Enhancing the prebiotic properties of malted barley

An investigation of the potential to utilize yeast fermented barley to promote probiotic bacteria and improve gut health

Master of Science Thesis in Biotechnology

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Abstract

Prebiotics can improve health indirectly by promoting probiotic bacteria in the gut and production of beneficial metabolites by the gut microbiota. In this project the possibility to enhance the potential prebiotic properties of malted barley was explored.

The aim was to find yeast strains with high potential to modify the polysaccharide composition, in particular the β-glucan profile, in malted barley and investigate if fermentation of barley malt materials by these yeast strains can promote growth of probiotic bifidobacteria and the production of organic acids.

Firstly, the β-glucan content in Brewers' Spent Grain was determined. Thereafter, yeasts were screened for production of β-glucanase enzyme. The growth of strains showing this ability was studied in the malt materials in order to select optimal strains to be used in further experiments with bifidobacteria. *Pichia butronii* TY01 and *Pichia kudriavzevii* TY3 were found to be most suitable for fermentation of the malt materials based on β-glucanase activity and highest increase in cell numbers during growth in malt materials.

The potential impact of the fermented malt materials on gut health was evaluated by analysing growth of *Bifidobacterium bifidum* and *Bifidobacterium breve* in malt-based media and the subsequent production of organic acids, in particular lactic acid. Indications of increased growth of *Bifidobacterium* in fermented malt was observed. Furthermore, a higher concentrations of lactic- and acetic acid was found in fermented malt compared to unfermented samples. Brewers' Spent Grain generated lower organic acid concentrations compared to malted barley, possibly due to differences in the β-glucan content.

In conclusion, this study showed increased prebiotic properties of barley malt materials fermented by β-glucanase producing yeast strains compared to unfermented materials.

Sammanfattning

Prebiotika kan förbättra hälsan indirekt genom att gynna tillväxt av probiotiska bakterier i tarmen och öka tarmflorans produktion av hälsofrämjande metaboliter. I detta examensprojekt undersöktes möjligheten att förhöja de potentiella prebiotiska egenskaperna hos maltat korn.

Syftet var att identifiera jäststammar med hög pontential att modifiera kompositionen av polysaccharider, speciellt β-glukan profilen, i maltat korn och undersöka om fermentering av kornmaterialet med hjälp av dessa jäststammar kan gynna tillväxten av probiotiska bakterier samt öka produktionen av organiska syror.

Innehållet av β-glukan i mäsk från ett lokalt bryggeri fastställdes. Därefter genomfördes en screen av jäststammars förmånga att producera enzymet β-glukanase. Tillväxten av stammar med denna förmånga studerades i maltmaterialet för att välja optimala stammar för använding i vidare experiment med bifidobakterier. Baserat på β-glukanase aktivitet och högst slutgiltiga cellkoncentration efter tillväxt i maltmaterialet valdes *Pichia butronii* TY01 och *Pichia kudriavzevii* TY3 som mest lämpade för fermentering av maltat korn.

Den potentiella effekten av det fermenterade maltmaterialet på tarmhälsan utvärderades genom att studera tillväxt av *Bifidobacterium bifidum* och *Bifidobacterium breve* i maltbaserat medium och produktionen av organiska syror, framförallt lactat. Resultatet visade en ökad tillväxt av *Bifidobacterium* i det fermenterade maltmaterialet. Dessutom genererade fermenterad malt högre koncentrationer av lactat och acetat jämfört med ofermenterad malt. Mäsk gav lägre koncentration av organiska syror jämfört med maltat korn, vilket skulle kunna bero på skillnader i innehållet av β-glukan.

Slutsatsen av denna studie var att maltat korn som fermenterats med hjälp av β-glukanase-producerande jäststammar visade ökade prebiotiska egenskaper jämfört med ofermenterad malt.

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1. Introduction

The raised interest in alternative ways to improve health and treat disease has made concepts such as probiotics and functional foods, with health benefits beyond their nutritional value, increasingly popular. "Good bacteria" are currently added to countless food products. However, foods containing microorganisms e.g. beer, bread, cheese, vegetables and yoghurt, have been a part of the human diet for thousands of years. Fermentation was initially a way to preserve the food and until recently the cause of the observed health benefits from consuming fermented foods has remained unknown. Excitingly enough, researchers have now begun to unravel the reason behind these health promoting effects.

During the last decades advances in DNA sequencing technologies has enabled mapping of the gut microbiota. Furthermore, an increased understanding of the interactions between host and the microorganisms that inhabit the gut has shed light on the importance of this relationship and its impact on our health. In addition, its potential imbalance may play a significant role in the development of for example allergies, cancer, auto-immune diseases and inflammatory disorders (Umu *et al.*, 2013, Cénit *et al.*, 2014).

Inflammatory Bowel Disease (IBD) is a collective name of the chronic inflammatory disorders Ulcerative colitis (UC) and Crohn's disease (CD) affecting the gastrointestinal tract (GIT). The cause of IBD is unknown but is believed to be linked to a reaction to the commensal intestinal microflora (Xavier *et al.*, 2007). Furthermore, a decreased diversity of the microbiota can be observed in most patients suffering from IBD (Cénit *et al.*, 2014). Despite a variety of different treatments, including antibiotics, no effective cure is available and a prolonged inflammation may ultimately end up in surgical removal of the affected tissue. In addition, many of the current treatments have severe side-effects (Pithadia *et al.*, 2011). Therefore, it is of great importance to find better ways to alleviate and hopefully cure this disease.

Dietary fiber are indigestible polysaccharides and resistant starch derived from edible parts of plants. One of the most important groups of dietary fiber and the major fiber constituent of barley is β-glucans. Some dietary fiber have prebiotic properties and β-glucans are a prominent example of this (Izydorczyk *et al.*, 2008). The term prebiotic is defined as a non-digestible compound that can act as nutrient for the microbiota, and give a positive health effect due to modulation of the microbiota or improvement of the environment in the gut (Laparra *et al.*, 2010). Thus, malted barley and Brewers' Spent Grain (BSG), a by-product from beer brewing consisting of the outer seed layer of malted barley grains, are of interest due to their high β-glucan content (Bränning *et al.*, 2011) and therefore potential use in functional foods.

The major end products of bacterial fermentation in the colon are short chain fatty acids (SCFAs); mainly acetic-, propionic- and butyric acid as well as the organic acid lactic acid (Brahe *et al.*, 2013). The SCFAs are rapidly absorbed by the epithelial cells in the gut and are an important energy source and essential for normal metabolism of the intestinal mucosa. Additionally, they stimulate cellular proliferation and differentiation in the gut (Rossi *et al*., 2010), and cause apoptosis in colonic cancer cells (Soldavini *et al.*, 2013). SCFAs have been associated with various protective and health promoting properties of which butyrate is believed to be the main reason (Rossi *et al.*, 2010). However, the mechanisms behind these effects are not yet understood.

Studies have shown that intake of fiber-rich foods has an anti-inflammatory effect which could be linked to elevated production of SCFAs by probiotic bacteria in the gut. Furthermore, BSG has been shown to reduce inflammation of the colon in patients suffering from UC (Kanachi *et al.*, 2003). This preventative effect has been linked to the high content of the amino acid glutamine and elevated levels of butyric acid production in the colon.

Interestingly, not only the amount of dietary fiber consumed but the specific polysaccharides and their molecular weight affects the species as well as the total number of microorganisms present in the GI (Shen et al., 2012). In addition, the fiber composition seems to give rise to production of different amounts and patterns of SCFAs (Berggren et al., 1993; Henningsson et al., 2002). For example, β-glucans in cereal based foods have been shown to influence the production of SCFAs and increase the population of the probiotic bacteria *Lactobacillus* and *Bifidobacterium* in the colon (Shen *et al.*, 2012). Moreover, the β-glucan profile has a significant impact on the ratio of SCFAs that are produced (Immerstrand *et al.*, 2010).

This master thesis is a part of a larger study with the aim to identify the factors affecting the concentration of butyric and propionic acid during fermentation of dietary fiber in the colon. However, the topic of this project is related to the impact of the β-glucan composition on probiotic bacteria and the subsequent production of SCFAs.

The hypothesis that will be explored in this project is based on the assumption that polysaccharides of different molecular weight serve as substrate for certain bacteria in the gut microbiota and the polysaccharide profile dictates which SCFAs that are subsequently produced by these bacteria (Berggren et al., 1993; Henningsson et al., 2002, Shen et al., 2012). Thus, modifying the polysaccharide composition to an optimal mix of low-, medium- and high weight compounds could favour growth of probiotic bacteria in the colon and improve gut health indirectly by increased production of certain beneficial SCFAs.

An affirmative result of this study could lead to the development of a barley based functional food product with the aim of improving gut health by promoting probiotic bacteria, restoring barrier

integrity, and providing an anti-inflammatory effect in the colon. This product could potentially be used to prevent colon cancer, and treat disorders such as IBD.

1.1 Aim

The aim of this master thesis project was to find yeast strains with high potential to modify the polysaccharide composition, in particular the β-glucan profile, in malted barley and investigate if fermentation of barley malt materials by β-glucanase producing yeast strains can enhance their potential prebiotic properties by promoting growth of probiotic bifidobacteria and the production of organic acids.

1.2 Delimitations

In vivo studies was not included in this project. The microbiota was represented by one species of probiotic bacteria instead of the complex mix that is normally found in the gut and a simple *in vitro* experiment was done to study the potential effects in the colon.

Furthermore, there are many species of bacteria and fungi that are known to produce β-glucanase enzymes. However, in the present thesis project different yeast strains were chosen with regards to the long-term aim to develop food products.

The impact of the selected yeast strains on other polysaccharides and constituents in malted barley will not be investigated. It is highly likely that there is a great change in the nutrient composition during fermentation and the effect this has on the bifidobacteria may or may not be greater than the impact of the modified β-glucan profile. However, any beneficial effects or changes observed in this study can be considered a success and the cause of these effects should be investigated in future research.

In addition, the scope of this study does not include:

- Health benefits of other nutrients e.g. the amino acid glutamine
- Evaluation of other cereals with high β-glucan content such as oat

2. Background

Microbiota and health

Our lives begin in the sterile environment of the uterus but immediately after birth we are colonised by microorganisms. The microbiota consists of ten times as many cells as the total amount of human cells in the body and over 100 times as many genes as the human genome (Cénit, 2014). These microorganisms inhabit all mucosal surfaces of the body with the highest densities in the gastrointestinal tract (GI). The bacterial community in the gut comprises of over 800 different species (Kelly, 2005; Laparra, 2010) of which most are strictly anaerobic (Cénit, 2014). The composition of the gut microbiota is affected by mode of delivery; vaginal or cesarean section, and to a certain extent by the diet.

The commensal relationship between the microbial community and host has evolved over time and contributes to our health, in contrast to pathogenic microorganisms which possess virulence factors that give rise to inflammation and disease. During the last decades researchers have discovered the importance of these interactions but we have only begun to unravel the mechanisms involved.

Role of the gut microbiota

The gut microbiota is important for our health through their metabolic activities which mediate digestion of dietary compounds, salvage of energy, supply of (micro)nutrients, and transformation of xenobiotics (Laparra, 2010). Another essential interaction is the cross-talk between the microbiota and the host immune system which modulate and strengthen the immune defense (Kelly, 2005). Lastly the commensal microflora competes with pathogens which prevents them from colonising the mucosal surfaces and invading tissues (Cénit, 2014).

