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Fermentation of raffinose family oligosaccharides by different yeast and bacteria in sour beer cofermentations

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Abstract

Legumes are a widespread and nutritious food source, ingested worldwide and used to produce a variety of products. They are however also known for producing gas and bloating when ingested. This is due in part to raffinose family oligosaccharides (RFOs), which cannot be fermented by humans. They are instead fermented by gut bacteria, which causes the symptoms. To improve legume products, RFOs can be removed and utilised in other products. The utilisation of RFOs as adjunct in sour beer was assessed in this study, as well as the impact on the product.

An important factor of this was the fermentation of RFOs by different yeast or bacteria. A range of *Lactococcus cremoris* strains and a *Lactiplantibacillus rhamnosus* strain were selected, alongside *Brettanomyces claussenii* and *Saccharomyces cerevisiae* yeast. These strains were tested for growth in media with RFOs, beer media and media with known concentrations of growth inhibitors.

Several strains showed growth in these conditions and were selected for small and large batch sour beer cofermentations. The produced beers were assessed for chemical and physical properties, using commercial sours beers for comparison. Cofermentations with *Lc. cremoris* TF121 and both yeast strains produced organic acids, which were present in substantially higher amounts when RFOs were added. Sugar utilisation combined with the organic acid profile in small and large batch sour beers showed signs of RFO fermentation. The large batch beers were also tested for sensory difference, using a Tetrad test which showed that a significant number of participants could differentiate between samples. The study shows promising results for the utilisation of legume by-products from food production.

Sammendrag

Belgfrukter er næringsrik matkilde som spises i store deler av verden, og er brukt for å lage flere produkter. De er også assosiert med gassproduksjon og oppblåsthet ved konsum. Dette skyldes til dels oligosakkarider i raffinosefamilien (RFOs), som er ufordøyelige for mennesker. RFO blir istedenfor fermentert av tarmbakterier, som forårsaker de ovenfornevnte symptomene. For å forbedre belgfrukt-produkter kan RFO fjernes, for deretter å benyttes i andre produkter. Bruk av RFO som en adjunkt i surøl-produksjon ble vurdert i denne studien, samt påvirkningen denne adjunkten hadde på det endelige produktet.

En viktig faktor var fermentasjon av RFO ved hjelp av forskjellige gjær og bakterier. Et utvalg av *Lactococcus cremoris* stammer samt en *Lactiplantibacillus rhamnosus* stamme ble valgt, sammen med *Brettanomyces claussenii* og *Saccharomyces cerevisiae* gjær. Disse stammene ble testet for vekst i media med RFO, øl-media og media med kjente konsentrasjoner av vekst-inhibitor-stoffer.

Flere av stammene viste vekst under disse forholdene, og ble valgt for surøl-fermentering i små og store parti. Surølet som ble produsert ble analysert for kjemiske og fysiske egenskaper, med kommersiell surøl til sammenligning. Kofermentasjon med *Lc. cremoris* TF121 og begge gjærstammene produserte organiske syrer, og i langt større kvanta ved tilsetning av RFO. Sukkerforbruk kombinert med organisk syre-profil i små og store parti av surøl viste tegn til fermentasjon av RFO. De store partiene med surøl ble også ved bruk av en Tetrad test analysert for sensorisk forskjell. Testen viste at et signifikant antall deltagere kunne skille mellom prøvene. Studien viser lovende resultater for bruk av biprodukter fra matproduksjon.

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1. Introduction

There has in recent years been a rise in interest towards using legumes and their pulse seeds for food and food production. Legumes are nutrient-rich sources with low production costs, and their cultivation requires less water than animal husbandry (protein/ g) (Mekonnen & Hoekstra, 2010). They are also capable of fixating nitrogen from the otherwise unavailable N_2 in air through a symbiotic relationship with *Rhizobia* bacteria in their roots, eliminating the need for fertilization (Wang et al., 2018). Different species of legumes can grow in almost all climates across the globe, thereby also reducing transport (Li et al., 2022).

Despite these beneficial traits, legumes have a reputation for causing gas and bloating when ingested (Ispiryan et al., 2022). This is due to a group of carbohydrates in pulses called raffinose family oligosaccharides (RFOs). Humans do not have the enzymes necessary to break down RFOs, and they are instead fermented by bacteria in the large intestine, which produces gas (Gibson & Shepherd, 2010). This qualifies them as dietary fibres, and at a low concentration RFOs can be used prebiotically, aiding and strengthening the natural gut flora (Kanwal et al., 2023). However, as the cut-off point above which RFOS cause gastrointestinal discomfort is yet unclear, several strategies to remove the RFOs from legumes and legume products have been devised. This is also beneficial to those suffering from irritable bowel syndrome, who may be extra sensitive to RFOs (Gibson & Shepherd, 2010; Ispiryan et al., 2022).

The Green Plant Food project (GPF) is a cooperation between several academic and industrial partners, which primary goal is to restructure the farm-to-fork systems in Norway towards a more sustainable value chain (Nofima, 2023). One of the objectives within this project is valorisation of side streams. For example, fermentation (e.g., sourdough production) (Loponen & Gänzle, 2018) has been suggested for reducing the fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs) contents as RFOs are a viable carbon source for the metabolism of bacteria and yeast provided they have suitable carbohydrases (Nyyssölä et al., 2020). One previously unexplored product category for such a purpose is sour beer. Not only has the commercial and academic interest in sour beer recently increased, but its production also involves both yeast and lactic acid bacteria (LAB), which have been shown to ferment RFOs (Álvarez-Cao et al., 2019; Zartl et al., 2018).

This master thesis is a part of GPF and investigated the metabolism of RFOs obtained from Norwegian peas in LABs and yeast. The goal was to assess whether RFOs concentrates could be utilized as an adjunct in sour beer brewing, utilizing a side stream product.

2. Literature

2.1 Peas and pea oligosaccharides

Peas (*Pisum sativum*) are a common type of legumes from the *Fabaceae* family. They are one of the larger legume crops by yield and grown in the temperate parts of the world (Allen, 2013). In 2021, the total yield of green peas worldwide was 20.5 megatons, with an average yield of 7.78 T/ha (FAOSTAT, 2021). The legume product consists of a fibrous pod, containing pulse seeds within. Both can be eaten, but the term pea refers primarily to the pulse seed. Dried peas contain low levels of fat (1 - 2.5%), and are high in carbohydrates (60 %) and protein (23%) (Zeece, 2020). This allows for the production of pea flour, as well as pea protein concentrates and isolates (Zeece, 2020). Peas are also a good source of minerals, such as iron, magnesium, phosphorous, potassium and zinc (Matvaretabellen, 2022). While the carbohydrate fraction is primarily starch, approximately (20-25%) are dietary fibres. In peas there also some antinutritional factors such as lectins, tannins, phytate, enzyme inhibitors and RFOs (Laurentin & Edwards, 2012; Martinez-Villaluenga et al., 2008)

The RFOs are fermentable oligosaccharides but indigestible to humans and other monogastric animals (Gulewicz et al., 2014). This is due to their chemical structure, where 1-4 galactose molecules are connected to a sucrose molecule via α-1,6-glycosidic bonds (α-galactosyl derivatives of sucrose) (Zhang et al., 2019). Organisms that digest RFOs do so primarily by using α-galactosidase (EC. 3.2.1.22), which monogastric animals do not produce (Nyyssölä et al., 2020). Instead, the RFOs are fermented by microbiota in the large intestine. This can in smaller doses be beneficial, as the RFOs may have a prebiotic effect (Elango et al., 2022; Hachem et al., 2012; Martinez-Villaluenga et al., 2008). In larger doses, the ingestion of RFO-containing legumes leads to bloating, gas, and general gastrointestinal discomfort. This is especially an issue for those suffering from irritable bowel syndrome, and removal of RFO-containing foods from their diet can give effective relief from the symptoms (Gibson & Shepherd, 2010).

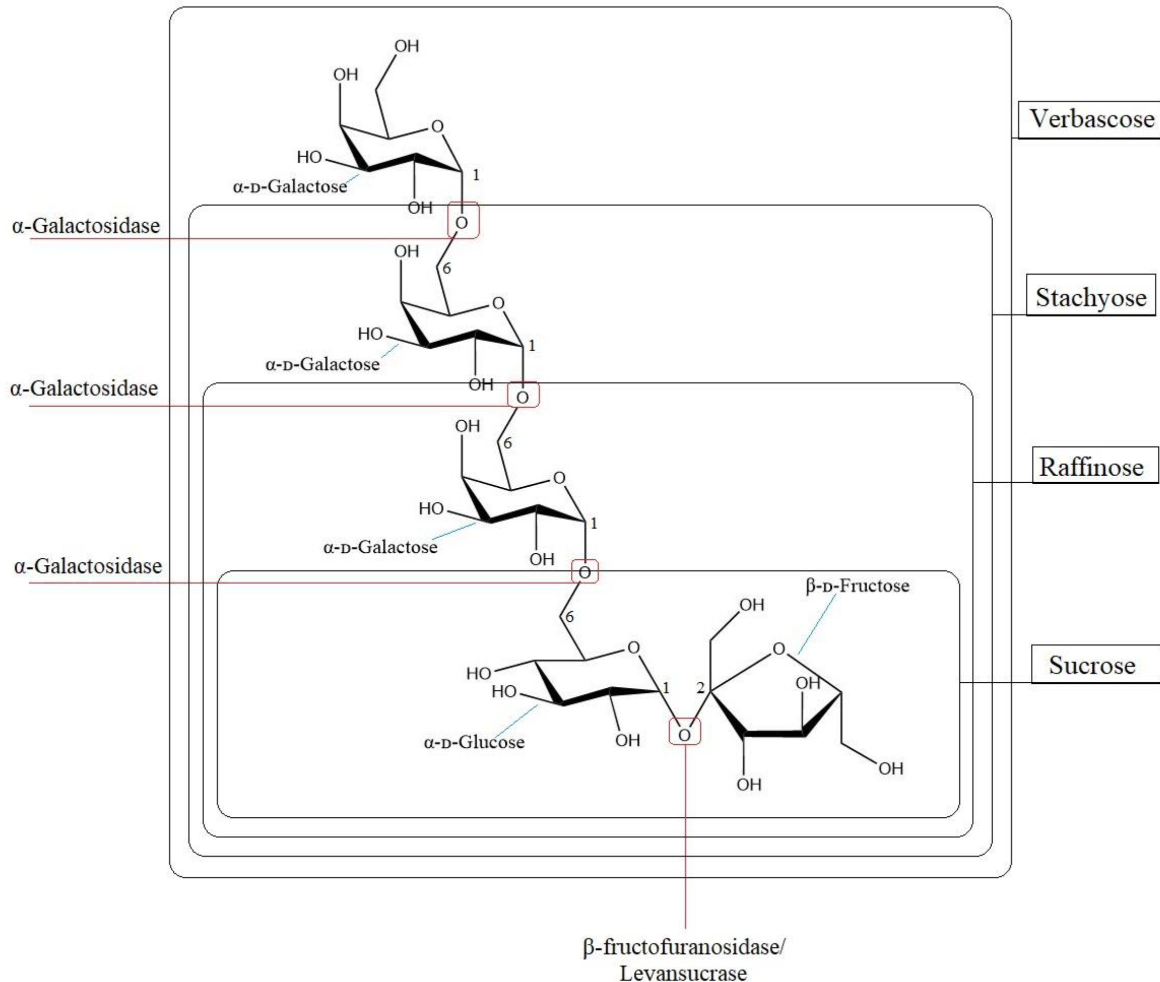

Figure 1: The composition and structure of raffinose family oligosaccharides, and their enzymatic degradation enzymes. Figure inspired by Zhang et al. (2019).

In plants RFOs are utilized as energy storage, for desiccation tolerance, and seed longevity (Nyyssölä et al., 2020). The major enzymes that synthesize raffinose, stachyose and verbascose in plants are galactinol-, raffinose-, stachyose- and verbascose synthetase (Kannan et al., 2018; Sengupta et al., 2015). These transfer a galactinol molecule from galactinol to sucrose, producing raffinose which again is the substrate for further galactinol additions. During germination, these oligosaccharides are hydrolysed by α-galactosidases in the peas, giving energy and carbon sources to the growing plant (Blöchl et al., 2008).

Several studies have focused on the RFO content of different legumes. In these, it has been observed that the content varies between species, but also among the different cultivars and varieties. In a study of 18 pea cultivars, the total amount of RFOs in peas ranged from 22.6 g/kg dry matter (DM) to 63.4 g/kg DM (Vidal-Valverde et al., 2003). The contents of stachyose and verbascose (10.7-26.7 g/kg and 0.0-26.7 g/kg DM) were for most cultivars significantly higher

than for raffinose (4.1-10.3 g/kg DM). These findings agree with other studies, that reported stachyose and verbascose as the main RFOs in peas (Gawłowska et al., 2017; Jones et al., 1999; Kasprowicz-Potocka et al., 2022; Kozlowska et al., 2000; Nyyssölä et al., 2020).

The cleaving of RFOs occurs either at the α-galactosyl terminal end by α-galactosidase (Hachem et al., 2012), or at the α-1,2 glycosidic bond between fructose and glucose by levansucrase (also known as β-fructofuranosidase or invertase) (Gänzle & Follador, 2012; Teixeira et al., 2012). The use of enzymes to remove RFOs from legume products has been proposed and tested (as reviewed by Nyyssölä et al. (2020); Zhang et al. (2019)) for both food and feed. The enzymatic processing can either be through free enzymes included in the feed, or fermentation of the RFOs through lactic acid bacteria (LAB) and/or yeast (Katrolia et al., 2014). Other methods used to remove RFOs include germination, soaking, cooking, roasting, frying, autoclaving and chemical treatment, with varying degrees of success (Zhang et al., 2019).

2.2 Sour beer

Sour beer primarily differs from regular beer in the addition of lactic acid bacteria, as well as the alteration of malt and storage. The common denominator for sour beer is, according to Dysvik et al. (2020b) a higher concentration of organic acids and reduced pH compared to regular beer (pH 3-3.9). The additional acidity from the bacteria produces an environment which is less habitable to other microbes. It is the Belgians that receive most praise for their sour beer traditions, with styles such as Oud Bruin, Flanders red ale, Lambic, Kriek and Geuze (Mosher & Trantham, 2021a). These represent the two greater categories within sour beer, namely spontaneous fermentation, and inoculated fermentation beer. The brewing of sour beer will be discussed in the next section.

2.2.1 Sour beer brewing

Sour beer brewing is similar to traditional beer brewing. In Lambic sour beer, barley malt and unmalted wheat are milled, increasing the surface area of starch allowing enzymes to degrade more of the starch (Briggs, 1978; Marshall et al., 1984). The milled grains are then transferred to a mash tun and combined with warm water. The temperature of the water is decided by the brewer based on preferred enzymatic activity, balancing between starch and protein degrading enzymes (Bamforth, 2009). In traditional sour beer brewing the mashing includes stepwise heating (Spitaels et al., 2017). The mash is then adjusted to about pH 5.2-5.5, the optimum for these enzymes (Mosher & Trantham, 2021b).

When mashing is done, the wort is heated to 75-80°C for a few minutes, which denatures the enzymes present. The spent grain is then sparged with hot water to increase the yield of sugar (Bamforth, 2009; Mosher & Trantham, 2021b). To sterilize the wort and further increase the sugar to water ratio, it is boiled. It is common to add hops at the beginning of the boil. In addition to implementing flavours and aroma, hops add iso-α-acids which are antimicrobials (Bokulich & Bamforth, 2013; Ting & Ryder, 2017; Yang et al., 2021). The boil has the added effect of precipitating high molecular proteins.

Post boil, traditional Lambic sour beer is cooled overnight, while modern Lambic sour beer is chilled quickly (Spitaels et al., 2017). In regular beer, yeast is added post-boil, which is not the case for spontaneous fermentations such as Lambic. They are traditionally left in the cooling tun to be fermented by bacteria and yeast in the air (Spitaels et al., 2017).

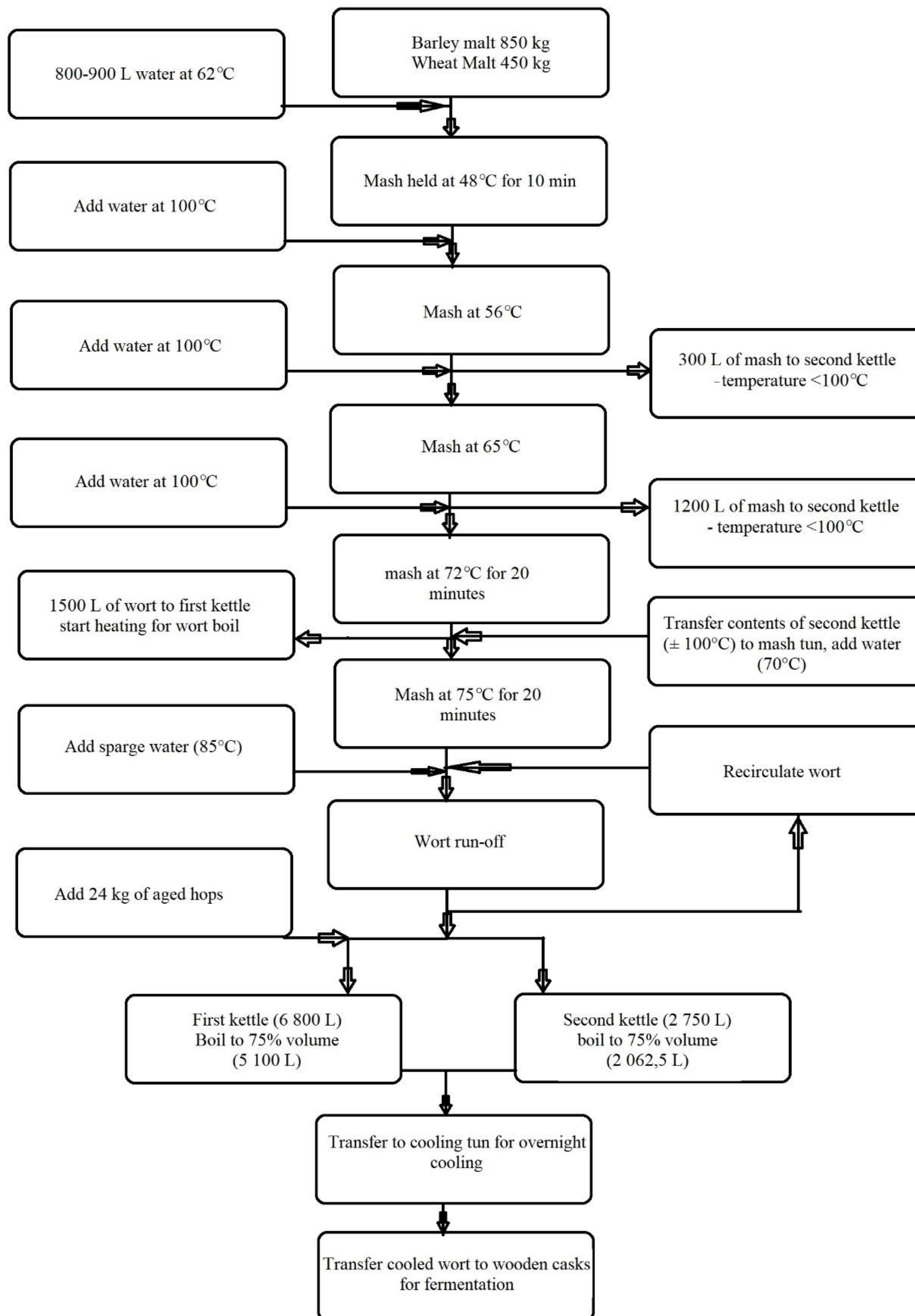

Figure 2 An example of traditional Lambic brewing scheme, adapted from Spitaels et al. (2017).

According to Spitaels et al. (2017) Lambic beer is traditionally produced between October and March, as the wort needs to cool down to 20°C within one night. They further state that the Lambic beer is traditionally produced with 2/3 malted barley and 1/3 unmalted wheat. Due to the method used when producing Lambic beer (a combination of English infusion and German decoction) called turbid mashing combined with the wheat that is added, the wort becomes rich in malto-oligosaccharides (dextrins). This benefits *Brettanomyces* yeast, which unlike the regular *Saccharomyces cerevisiae* can ferment dextrins (Spitaels et al., 2017). In Lambic beer large amounts of aged hops are added to produce a flavour high in hops notes, but low in α-acid notes. This is due to the oxidation of the hops upon aging, which reduces the α-acid levels (Spitaels et al., 2017).

Although the traditional Lambic still has a place in Belgian tradition, the time aspect of upwards to three years maturation has paved the way for inoculated fermentation sour beers (Bokulich & Bamforth, 2013). Red-brown acidic ales are somewhat controlled, in that they are re-pitched with yeast containing LAB from previous brews (Spitaels et al., 2017). The method is closer to regular beer brewing, in that the bacterial cultures and yeast are added to the beer. The bacterial culture can either be added prior to boiling, or post-boil. The first alternative is called kettle souring and allows the bacteria to acidify the wort before they are killed in the boil. Due to the short lifespan and rigorous boil, this method produces little to no aromatic complexity. If the bacteria are added post-boil, the process is called cofermentation (Bossaert et al., 2019; Dysvik et al., 2019). The process allows for a more controlled fermentation than in spontaneous fermentation, giving consistency between batches. These and other methods have been seen as a possibility to speed up the fermentation and maturation process.

Dysvik et al. (2020a) used single *Lactobacillus* strains together with *S. cerevisiae* in beer, with the intention of testing stress tolerance. Their testing for a 21-day cofermentation of *S. cerevisiae* US-05 and *Lactobacillus brevis* BSO464 was comparable to a Belgian Gueuze as determined by a tasting panel. In the same paper, Dysvik et al. (2020a) reported results pointing to the possibility that *Lactobacillus* strains could be metabolically affected by the hurdle effect. This is a much-used technology in industrial food production. Several smaller inhibitory factors (hurdles) applied together, form a barrier which inhibits microbial growth and metabolism (Leistner & Gorris, 1995). It is thus important to balance the hurdles in such a way that the metabolism of the wanted microorganisms are not negatively affected, while suppressing growth of unwanted microorganisms.

2.2.2 Yeast

Of the approximately 1 500 known yeast species, *Saccharomyces cerevisiae* and its strain varieties are the most well-known (Britannica, 2023). Willaert (2012) states that the three major fermentable sugars in barley wort are glucose, maltose and maltotriose, of which maltose comprises 50-70%. The sugars are consumed by yeast in order from simple to more complex formation.

The metabolism of yeast is complex and involves several intermediates and processes. The initial uptake of glucose, maltose and maltotriose from the wort by yeast is accomplished by a range of substrate-specific transporters in the cell membrane. The sugars are shifted through these into the cytosol. Yeast, as most microorganisms, uses glucose prior to degrading maltose and maltotriose (Boulton, 2017). Glucose also has an inhibitory effect on α-glucosidase, the enzyme that degrades the polymerised sugars in the cytosol. Maltose is depolymerised by α-glucosidase to glucose, as is maltotriose (Pires & Brányik, 2015a). Sucrose is cleaved to glucose and fructose outside the cell by sucrose-specific invertase (β-fructofuranosidase (EC.3.2.1.26)). The uptake and use of maltotriose is less preferred in yeast, compared to glucose and maltose (Willaert, 2012). The incomplete uptake of maltotriose from the wort may cause material loss, uncommon beer flavours and unwanted microbial growth.

When exhibiting fermentative sugar metabolism under anaerobic conditions, yeast cells produce ethanol and CO_2 as dominant fermentation products (Jouhten & Penttilä, 2014; Pronk et al., 1996). Additionally, higher alcohols, esters and aldehydes are all by-products of the metabolism. Within moderate amounts, these are important flavour compounds in beer. However, when produced at higher levels, they can also be a source of off-flavours and aroma.

The production of ethanol by *Saccharomyces cerevisiae* and other yeast cells occurs due to ATP production under substrate level phosphorylation in the glycolysis. A molecule of glucose is fermented via several intermediates and enzymatic reactions into two molecules of ethanol, yielding a net positive of two ATP. This is less effective than aerobic oxidation, which yeast compensates for by increasing the speed of fermentation (Jouhten & Penttilä, 2014). Yeast requires NADH to be oxidised to NAD^+, which occurs during anaerobic conditions when the electron acceptor acetaldehyde is present. Acetaldehyde is formed from pyruvate by the enzyme pyruvate decarboxylase (EC 4.1.1.1), along with a CO_2 molecule. The acetaldehyde can then either be a substrate to produce ethanol or acetate, depending on the availability of oxygen (Pronk et al., 1996).

When *S. cerevisiae* is in aerobic conditions, it is able to synthesize most of its growth requirements. Under anaerobic conditions, both the membrane and cell wall adapt by implementing uptake of sterols and fatty acids from their surroundings (Jouhten & Penttilä, 2014; Willaert, 2012). This is one of the major reasons for aeration of the wort when inoculating with yeast. Yeast may also produce ethanol despite being in aerobic conditions. This is known as the Crabtree effect and may give *S. cerevisiae* a competitive advantage to other microbial lifeforms due to the toxicity of ethanol (Smart, 2017). Once glucose and other sugars are depleted, the ethanol can be reabsorbed and converted to acetyl-CoA (Jouhten & Penttilä, 2014; Käppeli, 1987; Pronk et al., 1996).

The previously mentioned CO_2 produced as a by-product along with ethanol to a certain degree diffuses into the wort where it reacts with water to produce carbonic acid (H_2CO_3). Together with production of several organic acids, such as citric, malic, acetic, and lactic acid, this lowers the pH.

Brettanomyces spp. Belong to the yeast genus and are found in alcoholic fermentation products such as wine, cider, and beer. The genus consists of 5 accepted species, and the teleomorph name for the genus is *Dekkera* (Lawton et al., 2021; Steensels et al., 2015). As such, the terms *Brettanomyces* and *Dekkera* are often used interchangeably. The most studies species of *Brettanomyces* is *B. bruxellensis*, while the species covered in this paper is the lesser studied *B. claussenii*. Common for both (but not all *Brettanomyces*) is a facultative anaerobic metabolism (Steensels et al., 2015). The genus has been regarded as a spoilage organism in beverage fermentations, particularly wine. Its sturdy nature allows for it to survive in low pH and high ethanol conditions, such as a fully fermented wine. Despite the lack of glucose, *Brettanomyces* spp. yeasts are able to survive on pentose sugars, producing phenolic off-flavours in the wine (Lawton et al., 2021).

Contrary to the wine industry, beer brewers and particularly craft beer brewers have begun using *Brettanomyces* spp. as a viable yeast. This is in part due to the same flavours produced in wine, which give beer a “Lambic” or “Gueze” style in the aroma profile. All *Brettanomyces* strains have been isolated from traditional spontaneously fermented Lambic beer, primarily in the latter stages of fermentation/maturation (Lawton et al., 2021). The flavours produced by *Brettanomyces* yeast are characteristic in several sour beers. The general description involves fruity and floral notes, which are a result of phenolic acid decarboxylase and vinyl phenol reductase. Other enzymes in the yeast are able to hydrolyse the monoterpenes available from

hops, and through further β-glucosidase activity produce flavour compounds in beer (Lawton et al., 2021).

The *Brettanomyces* spp. genus shares several common features with the standard brewing yeast *S. cerevisiae*. These include the ability to endure high osmotic and ethanol stress, low pH, and oxygen environments, as well as the ability to ferment sugar to alcohol in aerobic conditions (Crab tree effect) (Pronk et al., 1996; Steensels et al., 2015). While some of these traits are common in yeast, the accumulation of several in a single species is rare. Unlike *S. cerevisiae* which shows a Pasteur effect when there is little sugar available, the facultative anaerobic *Brettanomyces* species stop the fermentation of glucose to ethanol under total anaerobic conditions (Custers effect (Serra Colomer et al., 2019; Steensels et al., 2015)). This is due to limited amounts of glycerol-3-phosphate phosphatase (G3PP) activity, and the lack of available NAD^+.

Under aerobic conditions NADH is produced in an NAD^+-aldehyde dehydrogenase complex, which also forms acetic acid. This is reverted to NAD^+ with electron acceptors such as oxygen present, but quickly blocks itself when taken from an aerobic to anaerobic condition. If *Brettanomyces* had the ability to produce glycerol (via G3PP), this would alleviate the redox imbalance. Instead of this the yeast suffers a prolonged lag phase and slow growth. The fermentation begins when NADH is reoxidised intracellularly, via reduction of hydroxystyrenes or with the external electron acceptor acetoin (Steensels et al., 2015), These pathways are slower compared to the preferred aerobic pathway, and the yeast stops producing acetic acid as a result. (Spitaels et al., 2014; Steensels et al., 2015).

2.3 Lactic acid bacteria

The use of LAB in fermentations has been extensively studied, due to their importance in food processing. They are a large heterogenous group of bacteria, sharing the metabolic property of producing lactic acid from carbohydrate fermentation (Mayo et al., 2010). With a few exceptions LABs are generally recognised as safe (GRAS) for food. They are found naturally on plants, vegetables, cereals and in milk as well as in the gut flora of humans and animals (Mayo et al., 2010). The fermentation of food by LAB affects the final product's flavour, nutritional and rheological properties (Leroy & De Vuyst, 2004).

Due to the lack of a respiratory system, LAB obtain energy through substrate-level phosphorylation. For LAB this is either through the homofermentative pathway (glycolysis) or the heterofermentative pathway (phosphoketolase pathway). Glycolysis will under normal

circumstances produce lactic acid from glucose as its only by-product in energy production. The phosphoketolase pathway produces CO_2 and ethanol or acetate along with lactic acid as by-products. Other hexoses such as galactose, mannose or fructose are isomerized and phosphorylated prior to entering either of these pathways (Wright & Axelsson, 2019). The fermentation of disaccharides is similar to that of monosaccharides, but with the addition of cleaving enzymes such as β-galactosidase, phospho-β-ᴅ-galactosidase, and sucrose hydrolase (Wright & Axelsson, 2019).

Their metabolic pathways divide LAB into three fermenting groups. The first are the obligate homofermentative bacteria, which are unable to ferment pentoses. The secondary group is comprised of obligate heterofermentative bacteria, and third are the facultative heterofermentative bacteria which are homofermentative for hexoses but can ferment pentoses through heterolactic fermentation (Wright & Axelsson, 2019).

As the name suggests, lactic acid bacteria primarily produce lactic acid, with the heterofermentative bacteria being able to additionally produce CO_2 and ethanol/acetate. There are however many other substances that pyruvate, the intermediate of glycolysis, can be metabolized into. Several LABs produce diacetyl (butter aroma) and acetoin (2,3-butanediol) when pyruvate is at a surplus, while formate, ethanol, acetate, and lactate may be the by-products when substrate is limited (Wright & Axelsson, 2019). This can also occur with homofermentative species, aptly named mixed acid fermentations. Other pathways for pyruvate include the enzymes pyruvate oxidase and pyruvate dehydrogenase, which form acetic acid and acetate respectively as by-products.

