, inducing stress to the yeast, and affecting the production of metabolites, especially aldehydes and esters. One solution to this problem would be to separate

the ethanol fermentation performed by the yeast, and the lactic acid producing fermentation performed by the bacteria into two separate stages, beginning with the yeast. This will however increase the overall fermentation time, but it would simplify the process and yield more consistent results.

It is evident that further studies are needed to fully understand the complex interactions between RFOs, and the various microbial strains involved in sour beer production. Additionally, optimization of fermentation parameters, and the use of acid shock starter techniques to acclimate the yeast to acidic conditions, may prove beneficial in realizing the full potential of RFOs as a substrate in sour beer production. Overall, the incorporation of RFOs as a substrate in sour beer fermentation holds promise, but a more comprehensive understanding of the process and optimization of fermentation conditions are crucial to achieving consistent and desirable outcomes.

The sensory evaluation of Pea Beer V4, given the low attenuation, demonstrates that incorporating RFOs as an adjunct in the production of low-alcohol sour beers has promising potential. The experimental beer fermented with RFOs displayed a discernible differentiation from the beer fermented without RFOs, without increasing unwanted off-flavor associated compounds. Conducting a hedonic analysis in future trials may provide further insight into whether the metabolites generated by RFO fermentation are favored by tasters in comparison to sour beers produced without this supplement.

Future research should continue to examine the interactions between different microbial strains and their combined response to various fermentation parameters, in order to continue to further the understanding of the dynamics affecting these fermentations. This may involve a series of systematic experiments, taking into account strain-specific characteristics, substrate utilization, and the production of volatile compounds. A more complete understanding of these multifaceted interactions will be instrumental to determine the optimal fermentation conditions in mixed fermentations of sour beer, especially when working with novel strains. The potential incorporation of specialty malts to modify the flavor and color profile of sour beers was not examined in this thesis. Future studies could explore the impact of these malts on the overall sensory characteristics of the final product. Varying malts/beer styles would shed light on which possibilities you could have with a given combination of microbes in mixed fermentation.

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6 Appendices

6.1 MSR media

Table 19: *Recipe for MRS media dissolved in 850 mL water before adjusting pH to between 6.2 and 6.5 before brought to 1000 mL with distilled water. Subsequently, the mixture was autoclaved or sterile filtered.*

Amount	Ingredient
10 g	Peptone
8 g	Meat extract
4 g	Yeast extract
2 g	K_2HPO_4 (Dipotassium hydrogen phosphate
5 g	CH_3COONa (Sodium acetate $3H_2O$)
2 g	$C_6H_{14}N_2O_7$ (Diammonium hydrogen citrate)
0.2 g	$MgSO_4$-$7H_20$ (Magnesium sulphate)
0.05 g	$MnSO_4$-H_2O (Manganese sulphate)

6.2 YEP media

Table 20: *Recipe for YEP media dissolved in 850 mL water before adjusting pH to 7.0 before brought to 1000 mL with distilled water. Subsequently, the mixture was autoclaved or sterile filtered.*

Amount	Ingredient
10 g	Yeast extract
10 g	Peptone
5 g	NaCl

6.3 Beer media 1 recipe

Table 21: *Recipe for Beer Media 1. Brewed on BrewTools (Grimstad, Norway) B40 pro.*

Vitals	
Efficiency	71.66%
Batch volume	23 L
Boil time	60 minutes
Mash water	25 L
Sparge water	7.5 L
Sparge water temperature	78°C
Pre-boil gravity	1.032
Original gravity	1.039
Final Gravity	1.015
EtOH	3.27% (v/v)
Dissolved oxygen	3.3 ppm
Mash pH	5.2
Final pH after fermentation	4.22
EBC	4.57
IBU	6.9
Mash temperature	68°C
Mash time	60 minutes
Fermentation temperature	>16°C-18°C
Fermentation time	14 days
Malts	
Bestmalz Wheat malt 4 EBC	2 kg (50%)
Bestmalz Pilsen malt 3.5 EBC	2 kg (50%)
Rice hulls	6% (w/w)
Hops	
Saaz 4.1% α-acids	11.4 g – 60 min addition
Yeast	
Fermentis US-05	23 g
Other	
Lactic acid 80%	Added to mash to hit target pH
Protafloc (Carrageenan)	1 tablet. Added last 15 min of boil
Yeast nutrient (Wyeast)*	½ tsp. Added last 15 min of boil

*Contains a proprietary blend of vitamins, minerals, inorganic nitrogen, organic nitrogen, zinc, phosphates, and other trace elements.