Intestinal mucosal barrier

The GI is the largest mucosal surface and constantly interacts with the external environment by exposure to dietary antigens and microorganisms. The epithelia creates a leaky barrier which allows uptake of nutrients while protecting against pathogens. The permeability is regulated by stimuli such as nutrients, cytokines and microorganisms. A mucus layer consisting of heavily glycosylated proteins, mucins, is produced by goblet cells and prevents bacteria and larger particles from having direct contact with the epithelia (Turner 2014). Thus, the intestines are crucial sites of immune regulation and maintaining barrier integrity at the same time as allowing appropriate stimulation of immune defense and uptake of nutrients affects health both on a systemic and local level.

Fibre and health

Dietary fibre are indigestible polysaccharides and resistant starch derived from edible parts of plants. Some dietary fibre have prebiotic properties and β-glucans are a prominent example of this (Izydorczyk *et al.*, 2008). The term prebiotic is defined as a non-digestible compound that can act as

nutrient for the microbiota, and give a positive health effect due to modulation of the microbiota or improvement of the environment in the gut (Laparra *et al.*, 2010).

Fermentation of fiber

Short-chain fatty acids (SCFAs) are the major end products of bacterial fermentation of undigested polysaccharides and resistant starch in the colon. The three main SCFAs are acetic acid (C2:0), propionic acid (C3:0) and butyric acid (C4:0). The ratio of butyrate, propionate and acetate in the colon of healthy adults is 1:1:3 (Brahe *et al.*, 2013, Rossi *et al.*, 2010). In the mammalian colon, SCFAs from bacterial fermentation generally reach 100mM under normal physiological conditions (Kaji *et al*., 2014).

Gut hormones

Enteroendocrine cells have important roles in local and systemic signal transduction through gut hormone release (Kaji *et al.* 2014). Enteroendocrine L-cells are present throughout the intestinal tract with highest concentration in the distal ileum and colon. L-cells produce the hormones GLP-1 and peptide YY (PYY) in response to a meal. Hormone secretion can be triggered by sugars, amino acids, and fatty acids. However, only fatty acids can be found in high levels in the colon (Tolhurst *et al.* 2012). GLP-1 is one of two incretin hormones generating insulinotrophic effect in response to oral glucose and lipid ingestion. Release of GLP-1 has anorexigenic effect and reduces food intake, inhibits gastric acid secretion, decelerates gastric emptying, suppresses glucagon release from pancreatic alpha-cells (Diakogiannaki *et al.* 2012). PYY, on the other hand, affects satiety and reduces appetite.

Short-chain fatty acids

SCFAs are weakly acidic and absorption is affected both by the pKa of the fatty acid and the pH of the intestinal lumen. It is speculated that SCFAs prevent colonisation of pathogenic bacteria and stimulate growth of commensal bacteria in the gut by lowering the pH (Rossi *et al.*, 2010) Furthermore, SCFAs represent 60-70% of the energy requirement for colonic enterocytes (Rossi et al., 2010, Brahe et al., 2013) and are essential for normal metabolism of intestinal mucosa. In contrast, SCFAs have anti - neoplastic properties *in vitro* and have been shown to cause apoptosis in colonic cancer cells (Fauser *et al.*, 2011, Soldavini *et al.*, 2013).

SCFAs can act directly on cells through at least two mechansisms; histone deactetylase (HDAC) inhibition, affecting how tightly DNA is wrapped around histones, but most importantly receptor activation. SCFAs have been shown to activate free fatty acid receptors (FFAR)2 and FFAR3 in enteroendocrine cells (Brahe *et al.*, 2013, Soldavini *et al.*, 2013, Kaji *et al*., 2014). The highest density of FFAR receptors is in the colon, where the SCFA concentration is high due to bacterial fermentation (Diakogiannaki *et al.* 2012). The FFAR receptors are important for intestinal chemosensing (Soldavini *et al.*, 2013) and may modulate incretin hormone release (Diakogiannaki *et al.* 2012). Recent studies

suggest that SCFAs stimulate gut hormone secretion via SCFA-FFAR signaling (Tolhurst *et al.* 2012, Kaji *et al*., 2014).

SCFAs can regulate the production of cytokines, eicosanoids, and chemokines by white blood cells (leukocytes) in addition to influencing their ability to migrate to the site of inflammation (Vinolo *et al.* 2011). Furthermore, SCFAs stimulate proliferation and differentiation of endothelial cells in both colon and small intestine (Rossi *et al.*, 2010, Soldavini *et al.*, 2013). The mechanisms behind these effects are not yet understood. However, butyrate is believed to be the main reason for the trophic effect of SCFAs (Rossi *et al.*, 2010).

Butyric acid (C4)

Butyrate has many suggested beneficial effects, some more studied than others. The suggested and confirmed biological activities of butyric acid are described and listed below.

Intestinal barrier and cell proliferation

Butyrate strengthens the intestinal barrier by enhancing cell proliferation and inhibiting apoptosis. *In vitro* studies have shown that butyrate modulate the expression of tight junction proteins and up-regulate mucin expression (Soldavini *et al.* 2013). In addition, butyrate influences homeostasis in the human colonic epithelium through regulation of gene expression, cell differentiation and apoptosis (Brahe *et al.*, 2013).

- Key energy source (energy homeostasis in particular after loss of small intestinal surface/insufficient nutrient absorption)(Soldavini *et al.* 2013)
- Modulation of tight junction (TJ) proteins (Brahe *et al.*, 2013)
- Increase of mucins eg. mucin 2(Brahe *et al.*, 2013)
- Activation of peroxisome proliferator-activated receptor γ (γ-PPAR) – reduce paracellular permeability. (Viladomiu *et al.*, 2013)
- Enhance cell proliferation in colon and small intestine (Rossi *et al.* 2010)
- Increased Na+ and Cl- absorption. Mediator of absorption of Na/water. Up-regulation of membrane Na/H exchange 3(NHE3) both *in vitro* and *in vivo* (Kles *et al.* 2006)
- Decreased proliferation and induced differentiation in cancer cell lines. May act on histone deacetylase to promote differentiation in cancer cells (Kles *et al.*, 2006).
- Histone deacetylase (HDAC) inhibitor - pro-differentiation, pro-apoptosis and induce cycle growth arrest in cancer cells (Soldavini *et al.* 2013)

Gut endocrine system

Butyrate can affect insulin sensitivity by stimulation of the enteroendocrine cells to excrete incretins; gastric inhibitory polypeptide (GIP) and glucagon-like peptide 1 (GLP-1). Furthermore, butyrate

stimulate glucagon-like peptide 2 (GLP-2) release. GLP-2 is co-excreted with GLP-1 and affects intestinal growth and function (Brahe *et al.*, 2013).

- Increased GLP-2 secretion – intestinotrophic on intestinal epithelium and reduce permeability (Brahe *et al.*, 2013)
- Enhance intestinal adaptation by increase of glucose transporter 2, proglucan ormithine decarboxylase, GLP-2 (Brahe *et al.*, 2013)

Inflammation and immune modulation

Butyrate has clear anti-inflammatory properties. This effect may be due to FFAR2 and FFAR3 receptor activation and histone deacetylase (HDAC) inhibition (Vinolo *et al.* 2011, Soldavini *et al.* 2013).

- Leukocyte chemotaxis – migration to inflammation site (Soldavini *et al.* 2013)
- Chemoattractant agent for neutrophils *in vitro* (Vinolo *et al.* 2011)
- Prevention of chemotaxis and cell adhesion – immune cell infiltration (Brahe *et al.*, 2013)
- Suppress production of pro-inflammatory TNF-α, IL-6, NO (Vinolo *et al.* 2011, Soldavini *et al.* 2013)
- Enhances production of anti-inflammatory IL-10 (Vinolo *et al.* 2011, Soldavini *et al.* 2013)
- Activation of anti-inflammatory function of peroxisome proliferator-activated receptor γ (γ-PPAR) – reduce paracellular permeability (Kles *et al.*, 2006, Brahe *et al.*, 2013)
- Inhibition of NFκB pathway (Brahe *et al.*, 2013)
- Suppress NFκB activation/expression and deacetylase inhibition (Kles *et al.*, 2006). Reduction of NFκB in nucleus of macrophages (Lürs *et al.*, 2002).

3. Materials and Methods

The possibility of enhancing the prebiotic properties of malted barley was evaluated by investigating the ability of different yeast strains to grow on malted barley materials and the effect of the combination of bifidobacteria with the fermented barley material on production of organic acids.

This was done by

- Determining the β-glucan content in the malt material collected from a local brewery.
- Screening for extracellular β-glucanase activity to select suitable yeast strains for fermentation of the malt materials.
- Assessing the level of the background microbial activity in the malt material.
- Evaluating cell growth/viability of yeast in malt solutions to select optimal strain(s) for fermentation.
- Assessing the potential impact of the fermented material on gut health by analysing cell growth/viability of the two selected bifidobacteria strains and the subsequent production of organic acids.

If the results of the previous steps show indications of improved prebiotic properties, samples of the fermented malt may be sent to SLU for determination of the β-glucan profile.

3.1 Barley malt materials

The following barley malt materials were used in the study:

- *Ocean*, Brewers' Spent Grain (BSG) from the Ocean brewery in Göteborg. A mixture between caramel, crystal and pale ale malt originating from England, UK.
- *Cinnamon*, malted barley. The samples were produced in a pilot plant in Lahden Polttimo, Finland. Whole barley kernels were steeped at 35°C with 0.4% lactic acid, followed by germination for 72h.
- *Tipple*, malted barley. The samples were produced in the pilot plant in Lahden Polttimo with the same procedure as for Cinnamon malt.
- *Blend*, a mixture between Tipple malt (70%) and standard BSG (30%) from Viking malt in Halmstad.

Table 1. Content of 100g (dry weight) flour from the malted barley varieties Tipple and Cinnamon, a standard BSG and Blend (a mix of Tipple (70%) and std BSG (30%)).

Variety	Tipple	Cinnamon	Std BSG	Blend
Conditions	35°C + 0.4% LA		48/55/60 °C	-
% Protein (g/100g dw)	7.9	9.5	17.2	10.7
Dietary fiber (g/100g dw)	18.9	22.3	54.2	-
β-glucan (g/100g dw)	2.9	3.9	0.5	2.2
β-glucan Molecular Weight (g/mol)	$1.38*10^6$	$1.31*10^6$	$3.88*10^5$	$1.08*10^6$

Figure 1. Samples of Brewers' Spent Grain from the Ocean brewery (left) and Cinnamon malt (right).

3.2 Microorganisms and culturing media

The following yeast strains were used in the study:

- *Pichia burtonii* TY01, isolated from maize seeds
- *Pichia kudriavzevii* (*Issatschenkia orientalis*) TY2, isolated from millet[1] seeds
- *Pichia kudriavzevii* TY3, isolated from sorghum[2]-Togwa[3]
- *Pichia anomala* TY06, isolated from maize-Togwa
- *Sacchaomyces cerevisiae* TY08, isolated from sorghum-Togwa
- *Pichia guilermondi* TY09, isolated from maize-Togwa
- *Pichia kudriavzevii* TY13, isolated from sorghum-Togwa
- *Hanseniaspora guilermondi* TY14, isolated from sorghum
- *Klyveromyces marxianus* TY17 CBS11445
- *Saccharomyces cerevisiae* CBS7765, isolated from Rainbow trout

The Tanzanian Yeast (TY) strains and *S. cerevisiae* CBS7765 were isolated by Thomas Andlid (Hellström et al. 2010).