2.3.1 Lactococcus

The genus Lactococcus is morphologically identified as short chains of 0,5-1,5 µm cocci which are gram positive. They are with one exception mesophilic and homofermentative, producing lactic acid from hexose fermentation (Wright, 2019). Due to their importance in the dairy industry, genus *Lactococcus* has received much attention from the scientific community. The subspecies of *L. lactis* which are included in starter cultures are especially studied.

The ability of homofermentative bacteria to modify their fermentation pathway to that of mixed acid fermentation has been suggested to be vital for their adaptation to growth in beer (Geissler et al., 2016). This is according to Geissler et al. (2016) potentially due to pH homeostasis, in which the proton gradient outside the cell is equal to that of the cell. This has been shown in

Lactococcus lactis, where alternate electron transporters are utilized in mixed acid fermentation (Kowalczyk & Bardowski, 2007).

2.3.2 Lactobacillus

The genus *Lactobacillus* are rod-shaped bacteria, which are classified as LAB. As with *Lactococcus* they are gram positive and can be either aerotolerant or anaerobic. Unlike *Lactococcus,* several species of *Lactobacillus* are facultative/obligate heterofermentative, and are thus able to produce CO_2 and ethanol/acetate.

Lactobacillus ability to grow in beer has primarily been viewed as an issue in the scientific literature, and they are described among the most common beer spoiling bacteria (Pittet et al., 2011; Suzuki, 2011). They are able to grow and proliferate in the otherwise harsh conditions that beer provide (CO_2, low pH/nutrients, hop bitter acids, ethanol), producing acidity, turbidity, viscosity and off flavours (Fraunhofer et al., 2017). They have been found to be somewhat ethanol and hop resistant, which gives them a great advantage in beer media (Pittet et al., 2011; Suzuki et al., 2008).

2.4 Flavour

The testing of flavours in beer is an interdisciplinary art, combining chemistry with tasting. These allow for the use of both threshold testing (noticeable taste/aroma compounds) and methodical reference testing to accurately describe the product and its individual components. The taste and smell of beer are along with its brew-style the defining characteristics, and thus of vital importance. These hail from the raw materials used (type of malt and adjunct; amount, intensity, and variety of hops; water quality) as well as from the fermentations that take place (yeast and bacterial fermentation) along with the maturation and carbonation. These factors along with their interaction allows for a multitude of flavours and aromas, which can be chemically categorized into 6 sections: Alcohols, esters, organic acids, carbonyl compounds sulphur containing compounds and volatile phenol compounds (Willaert, 2012).

2.4.1 Alcohols

Alcohols, higher alcohols, or fusel alcohols as they are called are primarily produced through fermentation by yeast and to a lesser extent through the fermentation of LAB. In yeast, they are produced via either the Ehrlich (catabolic) or the amino acid pathway (anabolic). The Ehrlich pathway transaminates amino acids from the wort, giving the corresponding α-keto acid. The common amino acids leucine, valine and phenylalanine gives isoamyl alcohol, isobutanol and

phenylethanol, respectively, which are then decarboxylated into aldehydes. The aldehydes are however intermediaries in this, due to the alcohol dehydrogenase which reduces them to fusel alcohols, a term used for long-chain alcohols (Willaert, 2012).

The synthesis of amino acids from the carbohydrate source in the anabolic pathway is also the source of α-keto acids for fusel alcohol production. This pathway is important during the latter stages of fermentation and maturation, and of less importance to the higher polymerized alcohols (Willaert, 2012). The synthesis of fusel alcohols is also dependant on the growth factors of the yeast cell, such as nutrient availability and temperature.

According to Yonezawa and Fushiki (2002) the primary alcohols in beer are ethanol, isobutanol, 2- or 3-methylbutanol and 2-phenylethanol. These primarily give of an alcoholic flavour, although some are also described as vinous.

2.4.2 Esters

Esters are among the primary flavour compounds in beer, according to Horák et al. (2010). Ester formation is influenced by the fermentation parameters, yeast strain and wort composition. The formation of esters is linked to the fatty acid metabolism of yeast through the Acetyl-CoA molecule, which is also central in glycolysis. The formation of esters occurs by reaction between Acetyl-CoA and alcohol, catalysed by alcohol acyltransferases. The primary group of esters in beer is acetate esters, which form by the reaction between Acetyl-CoA and ethanol, 2-phenylethanol or isoamyl alcohol (Willaert, 2012).

The link between the fatty acid metabolism and esters is due to the need for oxygen to synthesize lipids. Knowing this, brewers may regulate the oxygenation of the brew accordingly to produce a brew high or low in esters (Pires & Brányik, 2015b). These can give a variety of fruity and floral aromas to the beer, with the most important being ethyl acetate, isoamyl acetate, isobutyl acetate, phenyl ethyl acetate, ethyl hexanoate and ethyl octanoate (Pires & Brányik, 2015b).

2.4.3 Organic acids

The production of organic acids is important both for the taste as well as the microbial safety of the beer. These are in normal beer produced by the yeast, with organic acids such as pyruvate, acetate, lactate, citrate, succinate and malate being present at a moderate concentration (Willaert, 2012). There are also certain medium chain fatty acids present, such as caproic, caprylic and capric, all of which attribute to a “goat-like” smell (Yonezawa & Fushiki, 2002).

Their synthesis in beer is particularly due to the incomplete Krebs cycle during fermentation (Li & Liu, 2015).

Higher levels of organic acids may be present in sour beers with homo/heterofermentative bacteria (Ciosek et al., 2020). Dysvik et al. (2020b) reported concentrations of ~2600 mg/L lactic acid, ~950 mg acetic acid and ~200 mg/L succinic acid for a cofermentation with *S. cerevisiae* and *L. brevis* while little or none of these acids were detected in a fermentation with *S. cerevisiae* alone.

2.4.4 Carbonyl compounds (aldehydes and vicinal diketones)

The overarching category of carbonyl compounds stretches from the green apple flavour of acetaldehyde to the buttery taste of diacetyl and 2,3-pentanedione. The production of the latter is a by-product of amino acid synthesis, as with fusel alcohols (Willaert, 2012). As the taste threshold is much lower for diacetyl, it is a bigger contributor to off-flavour, and several steps may be taken to either avoid the formation of these or reduce the amount prior to bottling (Pires & Brányik, 2015a; Willaert, 2012; Yonezawa & Fushiki, 2002).

As for the aldehydes, several short chain unsaturated aldehydes have a grassy/leaf-like flavour, while aroma notes for the longer chained aldehydes include insect/orange-like (nonanal) or paper, cardboard, oxidized (*trans*-2-nonenal) and often have low thresholds (below 0,1 ppm) (Yonezawa & Fushiki, 2002). Aldehydes also contribute to the flavour of food products other than beer. The beany off-flavours associated with peas is primarily ascribed to hexanal, which is a generated as a consequence of lipoxygenase-induced lipid oxidation (El Youssef et al., 2020).

2.4.5 Sulphur containing compounds.

The sulphur in beer is usually sourced from the raw ingredients but is on its own of little issue. The problem for brewers lies primarily in the formation of unwanted flavour compounds such as hydrogen sulphide, dimethylsulfide (DMS), diethyl sulfide and ethyl mercaptan. These have all a low threshold (below 0.1 ppm), and give flavours such as rotten egg, boiled cabbage and rotten leeks (Yonezawa & Fushiki, 2002). Of these, the DMS is the most studied, and several strategies have been outlined to avoid it during fermentation and maturation. These include rigorous boiling (Pires & Brányik, 2015a), using DMS-degrading yeast strains (Bokulich & Bamforth, 2013) reducing the DMSO (precursor) amounts in the wort and extended maturation periods for reuptake of DMS by the yeast cell.

2.4.6 Volatile phenolic compounds

In beer, phenolics are either in a monomeric or polymeric form and include flavanols, volatile- and phenolic acids (Ouellette, 1997; Vanbeneden et al., 2008). Phenolic acids found in beer include gallic-, vanillic-, ferulic-, and caffeic acid, which are derivatives of benzoic and cinnamic acid. These are however usually below the threshold levels for detection, and thus go unnoticed (Vanbeneden et al., 2008). Of the noticeable phenolic acids in beer, phenol, vanillin acetovanillone and more have been detected (Willaert, 2012). These are usually contained and released from the raw material, and only two can be produced by yeast during the fermentation (4-vinylguaiacol and 4-vinylphenol). A surplus production of these can cause a "phenolic off-flavour (Thurston & Tubb, 1981), described as clove-like.

2.5 Aim of study

The focus of this study is the utilisation of side stream products, in the form of RFOs from pre protein concentrate. The RFOs will be used as an adjunct in sour beer brewing, with a focus on the fermentation of the sugars. This will be assessed through the application of yeast and bacteria with the potential to ferment these sugars. Utilisation will be estimated primarily by growth in optimal media, beer media and media with known concentrations of growth inhibitors. Yeast and bacteria strains shown to grow in these media will be used for cofermentations in small-batch sour beers with RFOs. These beers will be analysed for chemical and physical properties. Promising strains from the small batch fermentations will be applied in a larger scale sour beer with and without RFOs. The beer will be assessed for chemical and physical properties and used in a Tetrad test, for significant sensory difference between sour beers. An overview of the thesis can be seen in figure 3.

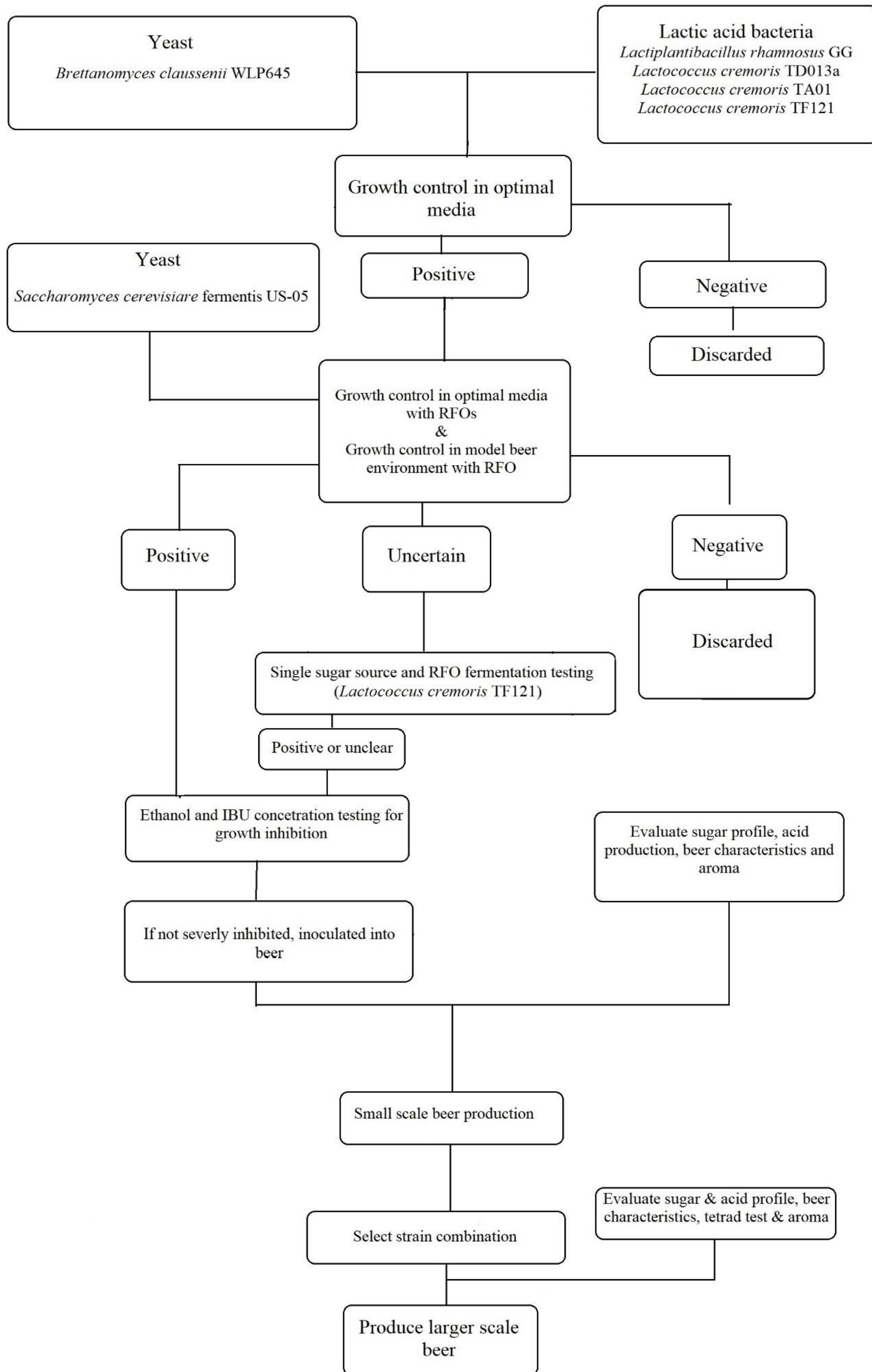

Figure 3: Flow chart of the process and steps of the thesis.

3. Materials and method

3.1 Bacterial/yeast cultures and substrate

The bacterial strains *Lactococcus cremoris* TA01*, Lc. cremoris* TD013a*, Lc. cremoris* TF121 (all formerly *Lactococcus lactis* subsp. *cremoris,* unknown ATCC/DSM due to confidential project) were donated from the Scifood group at NMBU and *Lacticaseibacillus rhamnosus* GG (ATCC 53103) from the PEP group at NMBU. The *Lactococcus* strains were in M17, and the *Lacticaseibacillus* strain in de Man, Rogosa and Sharpe (MRS) medium, both with added glucose (M17-G and MRS-G). The yeast strains *Brettanomyces claussenii* WLP645 (White labs, San Diego, California, USA) and *Saccharomyces cerevisiae* SafAle™ US-05 (Fermentis, Marquette-lez-Lille, France) (in future referenced as *S. cerevisiae* US-05) were bought as a wet mix and dry yeast, respectively.

All inoculation, reinoculation and sterile filtering was done using sterile equipment, in a sterile cabinet (Telstar™ AV-100 Vertical Laminar Flow Bench). Unless specified otherwise, *Lactococcus* strains were incubated at 30°C, *Lacticaseibacillus* at 37°c, yeast at room temperature (19-21°C) and sour beer at 24°C.

The RFOs were obtained from pea protein concentrate (AM nutrition, Stavanger, Norway), as summarized by Garbers et al. (2022).

Table 1: Yields of raffinose family oligosaccharide extracts obtained by Garbers et al. (2022) from pea protein concentrate.

Sample	RFO amount per 100 g
RFO mix 1	79.724 g
RFO mix 2	39,338 g
RFO mix 3	46,681 g
RFO mix 4	71,542 g

3.2 Fermentation studies

3.2.1 Culture testing and RFO utilization

Initial growth control

For growth testing, 50 µL of *B. claussenii* WLP645 mix was inoculated in 4.95 mL (1:100) of yeast extract peptone medium with 20g/L glucose (YEP-G) broth in a cell culture tube (CELLSTAR® Polystyrene Cell Culture Tubes 14 mL (Greiner Bio-One GmbH, Kremsmünster, Austria)). Testing the growth of *S. cerevisiae* US-05, 1 g of dry yeast was

solubilized in 5 mL of deionized water (Millipore Milli-Q, Merck). A sample of 50 µL from the solubilized yeast-water mixture was added to 4.95 mL YEP broth in a cell culture tube. Both yeast cultures were incubated for 72 hours at 30°C. The bacterial cultures were reinoculated into M17-G and MRS-G at a 1:100 ratio in cell culture tubes, as described above. These were incubated for 72 hours at 30°C (*Lactococcus* strains) and 37°C (*L. rhamnosus* GG). For reference the samples were also grown in media without carbon source.

Table 2: The ingredients used to produce yeast extract peptone, de Man Rogosa and Sharpe, and M17 broth/agar (BD; Himedia, 2016; Neogen).

Ingredients	**Yeast Extract Peptone (YEP) Broth 1 L**	**De Man, Rogosa, Sharpe (MRS) Broth 1 L**	**M17 Broth 1 L**
Bacto peptone	10 g/L	10 g/L	
Beef extract		5 g/L	5 g/L
Pancreatic digest of casein			5 g/L
Soy peptone			5 g/L
Yeast extract	10 g/L	10 g/L	2.5 g/L
Ascorbic acid (C6H8O6)			0.5 g/L
Dipotassium phosphate (K2HPO4)		2 g/L	
Disodium β-glycerophosphate (C3H7Na2O6P)			19 g/L
Magnesium sulphate (MgSO4)		0.2 g/L	0.25 g/L
Manganese sulphate (MnSO4)		0.05 g/L	
Sodium acetate (C2H3NaO2)		5 g/L	
Sodium chloride (NaCl)	5 g/L		
Triammonium citrate (C6H17N3O7)		2 g/L	
Agar (added when producing Agar plates)	15 g/L	15 g/L	11 g/L
Glucose (for a broth/agar with glucose)		20 g/L	20 g/L
RFO (for a broth/agar with RFO)		20 g/L	20 g/L
Milli-Q water	1000 ml	1000 mL	950 mL
Final pH at 25°C	7.00 ± 0.2	6.40 ± 0.2	6.90 ± 0.2

Freeze stock production

In order to keep a viable stock for the duration of the thesis work, 3 tubes of glycerol stock were made for each bacterial and yeast strain. The production of this stock follows the method described by Addgene. The stock was then frozen at -80°C and used as needed. For reanimation, a sterilized toothpick was inserted in the stock under sterile conditions and transferred to a

culture tube with media. The culture was then allowed to grow for 24-48 hours, before reseeding into fresh media.

RFO utilization control

The reinoculated cell cultures of *Lc. cremoris* TA01/TD013a/TF121 and *L. rhamnosus* GG were used to test whether the bacteria were able to utilize RFOs as a carbon source. M17 and MRS media was prepared using 20 g/L of RFO mix 1 (M17-RFO and MRS-RFO), as well as M17 and MRS media without a sugar source. Prior to inoculation, samples were vortexed (Fisherbrand™ ZX3 vortex mixer (Fisher Scientific, Roskilde, Denmark)) and inoculated at a ratio of 1:100 in 4.95 mL media in cell culture tubes. All inoculations were done in duplicate, and optical density (OD) was measured at 600 nm every 24 hours using a cell density meter (Ultrospec® 10 Cell Density Meter, Biochrom), for a total of 72 hours.

The *B. Claussenii* WLP645 and *S. cerevisiae* US-05 yeasts were inoculated from the initial stock into YEP-G (20 g/L glucose) at a 1:50 ratio, as well as YEP with 20 g/L RFO mix 1 and YEP without any sugar source. As initial growth had been proven slow, the mixtures were incubated at 30°C for 5 days and measured regularly.

Growth testing using single sugar sources

The growth of *Lc. cremoris* TF121 in media with specific substrates was evaluated by combining M17 media with 5 g/L RFO mix 1, 10 g/10 mL RFO mix1, 10 g/L of lactose, maltose, sucrose or raffinose, as well as 14 g/L of stachyose (of 70% purity). Cell culture tubes were set in duplicate, filled with 4.95 mL of each medium and inoculated with 50 µL of Lc. cremoris TF121. Growth was measured at regular intervals.

3.2.2 Growth in beer media

First base beer production

A base beer was produced as a growth medium, using a recipe with 2 kg wheat (Bestmalz Wheat malt 5 EBC, Heidelberg, Germany) and 2 kg barley malt (Bestmalz Pilsen malt 3,5 EBC, Heidelberg, Germany). The grains were milled to 1.2 mm, and 240 g of rice hulls were added post milling. In a Brewtools B40Pro (Grimstad, Norway), 25 L of mash water was heated to 68°C and adjusted to pH 5.2 using Brouwland Lactol (lactic acid 80% pure, Beverlo, Belgium).

At 68°C, the milled wheat and barley malt along with the rice hulls were added to the mash tun, and stirred in. Total mash time was 60 minutes, after which outmash occurred at 75°C for 15 minutes. The mash tun was after outmash raised from the Brewtools, and sparged with 7.5 L water maintained at 78°C. Post sparging, the wort was heated to 100°C and boiled for 60 minutes. At the beginning of the boil, 11.4 g of Saaz hops (4.1 % α-acids (Finest Co brewing supplies, Sofiemyr, Norway)) was added in a hop bag and left in the wort for the duration of the boil. During the last 15 minutes of the boil, 1 Tablet of protafloc (Finest Co brewing supplies, Sofiemyr, Norway) and ½ tsp. of Yeast nutrient (Wyeast laboratories Inc, Hood River Oregon USA) was added. The wort was cooled to >20°C using a water-cooled Counterflow Cooler Pro (Brewtools, Grimstad, Norway) after boiling, and 23 L were transferred into a 30 L fermentation vessel with yeast cap. Using 23 g of *S. cerevisiae* US-05, the batch was fermented for 14 days at >16°C, reaching an ethanol level of 3.27 % (v/v). The hops added a total of 6.9 international bitterness units (IBU.)

Beer medium testing

The first base beer (3.25% ethanol and 6.9 IBU) was sterile filtered into a 1 L glass laboratory bottle (Schott Duran Original round GL45 Clear Borosilicate glass laboratory bottle 1 L, VWR, Pennsylvania, USA) using a 0.22 µm PES membrane bottle top filter (Millipore, Burlington, Massachusetts, USA).

Half the medium was transferred to a 1 L sterile glass laboratory bottle and mixed with 20g/L RFO mix 1. Mixing was done using a sterilized mixer magnet and a magnetic stirrer at 700 RPM and room temperature until complete solubilization. After stirring in the RFO mix 1, the medium was sterile filtered again using the bottle top filter mentioned above.

For testing, cell culture tubes containing 4.95 ml of base beer 3.25% with/without RFOs were inoculated with 50 µL *Lc. cremoris* TA01/TD013a/TF121 and *L. rhamnosus* GG. All inoculations were done in duplicate. Total incubation time was 72 hours.

Second base beer production

To produce the second base beer, 23 L of water were heated to 62°C and adjusted to pH 5.3 using lactic acid (80%) in a Brewtools B40Pro. To the water 2.43 kg of Bestmalz Wheat malt (5 EBC) and Bestmalz Pilsen malt (3,5 EBC) were added. Mashing was done for 60 minutes, with additional outmash at 78°C for 15 minutes. The mash tun was raised out of the Brewtools, and sparged with 10 L of 75°C water. Boiling was done for 60 minutes, protafloc and yeast nutrition were added as described before (section 3.2.2). The batch was cooled to <20°C using

a water-cooled spiral cooler after boiling. Subsequently, 20 L of wort was transferred to a 30 L fermentation vessel and inoculated with 23 g of *S. cerevisiae* US-05 yeast. The batch was fermented for 14 days at ca. 16°C and reached a final ethanol percentage of 4.5% (v/v).

IBU and ethanol concentration testing

The *L. rhamnosus GG* strain, *S. cerevisiae* US-05 strain, *B. Claussenii* WLP645 strain and all *Lactococcus* strains were used during the first test with 2.25% and 4.5 % ethanol. All but the *B. claussenii* strain were used in subsequent 3.5%, 4.5% and 6% ethanol testing. For all tests the inoculation volume was 1:50, and all concentrations can be seen in Table 3.

To test the effect of IBU and ethanol percentage on the chosen cultures, a total of three tests were performed, as outlined in Table X. In the first test, beers of two ethanol concentrations (4.5% and 2.25%) were made with 0, 2.5, or 5 IBU. This was done by using the second base beer (diluted with water from 4.5% to 2.25% ethanol), and a hop concentrate with a known IBU of 185 IBU. All samples were made with 15 g/L RFO mix 1 added. For the second test, beers with three ethanol concentrations were made (3.5%, 4.5% and 6%). Beers with 4.5% or 6% ethanol (11.5 mL 96% ethanol added to 500 mL 4.5% beer) had the same IBU concentrations as for the first test, while the 3.5% ethanol concentration (diluted 4.5% ethanol beer) was made with 3.75 IBU. In this test, no RFOs were added.

For the third test, ethanol concentrations stayed the same, and the beers containing 6% and 3.5% ethanol were made with the same IBU as test 1 and 2. Beer with 4.5% ethanol but without IBU addition was used as a control. All samples were made with 15 g/L RFO.

After production, all media was sterile filtrated (0.22 µm) into autoclaved Schott flasks and pipetted into cell culture tubes (4.9 mL) in duplicates. The tubes were inoculated with 100 µL of culture, incubated and monitored with daily spectrophotometry. Samples (100 µL) were diluted (1:10) with Milli-Q when the culture measured 0.8 $OD_{600\ nm}$.

Table 3: Corresponding ethanol and international bitterness units (IBU) concentrations for Test 1, 2 and 3.

	2.25 % Ethanol	3.5% Ethanol	4.5 % ethanol	6% Ethanol
0 IBU	Test 1		Test 1/2/3	Test 2/3
2.5 IBU	Test 1		Test 1/2	Test 2/3
3.75 IBU		Test 2		
5 IBU	Test 1		Test 1/2	Test 2/3

3.2.3 Main beer experiment

Initial batches (v1-v3)

Using a Brewtools B-40Pro, 1.83 kg each of milled (1.2 mm) Bestmalz Best wheat malt (4 EBC) and Bestmalz Pilsen malt (3.5 EBC) along with 214 g of rice hulls were mashed at 62°C (65°C for Wort medium v2 and v3) for 1 hour in 22.5 L of water. After the addition of malt and rice husks, the mash was pH-adjusted to pH 5.3, using 9 mL of lactic acid (80%). When the mashing was done, the mixture was outmashed at 78°C for 15 minutes. The malt tun was post-outmash raised from the brewer, and sparged with 9.7 L of 75°C water. Temperature was raised to 100°C, and 20g of oxidized Archer hops (1.54% α-acids) were added in a hop bag. Anti foaming agent (0.5 mL) was also added.

When 15 minutes of the boil remained, yeast nutrition (2.2 g) and protafloc (1 Tablet) were added. Near the end of boiling (5 minutes left), 15 g/L of RFO mix 1 (wort v3 used mix 3) was added. The hop bag was removed upon completion of boiling and the wort cooled to >20°C using a cooling spiral with cold water. For batch v3, the wort was transferred to a 19 L Corneliusfat (AEB) and pressurised with CO_2 prior to use. Specifications regarding the brewing can be viewed in Table 4.

Final batch (v4)

Beer v4 was the final beer, made using 2,6 kg of each malt from v1. The mash volume was 19.1 L water, and sparge volume was 19.77 L water. The mash water profile was adjusted using 0.25 g $CaSO_4$, 1.72 g liquid $CaCl_2$ (33%) and 9 mL lactic acid (80%, turning the pH of the mash water to 5.3 at 65°C). Mashing was done at 65°C as with previous batches, in a Brewtools B-40Pro. The mashing was done for 1 hour, the temperature was then raised to 77°C for 15 minutes of outmashing. After this, the mash tun was raised from the brew, and sparged. The sparging water was corrected to a temperature of 75°C and pH 5.8. The water profile was corrected using 0.26 g $CaSO_4$ and 1.78 g of $CaCl_2$ solution (33%). The sparge water was added in batches of three L, being careful not to disturb the mash. Post sparging, the wort was boiled for 1 hour, adding 21.2 g of oxidised Archer hops (1,54 % α-acids) in a boiling bag. Anti-foaming agent was also added at the beginning of the boil. When there was 15 minutes left of the boil, 1 Tablet of Protafloc and 3 g of Wyeast Beer nutrition blend were added.

When the boil was done, 15 L wort was tapped into two 10 L Young Brew fermentation buckets washed with Suresan (Vitale Norge, Holter, Norway), 7.5 L wort in each. The buckets were added lids and yeast locks and set in a 2°C water bath with ice to cool down. The remaining 15 L of wort was added 225 g of RFO mix 4, which was blended at 90°C for 5 minutes. The 90°C blending was done to ensure that the substrate was sufficiently mixed in. The wort containing RFO was then cooled rapidly using a cooling spiral with cold water and tapped onto two 10 L buckets (7.5 L in each).

Table 4: Production parameters for sour beer samples TF121= *L. cremoris* TF121, WLP645= *B. claussenii* WLP645, US-05= *S. cerevisiae* US-05, LGG= *L. rhamnosus* GG.

	Base beer 1 (3.25%)	**Base beer 2 (4.5%)**	**Batch v1**	**Batch v2**	**Batch v3**	**Batch v4**
Malt base used (kg of Wheat/Malt (50/50))	4.00	4.86	3.66	3.66	3.66	5.20
Mash water (L)	25.0	23.0	22.5	22.5	22.5	19.1
Mash water pH	5.2	5.3	5.3	5.3	5.3	5.3
Mash temperature (°C)	68	62	62	65	65	65
Mash time (Minutes)	60	60	60	60	60	60
Outmash temperature (°C)	75	78	78	78	78	77
Outmash time (Minutes)	15	15	15	15	15	15
Sparge water (L)	7.50	10.00	9.70	9.70	9.70	19.77
Sparge water (°C)	78	75	75	75	75	75
Sparge temperature (°C)	75	78	78	78	78	77
Boil temperature (°C)	100	100	100	100	100	100
Boil time (Minutes)	60	60	60	60	60	60
Hops added (Grams)	11.4 (Saaz)	0	20 (Archer)*	20 (Archer)*	20 (Archer)*	21.2 (Archer)*
Protafloc (1 Tablet) and Wyeast nutrition (0.1 g/L)	Yes	Yes	Yes	Yes	Yes	Yes
RFOs added during boil (g/L)	0	0	15	15	15**	0***
Yeast or bacteria used (g or mL)	23 g US-05	23 g US-05	**1**: TF121 (60 mL) + US-05 (3 g) **2**: WLP645 (60 mL) **3**: US-05 (3 g)	**1**: LGG (60 mL) + US-05 (3 g) **2**: TF121 (60 mL)+ WLP645 (60 mL) + L. brevis (60 mL)	**1**: TF121 (6 mL) + US-05 (1 g) **2**: WLP645 (6 mL) **3**: US-05 (1 g) **4**: LGG (6 mL) + US-05 (1 g) **5**: TF121 (6 mL) + WLP645 (6 mL) + L. brevis (6 mL)	**1**: US-05 (2.2 g) **2**: TF121 (75 mL) + WLP645 (75 mL) **3**: US-05 (2.2 g) **4**: TF121 (75 mL) + WLP645 (75 mL)
Fermentation vol. (L)	23.0	20.0	3.0	3.0	0.3	7.5
Fermentation period (d)	14	14	19	19	19	14/19 ****
Planned fermentation efficiency (Brewhouse)	72%	70%	60%	66%	64%	72%
Final planned EtOH (v/v)	3.27	4.5	3.5	3.5	3.5	3.5
Final planned IBU	6.9	0.0	4.0	4.0	4.0	3.0

* Oxidized hops were used. ** Unlike batch v1/v2, v3 was added RFOs from mix 3. *** RFOs (mix 4) were added post boil, at 90°C. **** Samples containing *S. cerevisiae* US-05 were bottled after 14 days fermentation, while *L. cremoris* TF121 and *B. claussenii* WLP645 were bottled after 19 days.