6.4 Beer media 2 recipe

Table 22: *Recipe for Beer Media 2. Brewed on BrewTools B40 pro.*

Vitals	
Efficiency	69.64%
Batch volume	23 L
Boil time	60 minutes
Mash water	22 L
Sparge water	11.5 L
Sparge water temperature	78°C
Pre-boil gravity	1.042
Original gravity	1.047
Final Gravity	1.014
EtOH	4.49% (v/v)
Mash pH	5.32
Final pH after fermentation	10.55
EBC	4.69
IBU	0
Mash temperature	63°C
Mash time	60 minutes
Fermentation temperature	>19°C-22°C
Fermentation time	14 days
Malts	
Bestmalz Wheat malt 4 EBC	2.48 kg (50%)
Bestmalz Pilsen malt 3.5 EBC	2.48 kg (50%)
Rice hulls	6% (w/w)
Hops	
None	
Yeast	
Fermentis US-05	23 g
Other	
Lactic acid 80%	Added to mash to hit target pH
Protafloc (Carrageenan)	1 tablet. Added last 15 min of boil
Yeast nutrient (Wyeast)*	½ tsp. Added last 15 min of boil

*Contains a proprietary blend of vitamins, minerals, inorganic nitrogen, organic nitrogen, zinc, phosphates, and other trace elements.

6.5 Pea Beer Media V1-V3

Table 23: *Recipe for Pea Beer V1-V3. Brewed on BrewTools B40 pro.*

Vitals	
Efficiency V1	60.24%
Efficiency V2	66.26%
Efficiency V3	64.26%
Batch volume	23 L
Boil time	60 minutes
Mash water	22 L
Sparge water	10 L
Sparge water temperature	78°C
Pre-boil gravity V1	1.027
Pre-boil gravity V2	1.031
Pre-boil gravity V3	1.029
Original gravity V1 without RFO	1.030
Original gravity V1 with RFO	1.035
Original gravity V2 without RFO	1.033
Original gravity V2 with RFO	1.038
Original gravity V3 without RFO	1.032
Original gravity V3 with RFO	1.037
Final Gravity V1	1.007-1.009
Final Gravity V2	1.009-1.015
Final Gravity V3	1.011-1.018
EtOH V1	3.31-3.54%
EtOH V2	2.92-3.82%
EtOH V3	0.51-3.38%
Dissolved oxygen V1	3.1 ppm
Dissolved oxygen V2	3.1 ppm
Dissolved oxygen V3	2.4 ppm
Mash pH	5.3
Final pH after fermentation V1	3.64-4.18
Final pH after fermentation V2	3.52-4.54
Final pH after fermentation V3	3.91-5.17%
EBC V1	7.73-15.52 (unfiltered)
EBC V2	5.95-7.75
EBC V3	10.98-20.53
IBU V1	3.5
IBU V2	3.5
IBU V3	3.5
Mash temperature	65°C (62°C for V1)
Mash time	60 minutes
Fermentation temperature	24°C
Fermentation time	19 days
Malts	
Bestmalz Wheat malt 4 EBC	1.83 kg (50%)
Bestmalz Pilsen malt 3.5 EBC	1.83 kg (50%)
Rice hulls	6% (w/w)
Hops	
Archer 1.54% α-acid	20 g – 60 min addition
Yeast	
Fermentis US-05	
Other	
Lactic acid 80%	Added to mash to hit target pH
Protafloc (Carrageenan)	1 tablet. Added last 15 min of boil
Yeast nutrient (Wyeast)*	½ tsp. Added last 15 min of boil

*Contains a proprietary blend of vitamins, minerals, inorganic nitrogen, organic nitrogen, zinc, phosphates, and other trace elements.