The media used to culture the yeast was the complex media Yeast extract Peptone Dextrose (YPD) and the defined media Yeast Nitrogen Base (YNB).

The following bacteria strains were used in the study:

- *Bifidobacterium bifidum* B1906 – ”B1”, isolated from infant
- *Bifidobacterium breve* B2368 – ”B2”, isolated from infant

The media used to culture the bifidobacteria was Tryptone, Phytone, Yeast extract (TPY). TPY is normally used for the isolation and culturing for lactic acid bacteria and *Bifidobacterium*.

[1] Millet is a type of cereal, rich in minerals and widely as food and feed. Sv. Hirs
[2] Sorghum is a type of cereal widely used in Africa and Asia as food and feed. Sv. Durra, Sockerhirs
[3] Togwa is a non-alcoholic beverage from Tanzania, East Africa.

Table 2. Composition of culturing media. Concentrations of media components in 1L of distilled water.

YPD (yeast)	
Ingredients	**Concentrations (g/l)**
Yeast extract	10
Peptone	20
Glucose	20
Agar	*20*
YNB (yeast)	
YNB	6.7
NaOH	5.4
Succinate	8.91
TPY (bifidobacteria)	
Tryptone	10
Phytone	5
Glucose	15
Yeast extract	2.5
Tween 80	1 ml
Cysteine	0.5
K_2HPO_4	1.5
$MgCl•6H_2O$	0.5
Agar	*20*
Cycloheximid	*0.15*

3.3 Determination of β-glucan content

Brewers' Spent Grain (BSG) was obtained from the Ocean Brewery in Gothenburg. The β-glucan content of this material was determined using the Megazyme Mixed-linkage-β-glucan assay kit.

The method can be divided in three steps; preparation of the samples, enzymatic degradation of the β-glucan and colourimetric quantification of glucose.

Sample preparations

The BSG was milled to a fine powder in a centrifugal mill.

In the first experiment a small sample of the BSG material was milled (approximately 5ml), in contrast to the second run where a larger sample (approximately 15ml) was prepared and thoroughly mixed in order to obtain a more homogenous material.

Samples of the milled BSG (200mg), and reference flour (100mg) with known β-glucan content as control, were added to glass tubes (10ml capacity). The samples were soaked in ethanol (200µl, 50%) and incubated in boiling water for 1 min before being dissolved in sodium phosphate buffer (4ml) and incubated another 2 min in boiling water.

Enzymatic degradation

After equilibration in a 50°C water bath lichenase enzyme (200µl) was added to each tube and incubated 1h at 50°C under continuous stirring. The lichenase targets degradation of the [(1-3)(1-4)] glucosidic bonds in the β-glucan, cutting the molecules into smaller pieces, principle of the degradation shown in figure 2.

Sodium acetate buffer (2ml for BSG and 5ml for reference flour) was added before centrifugation. For each sample, the supernatant (100µl) was transferred to three new smaller glass tubes. β-glucosidase enzyme (100µl) was added to two of the tubes. This step frees the single glucose units from the β-glucan chains. The third tube was used as a reaction blank to measure the background glucose level in the samples and therefore acetate buffer was added instead of β-glucosidase. The tubes were incubated at 50°C for 10 min.

Triplicates of glucose standards with known concentrations of glucose (0, 0.5, 1) were prepared to enable a correlation between the absorbance of the samples and the glucose level to be established.

Glucose oxidase/peroxidase (GOPOD) reagent was added to all of the tubes and they were immediately incubated at 50°C for 30 min to start the colouring process, illustrated in figure 3.

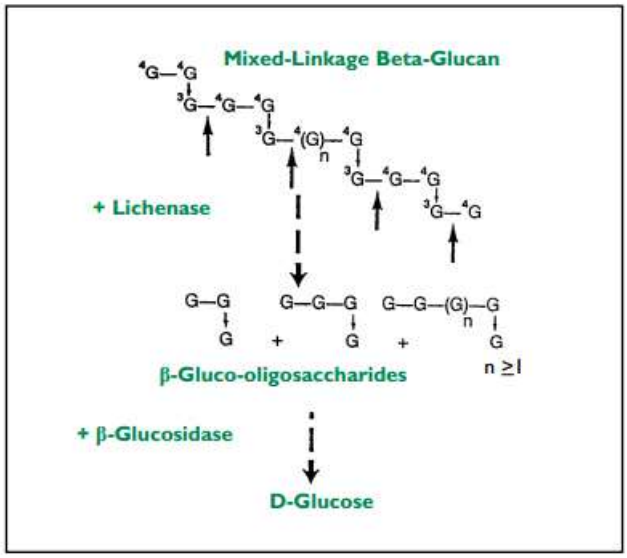


Figure 2. Principle of the Megazyme Mixed-linkage β-glucan assay[4]**.** In the first step the β-glucan is chopped into smalled pieces at the [(1-3)(1-4)] sites by endo 1,3(4) β-glucanase from *Bacillus subtilis*. β-glucosidase produced by *Aspergillus niger* is added in the second step to divide the β-gluco-oligosaccharides into D-glucose units.

[4] Illustration from Mixed-linkage β-glucan assay procedure.

Colourimetric quantification of glucose

The absorbance was measured at 510nm and necessary calculations were made to transform the absorbance readings into glucose concentrations.

$$\beta - glucan(\%) = \Delta A * F * \frac{1}{W} * X$$

ΔA = abs(sample) – abs(blank)

F = correlation factor for abs and glucose level = $\frac{100\mu g\ D-glucose}{Abs\ (100\mu g\ D-glucose)}$

W = dry weight of sample

X = volume correction factor; 8.46 for flour, 5.76 for malt

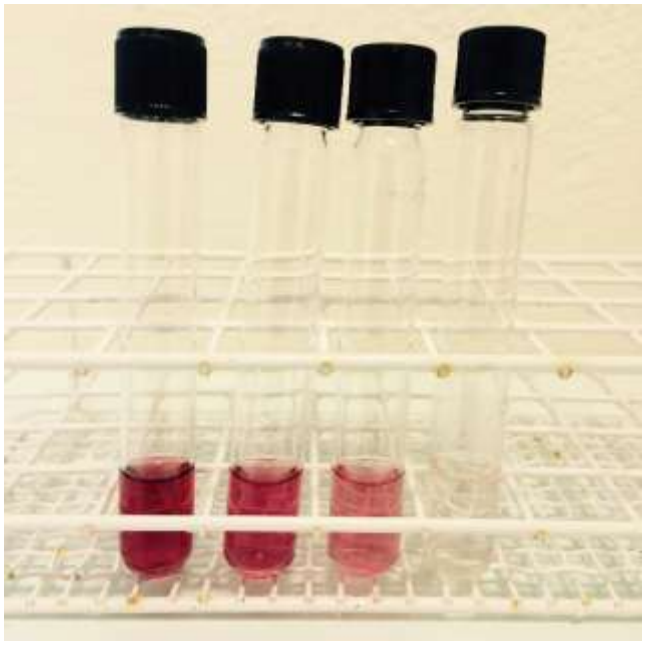


Figure 3. Glucose solutions incubated with GOPOD reagent (from left to right 1, 0.5, 0.25 and 0mg/ml glucose).

3.4 Screening of extracellular β-glucanase activity

3.4.1 Selection of yeast strains for the screening experiment

A literature study was done on β-glucanase producing microorganisms in order to identify species of interest for the screening experiment. 8 yeast strains were selected for screening. *Hanseniaspora guilermondi* and *Klyveromyces marxianus* were included based on their ability to produce β-glucanase observed in previous studies (ref.). The rest of the yeast (*P. anomala, P. burtonii, P. kudriavzevii, P. guilermondi*) were included in the due to their availability and the lack of literature regarding their β-glucanase activity. The two *S. cereviaiae* strains were included as negative controls based on their known inability to produce β-glucanase.

3.4.2 Screening of yeast strains for β-glucanase activity

In order to select suitable yeast strains for fermentation of the malt material, a screening of extracellular β-glucanase enzyme secretion was performed. The method chosen was a simple assay where the yeast was grown on agar plates containing 0.2% β-glucan and subsequent staining of the β-glucan with Congo Red dye visualized the potential degradation activity.

To investigate if the production of β-glucanase was affected by the glucose concentration two different media were used; YPD and YNB without (w/o) glucose. In addition, a screening was done on YNB plates containing β-glucan with the addition of yeast extract solution (100µl 50%, to make 10% in the solid media) to rule out that any nutrient necessary for β-glucanase enzyme production were missing in the YNB media.

Pre-culture

All of the different yeast strains were stored on YPD plates at 8°C. Pre-cultures were prepared the day before a screening experiment. A colony form each strain to be used in the experiment was picked and inoculated in 5ml liquid media, YPD or YNB with glucose depending on what solid media was to be used in the subsequent screening, in culture tubes (10ml) for 20h at 30°C in an incubation chamber with rotation wheel.

Preparation of screening plates

The cells were washed by discarding the supernatant after a centrifugation step and resuspending the pellet in NaCl (0.9%) solution. The cell concentration was determined by measuring the Optical Density of the cell solution in a spectrophotometer at wavelength nm.

After another centrifugation step the cells were resuspended in an appropriate volume of NaCl solution to make (OD) 2. Thereafter, a dilution series was created with 10^1, 10^2, 10^3, and 10^4.

10µl droplets of each dilution of the different yeast strains were added to the screening plate. Up to four different yeast strains were added to one plate. The plates were incubated for 24h at 30°C and subsequently stained to visualize potential β-glucanase activity.

An alternative method was used to confirm the result; all the screening experiments were repeated by simple streaking a colony of each yeast strain directly on the screening plate without pre-culturing in liquid media. *Saccharomyces cerevisiae* TY08 and CBS7765 were used as negative controls in these experiments.

Figure 4. Screening of β-glucanase activity on solid YPD media containing 0.2% β-glucan. Growth of yeast colonies on the screening plate, and staining of the β-glucan in the solid media after removal of the cells.

Staining of screening plates

The yeast colonies were scraped off and the plates were rinsed with distilled water to remove any remaining cells before the staining step. The plates were flooded with approximately 5ml of Congo Red solution (0.02%) and incubated for 15 min at room temperature.

The Congo Red dye binds specifically to the β-glucan in the solid media. Therefore, clear zones around the areas where the yeast colonies were growing indicates degradation of β-glucan by β-glucanase enzymes.

3.5 Fermentation of the malt materials by yeast

The next step in the project was to test the different yeast strains ability to ferment the malt material. The yeast strains showing β-glucanase activity in the screening experiment were chosen as being of interest for further investigation. The malt materials used in the test experiment were Ocean BSG, Blend-, Cinnamon-, and Tipple malt.

The yeast was incubated for 48h with malt as only nutrient source. The viability of the yeast was assessed by counting Colony Forming Units (CFU) at the starting point of the experiment, after 24h and 48h.

Pre-culture

The yeast was inoculated in 5ml liquid YPD media for 20-24h in an incubation chamber with rotation wheel.