Beer v1

After the production of wort v1, 3 L of wort was poured into 5 L glass fermenter with yeast cap, two in total. These were inoculated 1:50 with *Lc. cremoris* TF121 (with 1 g/L *S. cerevisiae* US-O5), and a control *S. cerevisiae* US-O5 (1 g/L). The fermenters were set to incubate at 24°C, with samples (50 mL) taken at 7, 14 and 19 days. These samples were used for high performance liquid chromatography (HPLC) analysis (saccharides and organic acids) and headspace gas chromatography (HSGC) for volatile compounds (see section 3.3.2). An additional sample of 200 mL was also taken after 19 days, which was characterized using an Anton Paar system (Anton Paar: Alcolyzer ME, pH meter, CarboQC ME, HAzeQC ME (turbidity meter), DMA 4500 M (Density meter), PFD filling device and Generation M software (version v2.42) (Graz, Austria)).

Beer v2

Similar to v1, 3 L of the v2 batch was added to 5 L glass fermenters with yeast caps, two in total. One was inoculated 1:50 with *L. rhamnosus* GG (with 1 g/L *S. cerevisiae* US-O5), while the other was a blend with 1:50 *Lc. cremoris* TF121, *B. claussenii* WLP645 and a *L. brevis* strain. The fermenters were incubated and sampled as above.

Beer v3

For batch v3 the monocultures and a blend from v1/v2 were re-evaluated in duplicate (1:50 inoculation volume), with 0.5 L glass bottles as fermenters. These were filled with 0.3 L of v3 wort. They were then mounted with 2-channel lids (in the case of both L. rhamnosus GG fermenters and 1 of 2 S. cerevisiae US-05 fermenters, lids were made using a 13 mm drill bit, and mounted with regular yeast caps), which were connected via silicone tubing to a 0.22 µm filter to avoid contamination and not create too much pressure. The fermenters were incubated and sampled beer v1/v2, but with smaller sample volumes (25 mL) for 7, 14 and 19 days.

Beer v4

The batch for v4 was inoculated with *S. cerevisiae* US-05 (2.2 g) for two fermenter buckets (one with and one without RFO) while the other two fermenter buckets were inoculated with 150 mL each of *B. claussenii* WLP645 and *Lc. cremoris* TF121. After inoculation, the fermenter buckets were placed in a 24°C heating cabinet. The cultures containing *S. cerevisiae* US-05 were fermented for 14 days, and the mixed culture batch for 19 days. The beer was sampled at 14 and 19 days and tested as above. After fermentation, the beer was inoculated with 0.1 g of

carbonation yeast and 7 g/L saccharose. The inoculated beer was bottled in 0.5 L glass bottles with metal caps and left to carbonate until tetrad testing (see section 3.3.3).

3.3 Analytical methods

3.3.1 Microbiological

Agar plating

Agar plating was performed for purity control, for aerobic/anaerobic testing with or without RFOs and for the determination cell forming units (CFUs).

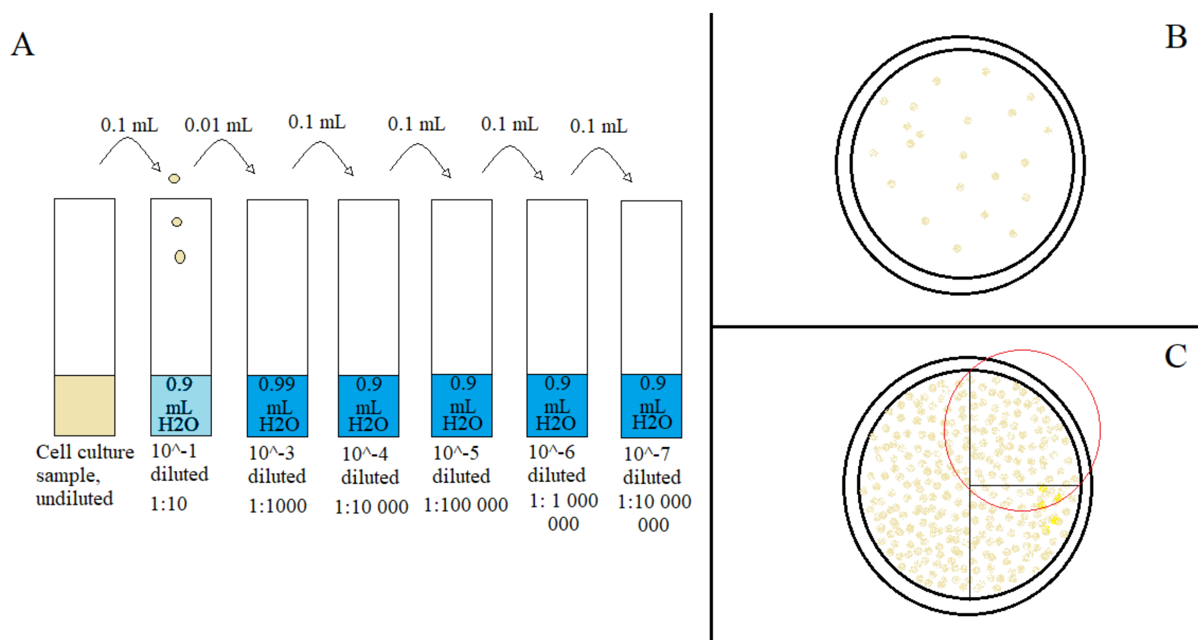

Figure 4: Dilutions and plate counting set-up. (A) Cell culture in broth was diluted to the various concentrations needed. Between dilution steps the mixtures were vortexed for 3 seconds. (B) A schematic example for a cell culture plate where all culture forming units (CFUs) could be counted (10-300). (C) Agar plate with high growth (above 300). CFUs were counted in either a quarter or half the plate and multiplied by 4 or 2, respectively. If the count exceeded 300 in a 1/4, the plate was deemed unreadable.

Aerobic and anaerobic testing of agar plates with glucose/RFO

The *Lactococcus* strains were tested for comparable growth in aerobic and anaerobic conditions, using either glucose or RFO mix 1 as sugar substrates. M17-G agar and M17-RFO agar were produced using 20 g/L of substrate, and 11 g/L of agar powder in M17 broth. The plates were made in plastic single-use petri dishes (20 mL in each), using a 25 mL pipette and pipette-boy under sterile conditions, and left to cool completely before inoculation. Using a glass spreader dipped in 70% ethanol and burned off with a Flame-boy propane torch, 100µl of culture dilutions was spread onto each petri dish. The culture *Lc. lactis* Ta01 was diluted at 1:10 000, 1:100 000 and 1:1 000 000 in Milli-Q. The other two *Lc. lactis* strains were diluted 1:100

000, 1:1 000 000 and 1:10 000 000 in Milli-Q. The plating technique used is described in method 4a of Sanders (2012).

All inoculation spreads were made in duplicate, with one half of the petri dishes subsequently placed in airtight plastic bags, filled with nitrogen gas (N2, AGA, Norway) and sealed. This was to simulate anaerobic growth conditions. They were left to grow for 7 days in 30°C, after which the plates were inspected, and cultures counted where possible (See Figure 3 B/C).

Purity control plating of *Lacticaseibacillus* strain on MRS-G Agar

The *L. rhamnosus* GG strain was tested for culture purity and growth on MRS-G agar upon receiving the strain. The agar was produced using 20 g/L glucose MRS broth with 15 g/L agar powder. Plate casting and culture spreading was done as described in previous section. The culture was diluted 1:100 000, 1:1 000 000 and 1:10 000 000. These dilutions were spread on individual plates in duplicate. Colony forming units (CFUs) were counted for each concentration (see Figure 3 B/C).

CFU count for final beer (v4)

To assess the CFU of the final beer (v4), 100 µL samples were taken from the culture used to inoculate the beer. These were diluted 1:100 000 / 1:1 000 000 for *Lc. cremoris* TF121, of which 100 µL were spread onto petri dishes with M17-G agar, which was incubated at 30°C for 90 hours.

3.3.2 Chemical analyses on beer and ingredients

Alpha acid determination

The hops had been oxidized for a total of 4 months prior to use, and thus the IBU was uncertain. To measure the IBU, method B400.18.110 from MEBAK was utilized (MEBAK, 2020). 1 L of water was heated in a 1 L flask in boiling water and infused with 60 g of hops in a hop boiling bag. The flask containing hops and water boiled for 60 minutes, after which the hops were removed, and the flask allowed to cool in a refrigerator (4°C). A 50 mL sample of the hop-water was transferred under sterile conditions to a Nunc tube and centrifuged at 10 000 RPM (4°C) for 15 minutes.

Post centrifugation, a 10 mL sample of the supernatant was taken and added to a Nunc tube along with 1 mL hydrochloric acid (3M) and 20 mL of isooctane. The mixture was subsequently shaken by hand for 15 minutes and centrifuged at 10 000 RPM for 15 minutes. A 350 µL sample of the supernatant was pipetted into a Corning® 96 well plate (Corning® 96-well clear flat

bottom polystyrene TC-treated microplate, New York, USA). The plate was measured at 275 nm in an Agilent BioTek Synergy H4 hybrid reader (Santa Clara, CA, USA) spectrophotometer, running Gen5 (Agilent, Santa Clara, CA, USA) software.

The method was also applied to samples containing fermented beer from v1-v4.

Starch Quantification

To test the amount of available starch in the base beers, a commercial starch kit was used according to the manufacturer's instructions (Total starch (α-amylase/amyloglucosidase) assay protocol, K-TSTA, analysis E-a and E-f, Megazyme, Ireland).

Titratable acidity

To find the total amount of organic acids in the final beer (v4), titratable acidity was measured. The test was done according to the beer method #8 protocol, from the American Society of Brewing Chemists as described by Lallemand (2023). The beer samples (*S. cerevisiae* US-05 with/without RFO, *Lc. cremoris* TF121 and *B. claussenii* WLP645 blend with/without RFO (14 and 19 days), Rodenbach Grand Cru) were prior to titration degassed by filtering through a 4 µm paper filter.

High Performance Liquid Chromatography (HPLC) of mono- and oligosaccharide

All samples were diluted 1:10 (100µL to 900µL) using deionized water in 1.5 mL Eppendorf tubes (Eppendorf Safe-Lock Tubes, Eppendorf SE). The closed Eppendorf tubes were then vortexed for 2-3 seconds each, and 200 µL were transferred to a marked 96 well filter plate (Millipore MultiScreen HTS HV Filter Plate, 0.45 µm, clear, non-sterile (Merck KGaA, Darmstadt, Germany)) with a 96 well plate (Nunc™ MicroWell™ 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplate (Thermo Fisher Scientific Inc.)) mounted beneath.

The samples were filtered through the plate using a filter pump (Millipore Chemical Duty Pump, 220 V/50 Hz (Merck KGaA, Darmstadt, Germany)) with a vacuum box. Samples were then transferred from the 96 well plate into HPLC vials (0.3ml PP Snap Ring Vials, VWR International, LLC.) and capped. The samples were then frozen at -20°C until the day of analysis.

On the days of analysis, the samples were injected into a Dionex ICS- 6000 system with eluent generator (EGC) and pulsed amperometic detection (PAD) detection, using a Dionex

carboPac™ PA210-Fast-4µm column equipped (150x2mm) with a Dionex Carbopac PA210 guard column (30x2mm). Column temperature during runs was 30 °C, operating pressure was about 4000 PSI at 0.2 mL/minute, when EGC was at 12mM KOH (Generated electrolytically via the EGC cartridge). Eluent was Milli-Q (degassed for 20 minutes), and sample injection volume was 0.4 µL.

HPLC of organic acids

The samples collected from batch v1-v3 was filtered into Nunc tubes (50 mL volume), using 4 µm folding filters from Whatman. The filtering was done in a chilled environment (4°C), and samples were frozen prior to testing (-20°C). On the day of testing, the samples were defrosted in room temperature (19-21°C). After defrosting, samples were centrifuged at 4700 RPM (4°C) for 15 minutes. These samples were used for section 2.3.4 and 2.3.5.

An internal NMBU protocol based on a method by Grønnevik et al. (2011) was followed for this analysis. Approximately 1 g of supernatant was weighed out for each sample and placed in individual 10 mL HPLC glass tubes. To each sample was added 2.5 mL of deionized water, 200 µL of 0.5 M H_2SO_4 (Merck, Germany) and 8 mL of acetonitrile (Merck). The HPLC tube was capped, and samples shaken by hand until homogenous. They were then placed in a MultiRS-60 BIOSAN turning machine (Montebello Diagnostics A/S, Oslo, Norway) for 30 minutes. The samples were then centrifuged at 3400 RPM (20°C) for 15 minutes in a Kubota 2010 centrifuge (Kubota Corporation, Tokyo, Japan).

The supernatant was then filtrated using a 0,2 mm PTFE membrane (Acrodisc CR 13 mm Syringe Filter, PALL, Great Britain) into HPLC glass tubes. The separation was undertaken using an Agilent Technologies 1260 Infinity II system (Agilent Technologies, Singapore) with a Refractive Index (RI) detector (Agilient technologies) for acetic acid and a diode array detector- ultraviolet (DAD-UV) monitoring at wavelength xx nm (Agilent technologies) for other organic acids. The software used was Openlabs CDS (Aglient technologies). For sampling, 25 µL of the sample was injected, and separated on a 300 x 7.8mm Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA). Prior to separation, the sample went through a precolumn (Cation-H refill (Bio Rad Laboratories)). During the separation, column temperature was 32°C, with a 5mM H_2SO_4 mobile phase (0.4 mL/minute).

For reference and calibration, standards were obtained from Sigma-Aldrich (citric acid, lactic acid, acetic acid, orotic acid, pyruvic acid, succinic acid, formic acid, uric acid, propionic acid, pyroglutamic acid) and Merck (maltose, lactose, fructose, glucose, galactose).

HeadSpace Gas Chromatography (HSGC) of volatile compounds

Using the prepared 50 mL samples described in 2.3.4, 10 g samples were transferred to headspace bottles (Machery Nagel, Dueren, Germany). The bottles were then sealed with Teflon-covered septa, with an aluminium ring (PTFA/Si septa, Agilent Technologies, Wilmington, DE, USA) and clamped shut. The method used for HSGC followed an internal method described by Dysvik et al. (2020c).

The testing was done using a Aglient Technologies 7679A automatic headspace sampler with a 6890 GC system (Aglient technologies) and a flame ionization detector. The software used was Open LAB EZChrom (Aglient technologies). As carrier gas, helium 6.0 (AGA, Norway) was used, with a flow of 5.0 mL/min. The headspace bath held 50°C, while the manifold was at 60°C. During the 45 minutes of equilibration, the samples were shaken at 70 shakes/minute. Prior to injection, the headspace bottles were pressurized to 10 PSI, and the injection time was 30 seconds. The samples were separated on a 25 m (0.53 mm inner diameter, 5 µm film thickness) CP-SIL 5CB GC column (Varian, Middelburg, Netherlands).

During the analysis, the following temperature gradient was used: 35 °C for 5 minutes, temperature raised to 40°C using a 10°C/min^{-1} raise for 2 minutes, temperature raised to 70°C using a 15°C/min raise for 2 minutes, temperature raised to 130°C using a 30°C/min raise for 4 minutes, temperature raised to 160°C using a 30°C/min^{-1} raise for 4 minutes, temperature raised to 180°C using a 10°C/min raise for 2 minutes, temperature raised to 200°C using a 10°C/min raise for 2 minutes.

External standard curves were used for quantification. Standards included acetaldehyde, diacetyl, ethyl acetate, 2-butanone, 2-hexanol, 2-methyl-butanal, 2-methyl-1-butanal, 2-methyl-1-propanal, 3-methyl-butanal, 3-methyl-1-butanol, 2-methyl-1-propanol, isobutyl acetate, hexanal, isoamyl acetate, ethyl hexanoate, 3-carene, *R*-(+)-limonene, ethyl heptanoate, ethyl octanoate, B-citronellol, ethyl nonanoate, ethyl decanoate, phenylethyl alcohol (Sigma-Aldrich), acetoin, acetone, ethanol, 1-butanol, 1-propanol, 2-butanol, dimethyl sulphate, 2.3-pentadione (all from Merck).

3.3.3 Sensory

Tetrad testing of final beer (v4)

The bottled beer (*S. cerevisiae* US-05 with or without RFO and *B. claussenii* WLP645/ *LC. cremoris* TF121 mix with/without RFO) was evaluated by 25 participants, using a tetrad sensory test. Samples were served in NMBUs sensory testing room with 6 sensory booths. The booths were equipped with a serving hatch, spit bucket, neutral plastic water glass and neutral light. Room temperature was 20-22°C. Samples were served in randomized order at room temperature, in 75 mL plastic containers with lid and a randomized 3-digit code. Participants were volunteers from the university (students & employees), with unknown previous sensory experience. Prior to the test, participants were informed of their task to differentiate between 4 samples, and group them 2 by 2 for each test. The data was recorded on an answer sheet (Nettskjema.no), which the participants accessed through their phones (See appendix, Figure S1).

3.4 Statical analysis

Statistical analysis was done using two-way analysis of variance (ANOVA) in excel for ethanol/IBU testing. Results of the tetrad test were calculated using a tetrad table from Rodgers (2017) .

4. Results

The main objective of the thesis was to assess the utilization of RFOs derived from peas in sour beer production and characterize the properties of the prepared beers. Thus, initial testing was done to select microorganisms suitable for use in sour beer that were able to ferment RFOs. For strains that showed growth in the media with RFOs as substrate, growth inhibition in model beer media was assessed. Based on these results, select strains were trialled in a series of small-scale beer fermentations, and the produced beer was assessed for physical and chemical properties. A final beer was made on a larger scale, which was assessed in a similar fashion to the previous beers and tested for noticeable differences by participants in a Tetrad test.

4.1 Negative and positive growth control

One yeast (*B. claussenii* WLP 645) and four bacteria strains (*L. rhamnosus* GG and the three *Lactococcus cremoris* (TD013a, TA01, TF121) strains) were tested for growth in media with (positive control) or without (negative) glucose (Figures 5, 6 and 7). All strains were viable for growth in media without sugar source but showed higher growth in media with glucose.

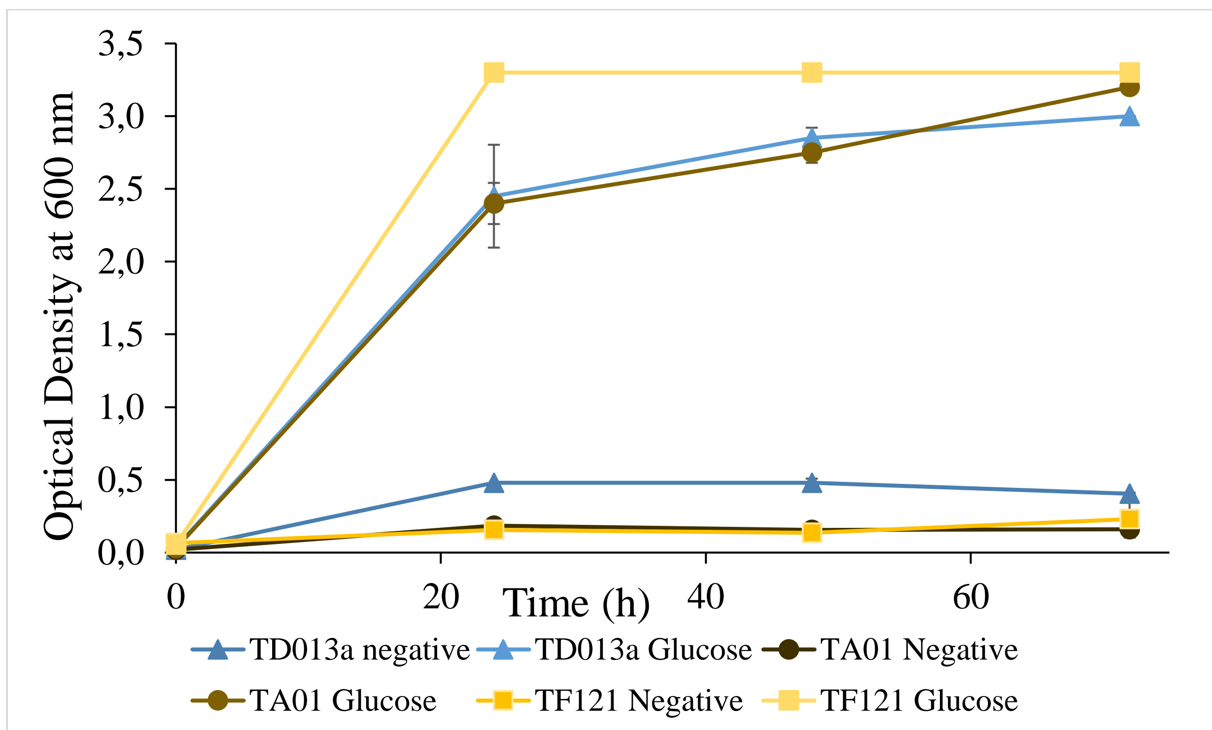

Figure 5: Growth curves of all *Lc. cremoris strains* (TD013a, TA01 and TF121) in M17 growth medium broth without sugar source (negative control) or 20 g/L glucose (positive control). Error bars indicate difference between duplicates (n=2) and were in some cases smaller than symbols used as markers.

For *Lc. cremoris,* the strains were observably inhibited by lack of a sugar source (Figure 5). Growth occurred primarily in the first 24 hours, both with and without glucose. For the sample

with glucose, *Lc. cremoris* TF121 grew more compared to the other two strains initially, but all samples reached a similar OD within 72 hours.

The *L. rhamnosus* GG culture (Figure 6) showed less growth inhibition in the negative control compared to the *Lc. cremoris* strains (Figure 5), reaching a higher OD. The growth in glucose reached an OD of 2 within 24 hours. While such high readings can lead to some measurement uncertainty, no dilutions were made since these experiments served as qualitative indicators of viability.

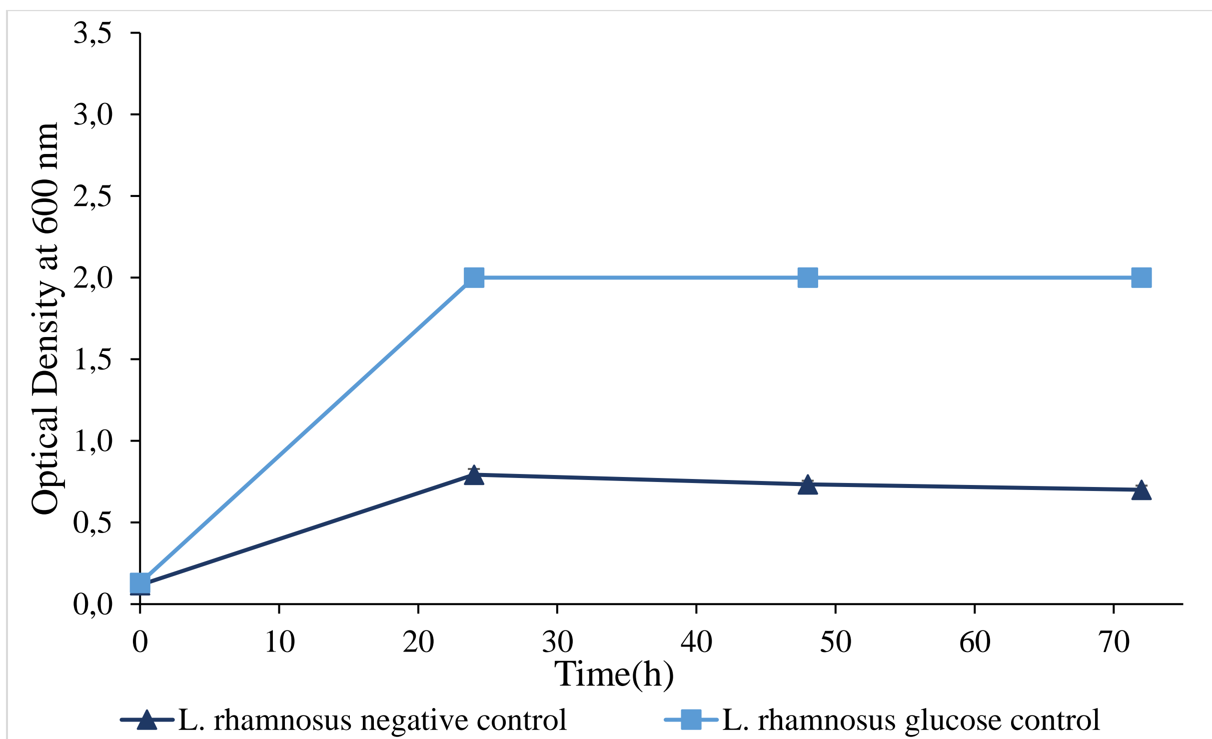

Figure 6: Growth curves showing OD for *L. rhamnosus* GG in De Man, Rogosa and Sharpe (MRS) growth medium broth without sugar source (negative control) or 20 g/L glucose (positive control). Error bars indicate difference between duplicates (n=2) and are in some cases smaller than marker symbols.

The initial growth of the *B. claussenii* WLP645 culture was slow in negative and positive controls with no observable growth in the first 24 hours (Figure 7). It was thus allowed to grow for longer than *Lc. cremoris* strains and *L. rhamnosus* GG, for a total of 120 hours. The growth phase for *B. claussenii* WLP645 in glucose YEP occurred after 48 hours, in which the density of the samples went from below 1 to above 6 OD.

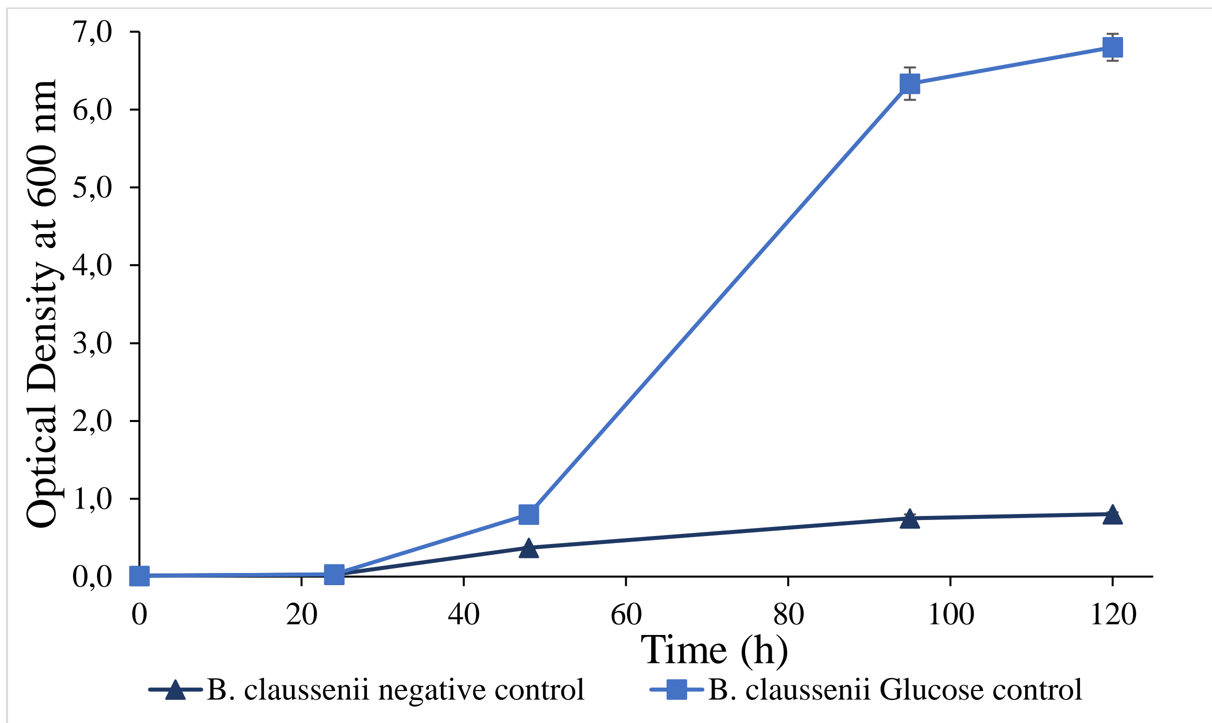

Figure 7: Growth curves showing OD for *B. claussenii* WLP645 in yeast extract peptone growth medium without sugar source (negative control) or 20 g/L glucose (positive control). Error bars indicate difference between duplicates (n=2), mean value is plotted.

Due to the observable growth for all cultures in medium with glucose, all strains were further tested in growth medium with RFOs.

4.2 RFO growth control

To assess their RFO-fermenting abilities, all strains were inoculated into medium that instead of glucose contained RFOs. The other conditions were analogous to the experiments with positive and negative controls.

The growth of *Lc. cremoris* strains and similar in OD to the negative control (Figure 8). After 48 hours, *Lc. cremoris* TF121 outgrew the other strains, reaching an OD comparable to that of growth with glucose (Figure 5). This contradicted the growth pattern of *Lc. cremoris* with glucose, in which most growth occurred during the first 24 hours.

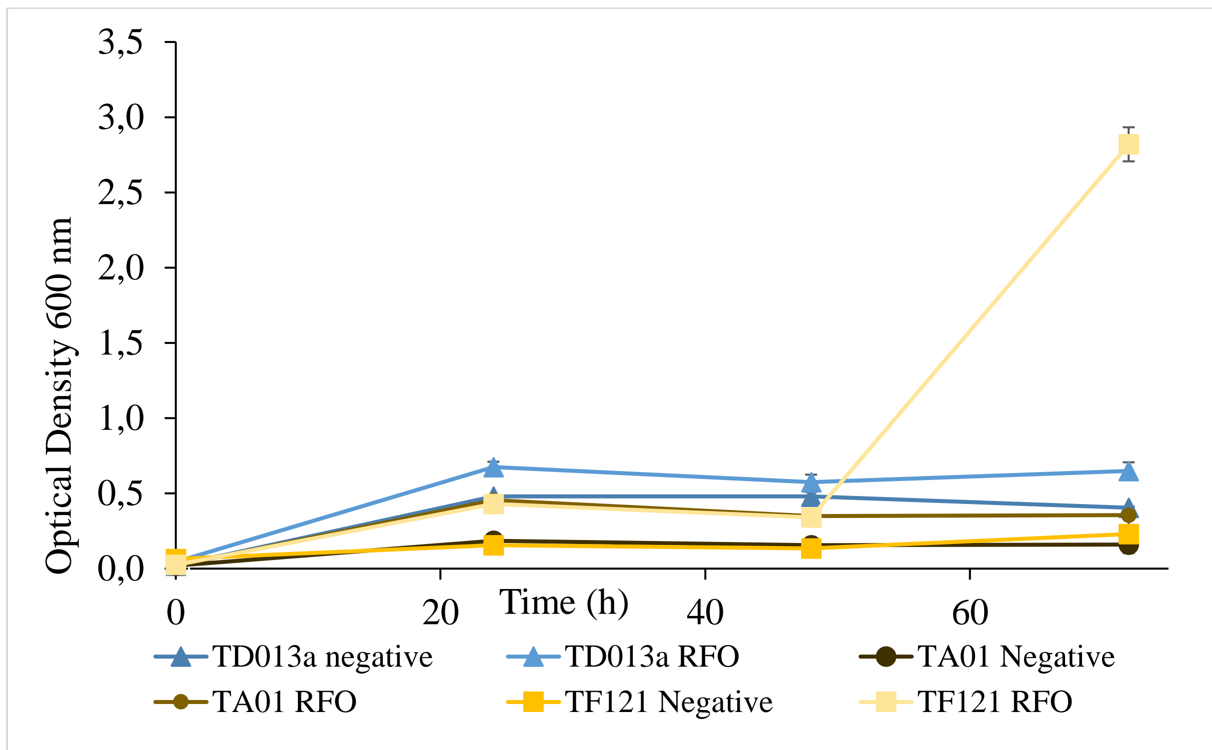

Figure 8: Measured growth in OD for *Lc. cremoris* strains (TD013a, TA01, TF121) in M17 medium without sugar (negative control) and with 20 g/L raffinose family oligosaccharides (RFO mix 1). Error bars indicate difference between duplicates (n=2), mean value is plotted.