6.6 Pea Beer media V4

Table 24: *Recipe for Pea Beer V4. Brewed on BrewTools B40 pro.*

Vitals	
Efficiency	71.60%
Batch volume	30 L
Boil time	60 minutes
Mash water	22 L
Sparge water	18.5 L
Sparge water temperature	78°C
Pre-boil gravity	1.034
Original gravity without RFO	1.036
Original gravity with RFO	1.041
Final Gravity bucket 1	1.008
Final Gravity bucket 2	1.010
Final Gravity bucket 3	1.030
Final Gravity bucket 4	1.031
EtOH bucket 1	3
EtOH bucket 2	3.45
EtOH bucket 3	0.82
EtOH bucket 4	1.25
Dissolved oxygen	2.5 ppm
Mash pH	5.33
Final pH after fermentation bucket 1	3.99
Final pH after fermentation bucket 2	4.62
Final pH after fermentation bucket 3	3.42
Final pH after fermentation bucket 4	3.46
EBC bucket 1	3.51
EBC bucket 2	6.07
EBC bucket 3	6.91
EBC bucket 4	8.67
IBU	3
Mash temperature	63°C for 30 min, 70°C for 30 min
Mash time	60 minutes
Fermentation temperature	24°C-26°C
Fermentation time	14 days US-05, 19 days BBB
Malts	
Bestmalz Wheat malt 4 EBC	2.41 kg (50%)
Bestmalz Pilsen malt 3.5 EBC	2.41 kg (50%)
Rice hulls	2% (w/w)
Hops	
Archer 1.54% α-acid	21.4 g for 60 min
Yeast/Bacteria	
Bucket 1	*S. cerevisiae*
Bucket 2	*B. claussenii, L. buchneri, L. brevis*
Bucket 3	*S. cerevisiae (RFO)*
Bucket 4	*B. claussenii, L. buchneri, L. brevis (RFO)*
Other	
Lactic acid 80%	Added to mash to hit target pH
Protafloc (Carrageenan)	1 tablet. Added last 15 min of boil
Yeast nutrient (Wyeast)*	½ tsp. Added last 15 min of boil

*Contains a proprietary blend of vitamins, minerals, inorganic nitrogen, organic nitrogen, zinc, phosphates, and other trace elements.

6.7 Calculating the new α-acid content of oxidized hops.

Average IBU values in samples using MEBAK® convention = 183 IBU

If the hops contained 3.7% α-acid the estimated IBU value (Brewfather software) if boiled at a concentration of 60 g/L for 1 hour = 440 IBU

$$100\%\ alpha\ acids = \frac{440\ IBU}{3.7\%}\ x\ 100\% = 11892\ IBU$$

$$New\ alpha\ acid\% = \frac{183\ IBU}{11892\ IBU}\ x\ 100\% = 1.54\%$$

6.8 Preparations Starch Assay

This procedure is adapted from the official protocol supplied by Megazyme.

1. Use the content of botte 1 as supplied. Bottle 1 contains thermostable α-amylase.
2. Use the contents of bottle 2 as supplied. Bottle 2 contains Amyloglucosidase.
3. Dilute the content of bottle 3 to 1 L of distilled water. Bottle 3 contains glucose oxidase/peroxidase (GOPOD) reactant buffer. Buffer (50 mL, pH 7.4) p-hydroxybenzoic acid and sodium azide (0.09%, w/v).
4. Dissolve the contents of bottle 4 in 20 mL of solution 3 and quantitatively transfer this to the bottle containing the remainder of solution 3. Bottle 4 contains the GOPOD reagent enzymes: Glucose oxidase plus peroxidase and 4-aminiantipyrine. Cover this bottle with aluminum foil to protect the reagent from light. This is now GOPOD reagent.
5. Use contents of bottles 5 and 6 as supplied. They include D-Glucose standard solution (5 mL, 1,0 mg/mL) in 0.2% (w/v) benzoic acid and standardized regular maize starch control, respectively.

In addition, the following reactants are required, but not supplied:

1. Sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM). To make this, 5.8 mL of glacial acetic acid is added to 900 mL of distilled water. Adjust the pH to 5.0 by adding 1 M sodium hydroxide solution (approximately 30 mL). Add 0.74 g of calcium chloride dihydrate and dissolve. Adjust the volume to 1 L and store the buffer at 4°C.