Preparation and incubation

Malt material (2%) was dissolved in 5ml succinate buffer (pH 5.5). The cells were washed with sterile NaCl (0.9%) solution and resuspended in succinate buffer. Appropriate amount of cell solution was added to the malt samples to make OD 0.5. The samples were placed in an incubation chamber with rotation wheel and incubated for 48h at 30°C.

Growth and viability

Samples were collected at time points 0, 24h and 48h. Dilution series were created for each sample and droplets of 10µl were added to YPD plates and incubated overnight at 30°C (four replicates of the two highest dilutions in the range 10^3-10^4 at 0h, 10^4-10^5 at 24h and 10^5-10^6 at 48h). The colonies were counted when visible and the amount of cells/ml of sample was calculated based on the average number of colonies formed.

$$c_{cells} = \frac{[dilution\ factor] * [average\ colonies]}{V_{droplet}}$$

Background microbial activity

The malt material used in this study was non-sterile and heat treatment was considered inappropriate due to unwanted degradation and the risk of altering the β-glucan composition. Furthermore, contamination may be undesirable or indifferent depending on what degree and what species of microorganisms. Therefore, it was necessary to determine the level of contamination in the malt material to be able to take this into account. The level of background contamination was assessed based on CFU. The malt material was dissolved in succinate buffer (2%) and diluted in four steps in the range 10^0 to 10^3. 10µl droplets were added to different solid media; YPD[5], LB[6], TSA[7], TSC[8], and MRS[9].

[5] YPD; Yeast extract, Peptone, Dextrose, is a growth medium for yeast.
[6] LB; Lysogeny broth, is a rich medium for bacteria.
[7] TSA; Trypticase Soy Agar, is a general-purpose medium for growing bacteria.
[8] TSC; Tryptose Sulfite Cycloserine agar, is a medium for growing sulfite-reducing anaerobic bacteria e.g. *Clostridium perfingens*.
[9] MRS; Man, Rogosa and Sharpe, designed for growth of *Lactobacilli*.

3.6 Growth of bifidobacteria in malt-based media

The potential impact of the fermented malt material on gut health was assessed by evaluating its effect on bifidobacteria, a species with known probiotic properties that is naturally present in the colon and commonly added to dairy products. The growth and viability of bifidobacteria was determined with CFU on solid TPY media at the start of the experiment and after 48h incubation under anaerobic conditions.

Preparation of malt based media

Freshly milled flour samples of the Ocean BSG, Cinnamon malt and Blend malt were weighed (exact weight was noted for each sample) and added to 4ml succinate buffer to make a concentration of 2% (80mg). Yeast, pre-cultured in liquid YPD media and washed with succinate buffer, was added to make OD 0.5 in the final solution. The samples were incubated for 48h at 30°C and stored in freezer until needed.

Controls (one set inoculated with bacteria and one without) were prepared by mixing freshly milled malt material with 4ml succinate buffer to make a 2% solution.

Before inoculation of the Bifidobacteria each previously prepared malt solution was transferred to glass tubes and 2ml of 3x TYP stock solution w/o glucose ("G"), alternatively 3x TYP stock solution w/o glucose, tryptone, phytone, and yeast extract ("GTPYE") was added. 1ml of each media was transferred to eppendorf tubes and stored in the feezer.

Thus, a set of different treatments and controls for each malt material was prepared for the two Bifido strains, shown in table 3.

Subculture of bifidobacteria

The strains *Bifidobacterium bifidum* B1906 and *B. breve* B2368 were stored frozen at -80°C in skim milk solution. A sample of each was inoculated in TYP media and incubated anaerobically at 37°C for 24h. The media was changed the next day by transferring the bacteria to 2-3 new tubes containing fresh TYP and incubated at 37°C for 48h.

Growth and viability in the malt-based media

The bacteria were washed with NaCl solution (0.9%) and inoculated in the malt-based media to make a final OD of 0.3. Samples of 100µl were collected from each tube except for controls without Bifidobacteria. Diltuion series were made for each sample and CFU plates were prepared.

The tubes were plated in a desiccator, shown in figure 6, for 1h. N_2 gas was let into the chamber to avoid any oxygen getting into the liquid before placing the tubes in the incubation jar. An anaerocult was soaked in water and placed together with the tubes to remove any remaining oxygen in the head space of the tubes. The tubes were incubated anaerobically at 37°C for 48h.

Table 3. The set of malt-based media that was prepared for the investigation of the three different malt material's impact on Bifidobacteria.

For each malt variety 8 different samples were made; four samples fermented with the two different yeast strains with the addition of either the TPY stock solution w/o glucose (G) or TPY stock solution w/o glucose, tryptone, phytone and yeast extract (GTPYE), and four controls without the addition of yeast/fermentation step. Bacteria was not added to one set of the controls, marked with *. In total, 24 samples of malt-based media was prepared for each Bifido strain.

Yeast strain	TPY Media
TY1	G
	GTPYE
TY3	G
	GTPYE
-	G
	GTPYE
-*	G
	GTPYE

When the incubation was completed 1ml of each sample was transferred to Eppendorf tubes and frozen (for analysis of organic acid content). Samples of 100µl were collected from each tube to create dilution series and determine the number of CFUs.

The CFU plates were placed in culture jars and incubated anaerobically at 37°C for 48h. The CFU analysis was made on selective plates with solid TPY media containing cycloheximid to inhibit growth of yeast.

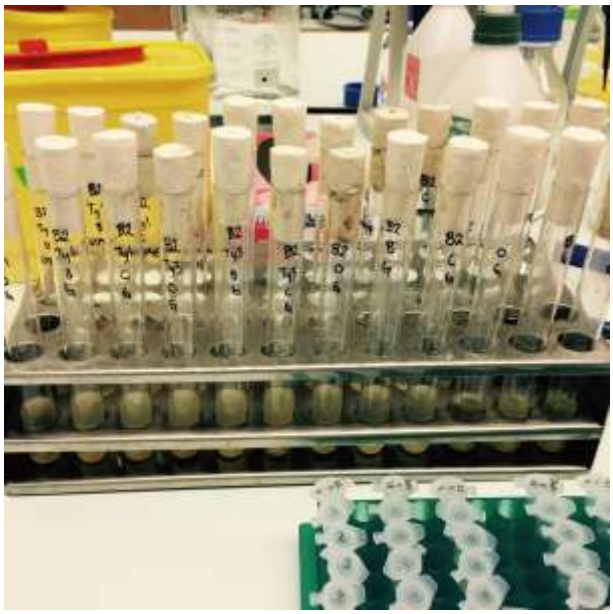


Figure 5. Sterile glass tubes containing malt-based media, ready for inoculation with bifidobacteria.

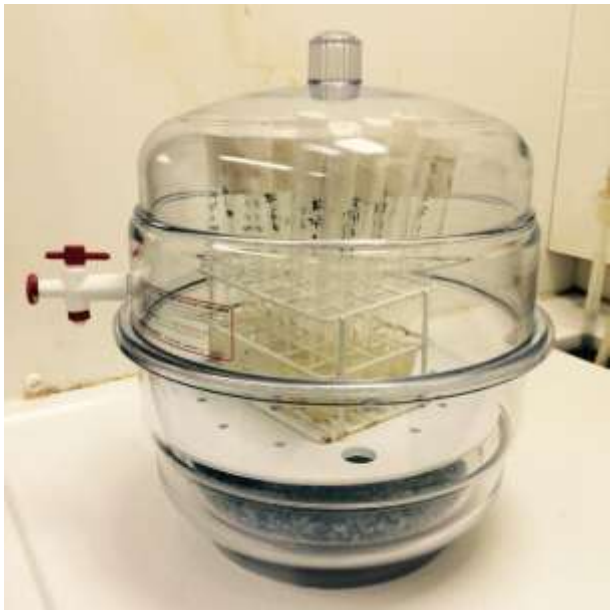


Figure 6. The desiccator where the tubes were placed 1h before incubation to remove the dissolved oxygen in the liquid.

3.7 Quantification of organic acids

The concentrations of organic acids in the samples from bifidobacteria grown in malt-based media were determined with a HPLC system.

Preparations

The previously frozen samples, from the 48h incubation of bifidobacteria in malt-based media, were thawed and centrifuged for 15 min at 12000g. The supernatant of each sample was diluted 5 times with MilliQ water to a final volume of 1ml and transferred to glass vials marked with a sample number, in total 48 samples, shown in figure 7. A standard solution containing 100ppm of the organic acids citric-, tartaric-, malic-, succinic-, lactic-, acetic- and propionic acid was prepared to enable identification and quantification.

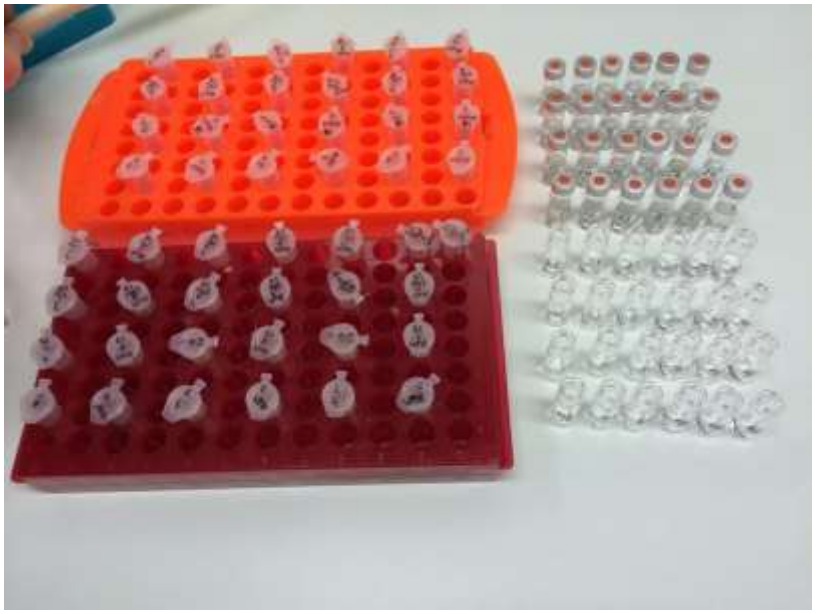


Figure 7. Preparation of the samples for HPLC analysis.

Experimental setup

The experimental setup can be viewed in figure 8. The mobile phase used for the analysis was 0.08 mmol/l sulfuric acid which was pumped through the column at a flow rate of 0.6 ml/min. The autosampler, "AS-2057 plus" injected 20µl of each sample for analysis.

The HPLC method was based on ion exclusion with a Thermasphere Hyper Rez xp column (8x300mm). The counter ions were H+ and particle size was 8µm. A small pre-column in the same material was used.

The detection was done with a UV-VIS Shimadzu SPP10A detector at wavelength 210nm. The computer program used was JASCO Chromatography Data Station.