The *L. rhamnosus* GG strain showed higher OD measurements while growing in RFO medium compared to the negative control (Figure 9). Growth was however lower than the OD reached for glucose (Figure 6). All observable growth occurred between 0 and 24 hours.

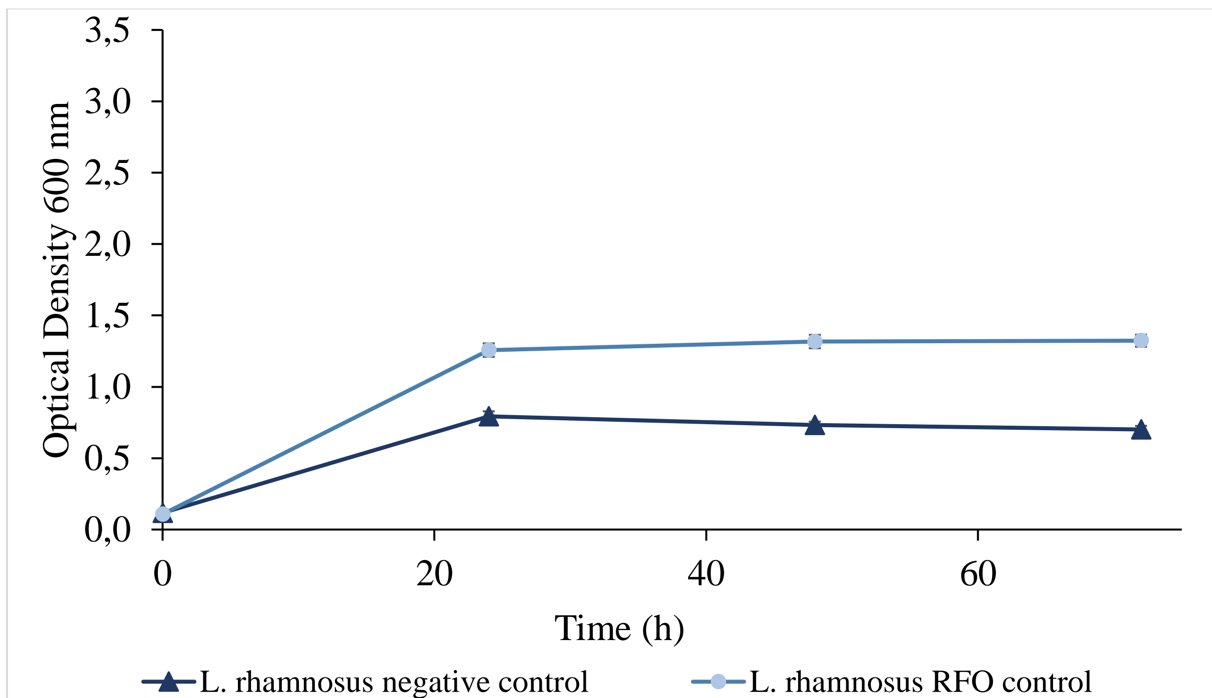

Figure 9: Measured growth in OD for *L. rhamnosus* GG in De Man, Rogosa and Sharpe (MRS) medium without sugar (negative) or with 20 g/L raffinose family oligosaccharides (RFO mix 1). Error bars indicate difference between duplicates (n=2), mean value is plotted.

The *B. claussenii* WLP645 yeast in medium with RFOs (Figure 10) showed a similar growth pattern to that of growth in glucose-containing medium (Figure 7) with a slow initial growth in the first 48 hours, and rapid increase in growth between 48 and 96 hours. Growth did however not occur in RFO medium after 96 hours as it did in glucose medium, and the highest reached OD was lower in RFO-medium than with glucose medium.

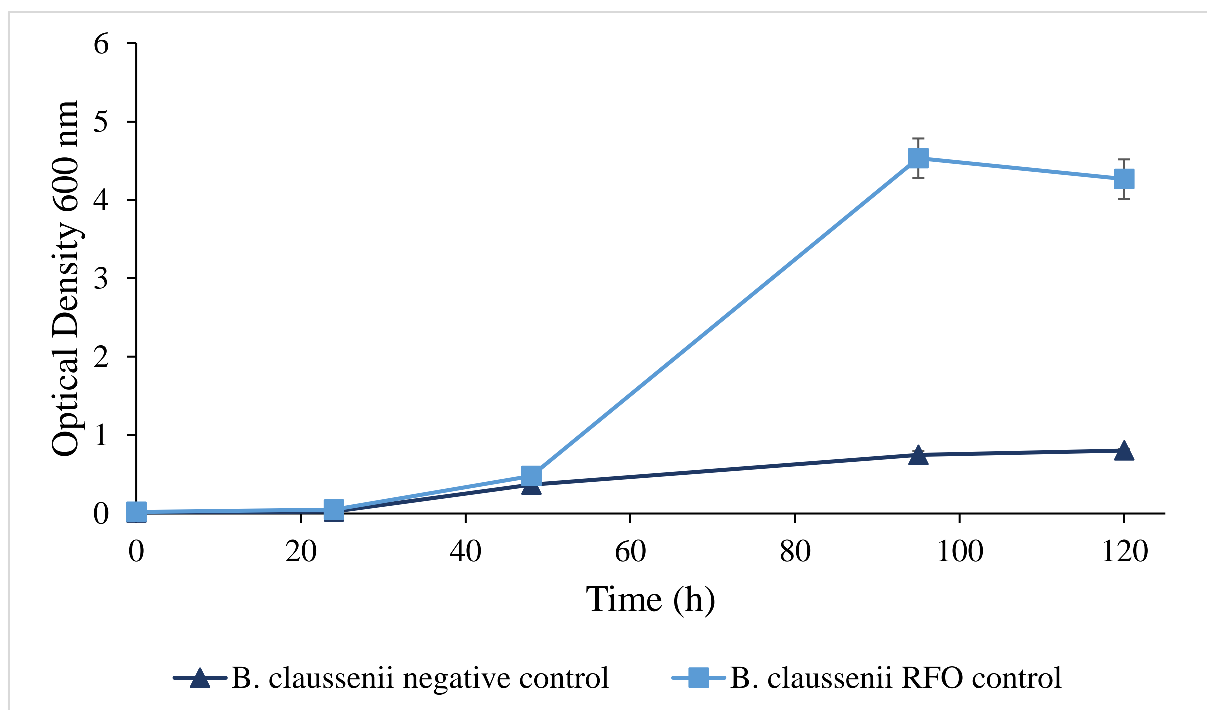

Figure 10: Measured growth in OD for *B. claussenii* WLP645 in yeast extract peptone (YEP) without sugar (negative) or with 20 g/L raffinose family oligosaccharides (RFO mix 1). Error bars indicate difference between duplicates (n=2), mean value is plotted.

The S. cerevisiae US-05 yeast showed high growth in medium containing RFOs compared to the negative without sugar (Figure 11). A similar initial growth to *B. claussenii* WLP645 was observed (Figure 7 and 10), with no apparent growth in the first 24 hours.

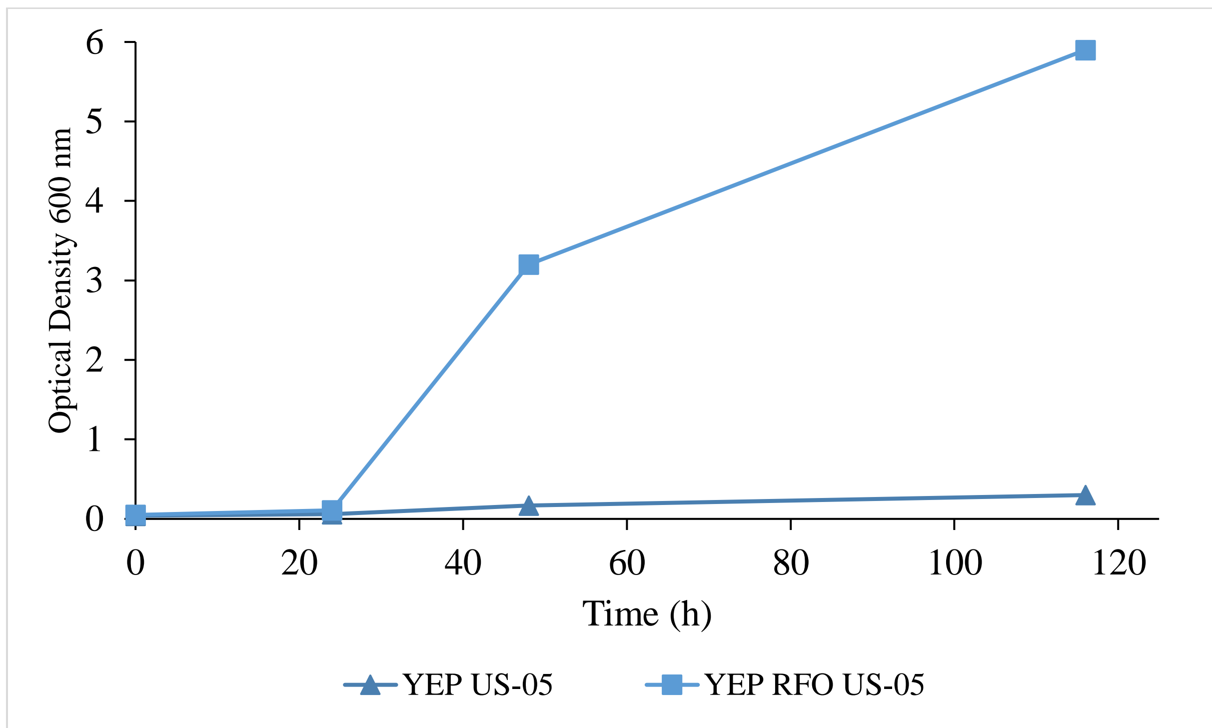

Figure 11: Measured growth in OD for US-05 = *S. cerevisiae* US-05 in yeast extract peptone (YEP) medium without sugar or with 20 g/L raffinose family oligosaccharides (RFO mix 1). Error bars indicate difference between duplicates which are sometimes smaller than the markers (n=2), mean value is plotted.

Both yeast strains showed more growth than the bacteria. For the *Lc. cremoris* strains, TF121 outperformed the two other strains, reaching a final OD of 2.82 on average.

After fermentation, the RFO media were evaluated for their sucrose and RFO contents using HPLC, to analyse if the strains had consumed them (Table 5). However, there were several complications with the HPLC method, which warranted careful interpretation of the results. These included shifts in retention times between runs and baseline issues. Moreover, some peaks fell outside the established linear range.

Table 5: Concentrations of di- and oligosaccharides for *Lc. cremoris* TF121, *L. rhamnosus* GG and *B. claussenii* WLP 645 (ppm).

Sample	**Sucrose**	**Raffinose**	**Stachyose**	**Verbascose**
Lc. cremoris TF121 RFO 0h	45	42.7	441.6	n.a
Lc. cremoris TF121 RFO 24h	35	38.4	403.6	n.a
Lc. cremoris TF121 RFO 48h	38.5	46.5	463.5	n.a
Lc. cremoris TF121 RFO 72h	36	36.5	400.5	n.a
L. rhamnosus GG 0h	200.4	190.2	264.5	185.7
L. rhamnosus GG 72h	163	189	269.6	179.6
B. claussenii WLP645 0h	73.4	183.5	234.6	98.2
B. claussenii WLP645 120h	180.6	96	222.4	67.7

Each strain exhibited a different pattern. A slight decrease in RFOs was observed in media fermented with *Lc. cremoris* TF121 over 72 hours. A decrease in sucrose and verbascose was present in media after fermentation with *L. rhamnosus* GG while raffinose remained similar, and stachyose increased. In contrast, after 5 days of fermentation the concentrations of raffinose and verbascose were substantially lower in media with *B. claussenii* WLP 645, while sucrose increased thus suggesting that galactose units may have been cleaved off the RFO structure. However, the concentrations of stachyose, the dominant RFO, were only marginally reduced.

4.3 Beer medium control

The first base beer described in section 3.2.2 containing 1.2g starch/100 g dry weight was used as a growth medium for the different *Lactococcus* strains and *L. rhamnosus* GG, to assess their viability in prefermented beer with and without additional RFOs. The yeasts were not utilized in this test.

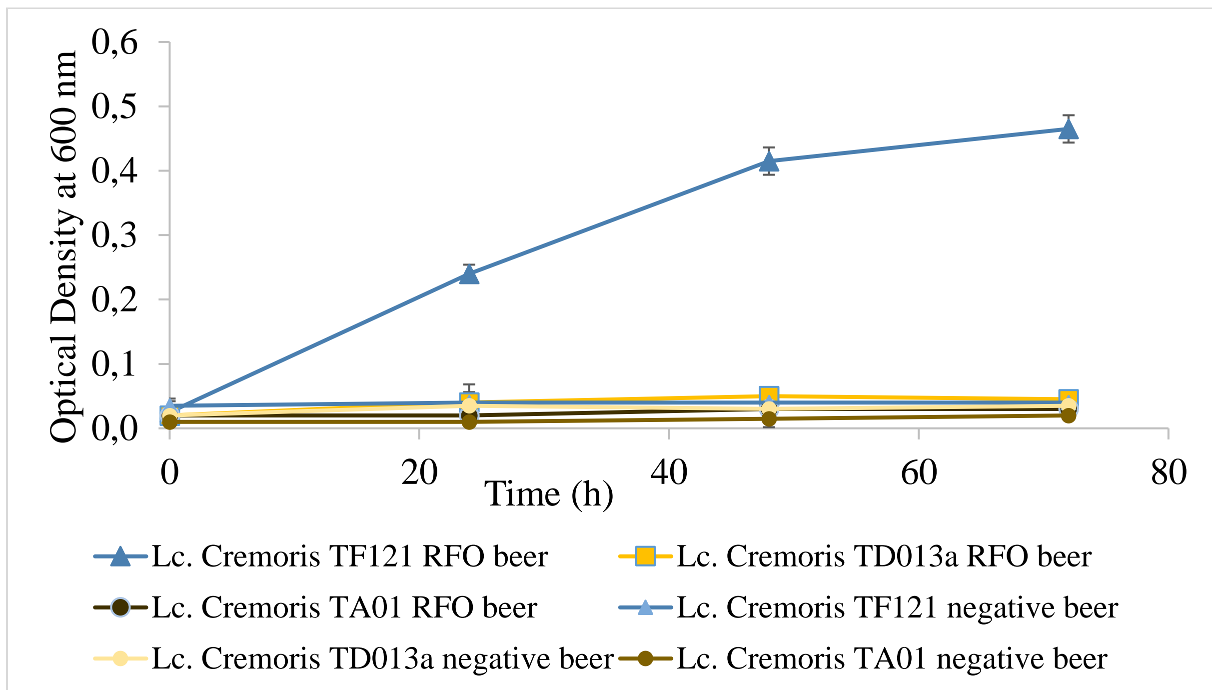

Figure 12: Beer inoculated with each *Lactococcus cremoris* strains (TD013a, TA01, TF121), without sugar source (negative) and with 20 g/L raffinose family oligosaccharides (RFOs, mix 1) added. Error bars indicate difference between duplicates (n=2), mean value is plotted.

The *Lc. cremoris* TF121 in beer medium with RFO was the only culture of the *Lc. cremoris* strains to show growth during the beer medium testing (Figure 12). The growth was low compared to testing in M17 medium (Figure 5 and 8) but had a steady incline over the 72 hours. The *Lc. cremoris* TD013a and TA01 strains were growth-inhibited.

The *L. rhamnosus* GG strain showed little growth during the first 24 hours, but subsequent measurements showed a higher growth in medium containing RFOs compared to the negative control (Figure 13).

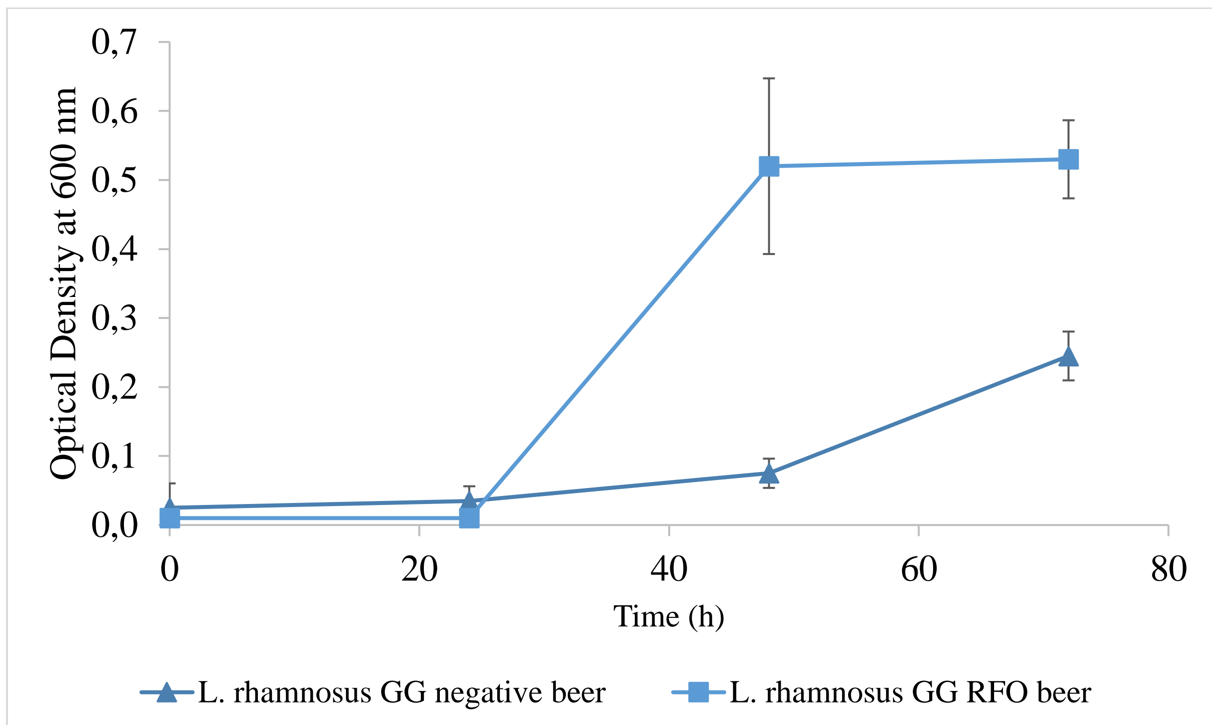

Figure 13: Beer inoculated with *L. rhamnosus* GG without sugar source (negative) and with 20 g/L raffinose family oligosaccharides (RFOs, mix 1). Error bars indicate difference between duplicates (n=2), mean value is plotted.

4.4 Single sugar and RFO growth testing of *Lc. cremoris* TF121

The late growth of *Lc. cremoris* TF121 in RFO medium warranted further testing. The strain was thus grown in M17 media containing either lactose (10 g/L), sucrose (10 g/L), maltose (10 g/L), raffinose (10 g/L), stachyose (14 g/L) or RFO mix 1 (5 or 10 g/L) (Figure 14 & 15). The growth was not recorded for OD above 2. The only culture to reach above 2 in OD was that grown in lactose media.

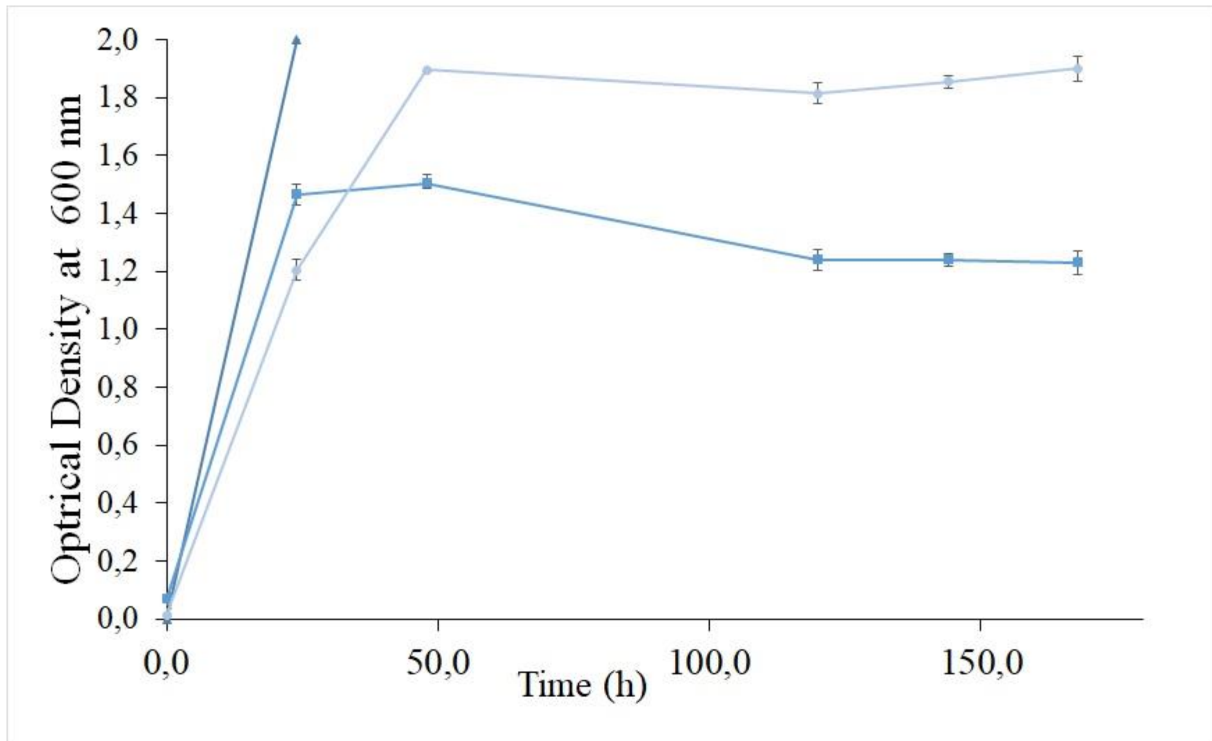

Figure 14: Growth graph of *Lc. cremoris* TF121 in M17 with 10 g/L of ▲: Lactose, ●: Sucrose and ■: Maltose. Growth was not recorded >2 OD. The error bars indicate difference between duplicates (n=2), mean value is plotted.

The culture grew well in all media with disaccharides, with the strain's growth in lactose media outperforming the other two. The growth in maltose reached the stationary phase within 24 hours, while the culture in sucrose required 48 hours to reach it. The OD measurements for RFO mix 1 (5 and 10 g/L), raffinose and stachyose (Figure 13) were lower compared to the disaccharides (Figure 12). The *Lc. cremoris* TF121 cultures grown in 5 or 10 g/L RFO mix 1 did also show comparably higher growth than cultures in raffinose or stachyose. There was little observable difference in OD between the 5 and 10 g/L sample of RFO. Between these two, the growth in media containing 70% stachyose outperformed the growth in 98% raffinose media.

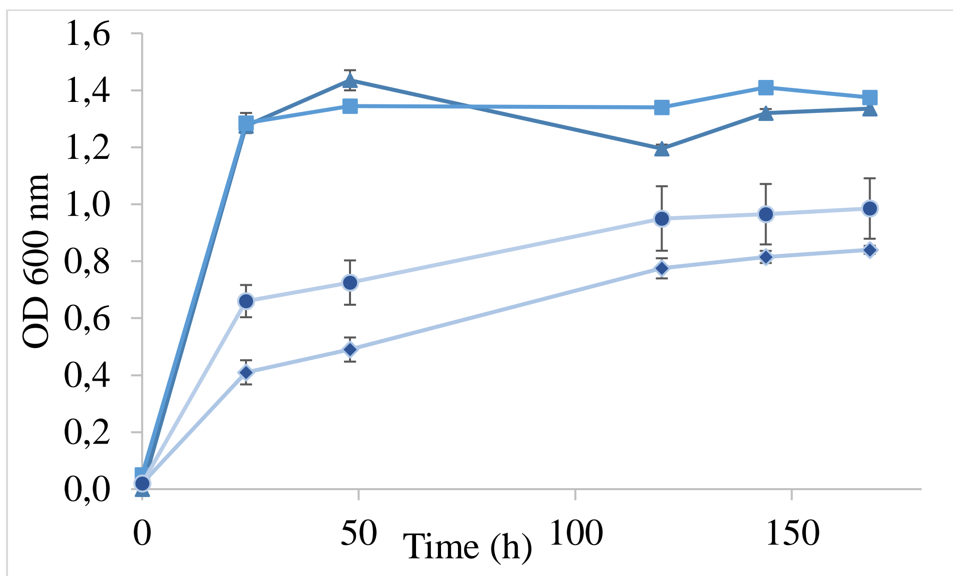

Figure 15: Growth graph of *Lc. cremoris* TF121 in M17 with p: 10 g/L raffinose family oligosaccharide (RFO) mix 1, u: 10 g/L Raffinose, l: 14 g/L Stachyose (70% pure) and n: 5 g/L RFO mix 1. Error bars indicate difference between duplicates (n=2), mean value is plotted.

HPLC analysis (Table S4 appendix) showed utilization of the different disaccharides, with all disaccharides in the different media decreasing over time.

Table 6: RFO content of prefermented and fermented samples of RFO (5 and 10 g/L), raffinose (10 g/L) or stachyose (14 g/L) by *Lc. cremoris* TF121. * Marks samples outside of calibration range.

	sucrose	raffinose	stachyose	verbascose
RFO 5 g/L 0h	141.33	73.70	121.03	53.57
RFO 5 g/L 167h	42.67	56.09	145.57	54.98
RFO 10 g/L 0h	247.20	145.83	193.35	110.50
RFO 10 g/L 167h	52.25	108.83	208.29	110.76
Raffinose 10 g/L 0h		562.80		
Raffinose 10 g/L 167h		563.35		
Stachyose 10 g/L 0h			313.81	
Stachyose 10 g/L 167h			336.16	

From the Table there is visible utilization of both sucrose and raffinose for 5 and 10 g/L RFO mix 1 by *Lc. cremoris* TF121, but an apparent accumulation of stachyose and verbascose. This was also the case for the media solely containing raffinose or stachyose, where accumulation occurred after fermentation. The HPLC results suffered similar issues to those in section 4.2, and careful interpretation was therefore needed. Moreover, it is possible that some peaks did not represent pure compounds due to coelution, which may have affected the peak areas.

4.5 IBU and ethanol testing

To assess the stress limits of the yeast and bacteria, the second base beer (see section 3.2.2) which contained 0.4 g starch/100 g dry weight was utilized with three different IBU amounts (Figure 16). Initial testing was done using 4.5 and 2.25 % ethanol, while the subsequent tests utilized 6, 4.5 and 3.75 % ethanol. For the initial test, yeast strains outperformed the bacteria in growth for most concentrations of IBU and ethanol, both reaching above 3 OD.

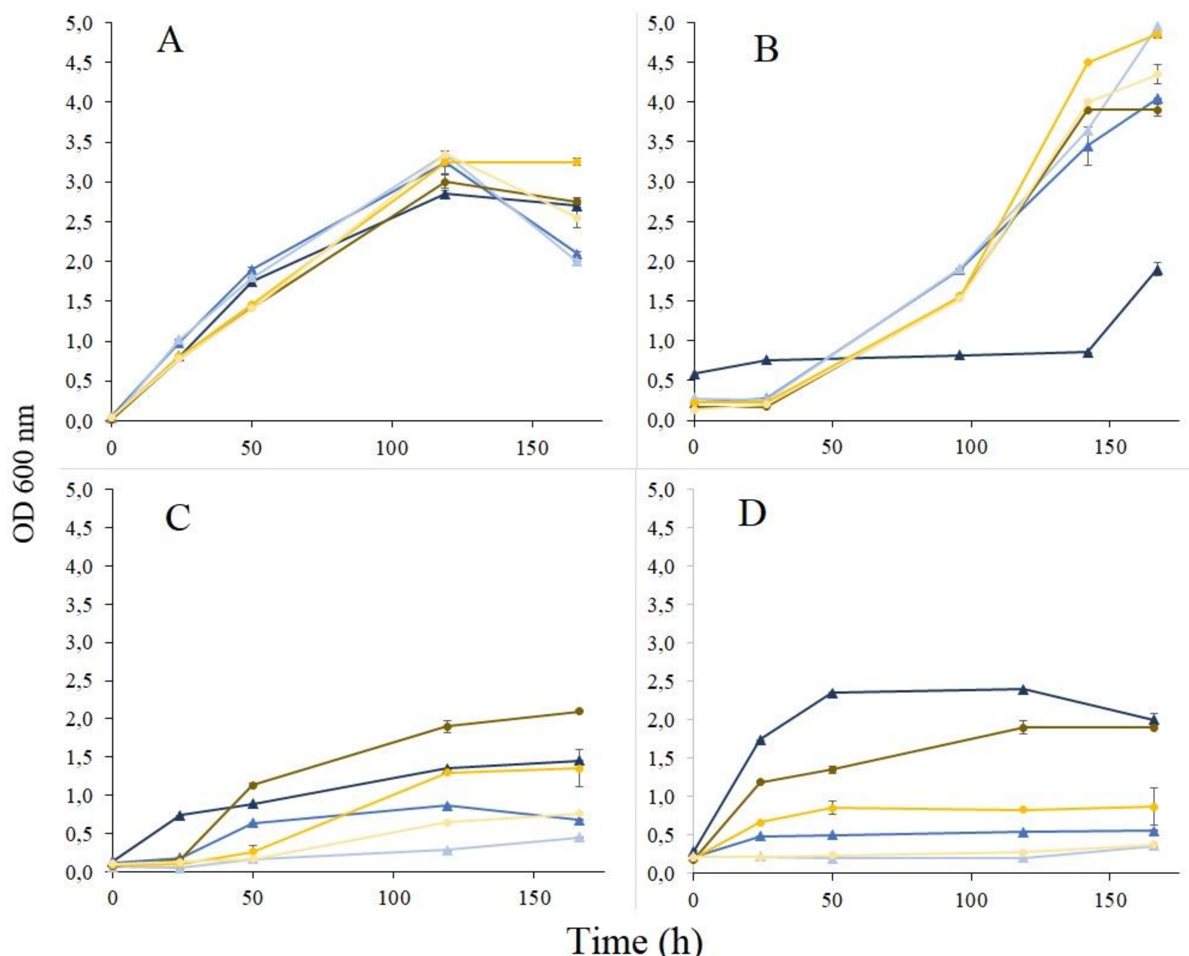

Figure 16: Growth in beer media 2 (20 g/L raffinose family oligosaccharides) with different concentrations of ethanol (EtOH) and IBU. Graphs for (A): *S. cerevisiae* US-05 (B): *B. claussenii* WLP645 (C): *Lc. cremoris* TF121 and (D): *L. rhamnosus* GG. ← Dark blue = 2.25 % ethanol, 0 IBU, ← Blue = 2.25 % ethanol, 2.5 IBU, ← Light Grey = 2.25% ethanol, 5 IBU. ● Brown = 4.5% ethanol, 0 IBU, ● Yellow = 4.5% ethanol, 2.5 IBU, ● Light yellow= 4.5% ethanol, 5 IBU. Error bars indicate difference between duplicates (n=2), mean value is plotted.