6.9 Preparations Raffinose Assay

This procedure is adapted from the official protocol supplied by Megazyme.

1. Use the content of bottle 1 as supplied. Bottle 1 contains Buffer (25 ml, pH 8.8) containing sodium azide (0.02% w/v).
2. Dissolve the content of bottle 2 in 12.2 ml of distilled water. Divide into aliquots, freeze, and store in polypropylene tubes. Bottle 2 contains NAD^+.
3. Use the content of bottle 3 as supplied. Shake before opening for the first time to remove any protein that could have settled on the rubber stopper. Store in an upright position. Bottle 3 contains D-galactose dehydrogenase plus mutarotase suspension (2.5 ml).
4. Dissolve the content of bottle 4 with 12 mL of distilled water and divide into aliquots of 3 mL. Store below -10°C and keep on ice during use. Bottle 4 contains α-galactosidase (pH 4.5), lyophilized powder.
5. Use the content of bottle 5 as supplied. Bottle 5 contains galactose standard solution (5 mL, 0.4 mg/ml) in 0.02% (w/v) sodium azide.
6. Weigh 0.5 g of the content of bottle 6 into a 25 mL volumetric flask, adjusting to the mark with distilled water, before divided into 10 mL aliquots and below -10°C. Bottle 6 contains raffinose control powder (~4% w/w raffinose in mannitol).

In addition, the following reactants were used:

1. The Carrez I solution was made by dissolving 3.6 g of potassium hexacyanoferrate in 100 mL of distilled water.
2. The Carrez II solution was made by dissolving 7.2 g zinc sulphate in 100 mL.
3. Sodium hydroxide (NaOH, 100 mM) was made by dissolving 4 g of NaOH in 1 L of distilled water.

6.10 HPAEC analysis of monosaccharides and short oligos on ICS-6000

Standard operating procedure adapted from Thermo Fisher SOP.

Analytical column: Dionex CarboPac™ PA210-Fast-4µm, 150 x 2 mm

Guard column: Dionex CarboPac PA210, 30 x 2 mm

Column precautions:

- Particle size diameter: 4 µm
- pH range: 0-14
- Temperature limit: 4-60°
- Pressure limit: 5000 psi

The normal operating pressure for this column including the guard at 0.200 mL/min is approximately 4000 psi when the EGC is at 12 mM KOH.

Operating conditions:

Idle/standby:

- Column temperature: 30°C
- Flow conditions: 0.150 mL/min
- KOH concentration: 4 mM
- Eluents: Milli-Q®, degassed for 20 min

Running sequences:

- Column temperature: 30°C
- Flow conditions: 0.200 mL/min
- Eluents: Milli-Q® water, degassed for 20 min
- Mode: Isocratic elution with 12 mM KOH electrolytically generated via EGC cartridge
- Injection volume: 0.4 µL

It is recommended before stating a run, to run a wash of the system for a couple of hours with 100 mM KOH, to eliminate baseline problems and carbonate contaminants.

Chromeleon™ CDS software from Thermo Fisher was used to operate the system.

6.11 HPLC analysis of organic acids

Adapted from SOP Organic acid and carbohydrate HPLC protocol from Kari Olsen (2017), NMBU, KBM.

Analytical column: Aminex HPX-87H, 300 x 7,8 mm, BioRad (Hercules, USA)

Guard column: Cation-H refill, 30 x 4,6 mm, BioRad

The column was connected to a 1269 Infinity II HPLC instrument from Agilent Technologies with pump, autosampler, column oven, Refractive Index (RI)-detector (used for acetic acid), and a Diode Array Detector-Ultraviolet (DAD-UV) (used on all the other organic acids).

Running sequences:

- Column temperature: 32°C
- Flow conditions: 0.40 mL/min
- Mobile phase: 5 mM H_2SO_4
- Detector: DAD-UV-detector, wavelength 210 nm
- RI-detector

Openlab CDS software from Agilent Technologies was used to operate the system.

6.12 HSGC of volatile aromatic compounds

Adapted from SOP Volatile Compounds HSGC protocol from Kari Olsen (2020).