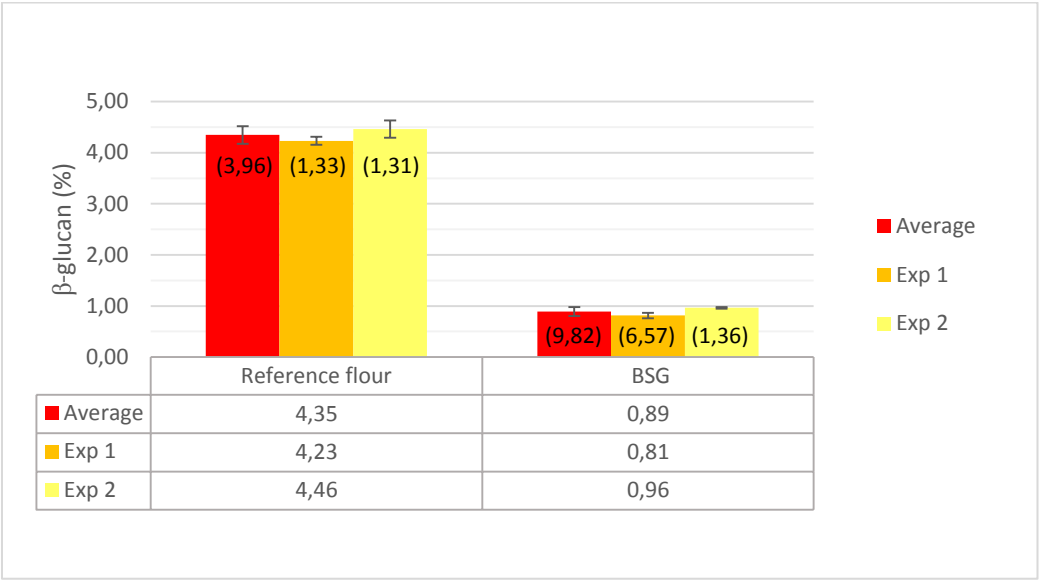
Figure 8. Setup of the HPLC equipment. From left to right; pump with eluent liquid, autosampler, column and UV-VIS detector.

4. Results

4.1 Determination of β-glucan content

The result of the Megazyme Mixed-linkage β-glucan assay showed an average β-glucan content of 0.89% in Brewers' Spent Grain from the Ocean brewery, shown in figure 9. A table with all of the results from the Megazyme Mixed-linkage β-glucan assay can be found in Appendix III.

The β-glucan content in different barley malt materials; Cinnamon and Tipple malted barley, standard and OceanBrewers' Spent Grain are compared in figure 10. The β-glucan content in BSG is lower compared to malted barley.



	Reference flour	BSG
Average	4,35	0,89
Exp 1	4,23	0,81
Exp 2	4,46	0,96

Figure 9. Determination of the β-glucan content in Brewers' Spent Grain.
The β-glucan content was determined with the Megazyme Mixed-linkage β-glucan assay kit. Two samples of the reference flour with known β-glucan content and the BSG from the Ocean brewery were prepared in each experimental run and the analysis of each of these samples was performed in duplicates. The samples were prepared differently in the two experimental runs; the sample size was approximately 3 times bigger in the second run and the material was thoroughly mixed before the analysis.
Red columns (Average) show the average of all the results from the two experimental runs. Orange columns (Exp 1) show average β-glucan content from the first experimental run. Yellow columns (Exp 2) show average β-glucan content from the second experimental run.
Standard deviation (%) is shown as error bars with the exact values in brackets below.

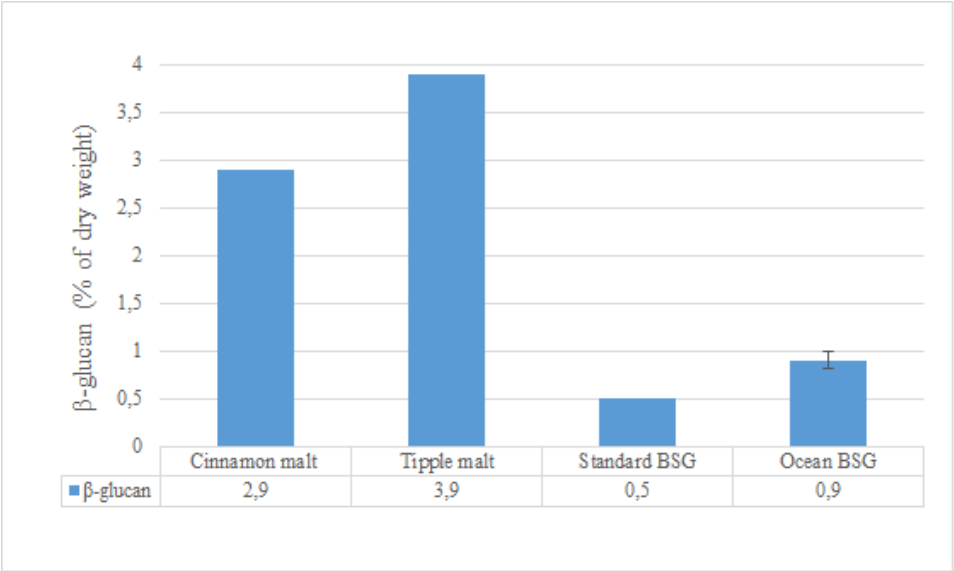


Figure 10. Comparison of the β-glucan levels in different barley malt materials. The result of the determination of β-glucan content in Ocean BSG by the Megazyme mixed-linkage β-glucan assay compared with levels found in Cinnamon malt, Tipple malt and Standard BSG. The β-glucan content in Cinnamon, Tipple and std BSG had previously been determined by Cristina Texeira.

4.2 Screening of extracellular β-glucanase activity

Table 4. Result of screening of β-glucanase activity on media containing 0.2% β-glucan.
Three different solid media were used; YPD, YNB without glucose and YNB without glucose with added yeast extract solution.
Yeast was added in droplets of diluted cell solutions or by streaking a colony directly on to the solid media.
Degree of degradation observed: (-) no degradation, (*) possible or low degradation, (**) degradation, (***) high level of degradation. No visible colony formation (x). *S. cerevisiae* was only included in the streak experiments.

Yeast strains	**YPD**		**YNB** w/o g	**YNB** w/o g w ye
	Droplets	Streak	Droplets	Streak
Pichia burtonii TY01	(***)	(*)	(-)	(-)
Pichia kudriavzevii TY2	(-)	(-)	(-)	(-)
Pichia kudriavzevii TY3	(***)	(*)	(-)	(-)
Pichia anomala TY06	(***)	(***)	(-)	(-)
Pichia guilermondi TY09	(-)	(**)	(x)	(x)
Pichia kudriavzevii TY13	(-)	(-)	(-)	(-)
Hansenula guilermondi TY14	(**)	(*)	(x)	(x)
Klyveromyces marxianus TY17	(**)	(*)	(-)	(-)
Saccharomyces cerevisiae TY08		(-)		(-)
Saccharomyces cerevisiae CBS7655		(-)		(-)

The following yeast strains showed β-glucanase activity on solid YPD media; *Pichia burtonii* TY01, *Pichia kudriavzevii* TY3, *Pichia anomala* TY06, *Hansenula guilermondi* TY14, and *Klyveromyces marxianus* TY17, shown in table 4 and figure 10.

Clear zones around the colonies of strains positive for enzyme activity could be observed. Furthermore, the strains *Pichia kudriavzevii* TY02, *Pichia kudriavzevii* TY13 and *Pichia guilermondi* TY09 had invasive growth and formation of hyphae.

However, the test where the yeast was streaked directly on the YPD plates containing 0.02% β-glucan did not show the same result (figure 11). The only strain with clear enzyme activity was *Pichia anomala* TY06. Possible positive results were observed for *P. burtonii* TY01, *P. kudriavzevii* TY3, *P. guilermondi* TY09, *H. guilermondi* TY14, and *K. marxianus* TY17.

The screening for β-glucan degradation on YNB media w/o glucose (figure 12) did not indicate any enzyme activity for the selected strains. Furthermore, *P. guilermondi* TY09 and *H. guilermondi* TY14 were not able to grow on these plates. The addition of yeast extract solution had no effect on the growth or enzyme activity (figure 13).

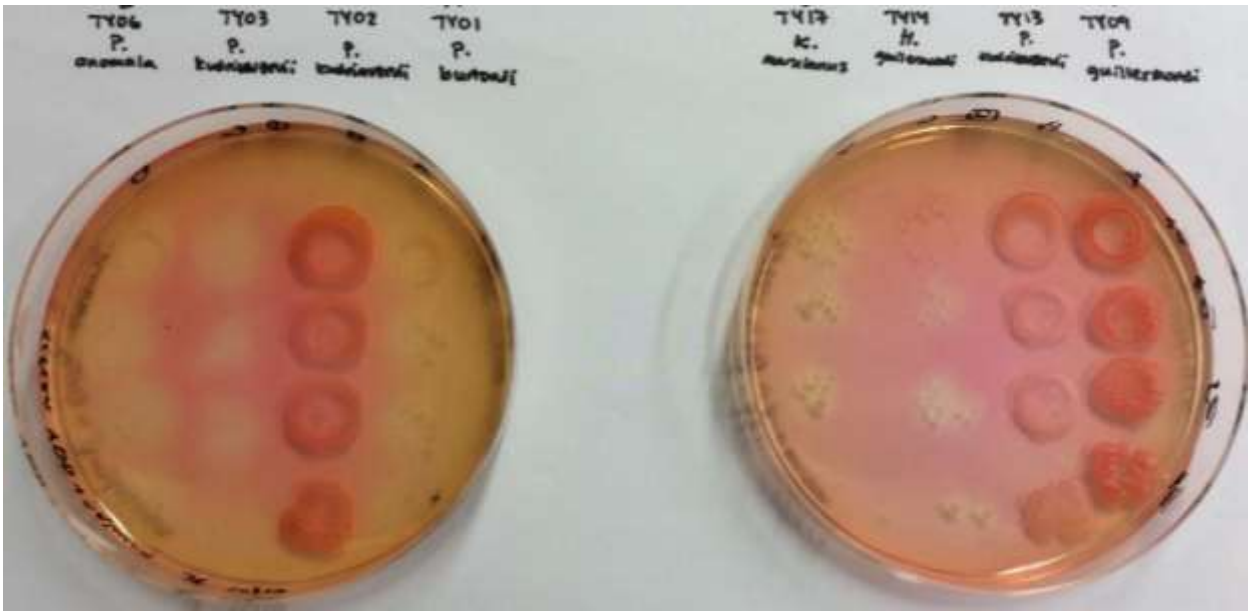


Figure 11. Screening of β-glucanase activity on solid YPD agar media containing 0.2% β-glucan.
The β-glucan was stained with Congo Red solution (0.02%). Dilutions in row 1-4; 10^1, 10^2, 10^3, 10^4.
Left petri dish from left to right column 1) *P. anomala* TY06 – positive, 2) *P. kudriavvzevii* TY3 – positive, 3) *P. kudriavzevii* TY02 – negative, *P. burtonii* TY01 – positive.
Right petri dish from left to right column 1) *K. marxianus* TY17 – positive, 2) *H. guilermondi* TY14 – positive, 3) *P.kudriavzevii* TY13 – negative, 4) *P. guilermondi* TY09 – negative.