The *S. cerevisiae* US-05 strains (Figure 16, A) were not affected by stressors in the exponential phase until 48 hours, with two-way ANOVA showing a significant ($p<0.01$) effect of ethanol. In the final measurement, cell lysis occurred for all samples of *S. cerevisiae* US-05 except the 4.5 EtOH/ 2.25 IBU sample. This did not occur in any of the other cultures. For the *B. claussenii* WLP 645 cultures (Figure 16, B) an observable difference was seen between the 2.25 EtOH/ 0

IBU strain and all other strains. The other strains were not affected by the stressors, with both 2.25 EtOH /5 IBU and 4.5 EtOH / 5 IBU growing to above 4 OD.

In the bacterial cultures stressors limited growth (Figure 16 C & D), with IBU as the main factor. For early growth (0-50 hours) there was an interaction effect between ethanol concentration and IBU concentration ($p<0.05$), while for the latter growth IBU was the main limiting factor ($p<0.001$) (For all Anova statistics see appendix Table X). The ethanol concentration did not affect the final OD in *Lc. cremoris* TF121 (Figure 16 C). There was however a connection between initial growth, IBU and ethanol, where the strain growing in 2.25 EtOH/ 0 IBU reached an initially high OD.

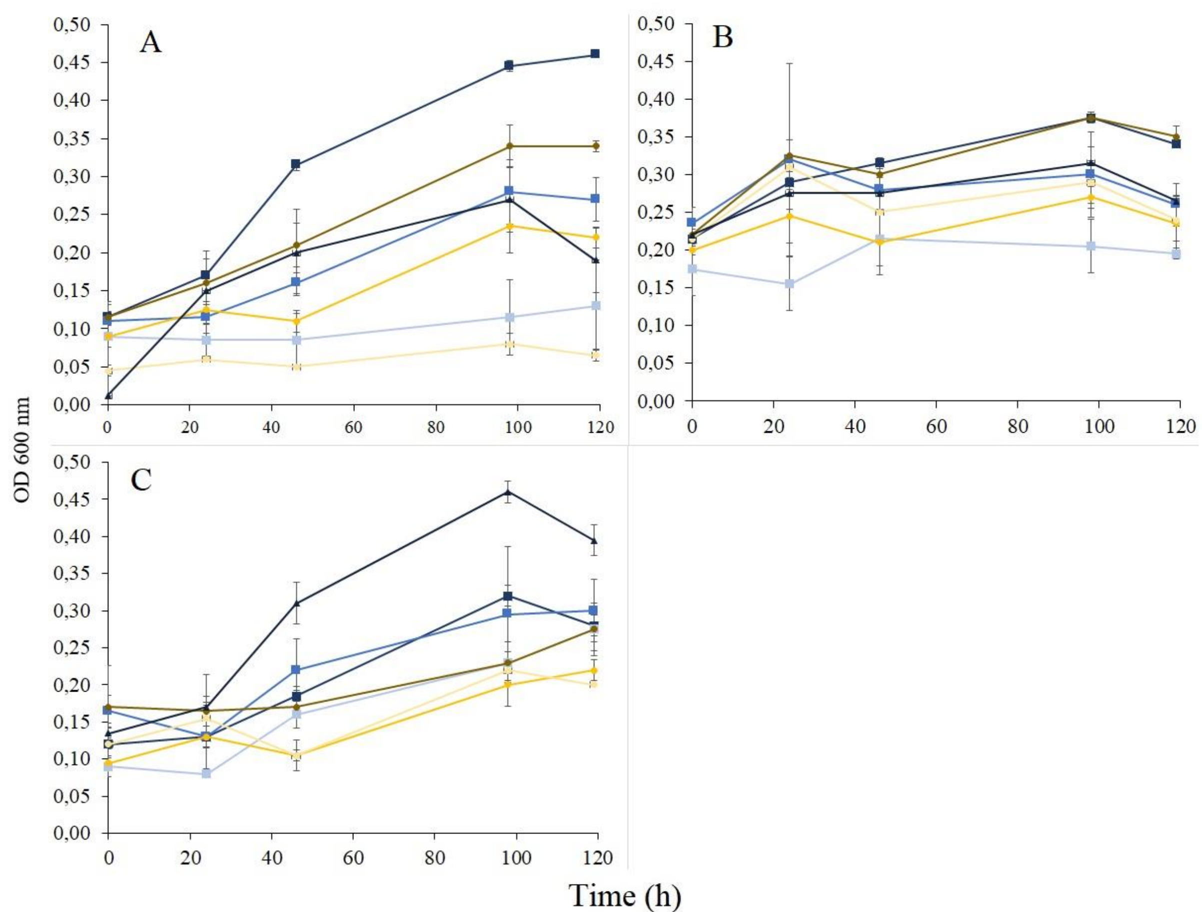

Figure 17: Growth in beer media 2 with different concentrations of ethanol (EtOH) and IBU without raffinose family oligosaccharides. Graphs for (A): *Lc. cremoris* TF121 (B): *L. rhamnosus* GG (C): *S. cerevisiae* US-05. ← Black = 3.5% ethanol, 3.75%. ■ Dark blue= 4.5% ethanol, 0 IBU, ■ Blue= 4.5 % ethanol, 2.5 IBU, ■ Light grey= 4.5% ethanol, 5 IBU. ● Brown= 6% ethanol, 0 IBU, ● Yellow= 6% ethanol, 2.5 IBU, ● Light yellow= 6% ethanol, 5 IBU. Error bars indicate difference between duplicates (n=2), mean value is plotted.

In beer media without RFO the growth was limited. The growth that occurred was due to fermentation of remaining nutrients in base beer 2. There was a significant ($p<0.05$) effect of IBU on *L. rhamnosus* GG (Figure 17 B) after 48 hours until the end of trial, while ethanol did not have a significant effect on growth. In contrast, *lc. cremoris* TF121 (Figure 17 A) was

significantly affected by IBU at all time points (p<0.05), while ethanol affected growth from 48 hours and until the end of trial (p<0.001).

The 3.5 EtOH /3.75 IBU media inoculated with *S. cerevisiae* US-05 (Figure 17 C) showed improved growth compared with the other strains of similar IBU. Ethanol was shown to significantly affect growth at 48 hours (p<0.01), 96 hours (p<0.05) and 120 hours (p<0.01). This indicated that the ethanol may have had a growth limiting effect when substrate was not present for *S. cerevisiae* US-05 (All Anova Tables can be viewed in the appendix, Figure S6).

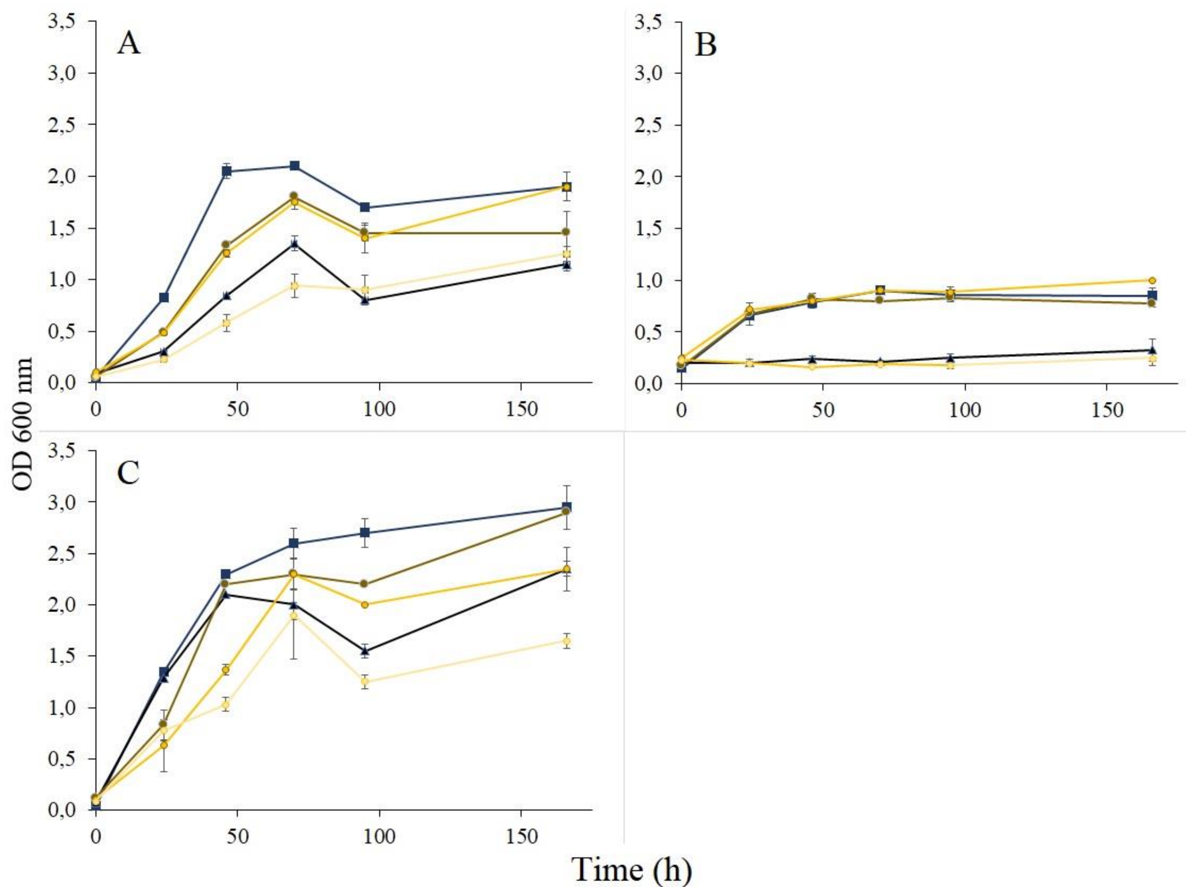

Figure 18: Growth in beer media 2 (20 g/L raffinose family oligosaccharides (RFOs, mix 1)) with different concentrations of ethanol (EtOH) and IBU. Graphs for (A): *Lc. cremoris* TF121 (B): *L. rhamnosus* GG (C): *S. cerevisiae* US-05. 🡐 Black = 3.5% ethanol, 3.75%. ■ Dark blue= 4.5% ethanol, 0 IBU. ● Brown= 6% ethanol, 0 IBU, ● Yellow= 6% ethanol, 2.5 IBU, ● Light yellow= 6% ethanol, 5 IBU. Error bars indicate difference between duplicates (n=2), mean value is plotted.

The effect of IBU inhibiting growth in *L. rhamnosus* GG was evident (Figure 18 B), where the strains in IBU above 2.5 showed lower growth compared to the samples of 2.5 IBU and below. This effect was also evident for *S. cerevisiae* US-05 (Figure 18 C) and *Lc. cremoris* TF121 (Figure 18 A), though ethanol content also seemed to affect initial growth. Ethanol had an effect on *L. rhamnosus* GG for 4.5% and 6% ethanol, showing lower growth overall compared to Figure 16 D, which for the strain in 4.5 EtOH / 0IBU was contradictory. In Figure 16D, this mixture reached a final OD of 1.8 after 167 hours, compared to a final OD of 1 in figure 18B.

Based on the result of the ethanol and IBU concentration tests, all strains were deemed viable for further use in beer sour beer fermentations. *S. cerevisiae* US-05 was chosen for further testing in v1 wort by itself as well as in cofermentation with *Lc. cremoris* TF121.

In v2, *B. claussenii* WLP645 and *Lc. cremoris* TF121 were combined to test difference in cofermentation from v1 with different yeast along with a *Lactobacillus brevis* strain which had shown promising growth in previous unpublished work (outside of this thesis). *L. rhamnosus* GG was combined with *S. cerevisiae* US-05 to test for difference compared to *Lc. cremoris* TF121 & *S. cerevisiae* US-05 cofermentation from v1.

4.6 Evaluation of single vs mixed culture fermentation

4.6.1 CFU counts

The aerobic and anaerobic conditions for growth of *Lc. cremoris* TD013a/TA01/TF121 both showed detectable growth (Tables S1-3 in the Appendix). There was an observable effect of the aerobic and anaerobic conditions for the growth of *Lc. cremoris* TF121 when diluted 1:1000000. This was not observed for *Lc. cremoris* TD013a and *Lc. cremoris* TA01. The overall growth was similar in both media. There was however growth of a secondary culture in the lowest concentration plates containing RFO for both aerobic and anaerobic conditions across all cultures. The secondary culture had a rod/cocci morphology under the microscope, and a yellow tint on the agar. These cultures were not considered when counting CFU.

The *L. Rhamnosus* GG grew monocultures for all plates, with a CFU of $1.56*10^9$/mL.

For the final batch, the *Lc. cremoris* TF121 had an average CFU of $1.895*10^8$/mL, making the total amount of cells in the inoculation approximately $2.842*10^{10}$.

4.6.2 Quantification of alpha acids

Prior to brewing, it was assessed whether the hops had undergone any oxidation during storage. This was done by initiating oxidation in a batch. The hops that were not further oxidized had a final concentration of 185 IBU, corresponding to 1.55 % α-acids, compared to the value of 3.7% stated by the manufacturer. The oxidised hops were similar, having a calculated α-acid percentage of 1.52%. These indicated that oxidative degradation of the hops had occurred, and results were used to adjust the added amounts of hops.

Table 7: Quantified international bitterness units (IBU) and α-acids in oxidised and not oxidised hop boil samples (n=3 replicates), and v1-v4 beers (n=4 replicates).

Sample	Oxidised hops	Not Oxidised hops	V1	V2	V3	V4
IBU	181	185	3.16	4.99	6.16	3.95
α-acids in %	1.52	1.55	0.027	0.042	0.052	0.033

The samples from v2, v3 and v4 had a higher calculated IBU than targeted (Table 4) (4 IBU (v2/v3) and 3 IBU (v4)), while the sample from v1 was lower (4 IBU) (Table 7).

4.7 v1, v2 and v3 testing

The initial batches v1 and v2 were produced in 5 L glass fermenters, with different cultures for a total of 19 days. Due to the limited availability of fermenters, the v3 experiment was conducted in 0.5 L glass bottles, with 0.22µm filters to avoid contamination. After completion of the experiment, it became apparent that the set-up had failed to exclude oxygen from the vessels. While results are included in Table X, they need to be interpreted with caution.

Table 8: The chemical and physical properties for beers v1/v2/v3. ADF= Apparent degree of fermentation. V1 and v2 were made with raffinose family oligosaccharide (RFO) mix 1, while RFO mix 3 was used for v3.

	Beer sample						
Batch number	v1	v1	v2	v2	v3 *	v3	v3
Yeast	*S. cerevisiae* US-05	*S. cerevisiae* US-05	*S. cerevisiae* US-05	*B. claussenii* WLP645	*S. cerevisiae* US-05 1	*S. cerevisiae* US-05	*S. cerevisiae* US-05
Bacteria		*Lc. cremoris* TF121	*L. rhamnosus* GG	*L. cremoris* TF121, *L. brevis*		*L. rhamnosus* GG	*Lc. cremoris* TF121
Alcohol (% v/v)	3.5	3.44	3.75	2.92	3.38	3.19	2.72
Present gravity	7.48	8.1	9.01	14.56		11.12	10.7
Original gravity	34.74	35.32	38.16	37.35		35.97	31.94
ADF (% w/w)	77.76	75.1	75.57	59.87		68.13	65.59
Colour value	8.11	7.73	5.96	6.95	11.88	11.7	18.84
pH-value	4.18	3.84	4.54	3.52	4.66	4.63	5.06
Sugar concentration (Brix)	1.91	2.22	2.3	3.72		2.85	2.73

*Incomplete data due to instrumental error.

The higher sugar concentration (Brix), ADF and PG (Table 12) of beers containing *S. cerevisiae* US-05 than the beer containing *B. claussenii* WLP645 showed that it fermented more of the available sugars. Comparing v1 and v2 with v3, less fermentation of the available sugars occurred when v3 was used. The pH was also higher for the v3 beers than it was for v1 or v2, indicating less production of organic acids from the bacteria.

After 7 or 14 days of fermentation, no maltose, glucose, or fructose were detected in either of the v1 beer samples, compared to 34742 ppm maltose, 4972 ppm glucose and 2285 ppm in the unfermented wort. However, samples fermented for 19 days contained traces of maltose (788 ppm for the sample containing *Lc. cremoris* TF121 and *S. cerevisiae* US-05 and 88 ppm for the *S. cerevisiae* US-05 sample). For all data, see Table S5 in the appendix.

Lactic acid was the dominant acid in the sample with *Lc. cremoris* TF121 and *S. cerevisiae* US-05 but not detected in beer containing only yeast (Figure 14). The concentrations of citric acid were similar in all samples (including the wort) at all time points. Low amounts of acetic acid were present in the wort. While acetic acid levels increased in all samples over fermentation time, the samples fermented with *Lc. cremoris* TF121 and *S. cerevisiae* US-05 contained higher values at each time point than samples with just yeast.

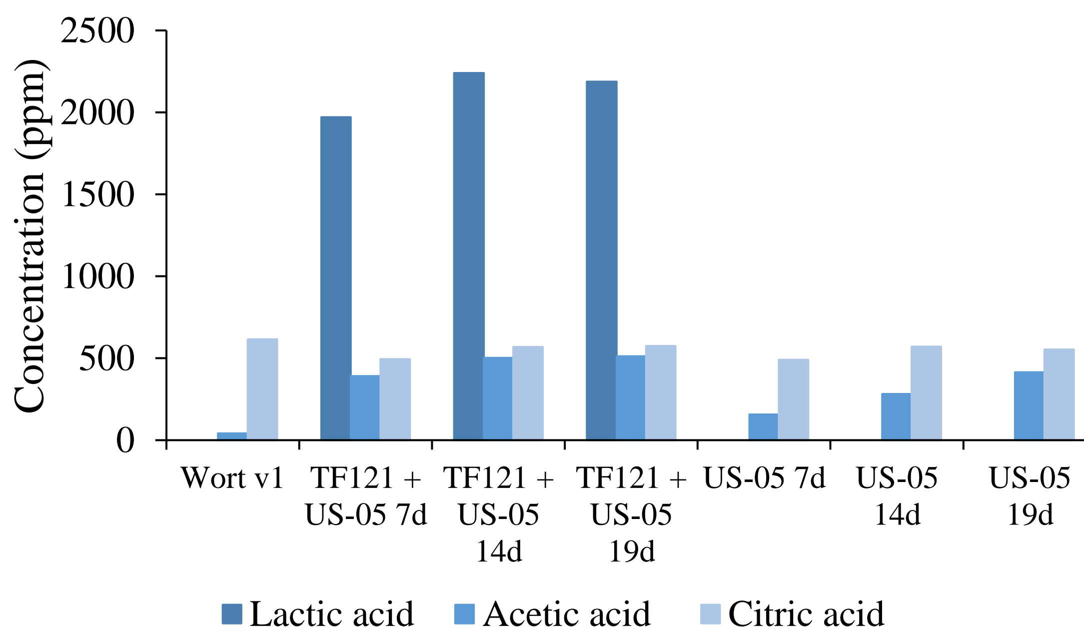

Figure 19: Lactic, acetic, and citric acid amounts (ppm) in beer v1 fermented with US-05= *S. cerevisiae* US-05 (US-05) alone or in combination with *Lc. cremoris* TF121 (TF121+ US-05).

The *Lc. cremoris* TF121 and *S. cerevisiae* US-05 beer from v1 produced both acetaldehyde and dimethylsulfide above the sensory threshold, but in the 19-day samples only acetaldehyde was present above threshold (table 9). Acetaldehyde was also initially produced in the *S. cerevisiae* US-05 beer, but its content decreased as the fermentation progressed. No other evaluated volatile components were detected above the sensory threshold values.

In the samples from beer v2, *B. claussenii* WLP645*, L. cremoris* TF121 and *L. brevis* were slow to utilise the available sugars. The beer contained over 30000 ppm of maltose, 4000 ppm of fructose, and 4500 ppm of glucose (Figure 20) after 7 days. However, after 14 days a large portion of these sugars were utilized, with 3240 ppm maltose, 35 ppm glucose and no fructose remaining. By day 19, monosaccharides had been completely fermented, and only a small fraction of maltose remained. The *L. rhamnosus* GG and *S. cerevisiae* US-05 beer samples had low amounts of maltose and fructose remaining throughout the fermentation.

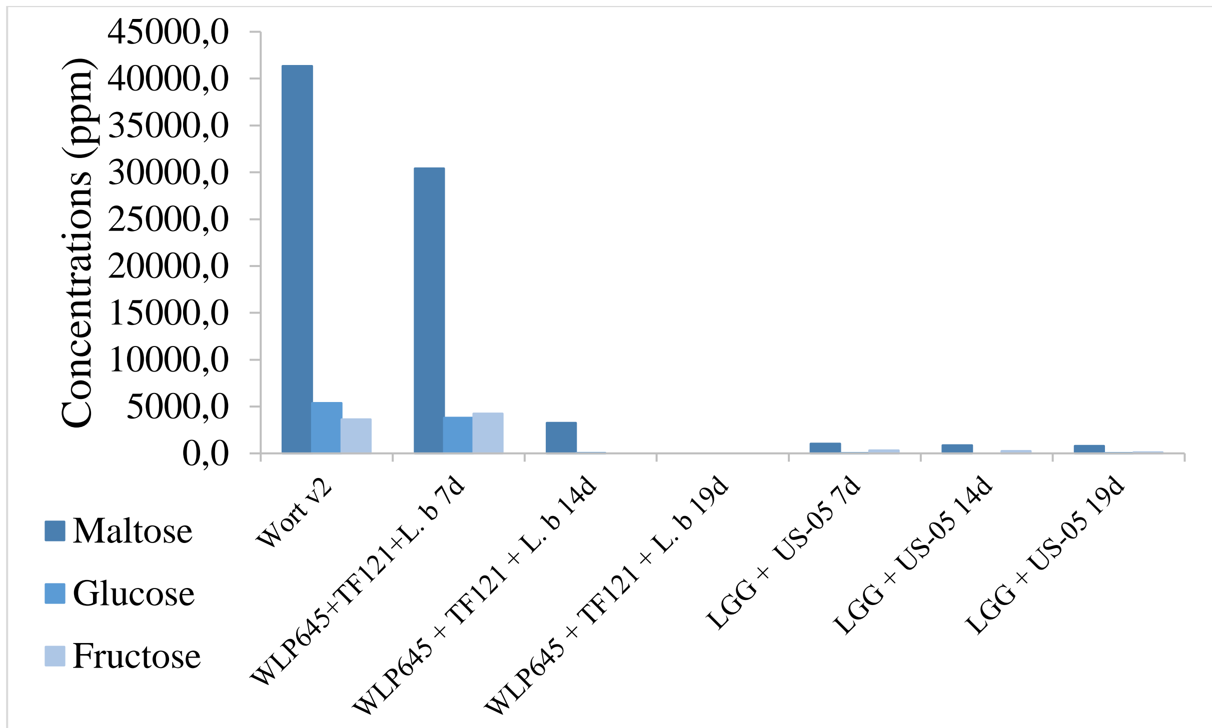

Figure 20: Maltose, glucose, and fructose for WLP645 + TF121+ L. b = *B. claussenii* WLP645, *L. cremoris* TF121 and *L. brevis*. LGG + US-05= *L. rhamnosus* GG and *S. cerevisiae* US-05

The mono- and disaccharides which were fermented in the *L. rhamnosus* GG and *S. cerevisiae* US-05 samples were not converted to organic acids. As can be seen in figure 21, no new organic acids were present until the final sample, where a small amount of acetic acid had been produced. This was in contrast to the beer containing *B. claussenii* WLP645, *L. cremoris* TF121 and *L. brevis* which produced high concentrations of both acetic and lactic acid. The citric acid was also fermented before the 7-day sample, likely as an intermediary in the citric acid cycle.

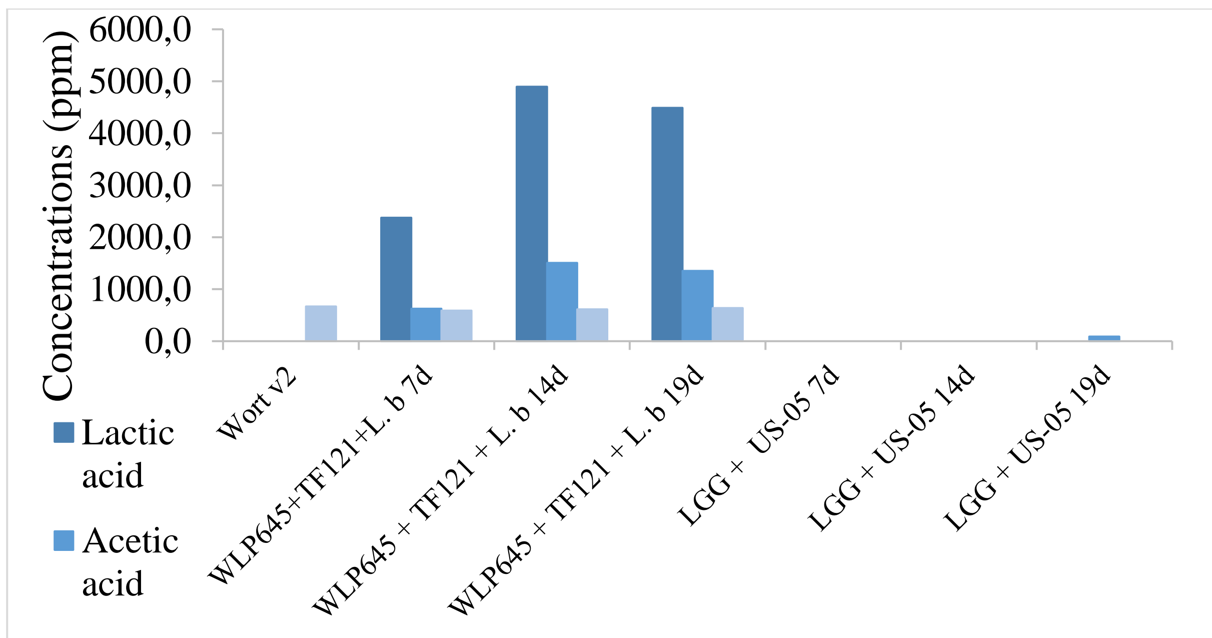

Figure 21: Lactic, acetic, and citric acid amounts in ppm for. WLP645 + TF121+ L. b = *B. claussenii* WLP645, *L. cremoris* TF121 and *L. brevis*. LGG + *US-05*= *L. rhamnosus* GG and *S. cerevisiae* US-05.

Dimethylsulfide was observed above the sensory threshold for v2 beers but was below the threshold in the 19- day samples (Table 9). Ethyl acetate was observed to be above the sensory threshold for all samples of *B. claussenii* WLP645, *L. cremoris* TF121 and *L. brevis*, with a slight decrease over time. None of the other volatile components tested for were above the sensory threshold values.

The v3 beers were not assessed for remaining sugars, organic acids, or volatile components, due to complications with fermentation. *B. claussenii* WLP645 and *Lc. cremoris* TF121 were selected for the final beer, as these had shown promising results in growth controls, as well as growth in the wort of v2, and had the potential to create a flavour more alike that of sour beer.

Table 9: Headspace gas chromatography results for the v1 and v2 beers. Results over the sensory threshold are bold and underlined. n.d= not detected A= (Meilgaard, 1975) B= (Tan & Siebert, 2004) C= (Meilgaard, 1993).

Sample name	Acetaldehyde	Dimethylsulfide	2-methyl-propanal	1-propanol	2-butanon	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
Threshold	10-33 ppm	0,024 ppm (B)	200 ppm (B)	800 ppm (A,	n.d	16 ppm (A, B)	30 ppm (A, B)	200 ppm (B)	0,6 ppm (A)	1,25 ppm (A)	50 ppm (B)	70 ppm (A)	65 pmm (A)	1,6 ppm (A)	0,350 (A)	400 ppm (A)	0,7-1,2 ppm
Wort v1	0,745	**0,053**	0,123	n.d.	n.d	n.d.	n.d.	0,451	0,041	0,04	n.d.	n.d.	n.d.	0,004	0,016	n.d.	0,017
Lc. cremoris TF121 + *S. cerevisiae fermentis* US-05 7d	6,707	0,018	n.d.	14,263	n.d	0,479	5,963	28,485	0,013	0,01	n.d.	24,275	5,241	0,001	0,002	0,529	0,083
Lc. cremoris TF121 + S. cerevisiae fermentis US-05 14d	12,41	**0,034**	0,03	15,464	n.d	0,493	7,257	31,589	0,016	0,011	n.d.	26,657	5,757	0,001	0,003	0,551	0,079
Lc. cremoris TF121 + S. cerevisiae fermentis US-05 19d	**29,437**	0,009	0,087	15,017	n.d	0,449	7,141	30,818	0,018	n.d	n.d	28,524	6,295	0,036	0,007	0,584	0,086
S. cerevisiae fermentis US-05 7d	**108,179**	0,017	0,433	19,6	n.d	n.d.	4,763	64,434	0,07	0,08	10,987	30,597	9,788	0,045	0,003	0,527	0,066
S. cerevisiae fermentis US-05 14d	**103,464**	0,018	0,379	19,017	n.d	n.d.	5,74	63,458	0,075	0,079	14,053	31,107	9,641	0,001	0,002	0,523	0,069
S. cerevisiae fermentis US-05 19d	**67,009**	0,008	0,197	18,595	n.d	n.d	6,449	63,623	0,044	0,069	8,116	34,611	10,704	0,03	0,004	0,537	0,097
Wort v2	0,457	n.d	0,063	n.d	0,056	n.d	0,026	0,275	0,022	0,029	n.d	0,244	n.d	0,037	0,023	n.d	n.d
B. claussenii WLP645+*Lc. cremoris* TF121+*L. brevis* 7d	3,206	**0,038**	n.d	3,6	0,083	0,778	**69,023**	1,911	0,029	0,267	n.d	4,137	1,001	0,098	0,008	0,462	0,065
B. claussenii WLP645 + *Lc. cremoris* TF121 + *L. brevis* 14d	2,528	0,019	n.d	6,114	n.d	0,827	**63,646**	7,312	0,009	0,182	n.d	20,982	4,772	0,071	0,009	0,142	0,09
B. claussenii WLP645 + *Lc. cremoris* TF121 + *L. brevis* 19d	3,254	n.d.	n.d.	4,493	n.d.	0,595	**60,853**	6,028	0,008	0,108		17,277	4,041	0,009	0,008	0,147	0,083
L. rhamnosus GG + *S. cerevisiae fermentis* US-05 7d	4,635	**0,024**	n.d	15,578	0,071	n.d	6,692	31,954	n.d	n.d	n.d	29,393	7,542	0,247	0,015	1,013	0,146
L. rhamnosus GG + *S. cerevisiae fermentis* US-05 14d	1,428	**0,031**	n.d	16,185	0,048	n.d	12,898	33,38	n.d	n.d	n.d	30,462	7,88	0,152	0,011	0,874	0,12
L. rhamnosus GG + *S. cerevisiae fermentis* US-05 19d	2,312	0,021	n.d.	15,051	n.d.	n.d.	22,835	32,364	0,009	n.d.		28,578	7,619	0,034	0,024	0,626	0,082

4.8 Mixed fermentations on a larger scale

Using one yeast and one bacteria strain per beer, samples were produced on a XXL scale in the presence or absence of RFOs. Compositional data and physical attributes of beer samples are presented in Table 10. A commercial sour beer sample is shown for comparison. Unfortunately, no sample of the fermented wort was available, meaning that the initial content of sugars, organic acid and volatile components could not be assessed. Results from v1-v3 indicated that most of the fermentation occurred over the initial 14 days. As such, a 14-day fermentation was planned for v4. However, when analysing the v4 beer after 14 days, the beers that did not contain *S. cerevisiae* US-05 exhibited a low ADF, and higher than expected amounts of sugars (Brix and PG). As such, it was decided that the v4 beer containing *B. claussenii* WLP645 and *L. cremoris* TF121 would be fermented for the full 19 days, as with prior beers.