Headspace autosampler:

- 7679A automatic headspace sampler, Agilent Technologies
- Headspace bath temperature: 50°C
- Manifold temperature: 60°C
- Equilibration time: 45 minutes at 70 shakes/min.
- Headspace bottle pressure before injection: 10 psi
- Injection time: 0.5 mL/min

Gas chromatography:

The system used for gas chromatography was a 6890 series GC system from Agilent Technologies which was connected to the autosampler and a flame ionization detector also from Agilent Technologies.

- **Column:** CP-SIL 5CB GC, Varian, 25 m x 0,53 mm, film thickness 5 µm
- Carrier gas: Helium 6.0 (Aga, Norway)
    - Flow rate: 5.0 mL/min
- Temperature program:
    - 35°C, 5 min

- Increase of 10°C/min until 40°C and kept for 2 min.
- Increase of 15°C/min until 70°C and kept for 2 min.
- Increase of 30°C/min until 130°C and kept for 4 min.
- Increase of 30°C/min until 160°C and kept for 4 min.
- Increase of 10°C/min until 180°C and kept for 2 min.
- Increase of 10°C/min until 200°C and kept for 2 min.

Openlab CDS software from Agilent Technologies was used to operate the system.

6.13 Water report from ALS Laboratory Group

ANALYSERAPPORT

Ordrenummer	: **NO2305728**	Side	: 1 av 3
Kunde	: **Norges Miljø- og Biovitenskapelige Universitet (NMBU)**	Prosjekt	: ----
Kontakt	: Lars-Fredrik Moen	Prosjektnummer	: ----
Adresse	: Postboks 5003 NMBU 1431 Ås Norge	Prøvetaker Sted Dato prøvemottak	: ---- : ---- : 2023-03-20 12:56
Epost	: lars.fredrik.moen@nmbu.no	Analysedato	: 2023-03-20
Telefon	: ----	Dokumentdato	: 2023-03-23 16:51
COC nummer	: ----	Antall prøver mottatt	: 2
Tilbuds- nummer	:	Antall prøver til analyse	: 2

Om rapporten

Forklaring til resultatene er gitt på slutten av rapporten.
Denne rapporten erstatter enhver foreløpig rapport med denne referansen. Resultater gjelder innleverte prøver slik de var ved innleveringstidspunktet. Alle sider på rapporten har blitt kontrollert og godkjent før utsendelse.

Denne rapporten får kun gjengis i sin helhet, om ikke utførende laboratorium på forhånd har skriftlig godkjent annet. Resultater gjelder bare de analyserte prøvene.

Hvis prøvetakingstidspunktet ikke er angitt, prøvetakingstidspunktet vil bli default 00:00 på prøvetakingsdatoen. Hvis datoen ikke er angitt, blir default dato satt til dato for prøvemottak angitt i klammer uten tidspunkt.

Kommentarer

pH og ledningsevne: Tidssensitive parametere analyseres uakkreditert da tiden fra prøvetaking overstiger analysens krav

Underskrivere	*Posisjon*
Torgeir Rødsand	DAGLIG LEDER

Analyseresultater

Submatriks: **DRIKKEVANN**				*Kundes prøvenavn*	**Slange-venstre**			
				Prøvenummer lab	*NO2305728001*			
				Kundes prøvetakingsdato	*2023-03-08 00:00*			
Parameter	**Resultat**	MU	*Enhet*	*LOR*	*Analysedato*	*Metode*	*Utf. lab*	*Acc.Key*
Totale elementer/metaller								
Ca (Kalsium)	**21.4**	± 2.10	mg/L	0.0500	2023-03-22	W-METMSFX6	PR	a ulev
Fe (Jern)	**0.0044**	± 0.0004	mg/L	0.0020	2023-03-22	W-METMSFX6	PR	a ulev
Mg (Magnesium)	**3.22**	± 0.30	mg/L	0.0030	2023-03-22	W-METMSFX6	PR	a ulev
Mn (Mangan)	**0.00149**	± 0.0001	mg/L	0.00050	2023-03-22	W-METMSFX6	PR	a ulev
Anioner								
Klorid (Cl-)	**35.8**	± 5.36	mg/L	0.500	2023-03-22	W-ANI-ENV	PR	a ulev
Sulfat (SO4)	**46.3**	± 6.95	mg/L	0.500	2023-03-22	W-ANI-ENV	PR	a ulev
Fysikalsk								
Temperatur	**20**	----	°C	1	2023-03-20	W-PH-PCT	NO	*
pH-verdi	**8.6**	----	-	0.1	2023-03-20	W-PH-PCT	NO	*
Ledningsevne (konduktivitet)	**30.7**	----	mS/m	0.100	2023-03-20	W-CON-PCT	NO	*
Alkalinitet pH 4.5	**0.834**	± 0.10	mmol/L	0.150	2023-03-22	W-ALK-PCT	PR	a ulev
Alkalinitet pH 8.3	<0.150	----	mmol/L	0.150	2023-03-22	W-ALK-PCT	PR	a ulev
Hardhet	**0.667**	----	mmol/L	0.00150	2023-03-22	W-HARD-FX	PR	a ulev