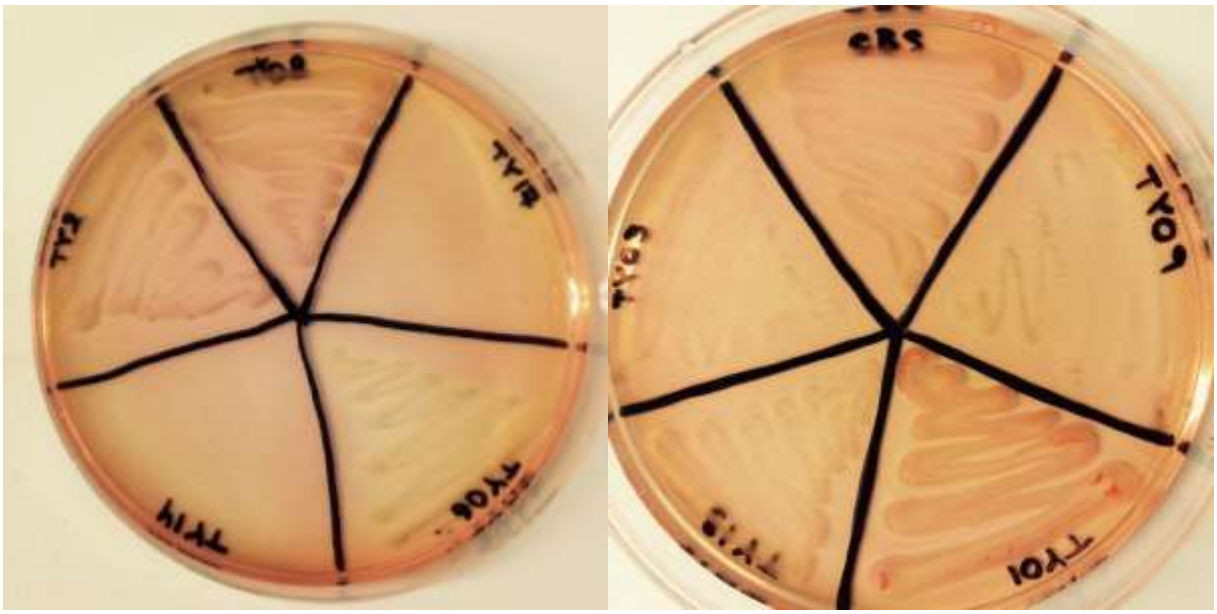


Figure 12. Screening of β-glucanase activity on solid YPD agar plates containing 0.2% β-glucan.
The β-glucan was stained with Congo Red solution (0.02%). Each section represents a different yeast strain. Left; *P. anomala* TY06 (lower right) - positive. *H. guilermondi* TY14 (lower left) and *K. marxianus* TY17 (upper right) – slight activity. *P. kudriavzevii* TY02 (upper left) - negative. Negative control – *S. cerevisiae* TY08 (upper middle). Right; *P. burtnoii* TY01 (lower right), *P. guilermondi* TY09 (upper right) and *K. marxianus* Ty03 (upper left) – slight acitvity. *P. kudriavzevii* TY13 (lower left) – negative. Negative control – *S. cerevisiae* CBS (upper middle).

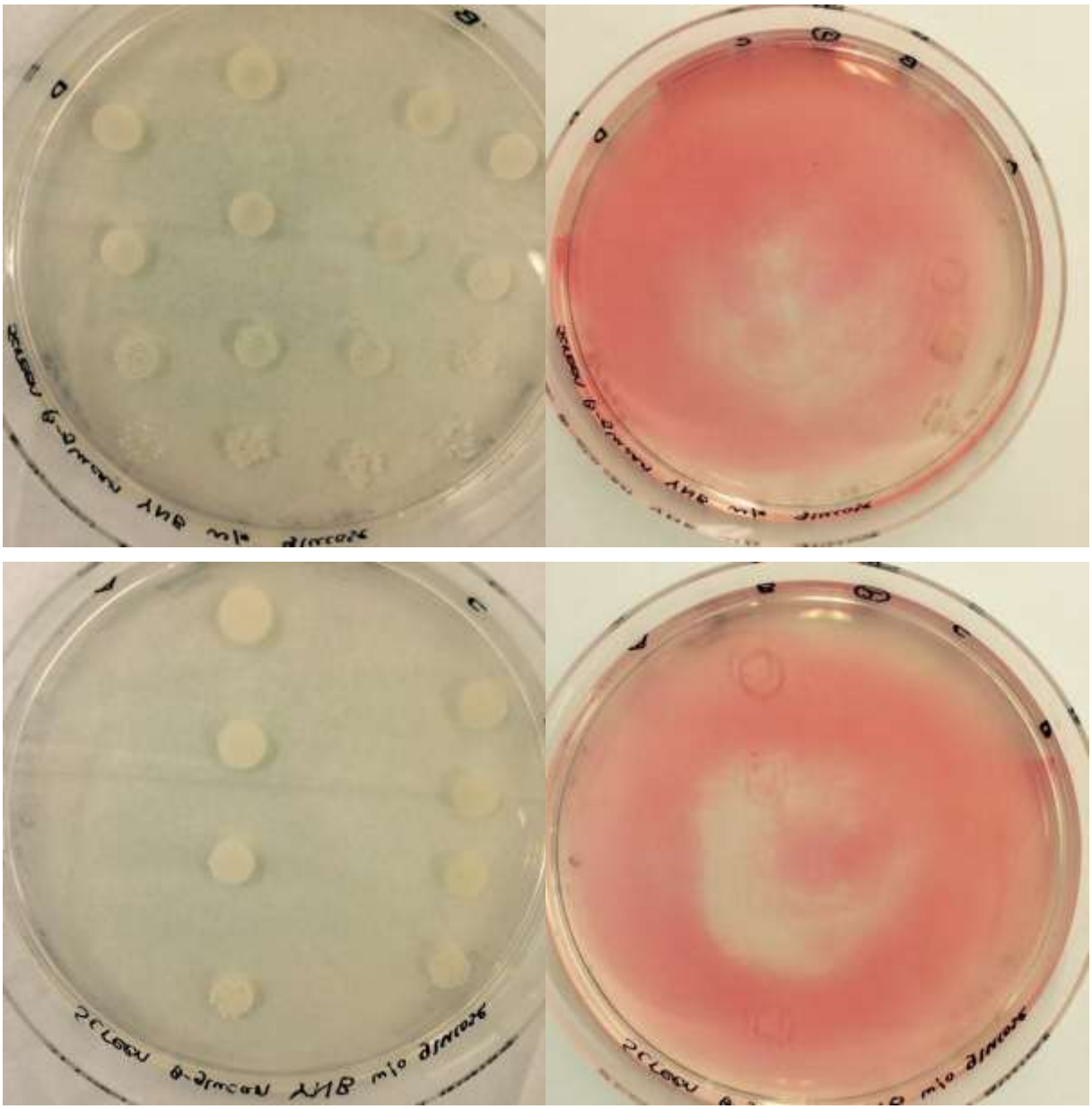


Figure 13. Screening of β-glucanase activity on solid YNB w/o glucose.

Dilutions in row 1-4; 10^1, 10^2, 10^3, 10^4.

Upper left; clearly visible growth of all the yeast strains, from left to right – *P. anomala* TY06, *P. kudriavzevii* TY3, *P. kudriavzevii* TY02, *P. burtonii* TY01.

Upper right; result after washing and staining with Congo Red – no degradation of β-glucan observed.

Lower left; the yeast strains from left to right *P. guilermondi* TY09 – no visible colonies, *P. kudriavzevii* TY13 – clear growth, *H. guilermondi* TY14 - no visible colonies, *K. marxianus* – clear growth.

Lower right; result after washing and staining with Congo Red – no degradation of β-glucan observed.

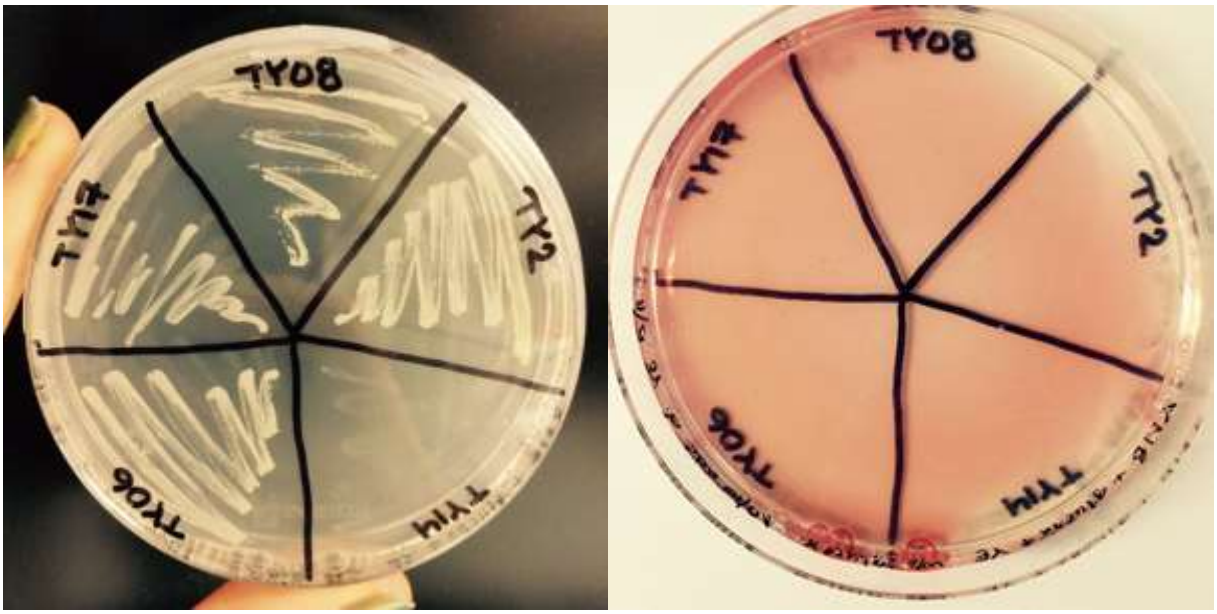


Figure 14. Screening of β-glucanase activity on solid YNB w/o glucose, w yeast extract (10%).

Each section represents a different yeast strain.

Left; clearly visible growth of all the yeast strains except *H. guilermondi* TY14 on the plate after 1 day incubation at 30°C.

Right; result after washing and staining with Congo Red solution (0.02%) – no degradation of β-glucan observed.

4.3 Fermentation of the malt material by yeast

4.3.1 Growth of yeast in the malt materials

The yeast strains that were used in this experiment were selected based on their presumed ability to produce β-glucanase enzyme. The growth curves generated from CFU counts during fermentation of the malt solutions by the different yeasts are shown in figure 15.

All the yeast strains were able to grow in the different malt solutions without addition of any extra nutrients. *P. burtonii* TY01 and *P. kudriavzevii* TY3, were selected for use in further experiments. The growth curves of *P. burtonii* TY01 and *P. kudriavzevii* TY3 in the different malt solutions are shown in figures 16 and 17 respectively. Both species had lowest growth in Ocean BSG. *P. burtonii* TY01, reached cell densities above 10^9 cell/ml in both Cinnamon, Tipple and Blend, in contrast to *P. kudriavzevii* TY3, which only reached above 10^9 cell/ml in Cinnamon malt.