Table 10: Physical and chemical characteristics of v4 beers.

Beer type	**Mixed fermentations**						**Commercial control**
Yeast	*S. cerevisiae* US-05 v4	*S. cerevisiae* US-05	*B. claussenii* WLP645	*B. claussenii* WLP645	*B. claussenii* WLP645	*B. claussenii* WLP645	Rodenbach Grand Cru
Bacteria	-	-	*L. cremoris* TF121	*L. cremoris* TF121	*L. cremoris* TF121	*L. cremoris* TF121	
RFO mix	-	yes	-	yes	-	yes	
Fermentation time			14	14	19	19	
Alcohol (%v/v)	3.28	4.02	0.59	1.22	0.87	1.51	6
Present gravity	8.1	11.7	35.8	35.4	34.2	34.9	
Original Gravity	33.7	43.0	40.5	45.0	41.1	46.8	
Apparent degree of fermentation (% w/w)	75.3	71.7	11.0	20.3	15.9	24.2	
Colour value (EBC)	3.34	5.69	4.00	5.23	5.62	11.09	
pH-value	4.02	4.06	3.67	3.40	3.63	3.38	3.29
Concentration Sugar (Brix)	2.07	2.99	8.97	8.87	8.61	8.77	
Total acidity* (mol/L)	0.020	0.058	0.046	0.145	0.049	0.146	0.102

* Expressed as lactic acid

The v4 beer containing *S. cerevisiae* US-05 was similar to that of v1, reaching 0.2 % ethanol less without RFOs and 0.5% ethanol more with RFOs, but with a similar ADF (77% v1, 75% v4 without and 71% v4 with respectively) and pH. The *S. cerevisiae* US-05 containing RFOs had a higher ethanol percentage than the sample without in v4, as well as the sample in v1 which did contain RFOs. This was likely due to the higher original gravity in the v4 beer, caused by the addition of RFO. This was also visible in the *B. Claussenii* WLP645, *L. cremoris* TF121 beer containing RFO for both 14 and 19 days. However, these samples had a higher ADF than their non RFO counterparts, despite the higher OG.

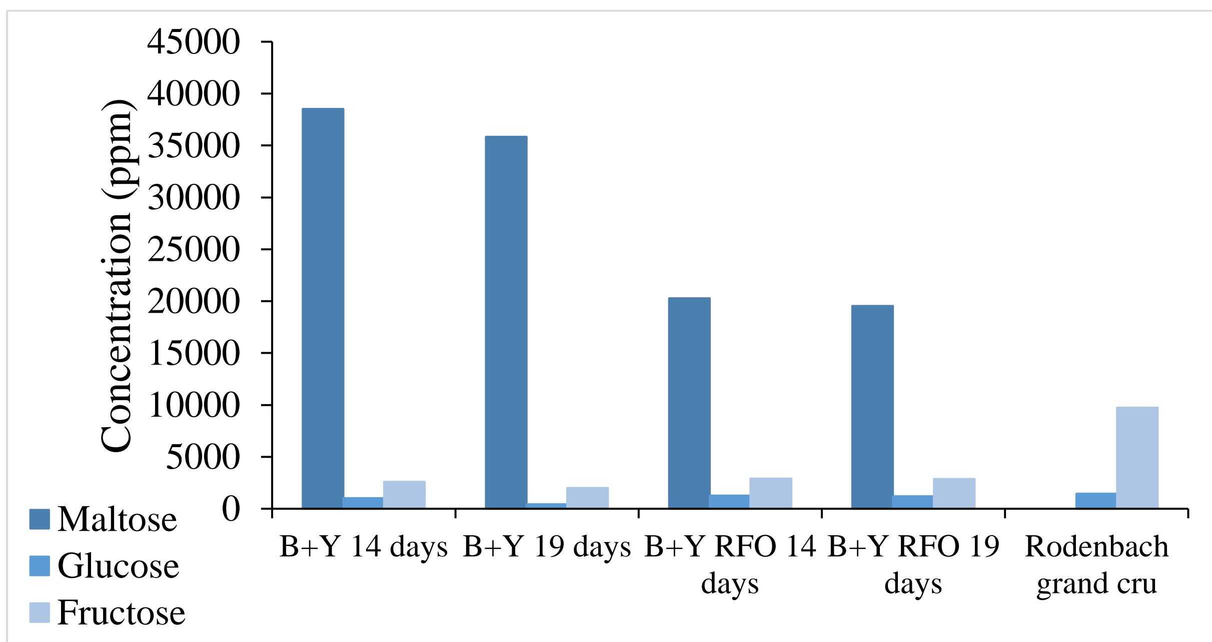

Figure 22: Maltose, glucose, and fructose in v4 beer samples with or without raffinose family oligosaccharides (RFOs) and a commercial sour beer, detected by high performance liquid chromatography. B+Y= *B. claussenii* WLP645, *L. cremoris* TF121

No remaining mono and disaccharides were detected in beer containing *S. cerevisiae* US-05 beer after 14 days, whereas quantifiable amounts of all three sugars were present in beer with *B. claussenii* WLP645 & *L. cremoris* TF121 beer, after 19 days (Figure 22). The analysis of commercial sour beers showed glucose and fructose present in the Rodenbach grand cru, while the Oude Geuze Boon did not contain any of the evaluated sugars. All beers containing *B. claussenii* WLP645 and *L. cremoris* TF121 had a lower pH than the *S. cerevisiae* US-05 indicating that more organic acid production occurred in these samples.

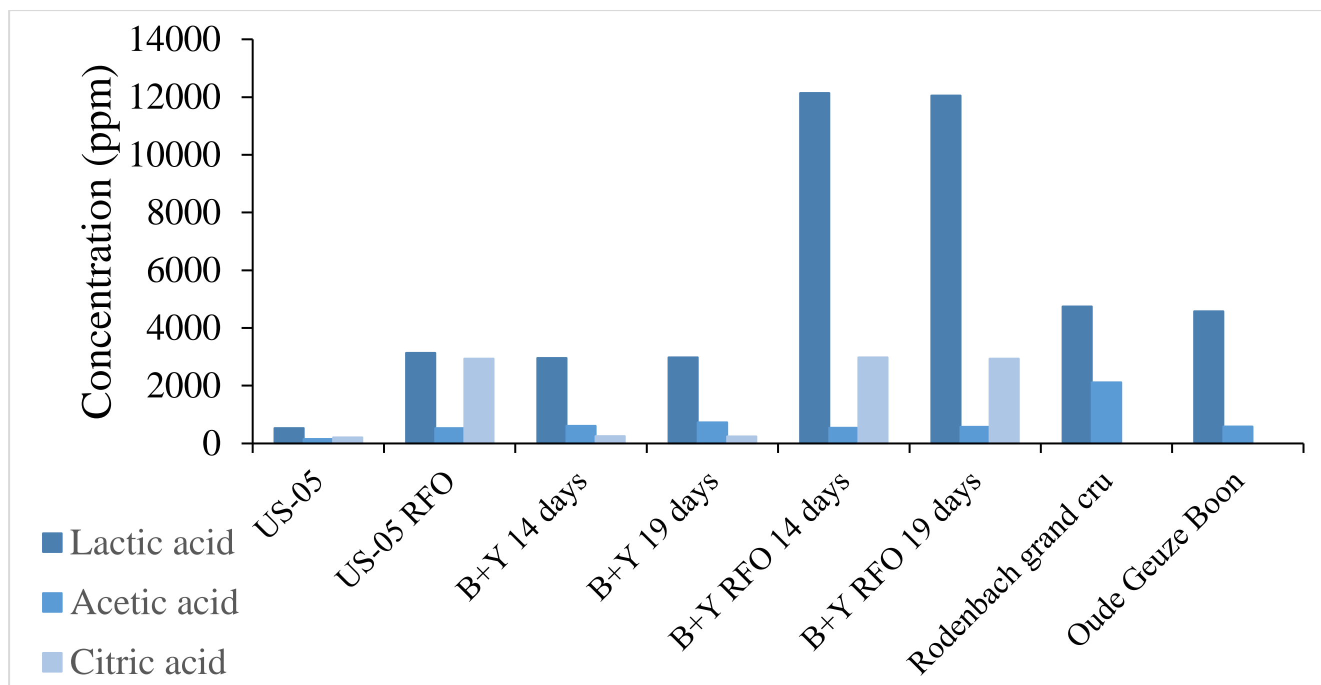

Figure 23: Lactic, acetic, and citric acid amounts (ppm) in beer containing combinations of yeast and lactic acid bacteria with or without raffinose family oligosaccharides (RFOs). US-05= *S. cerevisiae* US-05. B+Y= *B. claussenii* WLP645 & *L. cremoris* TF121.

There was a noticeable difference in acid production between the samples containing RFOs, and those without RFOs (Figure 23). This was primarily evident from the higher amounts of lactic acid in samples with RFOs, which reached 12 000 ppm. The amount of citric acid present in all samples containing RFO, both for the *S. cerevisiae* US-05 and *B. claussenii* WLP645 & *L. cremoris* TF121 beer indicated that this was present in the RFO mix (RFO mix 4, see Table 1) and neither sample utilized this organic acid. The amount of acetic acid was similar with and without RFO for the *B. claussenii* WLP645 and *L. cremoris* TF121 beer, while the *S. cerevisiae* US-05 beer containing RFO had substantially more acetic acid than the *S. cerevisiae* US-05 without RFOs

The disparity in lactic acid production between the *B. claussenii* WLP645 and *L. cremoris* TF121 with and without RFOs was also visible in the total acidity. The 19-day sample with RFOs had 0.146 mol acid per litre, compared to 0.049 mol acid per litre in the 19-day sample without RFOs. This was also high compared to the 0.102 mol acid per litre in the Rodenbach Grand Cru. There was a slight increase in total acidity for both 19-day samples compared to the 14-day samples, but the primary acid producing activity was prior to 14 days.

For volatile components (Table 10), *S. cerevisiae* US-05 with and without RFO produced acetaldehyde above the sensory threshold. All samples except the commercial Rodenbach grand cru produced dimethylsulfide above the threshold, with increasing concentrations for both *B. claussenii* WLP645 & *L. cremoris* TF121 beers after 19 days compared to the 14-day samples. The commercial beers were both well above the threshold for ethyl acetate and 3-methyl-1-butanol. The only non-commercial sample to show an above threshold value for ethyl acetate was the 14-day sample of *B. claussenii* WLP645 & *L. cremoris* TF121 without RFO, but this value decreased to below the threshold for the 19-day sample. No other above sensory threshold values for the volatile components were detected.

Table 11: Headspace gas chromatography table for the different v4 cultures and commercial beers. All ppm values are averages of n=2 samples, reads above threshold value in bold. A= (Meilgaard, 1975) B= (Tan & Siebert, 2004) C= (Meilgaard, 1993).

Horizontal (Category) Axis

Sample name	Acet-aldehyde	Dimethyl-sulfide	2-methyl-propanal	1-propanol	2-butanol	Ethyl acetate	2-methyl-1-propanol	3-methyl-butanal	2-methyl-butanal	3-methyl-1-butanol	2-methyl-1-butanol	Isobutyl acetate	Hexanal	2-hexanol	Isoamyl acetate
Threshold (ppm)	10-33 ppm (A,B,C)	0,024 ppm (B)	200 ppm (B)	800 ppm (A, B)	16 ppm (A, B)	30 ppm (A, B)	200 ppm (B)	0,6 ppm (A)	1,25 ppm (A)	70 ppm (A)	65 pmm (A)	1,6 ppm (A)	0,350 (A)	400 ppm (A)	0,7-1,2 ppm (A,B)
US-05	**36,036**	**0,026**	0,148	25,471	0,236	12,048	94,109	0,040	0,055	61,723	17,637	0,077	0,003	1,412	0,451
US-05 RFO	**30,380**	**0,024**	0,162	34,505	0,312	13,645	107,612	0,013	0,040	55,681	17,412	0,089	0,005	1,348	0,269
B+Y 14 days	2,571	**0,071**	n.d.	2,628	0,224	**61,138**	2,067	0,010	0,021	3,096	2,210	0,010	0,005	n.d.	0,038
B+Y 19 days	2,475	**0,093**	n.d.	3,322	0,264	26,158	2,857	0,012	0,042	4,231	3,002	0,011	0,009	n.d.	0,039
B+y RFO 14 days	1,916	**0,054**	n.d.	1,651	0,162	7,296	0,902	0,009	n.d.	1,359	1,171	0,008	0,007	0,191	n.d.
B+Y RFO 19 days	2,148	**0,106**	n.d.	1,691	0,158	28,195	0,992	0,010	0,027	1,485	1,305	0,010	0,008	0,257	n.d.
Rodenbach Grand Cru	4,048	n.d.	0,130	45,995	0,788	**173,221**	16,304	0,033	0,038	**99,583**	13,686	0,051	0,012	1,610	0,517
Oude Geuze Boon	6,189	**0,037**	0,105	17,214	n.d.	**46,601**	14,523	0,022	0,014	**86,973**	12,045	0,032	0,020	1,663	0,231

4. 9 Tetrad test

The tetrad test had a total 25 participants (18 women, 7 men) in the age span of 18-59, where 17 had previous experience with sensory testing. They were asked to differentiate and pair samples based on similarity. The four sample pairs were *S. cerevisiae* US-05 with and without RFO, *S. cerevisiae* US-05 against *B. claussenii* WLP645 and *lc. cremoris* TF121 without RFO, *B. claussenii* WLP645 and *lc. cremoris* TF121 with and without RFO, and *S. cerevisiae* US-05 against *B. claussenii* WLP645 and *lc. cremoris* TF121 with RFO. The results of the tetrad test are shown in figure 24.

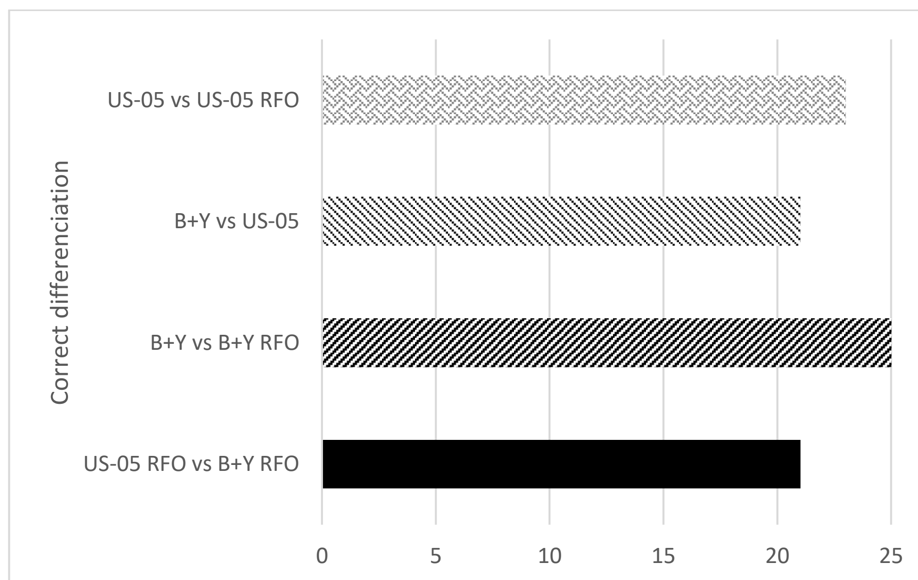

Figure 24: The number of participants which correctly identified the two samples for each test during the tetrad testing. Testing was done on v4 beers with and without raffinose family oligosaccharides (RFOs). US-05= S. cerevisiae US-05, B+Y= *B. claussenii* WLP645 & *Lc. cremoris* TF121. All results were statistically significant ($p<0.01$).

The presence of RFOs induced sensory differences that allowed the majority of participants to assign which two samples were the same for each of the four beers. In the case of the beer fermented with *B. claussenii* WLP645 & *Lc. cremoris* TF121, all participants correctly identified the corresponding pairs with and without RFOs. Even the combination with the lowest score of correct answers, *S. cerevisiae* US-05, *B. claussenii* WLP645 and *lc. cremoris* TF121, was still correctly identified by 21 participants.

After the testing, one participant notified the testing personnel that there might have been sour beers in all tests, as no pairing was without acidity. It was suspected that the *S. cerevisiae* US-05 with RFO had been contaminated with an acid producing microorganism.

5. Discussion

The initial goal of the thesis was to assess whether RFO concentrates could be utilized as an adjunct for sour beer brewing, substituting part of the malt. This included the exploitation of fermentation of the FODMAPs raffinose, stachyose and verbascose by specific bacteria or yeast. In this thesis, bacteria and yeast were selected for growth with RFO as a substrate. This was assessed by monitoring the OD and utilizing HPLC to determine decrease in RFOs due to fermentation. Subsequently, strains were selected and tested for tolerance and inhibition of growth in beer media. They were used as starter culture in wort with RFOs where fermentation products, as well as chemical and physical properties were assessed. For these analyses HPLC, HSGC, titratable acidity, IBU content and a general beverage analysis were applied. The final beers were also assessed by 25 participants for noticeable difference between beer brewed with and without RFOs.

Growth behaviour

Negative and positive growth control

The initial testing of the cultures (*B. claussenii* WLP645, *L. rhamnosus* GG and *Lc. cremoris* TD013a/ TA01/ TF121) gave results which signified the importance of a sugar source for the growth of both yeast and bacteria. In testing without any sugar source growth stagnated within 24 hours for the bacterial samples (Figure 5 and 6), while B. *claussenii* WLP645 grew slowly (Figure 7). The growth was likely due to fermentation of the yeast extract in the growth medium, which dependant on which media the yeast was grown in and the treatment process used, could contain both sugar and fat (Tao et al., 2023). The growth was substantially higher for all cultures when glucose was present (Figures 5-7). The *Lc. cremoris* strains reached stationary phases within 24 hours, as they also did without sugar source (Figure 5). For the *L. rhamnosus* GG strain growth was substantially improved with glucose. These samples were due to an oversight not diluted, and as such it was difficult to assess the exact growth pattern (Figure 6). The absorbance maximum of the spectrophotometer was 2, and standard lab practice was to dilute samples which reached or were close to this OD. This practice does not give as precise measurements, but a close estimation.

RFO growth control

While the *L. rhamnosus* GG culture grew in RFO-containing medium and reached a stationary phase within 24 hours (Figure 9), it however did not reach as high of an OD as with glucose-

containing medium (Figure 6). These observations pointed to that RFO mix 1 was a less optimal substrate for *L. rhamnosus* GG than glucose, but that the bacteria able to ferment it partially. The strain has previously been shown to contain four individual α-galactosidase encoding genes (WCN76399.1, WCN78003.1, CAR86896.1 and CAR87974.1) (Al-Nakeeb et al., 2023a, 2023b; Kankainen et al., 2009), two of which (WCN76399.1, WCN78003.1) were found in the *L. rhamnosus* LGG (DSMZ: 33156) from CHR Hansen AS. This strain has however been reported as raffinose and sucrose fermentation negative (Hansen, 2019). It is thus possible that the existence of the α-galactose genes alone is not sufficient to conclude that an organism is able to ferment RFOs.

Lc. cremoris TD013a and TA01 showed low ability to grow in media with RFO mix 1. The *Lc. cremoris* TF121 strain exhibited a similar growth pattern to the other strains for the first 48h but increased in OD between 48 and 72 hours (Figure 8). This growth indicated that the TF121 strain was potentially able to ferment a substance in the substrate, given time. It was also theorised that the datapoint could be an outlier, which was disproven by the subsequent testing.

Studies on the use of LABs to ferment RFOs have focused primarily on the strains which were prior to use proven to have α-galactosidases, but as mentioned above, this may not be the only factor required. Boucher et al. (2003) stated that the presence or absence of an α-galactosidase metabolism in *Lactococcus* strains was likely related to the ecological niche they were adapted to. This statement is supported by findings showing that *Lactococcus* strains found on fruits and vegetables can ferment RFOs. This gives the bacteria an ecological benefit, utilizing a substrate not available to all bacteria (Kelly et al., 1998a; Kelly et al., 1998b). According to Kelly and Ward (2002), *Lc. cremoris* (in the article referred to as *Lactococcus lactis* subsp. *lactis* (cremoris phenotype)) is widely used as a dairy starter and adapted to this environment (Lambie et al., 2014). To the best of the authors knowledge, no genetic sequences of *Lc. cremoris* with α-galactosidase or β-fructofuranosidase genes have been published and testing of RFO growth with *Lc. cremoris* has shown negative results (Fritsch et al., 2015).

Another aspect to consider is the potential effect of enzyme inhibitors. Boucher et al. (2003) described the α-galactosidase in *Lactococcus raffinolactis* and reported that glucose had an inhibitory effect on the enzyme activity. Similar inhibitory effects of glucose and sucrose have been reported for *Lactiplantibacillus plantarum* and *Streptococcus pneumoniae* respectively (Rosenow et al., 1999; Silvestroni et al., 2002). In the RFO substrate used for the RFO growth

control sucrose was available, along with other unknown substances. Thus, fermentation experiments with single sugars and RFO control were performed.

Both yeast strains grew better in the YEP RFO media (Figure 10 and 11) compared to the YEP media without sugar, due to the available sugar source. The *B. claussenii* WLP645 strain showed lower final OD in YEP medium with RFOs compared to YEP medium with glucose (Figure 7) and seemingly reached stationary phase after 96 hours in all scenarios. The growth of the *B. claussenii* WLP645 strain was slow for the first 48 hours in all media, indicating either a long generation time or lag phase. The *S. cerevisiae* US-05 yeast reached the highest OD measured for growth in RFO media (Figure 11) and grew regularly between each measurement after an initial lag phase. This phase may be due to the glucose repression during the shift from the original medium where it was grown pre-inoculation with glucose (Rowan-Nash et al., 2019).

Utilizing the mono- and oligosaccharide HPLC for verification of RFO fermentation proved to be difficult. In some instances, the amount of RFOs in a sample were far outside the calibration of the test. For these samples the results required careful interpretation, and biological data were trusted over the HPLC results. For other samples the baseline was far outside the normal accepted range and had to be heavily adjusted before data could be collected. It was also a concern that the HPLC method for mono- and oligosaccharides was developed for dairy products rather than beer. The difference between these matrixes is quite substantial, which was reflected in the results, particularly for beer samples.

Single sugar source control

The *Lc. cremoris* TF121 strain had higher growth with maltose, sucrose, and lactose, with the latter reaching > 2 OD within 24 hours (Figure 14), compared to growth in raffinose, stachyose and RFO (5 and 10 g/L) media (Figure 15). The preference for lactose over sucrose and maltose indicted that the strain was isolated from an environment rich in lactose, such as dairy products. The growth patterns in media with disaccharides were similar to the growth of *Lc. cremoris* TF121 in glucose media (Figure 5), showing growth between 0 and 24 hours and subsequent stationary phase for the following measurements. A similar pattern was observed for *Lc. cremoris* TF121 in media containing 5 and 10 g/L RFO, where the growth of *Lc. cremoris* TF121 after 24 hours was limited (Figure 15). In media containing raffinose and stachyose, *Lc. cremoris* TF121 had a different growth pattern. The samples grew slowly for the duration of the experiment (figure 15), not showing the stationary phase seen when utilizing other

substrates. The growth was increased from what was observed in the negative control (figure 5), implying that the *Lc. cremoris* TF121 was able to somewhat utilize the substrate.

While the results of growing *Lc. cremoris* TF121 on medium with both raffinose and stachyose showed a measurable difference without a carbon source, the same was not clear in beer medium.

Beer medium control

In the first beer base medium (Figure 12) there was little apparent growth for any of the *Lc. cremoris* strains (TD013a, TA01,TF121) with or without RFOs, with the exception of *Lc. cremoris* TF121 with RFOs in the medium. The growth was substantially lower in this medium than M17 with RFO (Figure 8) for *Lc. cremoris* TF121, possibly due to inhibition by the ethanol concentration combined with hop α-acids. The growth in media with RFO but not without RFO for *Lc. cremoris* TF121 indicated the limiting factor for growth was a lack of substrate. It is uncertain whether *Lc. cremoris* TD013a and TA01 were inhibited by ethanol, α-acids from the hops, lack of other nutrients or a combination. That there was an inhibition was however evident, as the growth was low for these strains compared to the negative growth control (Figure 5) as well as the growth control with RFOs (Figure 8).

The *L. rhamnosus* had improved growth in the beer medium with RFOs compared to beer without RFOs (Figure 13), but showed low growth compared to MRS with RFOs (Figure 9). There was little discernible initial growth, but after 24 hours both samples showed an increase in OD. Growth in the beer without RFO was likely due to utilization of unfermented sugars from the wort.

The two strains (*Lc. cremoris* TF121 and *L. rhamnosus* GG) which showed growth in beer medium with RFOs was used for further experiments, while the other two (*Lc. cremoris* TD013a and TA01) were not used. The yeasts were not tested in beer medium as both were beer yeasts, and as so were expected to grow in this medium.

IBU and ethanol testing

As discussed by Dysvik et al. (2020b) the hurdles that exist in beer inhibit growth of most microorganisms. While testing the *Lactococcus* and *Lactiplantibacillus* strains in base beer medium it became evident that these hurdles inhibited the growth of these strains as well. It was thus necessary to determine which concentrations of ethanol and IBU were inhibitory for the strains in order to create a suitable beer recipe. A normal sour beer is low in alpha acids due to

the aging and oxidation of the hops (Spitaels et al., 2017), and ethanol percentage varies between 3-7%. There was however uncertainty on how much hops could be added before there was noticeable inhibitory effect on the bacteria and yeast. The ethanol could also act as a secondary hurdle, as well as having an interaction effect. It was thus important to find potential limitations of these two aspects, prior to brewing on a larger scale. The bacteria were along with the yeast first tested in 2.25% ethanol and 4.5% ethanol, where each ethanol concentration was combined with 0, 2.5 or 5 IBU of hop concentrate.

Optimal growth testing of *S. cerevisiae* has shown that the yeast is able to withstand 15% ethanol in media in pH 4-6 (Gao & Fleet, 1988). Later studies have found that 10% ethanol did have an inhibitory effect on growth, but this effect was not present when *S. cerevisiae* was grown in 5% ethanol (Claro et al., 2007). Similar tolerance to ethanol has been shown for strains of *Brettanomyces* in wine according to Ciani and Comitini (2014).

Ethanol percentage showed a significant effect ($p<0.01$) on *S. cerevisiae* US-05 after 48 hours in the first ethanol and IBU test. This was however not visible on the growth curve(Figure 12), where the strains grown in 4.5 % ethanol and 2.25 % ethanol showed similar growth. For the *B. claussenii* WLP645 strain, ethanol did not have an apparent effect on growth, as 2.25 % ethanol and 4.5 % ethanol, both with 5 IBU had the highest OD readings. The strain inoculated in 2.25% ethanol and 0 IBU showed growth inhibition until the final measurement, making it a statistical anomaly. This affected the Anova analysis, showing a constant effect of both ethanol and IBU for the duration of the test.

In the second test (Figure 17), all *S. cerevisiae* strains showed similar growth in media without RFOs. There was however a pattern, whereby similar concentrations of ethanol were grouped more, and the strain showing the highest growth had the lowest IBU. It is therefore possible that the US-05 were somewhat inhibited by the ethanol concentration, but not affected by IBU. This pattern was not in the third test, where RFOs were available. Here a combined effect of IBU and ethanol seemed more plausible, as the strain containing 3.5% ethanol and 3.75 IBU reached the same final OD as the strain in 6% ethanol with 2.5 IBU. The difference between the second and third test in OD showed positive signs that US-05 was able to ferment the substrate.

Narendranath et al. (1997) tested a range of LAB (including an *L. rhamnosus* strain) in wheat mash for their cofermentation ability with *S. cerevisiae.* When the LAB strains were inoculated alone, consumption of fermentable carbohydrates was below 1g/100 mL, but the cultures

remained viable. When the *L. rhamnosus* strain was inoculated with *S. cerevisiae* it did not remain viable and showed a marked decline in CFU/mL within 48 hours. The ethanol concentrations produced after 72 hours were 11.86-12.50 % ethanol, dependant on the inoculation volume of bacteria. Other studies have shown a significant effect of 4-5% ethanol on the growth rate of LAB (Capucho & San Romão, 1994; Chen et al., 2020; Zhu et al., 2015). Regarding IBU, testing on *Lactobacillus brevis* showed iso-α-acid levels of 20-50 µM to be inhibitory for growth (Schurr et al., 2015). This threshold was also evident for the *Lc. cremoris* TF121 and *L. rhamnosus* GG strains in testing.