Submatriks: **DRIKKEVANN**				*Kundes prøvenavn*	**Vask-høyre**			
				Prøvenummer lab	*NO2305728002*			
				Kundes prøvetakingsdato	*2023-03-08 00:00*			
Parameter	**Resultat**	MU	*Enhet*	*LOR*	*Analysedato*	*Metode*	*Utf. lab*	*Acc.Key*
Totale elementer/metaller								
Ca (Kalsium)	**21.5**	± 2.20	mg/L	0.0500	2023-03-22	W-METMSFX6	PR	a ulev
Fe (Jern)	**0.0118**	± 0.001	mg/L	0.0020	2023-03-22	W-METMSFX6	PR	a ulev
Mg (Magnesium)	**3.30**	± 0.30	mg/L	0.0030	2023-03-22	W-METMSFX6	PR	a ulev
Mn (Mangan)	**0.00099**	± 0.00010	mg/L	0.00050	2023-03-22	W-METMSFX6	PR	a ulev
Anioner								
Klorid (Cl-)	**32.2**	± 4.83	mg/L	0.500	2023-03-22	W-ANI-ENV	PR	a ulev
Sulfat (SO4)	**42.0**	± 6.30	mg/L	0.500	2023-03-22	W-ANI-ENV	PR	a ulev
Fysikalsk								
Temperatur	**20**	----	°C	1	2023-03-20	W-PH-PCT	NO	*
pH-verdi	**7.9**	----	-	0.1	2023-03-20	W-PH-PCT	NO	*
Ledningsevne (konduktivitet)	**30.8**	----	mS/m	0.100	2023-03-20	W-CON-PCT	NO	*
Alkalinitet pH 4.5	**0.798**	± 0.10	mmol/L	0.150	2023-03-23	W-ALK-PCT	PR	a ulev
Alkalinitet pH 8.3	<0.150	----	mmol/L	0.150	2023-03-23	W-ALK-PCT	PR	a ulev
Hardhet	**0.672**	----	mmol/L	0.00150	2023-03-22	W-HARD-FX	PR	a ulev