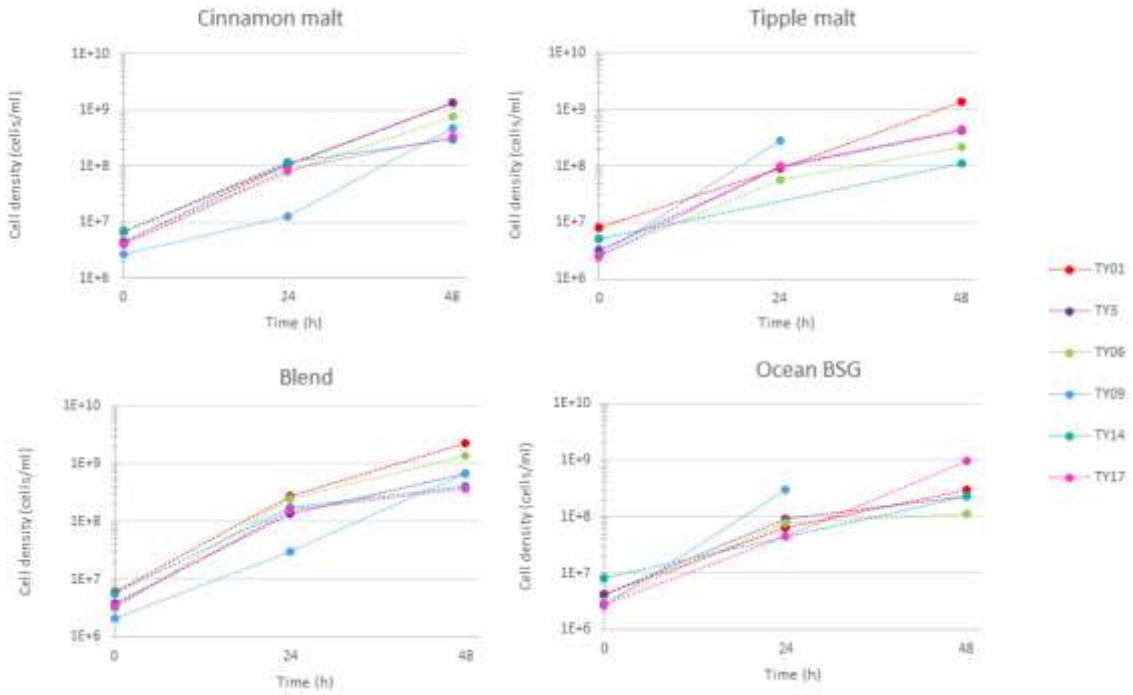


Figure 15. Growth of the yeast strains with different malt solutions (2%) as only nutrient source.

The cell density is based on CFU on YPD plates, with samples collected at time points 0h, 24h and 48h after incubation. The yeast strain were *P. burtonii* TY01 (red), *P. kudriavzevii* TY3 (dark purple), *P. anomala* TY06 (light green), *P. guilermondi* TY09 (light blue), *H. guildermondi* TY14 (turquoise), *K. marxianus* TY17 (magenta).

Cinnamon malt; The highest cell count observed in Cinnamon was for *P. burtonii* TY01 and *P. kudriavzevii* TY3, both reaching above 10^9 cells/ml.

Tipple malt; The highest cell count observed in Ocean was for *P. burtonii* TY01, reaching above 10^9 cells/ml. However, the result of the final CFU count at 48h was inconclusive for *P. guilermondi* TY09 because of too densely growing colonies.

Blend malt; The highest cell count observed in Blend was for *P. burtonii* TY01 and *P. anomala* TY06, both reaching above 10^9 cells/ml.

Ocean BSG; The highest cell count observed in Ocean was for *K. marxianus* TY17, just below 10^9 cells/ml. However, the result of the final CFU count at 48h was inconclusive for *P. guilermondi* TY09 because of too densely growing colonies.

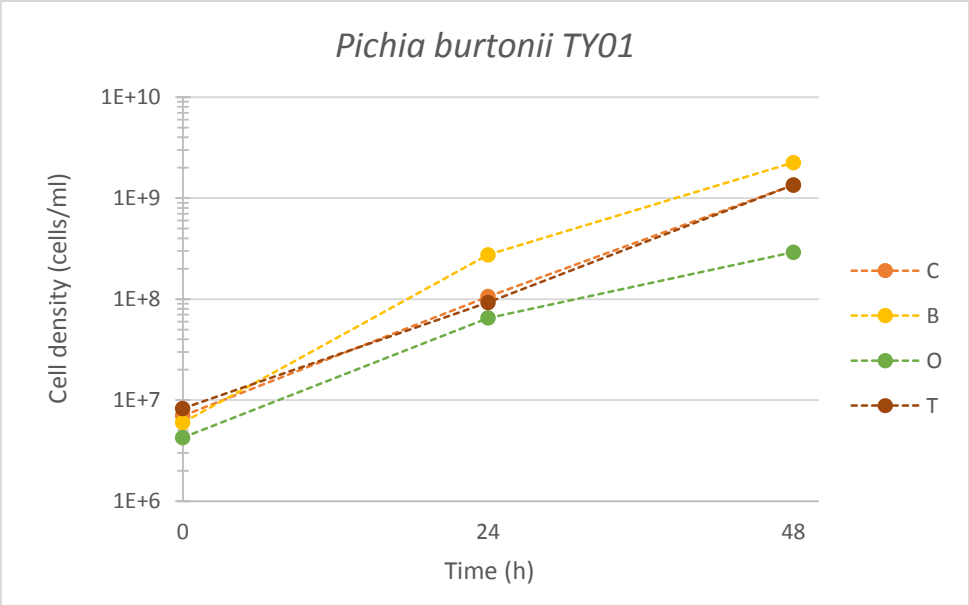


Figure 16. Growth of *P. burtonii* TY01 in the different malt solutions.

The values of the cell densities are based on the number of CFU on solid YPD media at the start, after 24h and at the end of the incubation time. The different malt solutions (2%) were Cinnamon malt (orange), Blend malt (yellow), Ocean BSG (green), Tipple (brown).

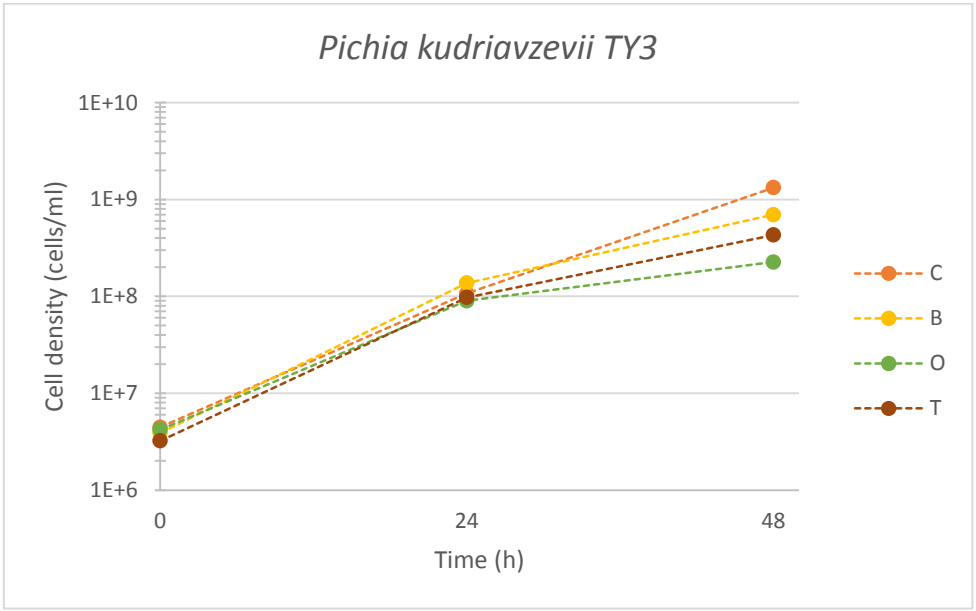


Figure 17. Growth of *P. kudriavzevii* TY3 in the different malt solutions.

The values of the cell densities are based on the number of CFU on solid YPD media at the start, after 24h and at the end of the incubation time. The different malt solutions (2%) were Cinnamon malt (orange), Blend malt (yellow), Ocean BSG (green), Tipple (brown).

4.3.2 Characterization of background microbial levels

There were high background levels of microorganisms present in all malt materials except Ocean BSG. New malt materials were obtained which showed lower levels of microorganisms, shown in figure 18.

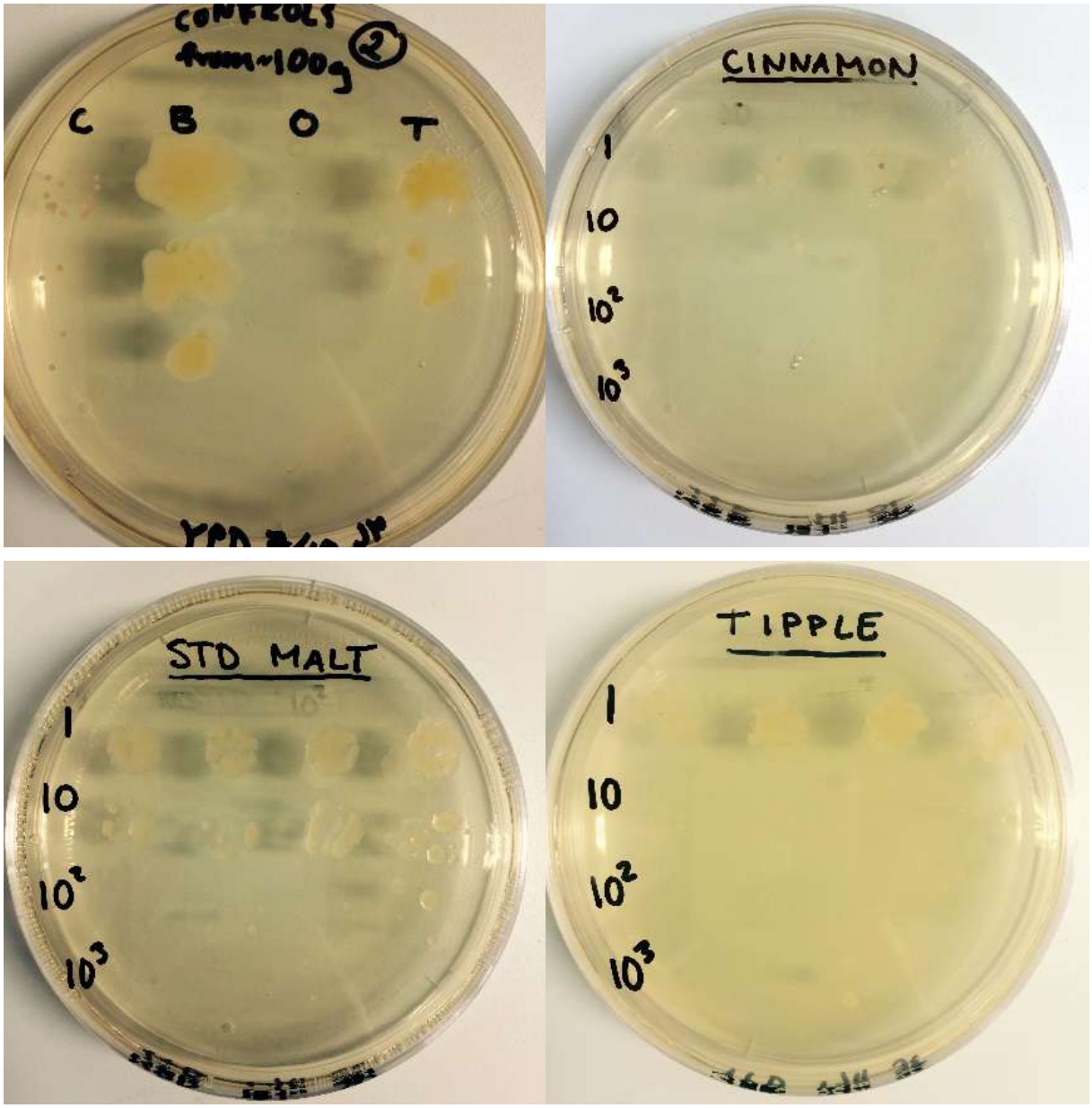


Figure 18. Evaluation of background microbial levels. The different malt varieties and the BSG to be used in the study was added to on solid YPD media and CFUs noted. Dilutions of the malt solutions in row 1-4; 10^0, 10^1, 10^2, 10^3.
Upper left; Initial assessment of the malt flour and BSG. Columns from left to right; Cinnamon, Blend, Ocean and Tipple. Observations - growth of red colonies in the Cinnamon malt 10^0-10^2, growth in Blend 10^0-10^2, no growth in Ocean, growth in Tipple 10^0-10^1.
Upper right; New Cinnamon malt stored as grains in freezer. Sporadic colonies visible in 10^0.
Lower left; New Blend stored as grains in freezer. Observations – growth in 10^0-10^1, sporadic colonies in 10^2.
Lower right; New Tipple malt stored as grains in freezer. Observations – growth in 10^0, sporadic colonies in 10^1.