In the first ethanol and IBU test (Figure 12) the bacterial strains were limited in growth primarily due to IBU. This was evident by the 2.25 % ethanol and 5 IBU media showing lower growth than any other strain for both bacteria. Ethanol concentration seem to be less of an inhibitory factor, with equal IBU concentrations showing similar growth. The IBU effect was also evident for the second and third test (Figure 13 A/C and 14 A/C) where the grouping of strains was mostly independent of ethanol concentration. The odd observation here was the strain in 6% ethanol and 2.5 IBU for both bacteria (Figure 14 A/C) which showed comparable growth to the strains in 0 IBU media (4.5 % and 6% ethanol). This may however be due the utilization of the same hop concentrates from the first ethanol and IBU test, where α-acid may have been lost over time. This would have caused the actual IBU to be lower.

Colony Forming Units

The aerobic and anaerobic test of CFU for *Lc. cremoris* TD013a, TA01 and TF121 gave monocultures on glucose agar and on the higher inoculations of RFO agar. On the RFO agar plates CFU was higher under aerobic conditions, suggesting that the bacteria preferred aerobic conditions. For the 1:1 000 000 inoculation of RFO agar, two different cultures grew, and one of these were believed to be a contaminant in the most diluted samples. Alternately it could a contaminant from the lab, which in RFO agar had a better chance of competing with the *Lc. cremoris* strains. When assessed under microscope, the culture had a diffuse morphology, being somewhat both rod and cocci.

Quantification of alpha acids

The oxidation test on aged hops showed some variation in the samples taken, but little variation between the means of oxidised and not oxidised. Both showed a marked decrease in α-acid level, compared to the values stated by the producer which showed that the packed hops already had undergone oxidation. Further testing of specific components within the hops may have

elucidated more information, such as Schurr et al. (2015)s experimental design where they isolated the minimum inhibitory concentrations of methyl-iso-α-acids, humulonic acids, cis- and methyl-cis-iso-cohumulone.

Small and large-batch fermentations

V1 and v2 experimental beers

The v1 and v2 beers were designed to test cofermentation of yeast and bacteria in wort medium, with the intention of producing a beer while utilizing RFOs as an adjunct. The previous experiments were rather designed as secondary fermentations. The beers were formulated based on the results gathered from control growth (Figures 5-13) and the first ethanol/ IBU testing (Figure 14 and 15). For v1, *S. cerevisiae* US-05 was tested on its own as a control, and with *Lc. cremoris* TF121 for a cofermentation. There was noticeable decrease in pH of the *Lc. cremoris* TF121 and *S. cerevisiae* US-05 sample compared to the control sample, indicating that the *Lc. cremoris* TF121 fermented some of the substrate and produced organic acids. This was supported by the similarities in ethanol concentration (3.5% and 3.4% (v/v) respectively) and the remaining sugars (1.91° and 2.22° brix) (Table 8), meaning *S. cerevisiae* US-05 had been able to ferment equal amounts of ethanol. This may then have been fermentation of the RFOs, or other oligosaccharides remaining in the wort.

The v1 beer was measured to have 3.1 IBU (Table 9), which could be slightly inhibitory in accordance with the results from ethanol and IBU testing (Figures 16-18). The HPLC showed that all maltose, glucose, and fructose present in the wort had been fermented within 7 days for the *S. cerevisiae* US-05 control as well as the cofermentation. The *Lc. cremoris* TF121 and *S. cerevisiae* US-05 contained primarily lactic acid (2000 ppm), but there were also substantial amounts of acetic acid (500 ppm).

Acetic acid is produced by *S. cerevisiae* US-05 during ethanol fermentation as a by-product (Mira et al., 2010). The amount of acetic acid in the *Lc. cremoris* TF121 and *S. cerevisiae* US-05 cofermentation was higher than the levels in beer with *S. cerevisiae* US-05 alone, indicating that a percentage could have been produced by mixed acid fermentation in *Lc. cremoris* TF121 (Vinderola et al., 2019). As testing did not include formate as a product, this cannot be confirmed. There was also citric acid in the *Lc. cremoris* TF121 and *S. cerevisiae* US-05 beer. Though citric acid can be a by-product from *S. cerevisiae* fermentation (Li & Liu, 2015), the citric acid in v1 was accounted for in the wort (Figure 15). The origin of the citric acid may be

the RFO substrate, where citric acid was utilized to solubilize the oligosaccharides in pea protein concentrate (Garbers et al., 2022).

The occurrence of acetaldehyde above the threshold for human perception for both the control *S. cerevisiae* US-05, and the cofermentation with *Lc. cremoris* TF121 was likely to be caused by fermentation of substrate by the *S. cerevisiae* US-05 strain (Table 9). Acetaldehyde formation as a result of fermentation has previously been shown for yeast in wine (Walker, 2014), sake (Shimoi, 2014) and beer (Liu et al., 2018). The volatile component accumulates during the growth phase of the yeast, when it is produced by decarboxylation of pyruvate as an intermediary of ethanol, and a subsequent decrease is standard when the growth reaches stationary phase (Stewart, 2014). There was an above threshold (0.024 ppm) amount of dimethylsulfide detected in the wort for v1, as well as in the 14-day sample of the *S. cerevisiae* US-05 and *Lc. cremoris* TF121 cofermentation (Table 9). This is an unwanted flavour compound which can cause the beer to smell of boiled cabbage and rotten leaks (Yonezawa & Fushiki, 2002). The compound was not detected in the final day samples, so it is possible that the US-05 degraded it (Bokulich & Bamforth, 2013). For v1 as well as v2, none of the compounds associated with unwanted beany flavour (hexanal, 3-methyl-1butanol) from peas (Trindler et al., 2022) were found above threshold.

The v2 beers were both made to be cofermentations. The cofermentations were done using *L. rhamnosus* GG with *S. cerevisiae* US-05, and *Lc. cremoris* TF121 with *L. brevis* and *B. claussenii* WLP645. The production of ethanol was higher for the sample containing *L. rhamnosus* GG and *S. cerevisiae* US-05 with 3.75 % ethanol, compared to the 2.92 % ethanol in the sample with *Lc. cremoris* TF121, *L. brevis* and *B. claussenii* WLP645 (Table 8). The latter sample had also utilized less sugar, with a final apparent degree of fermentation (ADF) of 59.87 % and 3.72° Brix remaining, compared to 75.57 % ADF and 2.3° Brix for the *L. rhamnosus* GG and *S. cerevisiae* US-05 cofermentation. The lower production of ethanol and utilization of sugars were likely due to the slower growth rate of *B. claussenii* WLP compared to *S. cerevisiae* US-05 (Abbott et al., 2005). This is also supported by the observation that there were substantial amounts of residual unfermented sugar in *Lc. cremoris* TF121 with *L. brevis* and *B. claussenii* WLP645 beer for the 7 and 14- day samples (Figure 20).

The beer containing *Lc. cremoris* TF121, *L. brevis* and *B. claussenii* WLP645 produced > 4000 ppm lactic acid and >1000 ppm acetic acid in the 19-day sample. This implied that the bacteria present were not inhibited by the increased IBU (4.98) of the v2 beer. In comparison, *L.*

rhamnosus GG failed to produce lactic acid throughout the fermentation, likely inhibited by the IBU.

The sample showed an above threshold value for ethyl acetate which is associated with a vinegar taste in wine (Shimoi, 2014), while it in beer imparts a fruity, sweet and solvent like flavour (Meilgaard, 1975). The *B. claussenii* WLP645 did produce ethanol (2.92 %), indicating that it was active.

The v3 beer

The v3 beers were designed to be reproduced biological duplicates of the beers from v1 and v2. The instrumental setup of the experimental conditions was however flawed, as the smaller fermentors quickly formed a thick layer of white/brown foam on top. It became apparent that the problem causing difference from v1 and v2 were the lack of yeast lock, but rather a 0.22 µm air filter. This probably gave free access to oxygen for the fermentation, and no internal pressure in the fermenter. As such, it was decided that the results would vary too much from v1 and v2, and thus little analysis was done for v3. The few results that were gathered proved this theory, as the samples of 7, 14 and 19 days of *S. cerevisiae* US-05 showed above threshold levels of acetaldehyde, diacetyl and ethyl acetate higher than in any previous sample (appendix Table Sx). The pH of these samples were also different for v3 (Table 12), all being comparatively higher than in v1 and v2.

V4 beer

For the v4 beer, a control fermentation consisting of *S. cerevisiae* US-05 with and without RFOs and a experimental cofermentation of *B. claussenii* WLP645 and *Lc. cremoris* TF121 with and without RFOs were produced. The control produced 3.3% ethanol in the sample without RFOs and 4% in the sample with RFOs. This ethanol level and the ADF of the samples were comparable to the *S. cerevisiae* US-05 fermentation in v1 which had RFOs. The main difference between these two samples were in the organic acids, where the v4 control with and without RFOs contained lactic acid, while the v1 control with RFOs did not. The v4 control with RFOs contained higher levels of lactic and acetic acid compared to the control without RFO, and a high level of citric acid which was theorised to orginate from the RFOs substrate as mentioned previously.

The *S. cerevisiae* US-05 with RFOs had a lower ADF (71.7% and 75.2%) than its counterpart without RFOs, despite the higher ethanol level (Table 10). This could indicate that while there

was more sugar in the wort due to the addition of RFOs (2.99° Brix compared to 2.07° Brix) these were not available to the *S. cerevisiae* US-05.

For the experimental cofermentation of *B. claussenii* WLP645 and *Lc. cremoris* TF121, the ethanol production was low (Table 11). There was a clear increase in ethanol between the sample containing RFOs and the one without, as well as differences in the pH and ADF. The beer with RFOs had a higher ADF despite the fact that there was more available sugar indicating an improved fermentation when RFOs were available. An increase was also visible for the 19-day samples compared to the 14-day samples. Viewing the remaining sugars in the samples (Figure 22), it was evident that the fermentation was slow. As previously remarked, *B. claussenii* WLP645 is a slower growing yeast compared to *S. cerevisiae* (Abbott et al., 2005). LABs has been shown to ferment little of the substrate in wort (Narendranath et al., 1997), and thus produce low levels of ethanol.

Comparing the samples with and without RFOs, there was a substancial difference in lactic acid produced. These were also high compared to the commercial sour beers, which had approximately a third of the lactic acid observed in the 14 and 19 day samples of the RFOs cofermentation. These results indicate that the primary fermentation in the *B. claussenii* WLP645 and *Lc. cremoris* TF121 v4 beer with RFOs were mainly acid producing, rather than ethanol producing. The lactic acid in the *B. claussenii* WLP645 and *Lc. cremoris* TF121 beer with RFOs were dramatically higher than the beer without RFOs. This, combined with fact that the beer with RFOs had a higher ADF percentage points to a potential utilisation of the RFOs in the wort.

The acetic acid levels were similar between the samples with and without RFOs for *B. claussenii* WLP645 and *Lc. cremoris* TF121. In total the organic acid profile of the cofermentation was similar to that described for Belgian red-brown adicic ales by Snauwaert et al. (2016), though the characteristic volatile components ethyl acetate and isoamyl acetate were not as prominent. The titratable acidity of the beer containing RFOs were also higher, but the reductions in pH were less pronounced, and in the case of *S. cerevisiae* US-05 the pH of beers made with and without RFOs were almost the same (appendix Table S7).

The flavour variation of the beer was not considered an important objective on the onset of the thesis work. As such, the focus on variations within volatile compounds such as aldehydes, esters, higher alcohols and hop bitters were of a chemical concern rather than liking. Sensory threshold values were utilized, as few other comparative methods exist.

Tetrad testing

The tetrad testing of the beers from v4 was done to see whether there was a significant noticeable difference between beer with and without RFOs. This was shown to be the case, as a significant result was achieved in differentiating between each pairing (Figure 24) . A likely characteristic that may have helped the panellists on this matter was the differences in titratable acidity for each beer, as titratable acidity has been shown to be more in line with sensory analyses in other studies than pH (Tyl & Sadler, 2017). The differences in volatile components were less substantial on the other hand. The participants were seemingly unaffected by "adaptation" (sensory fatigue) to the sour taste, which is a common issue (Ford, 2017). The observation of one participant was that there was sour beer in all tests can be explained by the increased organic acids in US-05 with RFOs (Figure 23). This sample may have been subject to a contamination of acid producing microorganisms, which would explain the increased acidity.

The tetrad testing gave clear results to that there a perceivable difference between all samples. However, for a better and more comprehensive understanding of the flavours and the beers viability as products, further tests should be done with a trained panel for descriptive analysis, as well as a liking test on a diverse group of subjects.

6. Conclusion and further works

The utilisation and valorisation of side stream products are an important step towards environmental sustainability, as well as the economic and nutritional benefits gained. The use of RFOs as an adjunct to sour beer adheres to these goals, offering additional sugar that can impart a positive benefit to the microorganisms in the beer.

In the initial growth controls, the bacteria *Lc. cremoris* TF121 and *L. rhamnosus* GG, as well as the yeasts *S. cerevisiae* US-05 and *B. claussenii* WLP645 showed growth on RFOs in optimal media. These results were confirmed by HPLC analysis of the utilized sugars for *B. claussenii* WLP645, while the data for *Lc. cremoris* TF121 and *L. rhamnosus* GG were inconclusive. Further method development, involving sample clean-up prior to injection, may be necessary to remove matrix effects. Other methods available such as NMR, LC-MS or GC-MS may be better suited to verify the results. Further testing showed positive growth in model beer media for both strains, and growth on isolated raffinose and stachyose were observed for *Lc. cremoris* TF121.

Prior to beer production, the bacteria and yeasts were tested for potential IBU and ethanol growth inhibition. It was statistically shown that IBU was a considerable inhibitor for the bacteria at high concentrations, while this was not a factor for the yeasts. Ethanol was at higher concentration limiting for growth, but no apparent mass cell lysis was observed. In beer testing, the RFO concentrate was easily dissolved in the boiling wort, but showing utilization of the substrate was a challenging task, and further method development for HPLC on beer media is required. In cofermentations with *Lc. cremoris* TF121 and yeast, lactic and acetic acid was produced. Similar results were not obtained for *L. rhamnosus* GG, which seemed to be inhibited in the beer media.

The *B. claussenii* WLP645 yeast was comparably slower than *S. cerevisiae* US-05 to ferment the sugars available in the wort. This was particularly evident in the larger scale beer. Here the cofermentation beer containing *Lc. cremoris* TF121 and *B. claussenii* WLP645 had 0.6% ethanol in beer without RFOs and 0.9 % ethanol in beers with RFOs, compared to 3.3% and 4% respectively for the beers with *S. cerevisiae* US-05 after 14 days. The beers with *Lc. cremoris* TF121 and *B. claussenii* WLP645 were however high in organic acid, having over 12 000 ppm lactic acid in the beer with RFOs, and 2 900 ppm lactic acid in the beer without. This difference in lactic acid between samples of *Lc. cremoris* TF121 and *B. claussenii* WLP645 with and without RFOs combined with improved ADF of beer with RFOs pointed to

an improved fermentation when RFOs were available. This again indicates that RFOs may be utilised by the yeast or bacteria during the fermentation, despite the availability of maltose.

The Tetrad testing of the final beer gave significant results for a noticeable flavour difference between samples, with and without RFOs as well as beer with *S. cerevisiae* US-05 compared to beer with *B. claussenii* WLP645 and *Lc. cremoris* TF121. Further analysis of the flavour compounds and a liking test should be beneficial in future works.

One possible alteration to the beer fermentations would be to prolong the fermentation and ageing of the beer, as this may alter the chemical and physical profile (Coelho et al., 2019). Most traditional sour beers are aged for longer periods of time, allowing the microorganisms to fully utilize the substrates available. This may in time allow the *B. claussenii* WLP645 and *Lc. cremoris* TF121 beer to produce the expected ethanol percentage of 3.5 %, instead of the 1.2-1.5% ethanol produced within the 19 days of fermentation. This could also have been achieved by initially fermenting the wort with *S. cerevisiae* for then to add *B. claussenii* WLP645 and *Lc. cremoris* TF121. For the final beer there should also have been a control of the beer containing only *B. claussenii* WLP645 and another containing only *Lc. cremoris* TF121, as this would have given more certainty of the utilization aspect.

There will always be uncertainties when applying living organisms for fermentation, as minute differences may have large consequences. These differences can be observed between samples, but also over time. Despite the apparent challenges that working with living organisms contribute and possible improvements available, growth in media was shown. Future research into applying RFOs in sour beer brewing should focus on method development regarding quantification of RFOs in beer media, as this is yet unavailable. Attention may also be given to the use of different strains of yeast and bacteria, for assessment of yield and utilisation. Finally, the scope may be opened further by exploring other side stream products from food production, which may otherwise not be used.

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Appendix

Obligatoriske felter er merket med stjerne *

Test nr 1

Instruksjon:

Du har nå fått 4 prøver.

Lukt og smak på prøvene fra topp til bunn (i forhold til rekkefølgen på skjermen).

Hak av prøvene du synes er like i samme gruppe, 2 i hver.

Dersom du ikke kjenner forskjell må du smake.

Når du har gruppert prøvene 2 og 2, kan du skylle munnen med vann og gå videre til neste side.

You have now recieved 4 samples.

Smell and taste the samples from top to bottom (according to the order on the screen).

Group the samples that taste/smell the same in each group, 2 in each.

If you do not taste/smell a difference you will have to guess.

When you have grouped the samples (2 in each), rinse your mouth with water and move to the next page.

	591	814	095	210
Gruppe A *	☐	☐	☐	☐
Gruppe b *	☐	☐	☐	☐

Figure S1: Example of answer sheet used during tetrad test.

Dilution	Aerobic plates with 20 g/L glucose						Anaerobic plates with 20 g/L glucose					
	Lc. cremoris T013a		*Lc. cremoris* TA01		*Lc. cremoris* Tf121		*Lc. cremoris T013a*		*Lc. cremoris* TA01		*Lc. cremoris* Tf121	
1:1 000	n.a	n.a	T.H	T.H	n.a	n.a	n.a	n.a	T.H	T.H	n.a	n.a
1: 10 000	T.H	T.H	T.H	8.12E+07	T.H	T.H	T.H	T.H	6.84E+07	7.26E+07	T.H	T.H
1: 100 000	5.6E+08	6.32E+08	3.90E+08	4.50E+08	5.98E+08	1.31E+08	9.78E+07	8.56E+07	2.94E+08	3.48E+08	1.26E+08	9.52E+07
1: 1 000 000	6.7E+08	7.2E+08	n.a	n.a	7.2E+08	7.4E+08	6.2E+08	5.9E+08	n.a	n.a	5E+08	5.6E+08

Table S1: CFU counts for petri dishes with M17 20 g/L glucose for all *Lactococcus* strains. n.a.= Not applicable. T.H= Too high to count.

Dilution	Aerobic plates with 20 g/L RFO						Anaerobic plates with 20 g/L RFO					
	Lc. cremoris T013a		*Lc. cremoris* TA01		*Lc. cremoris* Tf121		*Lc. cremoris T013a*		*Lc. cremoris* TA01		*Lc. cremoris* Tf121	
1:1 000	n.a	n.a	T.H	T.H	n.a	n.a	n.a	n.a	T.H	T.H	n.a	n.a
1: 10 000	T.H	T.H	T.H	T.H	T.H	T.H	T.H	T.H	9.86E+07	T.H	T.H	T.H
1: 100 000	4.98E+08	5.72E+08	2.57E+08	3.12E+08	9.28E+08	8.72E+08	6.24E+07	5.84E+07	2.12E+08	8.56E+07	8.98E+07	7.28E+07
1: 1 000 000	4.50E+08	8.70E+08	n.a	n.a	1.84E+09	1.24E+09	4.18E+08	3.38E+08	n.a	n.a	6.20E+08	6.50E+08

Table S2: CFU counts for petri dishes with m17 20 g/L RFO for all *Lactococcus* strains. n.a.= Not applicable. T.H= Too high to count. Boxes marked with orange had two morphologically distinct cultures.

Table S3: the Table shows dilutions and calculated CFU values for the *L.rhamnosus* GG purity testing.

Dilution	1 : 100 000	1 : 1 000 000	1 : 10 000 000
	5.04E+08	1.07E+09	2.00E+09
	9.60E+08	1.68E+09	3.30E+09

Table S4: high pressure liquid chromatography (HLPC mono and oligosaccharides) results for *Lc. cremoris* TF121. The numbers are area (nC*min) under peaks confirmed to be lactose, maltose, or sucrose. A decrease or increase in relative abundance is correlated to the amount available in the sample.

	0h	167h	Percentage utilized
Lactose 10 g/L	40,1621	0,7924	**78,1 %**
Maltose 10 g/L	27,1548	8,9327	**67,1 %**
Sucrose 10 g/L	7,8579	0,4792	**93,9 %**

Table 5: Anova Tables from ethanol testing of ethanol and international bitterness units testing for *S. cerevisiae* US-05, *B. claussenii* WLP645, *L. rhamnosus* GG and *Lc. cremoris* TF121. Measured data is in optical density.

Anova: Two-Factor with Replication

0 hour for s.cerevisiae

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	0,08	0,12	0,05	0,25
Average	0,04	0,06	0,025	0,041667
Variance	0,0002	0,0002	0,00045	0,000417
4,5				
Count	2	2	2	6
Sum	0,04	0,09	0,09	0,22
Average	0,02	0,045	0,045	0,036667
Variance	0,0002	0,00125	0,00405	0,001267
Total				
Count	4	4	4	
Sum	0,12	0,21	0,14	
Average	0,03	0,0525	0,035	
Variance	0,000267	0,000558	0,001633	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	7,5E-05	1	7,5E-05	0,070866	0,798992	5,987378

Columns	0,001117	2	0,000558	0,527559	0,615093	5,143253
Interaction	0,00095	2	0,000475	0,448819	0,658192	5,143253
Within	0,00635	6	0,001058			
Total	0,008492	11				

Anova: Two-Factor with Replication
24 hours for s.cerevisiae

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	1,61	1,96	2,04	5,61
Average	0,805	0,98	1,02	0,935
Variance	5E-05	0,005	0,0648	0,02443
4,5				
Count	2	2	2	6
Sum	1,56	1,62	1,56	4,74
Average	0,78	0,81	0,78	0,79
Variance	0,0018	0,0018	0,0008	0,00112
Total				
Count	4	4	4	
Sum	3,17	3,58	3,6	
Average	0,7925	0,895	0,9	
Variance	0,000825	0,0119	0,041067	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,063075	1	0,063075	5,09697	0,064757	5,987378
Columns	0,02945	2	0,014725	1,189899	0,367074	5,143253
Interaction	0,02405	2	0,012025	0,971717	0,430952	5,143253
Within	0,07425	6	0,012375			
Total	0,190825	11				

Anova: Two-Factor with Replication
50 hours for s.cerevisiae

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	3,5	3,8	3,6	10,9
Average	1,75	1,9	1,8	1,816667
Variance	0,005	0,02	0,08	0,025667

4,5				
Count	2	2	2	6
Sum	2,85	2,92	2,82	8,59
Average	1,425	1,46	1,41	1,431667
Variance	0,00245	0,0032	0,005	0,002657

Total			
Count	4	4	4
Sum	6,35	6,72	6,42
Average	1,5875	1,68	1,605
Variance	0,037692	0,072267	0,079033

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,444675	1	0,444675	23,07004	0,002991	5,987378
Columns	0,019317	2	0,009658	0,501081	0,629155	5,143253
Interaction	0,00665	2	0,003325	0,172503	0,845586	5,143253
Within	0,11565	6	0,019275			
Total	0,586292	11				

Anova: Two-Factor with Replication

119 hours for s.cerevisiae

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	5,7	6,5	6,7	18,9
Average	2,85	3,25	3,35	3,15
Variance	0,045	0,005	0,005	0,067
4,5				
Count	2	2	2	6
Sum	6	6,5	6,7	19,2
Average	3	3,25	3,35	3,2
Variance	0,08	0,125	0,045	0,076
Total				
Count	4	4	4	
Sum	11,7	13	13,4	
Average	2,925	3,25	3,35	
Variance	0,049167	0,043333	0,016667	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*

Sample	0,0075	1	0,0075	0,147541	0,71414	5,987378
Columns	0,395	2	0,1975	3,885246	0,082719	5,143253
Interaction	0,015	2	0,0075	0,147541	0,865864	5,143253
Within	0,305	6	0,050833			
Total	0,7225	11				

Anova: Two-Factor with Replication

166 hours for s.cerevisiae

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	5,4	4,2	4	13,6
Average	2,7	2,1	2	2,266667
Variance	0,02	0,02	0,02	0,126667
4,5				
Count	2	2	2	6
Sum	5,5	6,5	5,1	17,1
Average	2,75	3,25	2,55	2,85
Variance	0,045	0,045	0,125	0,147
Total				
Count	4	4	4	
Sum	10,9	10,7	9,1	
Average	2,725	2,675	2,275	
Variance	0,0225	0,4625	0,149167	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	1,020833	1	1,020833	22,27273	0,00326	5,987378
Columns	0,486667	2	0,243333	5,309091	0,047066	5,143253
Interaction	0,606667	2	0,303333	6,618182	0,030345	5,143253
Within	0,275	6	0,045833			
Total	2,389167	11				

Anova: Two-Factor with Replication

0 hour for B. claussenii

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	1,17	0,45	0,56	2,18
Average	0,585	0,225	0,28	0,363333
Variance	0,00245	0,00045	0	0,030667

4,5				
Count	2	2	2	6
Sum	0,35	0,45	0,27	1,07
Average	0,175	0,225	0,135	0,178333
Variance	0,00245	0,00045	0,00045	0,002297

Total			
Count	4	4	4
Sum	1,52	0,9	0,83
Average	0,38	0,225	0,2075
Variance	0,057667	0,0003	0,007158

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,102675	1	0,102675	98,568	6,04E-05	5,987378
Columns	0,072117	2	0,036058	34,616	0,000507	5,143253
Interaction	0,08645	2	0,043225	41,496	0,000306	5,143253
Within	0,00625	6	0,001042			
Total	0,267492	11				

Anova: Two-Factor with Replication

26 hours for B. claussenii

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	1,52	0,56	0,52	2,6
Average	0,76	0,28	0,26	0,433333
Variance	0,0008	0,0008	0,0002	0,064467
4,5				
Count	2	2	2	6
Sum	0,34	0,45	0,41	1,2
Average	0,17	0,225	0,205	0,2
Variance	0,0002	0,00125	0,00045	0,001
Total				
Count	4	4	4	
Sum	1,86	1,01	0,93	
Average	0,465	0,2525	0,2325	
Variance	0,116367	0,001692	0,001225	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0,163333	1	0,163333	264,8649	3,43E-06	5,987378
Columns	0,132817	2	0,066408	107,6892	1,99E-05	5,143253
Interaction	0,190817	2	0,095408	154,7162	6,88E-06	5,143253
Within	0,0037	6	0,000617			
Total	0,490667	11				

Anova: Two-Factor with Replication

96 hours for B. claussenii

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	1,64	3,8	3,82	9,26
Average	0,82	1,9	1,91	1,543333
Variance	0,0002	0,0002	0,0018	0,314387
4,5				
Count	2	2	2	6
Sum	3,12	3,12	3,05	9,29
Average	1,56	1,56	1,525	1,548333
Variance	0,0032	0,0018	0,00245	0,001817
Total				
Count	4	4	4	
Sum	4,76	6,92	6,87	
Average	1,19	1,73	1,7175	
Variance	0,183667	0,0392	0,050825	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	7,5E-05	1	7,5E-05	0,046632	0,836185	5,987378
Columns	0,760017	2	0,380008	236,2746	1,97E-06	5,143253
Interaction	0,81135	2	0,405675	252,2332	1,62E-06	5,143253
Within	0,00965	6	0,001608			
Total	1,581092	11				

Anova: Two-Factor with Replication

142 hours for B. claussenii

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	1,72	6,9	7,3	15,92
Average	0,86	3,45	3,65	2,653333

Variance	0,0008	0,245	0,045	1,995787

4,5				
Count	2	2	2	6
Sum	7,8	9	8	24,8
Average	3,9	4,5	4	4,133333
Variance	0,02	0	0	0,086667

Total			
Count	4	4	4
Sum	9,52	15,9	15,3
Average	2,38	3,975	3,825
Variance	3,087467	0,449167	0,055833

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	6,5712	1	6,5712	126,8571	2,93E-05	5,987378
Columns	6,206067	2	3,103033	59,90412	0,000108	5,143253
Interaction	3,8954	2	1,9477	37,60039	0,000403	5,143253
Within	0,3108	6	0,0518			
Total	16,98347	11				

Anova: Two-Factor with Replication

166 hours for B. claussenii

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	3,8	8,1	9,9	21,8
Average	1,9	4,05	4,95	3,633333
Variance	0,08	0,005	0,125	2,006667
4,5				
Count	2	2	2	6
Sum	7,8	9,7	8,7	26,2
Average	3,9	4,85	4,35	4,366667
Variance	0,08	0,045	0,125	0,230667
Total				
Count	4	4	4	
Sum	11,6	17,8	18,6	
Average	2,9	4,45	4,65	
Variance	1,386667	0,23	0,203333	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	1,613333	1	1,613333	21,04348	0,003742	5,987378
Columns	7,34	2	3,67	47,86957	0,000205	5,143253
Interaction	3,386667	2	1,693333	22,08696	0,00171	5,143253
Within	0,46	6	0,076667			
Total	12,8	11				

Anova: Two-Factor with Replication

0 hour for L. rhamnosus

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	0,58	0,43	0,44	1,45
Average	0,29	0,215	0,22	0,241667
Variance	0,0018	0,00045	0,0002	0,001897
4,5				
Count	2	2	2	6
Sum	0,36	0,41	0,42	1,19
Average	0,18	0,205	0,21	0,198333
Variance	0,0018	5E-05	0,0032	0,001217
Total				
Count	4	4	4	
Sum	0,94	0,84	0,86	
Average	0,235	0,21	0,215	
Variance	0,005233	0,0002	0,001167	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0,005633	1	0,005633	4,506667	0,077971	5,987378
Columns	0,0014	2	0,0007	0,56	0,598431	5,143253
Interaction	0,006667	2	0,003333	2,666667	0,148382	5,143253
Within	0,0075	6	0,00125			
Total	0,0212	11				

Anova: Two-Factor with Replication

24 hours for L. rhamnosus

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	3,48	0,97	0,43	4,88

Average	1,74	0,485	0,215	0,813333
Variance	0,0002	0,00045	0,00125	0,530187

4,5				
Count	2	2	2	6
Sum	2,37	1,32	0,42	4,11
Average	1,185	0,66	0,21	0,685
Variance	0,01805	0,0018	0,0002	0,19451

Total			
Count	4	4	4
Sum	5,85	2,29	0,85
Average	1,4625	0,5725	0,2125
Variance	0,108758	0,010958	0,000492

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,049408	1	0,049408	13,50569	0,010392	5,987378
Columns	3,312267	2	1,656133	452,7016	2,85E-07	5,143253
Interaction	0,289267	2	0,144633	39,53531	0,000351	5,143253
Within	0,02195	6	0,003658			
Total	3,672892	11				