Kort oppsummering av metoder

Analysemetoder	Metodebeskrivelser
W-CON-PCT	Bestemmelse av konduktivitet (ledningsevne) i rentvann, sjøvann og avløpsvann ihht. NS ISO 7888.
W-PH-PCT	Bestemmelse av pH i rentvann, bassengvann og avløpsvann ihht. NS-EN ISO 10523:2012. Sjøvann basert på NS-EN ISO 10523.
W-ALK-PCT	CZ_SOP_D06_02_072 (CSN EN ISO 9963-1,CSN EN ISO 9963-2, CSN 75 7373, SM2320) Bestemmelse av syrenøytraliserende evne (alkalinitet) ved potensiometrisk titrering og bestemmelse av karbonathardhet og bestemmelse av CO2-varianter ved utregning fra målte verdier inkludert utregning av total mineralisering.
W-ANI-ENV	CZ_SOP_D06_02_068 (CSN EN ISO 10304-1, CSN EN 16192) Bestemmelse av løst fluorid, klorid, nitritt, bromid, nitrat og sulfat ved IC og bestemmelse av nitritt-N og nitrat-N og sulfat-S ved utregning fra målte verdier inkludert utregning av total mineralisering.
W-HARD-FX	CZ_SOP_D06_02_001 (US EPA 200.7, ISO 11885, CSN EN 16192, US EPA 6010, SM 3120, CSN 75 7358 prøver opparbeidet i henhold til CZ_SOP_D06_02_J02 kap. 10.1 og 10.2) Bestemmelse av elementer ved AES med ICP og støkiometriske utregninger av konsentrasjonen til aktuelle forbindelser fra målte verdier inkludert utregning av total mineralisering og kalkulering av summen Ca+Mg. Prøven ble fiksert med salpetersyre før analyse.
W-METMSFX6	CZ_SOP_D06_02_002 (US EPA 200.8, CSN EN ISO 17294-2, US EPA 6020A, CSN EN 16192, CSN 75 7358 prøver opparbeidet i henhold til CZ_SOP_D06_02_J02 kap. 10.1 og 10.2) Bestemmelse av elementer ved MS med ICP og støkiometriske utregninger av konsentrasjonen til aktuelle forbindelser fra målte verdier inkludert utregning av total mineralisering og kalkulering av summen Ca+Mg. Prøven ble fiksert med salpetersyre før analyse.

Noter:

LOR = Rapporteringsgrenser representerer standard rapporteringsgrenser for de respektive parameterne for hver metode. Merk at rapporteringsgrensen kan bli påvirket av f.eks nødvendig fortynning grunnet matriksinterferens eller ved for lite prøvemateriale

MU = Måleusikkerhet

a = A etter utøvende laboratorium angir akkreditert analyse gjort av ALS Laboratory Norway AS

a ulev = A ulev etter utøvende laboratorium angir akkreditert analyse gjort av underleverandør

* = Stjerne før resultat angir ikke-akkreditert analyse.

< betyr mindre enn

> betyr mer enn

n.a. – ikke aktuelt

n.d. – Ikke påvist

Måleusikkerhet:

Måleusikkerhet skal være tilgjengelig for akkrediterte metoder. For visse analyser der dette ikke oppgis i rapporten, vil dette oppgis ved henvendelse til laboratoriet.

Måleusikkerheten angis som en utvidet måleusikkerhet (etter definisjon i "Evaluation of measurement data - Guide to the expression of uncertainty in measurement", JCGM 100:2008 Corrected version 2010) beregnet med en dekningsfaktor på 2 noe som gir et konfidensinterval på om lag 95%.

Måleusikkerhet fra underleverandører angis ofte som en utvidet usikkerhet beregnet med dekningsfaktor 2. For ytterligere informasjon, kontakt laboratoriet.

Utførende lab

	Utførende lab
NO	*Analysene er utført av:* ALS Laboratory Group avd. Oslo, Drammensveien 264 Oslo Norge 0283
PR	*Analysene er utført av:* ALS Czech Republic, s.r.o., Na Harfe 336/9 Prague 9 - Vysocany 190 00

6.14 Calculating the Na^+ concentration from the water report

Table 25: *Summary of ions in the brewing water from the ALS report*

Ion:	Atomic weight:
Ca^{2+}	40.1 g/mol
Mg^{2+}	24.3 g/mol
Na^+	23.0 g/mol
Cl^-	35.5 g/mol
SO_4^{2-}	96.1 g/mol

Recognizing that water is electrically neutral, with anions equating to cations, it is possible to calculate the concentration of Na^+ ions based on the information provided in the water report from ALS Laboratory Group.