Anova: Two-Factor with Replication

50 hours for L. rhamnosus

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	4,7	1	0,39	6,09
Average	2,35	0,5	0,195	1,015
Variance	0,005	0,0018	5E-05	1,08931
4,5				
Count	2	2	2	6
Sum	2,7	1,7	0,46	4,86
Average	1,35	0,85	0,23	0,81
Variance	0,045	0,0098	0,0008	0,26296
Total				
Count	4	4	4	
Sum	7,4	2,7	0,85	
Average	1,85	0,675	0,2125	
Variance	0,35	0,0447	0,000692	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0,126075	1	0,126075	12,11289	0,013137	5,987378
Columns	5,70125	2	2,850625	273,8791	1,27E-06	5,143253
Interaction	0,99765	2	0,498825	47,92554	0,000204	5,143253
Within	0,06245	6	0,010408			
Total	6,887425	11				

Anova: Two-Factor with Replication

119 hours for L. rhamnosus

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	4,8	1,08	0,4	6,28
Average	2,4	0,54	0,2	1,046667
Variance	0	0,0008	0,0002	1,122227
4,5				
Count	2	2	2	6
Sum	3,8	1,65	0,54	5,99
Average	1,9	0,825	0,27	0,998333
Variance	0,08	0,00605	0,005	0,567617
Total				
Count	4	4	4	
Sum	8,6	2,73	0,94	
Average	2,15	0,6825	0,235	
Variance	0,11	0,029358	0,003367	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0,007008	1	0,007008	0,456817	0,524297	5,987378
Columns	8,02805	2	4,014025	261,642	1,46E-06	5,143253
Interaction	0,329117	2	0,164558	10,72624	0,01044	5,143253
Within	0,09205	6	0,015342			
Total	8,456225	11				

Anova: Two-Factor with Replication

166 hours for L. rhamnosus

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	4	1,11	0,71	5,82

Average	2	0,555	0,355	0,97
Variance	0,08	0,00605	0,00845	0,66344
4,5				
Count	2	2	2	6
Sum	3,8	1,73	0,74	6,27
Average	1,9	0,865	0,37	1,045
Variance	0	0,00405	0,0392	0,49627
Total				
Count	4	4	4	
Sum	7,8	2,84	1,45	
Average	1,95	0,71	0,3625	
Variance	0,03	0,0354	0,015958	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,016875	1	0,016875	0,735027	0,424174	5,987378
Columns	5,57135	2	2,785675	121,3361	1,4E-05	5,143253
Interaction	0,08945	2	0,044725	1,948094	0,222869	5,143253
Within	0,13775	6	0,022958			
Total	5,815425	11				

Anova: Two-Factor with Replication

0 hour for Lc. Cremoris

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	0,27	0,24	0,13	0,64
Average	0,135	0,12	0,065	0,106667
Variance	0,00045	0,0008	5E-05	0,001347
4,5				
Count	2	2	2	6
Sum	0,15	0,17	0,21	0,53
Average	0,075	0,085	0,105	0,088333
Variance	0,00045	0,00005	0,00045	0,000377
Total				
Count	4	4	4	
Sum	0,42	0,41	0,34	
Average	0,105	0,1025	0,085	
Variance	0,0015	0,000692	0,0007	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0,001008	1	0,001008	2,688889	0,152161	5,987378
Columns	0,00095	2	0,000475	1,266667	0,347614	5,143253
Interaction	0,005417	2	0,002708	7,222222	0,025277	5,143253
Within	0,00225	6	0,000375			
Total	0,009625	11				

Anova: Two-Factor with Replication

24 hours for Lc. Cremoris

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	1,48	0,36	0,1	1,94
Average	0,74	0,18	0,05	0,323333
Variance	0,0032	0,0018	0,0002	0,108587
4,5				
Count	2	2	2	6
Sum	0,31	0,21	0,26	0,78
Average	0,155	0,105	0,13	0,13
Variance	0,00125	0,00005	0,0002	0,0008
Total				
Count	4	4	4	
Sum	1,79	0,57	0,36	
Average	0,4475	0,1425	0,09	
Variance	0,115558	0,002492	0,002267	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0,112133	1	0,112133	100,4179	5,72E-05	5,987378
Columns	0,298117	2	0,149058	133,4851	1,06E-05	5,143253
Interaction	0,242117	2	0,121058	108,4104	1,95E-05	5,143253
Within	0,0067	6	0,001117			
Total	0,659067	11				

Anova: Two-Factor with Replication

50 hours for Lc. Cremoris

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	1,77	1,27	0,33	3,37

Average	0,885	0,635	0,165	0,561667
Variance	0,00405	0,00125	0,00405	0,108777

4,5				
Count	2	2	2	6
Sum	2,27	0,522	0,33	3,122
Average	1,135	0,261	0,165	0,520333
Variance	0,01805	0,087362	5E-05	0,249625

Total			
Count	4	4	4
Sum	4,04	1,792	0,66
Average	1,01	0,448	0,165
Variance	0,0282	0,076163	0,001367

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,005125	1	0,005125	0,267847	0,6233	5,987378
Columns	1,479944	2	0,739972	38,67045	0,000373	5,143253
Interaction	0,197251	2	0,098625	5,154095	0,049801	5,143253
Within	0,114812	6	0,019135			
Total	1,797132	11				

Anova: Two-Factor with Replication

119 hours for Lc. Cremoris

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	2,7	1,73	0,57	5
Average	1,35	0,865	0,285	0,833333
Variance	0,005	0,00045	5E-05	0,228547

4,5				
Count	2	2	2	6
Sum	3,8	2,6	1,29	7,69
Average	1,9	1,3	0,645	1,281667
Variance	0,08	0,0072	0,00245	0,333137

Total			
Count	4	4	4
Sum	6,5	4,33	1,86
Average	1,625	1,0825	0,465
Variance	0,129167	0,065625	0,044033

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,603008	1	0,603008	38,0247	0,000835	5,987378
Columns	2,69495	2	1,347475	84,96952	3,97E-05	5,143253
Interaction	0,018317	2	0,009158	0,577509	0,589687	5,143253
Within	0,09515	6	0,015858			
Total	3,411425	11				

Anova: Two-Factor with Replication

166 hours for Lc. Cremoris

SUMMARY	0	2,5	5	Total
2,25				
Count	2	2	2	6
Sum	2,9	1,36	0,9	5,16
Average	1,45	0,68	0,45	0,86
Variance	0,005	0,0072	0,0032	0,22252
4,5				
Count	2	2	2	6
Sum	4,2	2,7	1,53	8,43
Average	2,1	1,35	0,765	1,405
Variance	0,02	0,245	0,00125	0,41151
Total				
Count	4	4	4	
Sum	7,1	4,06	2,43	
Average	1,775	1,015	0,6075	
Variance	0,149167	0,2337	0,034558	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,891075	1	0,891075	18,9826	0,004787	5,987378
Columns	2,80895	2	1,404475	29,91958	0,000757	5,143253
Interaction	0,07955	2	0,039775	0,847328	0,474118	5,143253
Within	0,28165	6	0,046942			
Total	4,061225	11				

Table S6: Anova analysis Tables of ethanol and international bitterness units for *L. rhamnosus* GG, *Lc. cremoris* TF121 and *S. cerevisiae* US-05

0h L. rhamnosus

Anova: Two-Factor with Replication

SUMMARY	0	2,5	5	Total
4,5				
Count	2	2	2	6
Sum	0,43	0,47	0,35	1,25
Average	0,215	0,235	0,175	0,208333
Variance	0,00045	0,00045	0,00125	0,001177
6				
Count	2	2	2	6
Sum	0,44	0,4	0,43	1,27
Average	0,22	0,2	0,215	0,211667
Variance	0,0002	0,0008	5E-05	0,000297
Total				
Count	4	4	4	
Sum	0,87	0,87	0,78	
Average	0,2175	0,2175	0,195	
Variance	0,000225	0,000825	0,000967	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	3,33E-05	1	3,33E-05	0,0625	0,810927	5,987378
Columns	0,00135	2	0,000675	1,265625	0,347869	5,143253
Interaction	0,002817	2	0,001408	2,640625	0,150446	5,143253
Within	0,0032	6	0,000533			
Total	0,0074	11				

0h for Lc. cremoris

Anova: Two-Factor with Replication

SUMMARY	0	2,5	5	Total
4,5				
Count	2	2	2	6
Sum	0,23	0,22	0,18	0,63
Average	0,115	0,11	0,09	0,105
Variance	0,00005	0	0,0018	0,00051
6				
Count	2	2	2	6
Sum	0,23	0,18	0,09	0,5
Average	0,115	0,09	0,045	0,083333
Variance	0,00045	0,0002	5E-05	0,001147

Total			
Count	4	4	4
Sum	0,46	0,4	0,27
Average	0,115	0,1	0,0675
Variance	0,000167	0,0002	0,001292

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,001408	1	0,001408	3,313725	0,11856	5,987378
Columns	0,004717	2	0,002358	5,54902	0,043213	5,143253
Interaction	0,001017	2	0,000508	1,196078	0,365454	5,143253
Within	0,00255	6	0,000425			
Total	0,009692	11				

24h L. rhamnosus

Anova: Two-Factor with Replication

SUMMARY	0	2,5	5	Total
4,5				
Count	2	2	2	6
Sum	0,58	0,64	0,31	1,53
Average	0,29	0,32	0,155	0,255
Variance	0	0,0162	0,00125	0,00967
6				
Count	2	2	2	6
Sum	0,65	0,49	0,62	1,76
Average	0,325	0,245	0,31	0,293333
Variance	0,00045	0,00125	0	0,001787
Total				
Count	4	4	4	
Sum	1,23	1,13	0,93	
Average	0,3075	0,2825	0,2325	
Variance	0,000558	0,007692	0,008425	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,004408	1	0,004408	1,381201	0,284418	5,987378
Columns	0,011667	2	0,005833	1,827676	0,239966	5,143253
Interaction	0,026467	2	0,013233	4,146214	0,073984	5,143253
Within	0,01915	6	0,003192			

Total	0,061692	11				

24h
Lc.cremoris

Anova: Two-Factor with Replication

SUMMARY	0	2,5	5	Total
4,5				
Count	2	2	2	6
Sum	0,34	0,23	0,17	0,74
Average	0,17	0,115	0,085	0,123333
Variance	0	0,00045	0,00045	0,001667
6				
Count	2	2	2	6
Sum	0,32	0,25	0,12	0,69
Average	0,16	0,125	0,06	0,115
Variance	0,0018	5E-05	0	0,00243
Total				
Count	4	4	4	
Sum	0,66	0,48	0,29	
Average	0,165	0,12	0,0725	
Variance	0,000633	0,0002	0,000358	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,000208	1	0,000208	0,454545	0,525293	5,987378
Columns	0,017117	2	0,008558	18,67273	0,002652	5,143253
Interaction	0,000617	2	0,000308	0,672727	0,545002	5,143253
Within	0,00275	6	0,000458			
Total	0,020692	11				

46h

46h L. rhamnosus

Anova: Two-Factor with Replication

SUMMARY	0	2,5	5	Total
4,5				
Count	2	2	2	6
Sum	0,63	0,56	0,43	1,62
Average	0,315	0,28	0,215	0,27
Variance	5E-05	0,0008	0,00125	0,00248
6				
Count	2	2	2	6
Sum	0,6	0,42	0,5	1,52

Average	0,3	0,21	0,25	0,253333
Variance	0	0,0018	0	0,001987

Total			
Count	4	4	4
Sum	1,23	0,98	0,93
Average	0,3075	0,245	0,2325
Variance	9,17E-05	0,0025	0,000825

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,000833	1	0,000833	1,282051	0,30073	5,987378
Columns	0,012917	2	0,006458	9,935897	0,012473	5,143253
Interaction	0,005517	2	0,002758	4,24359	0,07104	5,143253
Within	0,0039	6	0,00065			
Total	0,023167	11				

46h
Lc.cremoris

Anova: Two-Factor with Replication

SUMMARY	0	2,5	5	Total
4,5				
Count	2	2	2	6
Sum	0,63	0,32	0,17	1,12
Average	0,315	0,16	0,085	0,186667
Variance	5E-05	0,0002	0,00125	0,011307
6				
Count	2	2	2	6
Sum	0,42	0,22	0,1	0,74
Average	0,21	0,11	0,05	0,123333
Variance	0,0008	0,0002	0	0,005427
Total				
Count	4	4	4	
Sum	1,05	0,54	0,27	
Average	0,2625	0,135	0,0675	
Variance	0,003958	0,000967	0,000825	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,012033	1	0,012033	28,88	0,001705	5,987378

Columns	0,07845	2	0,039225	94,14	2,95E-05	5,143253
Interaction	0,002717	2	0,001358	3,26	0,110063	5,143253
Within	0,0025	6	0,000417			
Total	0,0957	11				

98h

98h L. rhamnosus

Anova: Two-Factor with Replication

SUMMARY	0	2,5	5	Total
4,5				
Count	2	2	2	6
Sum	0,75	0,6	0,41	1,76
Average	0,375	0,3	0,205	0,293333
Variance	5E-05	0,0032	0,00125	0,006707
6				
Count	2	2	2	6
Sum	0,75	0,54	0,58	1,87
Average	0,375	0,27	0,29	0,311667
Variance	5E-05	0,0002	0,0008	0,002697
Total				
Count	4	4	4	
Sum	1,5	1,14	0,99	
Average	0,375	0,285	0,2475	
Variance	3,33E-05	0,001433	0,003092	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,001008	1	0,001008	1,09009	0,33668	5,987378
Columns	0,03435	2	0,017175	18,56757	0,002691	5,143253
Interaction	0,007117	2	0,003558	3,846847	0,084119	5,143253
Within	0,00555	6	0,000925			
Total	0,048025	11				

98h Lc.cremoris

Anova: Two-Factor with Replication

SUMMARY	0	2,5	5	Total
4,5				
Count	2	2	2	6
Sum	0,89	0,56	0,23	1,68
Average	0,445	0,28	0,115	0,28

Variance	5E-05	0,0018	0,00245	0,02264

6				
Count	2	2	2	6
Sum	0,68	0,47	0,16	1,31
Average	0,34	0,235	0,08	0,218333
Variance	0,0008	0,00125	0,0002	0,014137

Total			
Count	4	4	4
Sum	1,57	1,03	0,39
Average	0,3925	0,2575	0,0975
Variance	0,003958	0,001692	0,001292

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,011408	1	0,011408	10,45038	0,01785	5,987378
Columns	0,174467	2	0,087233	79,9084	4,74E-05	5,143253
Interaction	0,002867	2	0,001433	1,312977	0,336537	5,143253
Within	0,00655	6	0,001092			

119h L. rhamnosus

Anova: Two-Factor with Replication

SUMMARY	0	2,5	5	Total
4,5				
Count	2	2	2	6
Sum	0,68	0,52	0,39	1,59
Average	0,34	0,26	0,195	0,265
Variance	0	0,0008	5E-05	0,00439
6				
Count	2	2	2	6
Sum	0,7	0,47	0,48	1,65
Average	0,35	0,235	0,24	0,275
Variance	0,0002	0,00125	0,0008	0,00383
Total				
Count	4	4	4	
Sum	1,38	0,99	0,87	
Average	0,345	0,2475	0,2175	
Variance	1E-04	0,000892	0,000958	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0,0003	1	0,0003	0,580645	0,474937	5,987378
Columns	0,03555	2	0,017775	34,40323	0,000516	5,143253
Interaction	0,00245	2	0,001225	2,370968	0,174263	5,143253
Within	0,0031	6	0,000517			
Total	0,0414	11				

119h Lc.cremoris

Anova: Two-Factor with Replication

SUMMARY	0	2,5	5	Total
4,5				
Count	2	2	2	6
Sum	0,92	0,54	0,26	1,72
Average	0,46	0,27	0,13	0,286667
Variance	0	0,0008	0,0032	0,022747
6				
Count	2	2	2	6
Sum	0,71	0,44	0,13	1,28
Average	0,355	0,22	0,065	0,213333
Variance	5E-05	0,0002	5E-05	0,016907
Total				
Count	4	4	4	
Sum	1,63	0,98	0,39	
Average	0,4075	0,245	0,0975	
Variance	0,003692	0,001167	0,002492	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0,016133	1	0,016133	22,51163	0,003176	5,987378
Columns	0,19235	2	0,096175	134,1977	1,05E-05	5,143253
Interaction	0,001617	2	0,000808	1,127907	0,383861	5,143253
Within	0,0043	6	0,000717			
Total	0,2144	11				

Anova: Two-Factor with Replication

0h S.cerevisiae

SUMMARY	0	2	5	Total
4,5				
Count	2	2	2	6
Sum	0,24	0,33	0,18	0,75
Average	0,12	0,165	0,09	0,125

Variance	0,0008	0,00045	0,0002	0,00143
6				
Count	2	2	2	6
Sum	0,34	0,19	0,24	0,77
Average	0,17	0,095	0,12	0,128333
Variance	0,0032	5E-05	0	0,001817
Total				
Count	4	4	4	
Sum	0,58	0,52	0,42	
Average	0,145	0,13	0,105	
Variance	0,002167	0,0018	0,000367	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	3,33E-05	1	3,33E-05	0,042553	0,843391	5,987378
Columns	0,003267	2	0,001633	2,085106	0,205335	5,143253
Interaction	0,008267	2	0,004133	5,276596	0,047622	5,143253
Within	0,0047	6	0,000783			
Total	0,016267	11				

Anova: Two-Factor with Replication

24 h S.cerevisiae

SUMMARY	0	2	5	Total
4,5				
Count	2	2	2	6
Sum	0,26	0,26	0,16	0,68
Average	0,13	0,13	0,08	0,113333
Variance	0,0002	0,0002	0	0,000747
6				
Count	2	2	2	6
Sum	0,33	0,26	0,31	0,9
Average	0,165	0,13	0,155	0,15
Variance	0,00245	0,0018	0,00045	0,0012
Total				
Count	4	4	4	
Sum	0,59	0,52	0,47	
Average	0,1475	0,13	0,1175	
Variance	0,001292	0,000667	0,002025	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0,004033	1	0,004033	4,745098	0,072228	5,987378
Columns	0,001817	2	0,000908	1,068627	0,400885	5,143253
Interaction	0,002817	2	0,001408	1,656863	0,267352	5,143253
Within	0,0051	6	0,00085			
Total	0,013767	11				

Anova: Two-Factor with Replication
48 h S.cerevisiae

SUMMARY	0	2	5	Total
4,5				
Count	2	2	2	6
Sum	0,37	0,44	0,32	1,13
Average	0,185	0,22	0,16	0,188333
Variance	5E-05	0,0018	0	0,001097
6				
Count	2	2	2	6
Sum	0,34	0,21	0,21	0,76
Average	0,17	0,105	0,105	0,126667
Variance	0,0008	0,00045	0,00005	0,001387
Total				
Count	4	4	4	
Sum	0,71	0,65	0,53	
Average	0,1775	0,1625	0,1325	
Variance	0,000358	0,005158	0,001025	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0,011408	1	0,011408	21,73016	0,003462	5,987378
Columns	0,0042	2	0,0021	4	0,078717	5,143253
Interaction	0,005067	2	0,002533	4,825397	0,056344	5,143253
Within	0,00315	6	0,000525			
Total	0,023825	11				

Anova: Two-Factor with Replication
96 h S.cerevisiae

SUMMARY	0	2	5	Total
4,5				
Count	2	2	2	6
Sum	0,64	0,59	0,58	1,81

Average	0,32	0,295	0,29	0,301667
Variance	0,0002	0,00845	0,0002	0,001977
6				
Count	2	2	2	6
Sum	0,46	0,4	0,44	1,3
Average	0,23	0,2	0,22	0,216667
Variance	0,0008	0,0008	0,0002	0,000547
Total				
Count	4	4	4	
Sum	1,1	0,99	1,02	
Average	0,275	0,2475	0,255	
Variance	0,003033	0,006092	0,001767	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,021675	1	0,021675	12,21127	0,012913	5,987378
Columns	0,001617	2	0,000808	0,455399	0,654439	5,143253
Interaction	0,00035	2	0,000175	0,098592	0,90755	5,143253
Within	0,01065	6	0,001775			
Total	0,034292	11				

Anova: Two-Factor with Replication

119. h S.cerevisiae

SUMMARY	0	2	5	Total
4,5				
Count	2	2	2	6
Sum	0,56	0,62	0,56	1,74
Average	0,28	0,31	0,28	0,29
Variance	0,0002	0,0008	0,0008	0,0006
6				
Count	2	2	2	6
Sum	0,55	0,44	0,4	1,39
Average	0,275	0,22	0,2	0,231667
Variance	0,00125	0,0002	0	0,001497
Total				
Count	4	4	4	
Sum	1,11	1,06	0,96	
Average	0,2775	0,265	0,24	
Variance	0,000492	0,003033	0,0024	

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,010208	1	0,010208	18,84615	0,004869	5,987378
Columns	0,002917	2	0,001458	2,692308	0,146386	5,143253
Interaction	0,004317	2	0,002158	3,984615	0,079239	5,143253
Within	0,00325	6	0,000542			
Total	0,020692	11				

Table S7: Titratable acidity data for v4 beer samples.

Sample	Sample size (mL)	NaOH used (mL)	Initial pH	Titrated pH	Total Acidity (mol/L)	Lactic acid (g/L)
Rodenbach Grand Cru	24	24.5	3.29	8.29	0.102	9.196
US-05	30	5.9	3.98	8.23	0.020	1.772
US-05 with RFO	21	12.2	4.06	8.26	0.058	5.232
B+Y 14 days	23	10.5	3.65	8.27	0.046	4.112
B+Y 14 days with RFO	25	36.3	3.43	8.22	0.145	13.080
B+Y 19 days	24	11.7	3.66	8.23	0.049	4.391
B+Y 19 days with RFO	23	33.6	3.44	8.26	0.146	13.160

Table S8: Values from high pressure liquid chromatography for organic acids of v1, v2 and v3 beers.

Sample name	Maltose	Glucose	Pyruvic acid	Fructose	Lactic acid	Acetic acid	Citric acid	DL - pyro-glutamic acid	Lactic : acetic
	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	Ratio
Wort v1	34740,26	4971,67	n.d.	2285,42	n.d.	41,73	616,55	72,36	n.a.
Lc. cremoris TF121 + *S. cerevisiae* US-05 7d	n.d.	n.d.	n.d.	n.d.	1970,31	390,85	494,13	61,62	5,04
Lc. cremoris TF121 + S. cerevisiae US-05 14d	n.d.	n.d.	n.d.	n.d.	2239,36	501,49	569,28	75,72	4,47
Lc. cremoris TF121 + S. cerevisiae US-05 19d	787,98	n.d.	n.d.	n.d.	2187,11	512,84	574,13	77,94	4,26
S. cerevisiae US-05 7d	n.d.	n.d.	407,79	n.d.	n.d.	157,16	489,73	61,37	n.a.
S. cerevisiae US-05 14d	n.d.	n.d.	361,10	n.d.	n.d.	281,12	569,74	65,68	n.a.
S. cerevisiae US-05 19d	88,50	33,67	n.d.	n.d.	n.d.	413,98	553,34	74,40	n.a.
Wort v2	41350,11	5364,29	n.d.	3645,56	n.d.	n.d.	666,08	84,31	n.a.

B. claussenii WLP645+*Lc. cremoris* TF121+*L. brevis* 7d	1030,26	11,80	n.d.	320,30	n.d.	n.d.	588,71	64,19	n.a.
B. claussenii WLP645 + *Lc. cremoris* TF121 + *L. brevis* 14d	850,66	n.d.	n.d.	250,43	n.d.	n.d.	615,46	70,40	n.a.
B. claussenii WLP645 + *Lc. cremoris* TF121 + *L. brevis* 19d	791,35	15,96	376,25	126,32	n.d.	88,21	639,31	70,8	n.a.
L. rhamnosus GG + *S. cerevisiae* US-05 7d	30420,54	3800,47	n.d.	4231,08	2375,99	625,64	n.d.	81,69	3,80
L. rhamnosus GG + *S. cerevisiae* US-05 14d	3240,63	35,15	n.d.	n.d.	4895,50	1504,35	n.d.	76,31	3,25
L. rhamnosus GG + *S. cerevisiae* US-05 19d	n.d.	n.d.	n.d.	n.d.	4489,67	1352,18	n.d.	65,36	3,32

Table S9: Values from high pressure liquid chromatography for organic acids of v4 beers. US-05= *S. cerevisiae* US-05, B+Y= *Lc. cremoris* TF121 & *B. claussenii* WLP645.

Sample name	**Maltose**	**Glucose**	**Pyruvic acid**	**Fructose**	**Lactic acid**	**Acetic acid**	**Citric acid**	**DL-pyroglutamic acid**
	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm
US-05	n.d	n.d	n.d	n.d	3137,38	535,68	2931,48	117,05
US-05 RFO	n.d	n.d	n.d	n.d	3137,38	535,68	2931,48	117,05
B+Y 14 days	38523,74	1030,93	n.d	2603,60	2955,69	603,25	247,93	99,26
B+Y 19 days	35842,22	424,63	n.d	1997,31	2981,96	726,24	242,00	96,72
B+Y RFO 14 days	20296,22	1277,07	n.d	2901,93	12142,79	540,18	2983,56	95,23
B+Y RFO 19 days	19532,22	1213,56	n.d	2858,93	12051,53	577,28	2935,11	95,07
Rodenbach grand cru	n.d	1462,11	n.d	9750,62	4739,76	2113,52	n.d	144,01
Oude Geuze Boon	n.d	n.d	n.d	n.d	4577,95	581,21	n.d	153,66

Table S10: statistics regarding the participants in the tetrad testing, concerning sex, age, and previous sensory experience.

NR	Kjønn/sex:_1	Kjønn/sex:_2	Alder/age	Alder/age	Alder/age	Har du tidligere vært med på en sensorisk test, enten på NMBU eller andre steder? Do you have any previous experience with sensory testing, NMBU or otherwise?_1	Har du tidligere vært med på en sensorisk test, enten på NMBU eller andre steder? Do you have any previous experience with sensory testing, NMBU or otherwise?_2
27075931	Kvinne/female		18-29			Ja/yes	
27076084	Kvinne/female		18-29			Ja/yes	
27076139	Kvinne/female		18-29			Ja/yes	
27076835	Kvinne/female		18-29			Ja/yes	
27077185		Mann/male		30-44		Ja/yes	
27077188	Kvinne/female		18-29				Nei/no
27077197	Kvinne/female		18-29				Nei/no
27079053		Mann/male		30-44		Ja/yes	
27079821	Kvinne/female			30-44		Ja/yes	
27080463	Kvinne/female		18-29			Ja/yes	
27080535	Kvinne/female		18-29			Ja/yes	
27080626		Mann/male	18-29			Ja/yes	
27080854	Kvinne/female			30-44			Nei/no
27080864	Kvinne/female				45-59	Ja/yes	
27081111		Mann/male	18-29			Ja/yes	
27081173	Kvinne/female		18-29				Nei/no
27081248		Mann/male	18-29			Ja/yes	
27081249		Mann/male	18-29			Ja/yes	
27081419	Kvinne/female		18-29				Nei/no
27081649	Kvinne/female		18-29			Ja/yes	
27082124	Kvinne/female				45-59	Ja/yes	
27082309		Mann/male	18-29			Ja/yes	

27082315	Kvinne/female	18-29		Nei/no
27082738	Kvinne/female	18-29		Nei/no
27082771	Kvinne/female		30-44	Nei/no

Table S11: Complete answer sheet from tetrad testing. Green cells indicate correct pairings, red cells indicate incorrect pairing.

NR	Gruppe A_1	Gruppe A_2	Gruppe A_3	Gruppe A_4	Gruppe b_1	Gruppe b_2	Gruppe b_3	Gruppe b_4
27075931		814		210	591		095	
27076084		814		210	591		095	
27076139	591		095			814		210
27076835	591			210		814	095	
27077185		814		210	591		095	
27077188	591		095			814		210
27077197		814		210	591		095	
27079053		814		210	591		095	
27079821	591		095			814		210
27080463	591	814					095	210
27080535	591		095			814		210
27080626	591		095			814		210
27080854		814		210	591		095	
27080864		814		210	591		095	
27081111		814		210	591		095	
27081173		814		210	591		095	
27081248	591		095			814		210
27081249	591		095			814		210
27081419	591		095			814		210
27081649	591			210		814	095	
27082124	591	814					095	210
27082309	591		095			814		210
27082315	591		095			814		210
27082738	591		095			814		210
27082771		814		210	591		095	

NR	Gruppe A_1	Gruppe A_2	Gruppe A_3	Gruppe A_4	Gruppe B_1	Gruppe B_2	Gruppe B_3	Gruppe B_4
27075931	327	618					943	156
27076084	327	618					943	156

27076139	327	618					943	156
27076835	327	618					943	156
27077185			943	156	327	618		
27077188	327	618					943	156
27077197	327	618					943	156
27079053	327	618					943	156
27079821			943	156	327	618		
27080463	327	618					943	156
27080535	327	618					943	156
27080626	327	618					943	156
27080854			943	156	327	618		
27080864	327	618					943	156
27081111	327	618					943	156
27081173			943	156	327	618		
27081248			943	156	327	618		
27081249	327	618					943	156
27081419			943	156	327	618		
27081649	327	618					943	156
27082124	327	618					943	156
27082309	327	618					943	156
27082315	327	618					943	156
27082738			943	156	327	618		
27082771			943	156	327	618		

NR	Gruppe A_1	Gruppe A_2	Gruppe A_3	Gruppe A_4	Gruppe B_1	Gruppe B_2	Gruppe B_3	Gruppe B_4
27075931		832	149		476			587
27076084		832		587	476		149	
27076139	476		149			832		587
27076835	476		149			832		587
27077185	476		149			832		587
27077188	476		149			832		587
27077197	476	832					149	587
27079053	476		149			832		587
27079821	476		149			832		587
27080463	476	832					149	587
27080535	476		149			832		587
27080626	476		149			832		587
27080854		832		587	476		149	
27080864	476		149			832		587
27081111	476		149			832		587
27081173	476		149			832		587
27081248		832		587	476		149	

27081249		832		587	476		149	
27081419	476		149			832		587
27081649	476		149			832		587
27082124	476		149			832		587
27082309	476		149			832		587
27082315	476	832					149	587
27082738	476	149				832		587
27082771	476	149				832		587

NR	Gruppe A_1	Gruppe A_2	Gruppe A_3	Gruppe A_4	Gruppe B_1	Gruppe B_2	Gruppe B_3	Gruppe B_4
27075931		542	919		386			123
27076084	386			123		542	919	
27076139	386			123		542	919	
27076835	386			123		542	919	
27077185	386			123		542	919	
27077188	386			123		542	919	
27077197	386			123		542	919	
27079053	386			123		542	919	
27079821	386			123		542	919	
27080463		542	919		386			123
27080535	386			123		542	919	
27080626	386			123		542	919	
27080854		542	919		386			123
27080864	386			123		542	919	
27081111		542	919		386			123
27081173	386			123		542	919	
27081248		542	919		386			123
27081249	386			123		542	919	
27081419	386			123		542	919	
27081649	386			123		542	919	
27082124	386		919			542		123
27082309	386			123		542	919	
27082315	386		919			542		123
27082738	386			123		542	919	
27082771		542	919		386			123

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