By dividing the concentration of a particular ion in the water by its atomic weight, the concentration in mmol/L can be determined. Subsequently, multiplying this value by the ion's charge yields the number of equivalents per liter.

$$Ca^{2+} = \frac{21.4\ mg/l}{40.1\ g/mol} = 0.53\ mmol/l\ x\ 2 = 1.07\ mEq/l$$

$$Mg^{2+} = \frac{3.22\ mg/l}{24.3\ g/mol} = 0.13\ mmol/l\ x\ 2 = 0.27\ mEq/l$$

$$Cl^- = \frac{35.8\ mg/l}{35.5\ g/mol} = 1.01\ mmol/l\ x\ 1 = 1.01\ mEq/l$$

$$SO_4^{2-} = \frac{46.3\ mg/l}{96.1\ g/mol} = 0.48\ mmol/l\ x\ 2 = 096\ mEq/l$$

The concentration of HCO_3^- is provided in the water report as total alkalinity at pH 4.5.

$$HCO_3^- = 0.83\ mmol\ x\ 1 = 0.83\ mEq/l$$

Knowing the number of equivalents per liter:

$$Positives = Negatives$$

$$Ca^{2+} + Mg^{2+} + Na^{+} = Cl^{-} + SO_4^{2-} + HCO_3^{-}$$

$$Na^{+} = 0.83 + 0.96 + 1.01 - 0.27 - 1.07 = 1.46\, mEq/l = 1.46\, mmol/l$$

$$\frac{[Na^{+}]}{23\frac{g}{mol}} = 1.46\, mmol/l$$

$$[Na^{+}] = 1.46\, mmol/l\, x\, 23\, g/mol = 33.58\, mg/l$$

It is assumed in these calculations that the concentrations of potassium, iron, nitrate, nitrite, and fluoride in the water is negligible.

6.15 Missing growth curves from figure 11

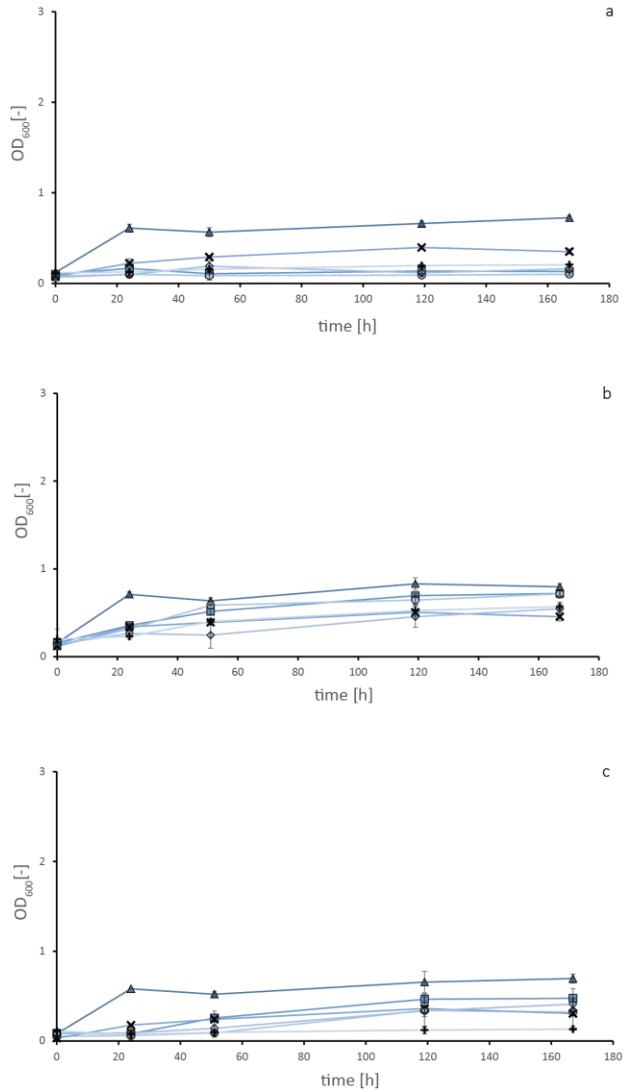
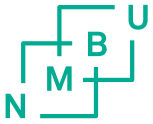


Figure 30: *Growth of bacterial strains in Beer Media 2 with different ethanol concentration and IBU.* ***(a)*** *W. confusa TM76* ***(b)*** *L. brevis* ***(c)*** *L. delbrueckii. ▲ 2.5% EtOH, 0 IBU, ■ 2.25% EtOH, 2.5 IBU, ● 2.25% EtOH, 5 IBU ✕ 4.5% EtOH, 0 IBU. ◆ 4.5% EtOH, 2.5 IBU. ✛ 4.5% EtOH, 5 IBU.*



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