4.4 Impact of the fermented malt material on probiotic bacteria

4.4.1 Growth of bifidobacteria

A more than 10-fold increase of the cell density was observed for some of the fermented samples with Cinnamon malt (table 5). Overall poor growth was observed for samples containing Ocean BSG. A table containing the complete result of the growth test can be found in Appendix III.

Table 5. Result of growth assessment of bifidobacteria in malt-based media. Samples showing a more than 10-fold increase in the number of cells.

Sample (bacteria-yeast-malt-media)	**Initial cell density** (cells/ml)	**Final cell density** (cells/ml)
B1-Ty1-C-G	$9*10^6$	$3.2*10^8$
B1-TY3-C-G	$4*10^6$	$2.3*10^8$
B1-TY3-C-GTPYE	$3*10^6$	$9*10^7$
B2-Ty1-C-GTPYE	$2.1*10^7$	$2.75*10^8$

4.4.2 Quantification of organic acid

The organic acid profiles resulting from incubation of bifidobacteria in Cinnamon malt-based media are shown in figures 19 and 20. A clear tend can be seen; lactic acid is not present or can be found in low concentrations in the controls as well as in the samples containing untreated malt. Furthermore, the concentrations of lactic acid in the samples with fermented malt is significantly higher than in the samples with untreated malt. The only exception to this trend is the sample "B2" in Cinnamon-based media without glucose, figure 19, which has a slightly higher level of lactic acid despite containing untreated malt.

The result for the Ocean BSG did not show as clear trends as for Cinnamon malt. In Ocean BSG-based media without glucose, shown in figure 21, lactic acid was found in the samples with BSG fermented by *P.* kudriavzevii TY3. However, lactic acid was also found in the control and in the unfermented BSG with added *Bifidobacterium breve* "B2". In Ocean BSG-based media without glucose, tryptone, phytone and yeast extract added, lactic acid was found in three of the four samples with fermented BSG. However, a relatively high level of lactic acid was observed in the unfermented BSG with added B2, shown in figure 22.

The result for samples based on Blend malt, shown in figures 23 and 24, are drastically different from Cinnamon malt and Ocean BSG. High lactic acid concentrations was found in all of the samples, including the controls.

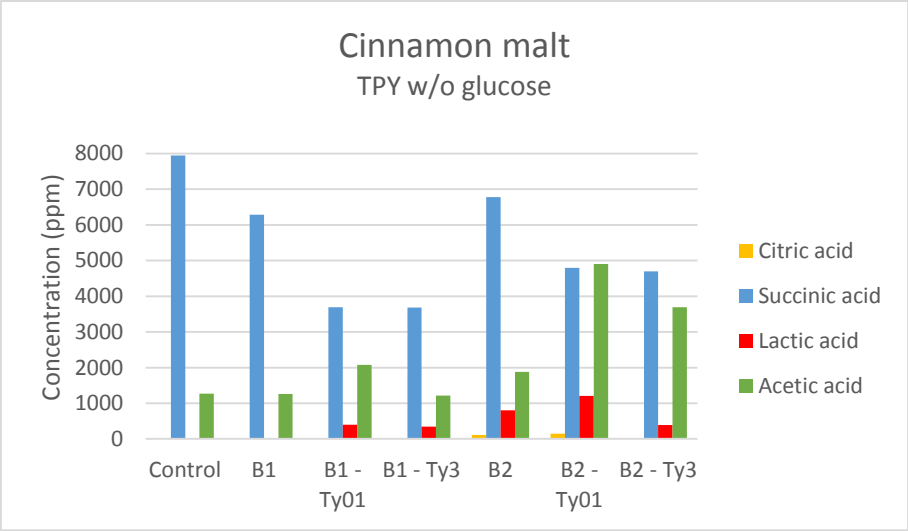


Figure 19. Organic acid profile in samples with Cinnamon malt and TPY media witout glucose.
Control – untreated Cinnamon malt incubated without Bifidobacteria.
(B1) - untreated Cinnamon malt incubated with *Bifidobacterium bifidum* B1906.
(B1 – TY01) – Cinnamon malt fermented by *P. burtonii* TY01 and subsequently incubated with *Bifidobacterium bifidum* B1906.
(B1-TY3) - Cinnamon malt fermented by *P. kudriavzevii* TY3 and subsequently incubated with *Bifidobacterium bifidum* B1906.
(B2) - unfermented Cinnamon malt incubated with *Bifidobacterium breve* B2368.
(B2 – TY01) – Cinnamon malt fermented by *P. burtonii* TY01 and subsequently incubated with *Bifidobacterium breve* B2368.
(B2-TY3) - Cinnamon malt fermented by *P. kudriavzevii* TY3 and subsequently incubated with *Bifidobacterium breve* B2368.

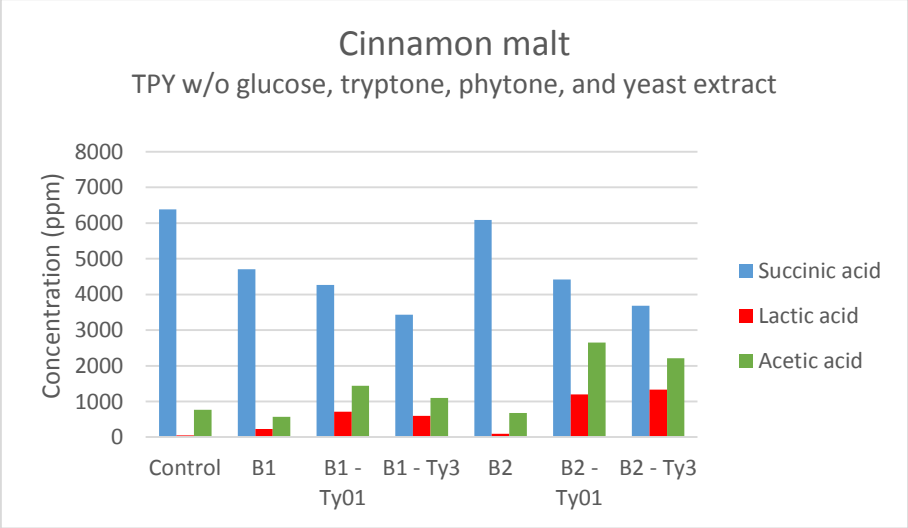


Figure 20. Organic acid profile in samples with Cinnamon malt and TPY media witout glucose, tryptone, phytone and yeast extract.
Control – untreated Cinnamon malt incubated without Bifidobacteria.
(B1) - untreated Cinnamon malt incubated with *Bifidobacterium bifidum* B1906.
(B1 – TY01) – Cinnamon malt fermented by *P. burtonii* TY01 and subsequently incubated with *Bifidobacterium bifidum* B1906.
(B1-TY3) - Cinnamon malt fermented by *P. kudriavzevii* TY3 and subsequently incubated with *Bifidobacterium bifidum* B1906.
(B2) - unfermented Cinnamon malt incubated with *Bifidobacterium breve* B2368.
(B2 – TY01) – Cinnamon malt fermented by *P. burtonii* TY01 and subsequently incubated with *Bifidobacterium breve* B2368.
(B2-TY3) - Cinnamon malt fermented by *P. kudriavzevii* TY3 and subsequently incubated with *Bifidobacterium breve* B2368.

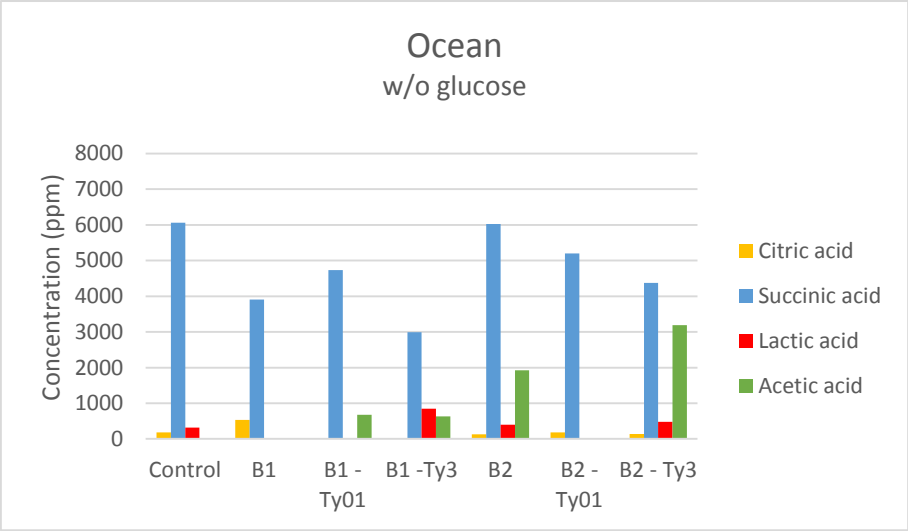


Figure 21. Organic acids identified in samples with Ocean BSG and TPY media witout glucose.
Control – untreated Ocean BSG incubated without Bifidobacteria.
(B1) - untreated Ocean BSG incubated with *Bifidobacterium bifidum* B1906.
(B1 – TY01) – Ocean BSG fermented by *P. burtonii* TY01 and subsequently incubated with *Bifidobacterium bifidum* B1906.
(B1-TY3) - Ocean BSG fermented by *P. kudriavzevii* TY3 and subsequently incubated with *Bifidobacterium bifidum* B1906.
(B2) - unfermented Ocean BSG incubated with *Bifidobacterium breve* B2368.
(B2 – TY01) – Ocean BSG fermented by *P. burtonii* TY01 and subsequently incubated with *Bifidobacterium breve* B2368.
(B2-TY3) - Ocean BSG fermented by *P. kudriavzevii* TY3 and subsequently incubated with *Bifidobacterium breve* B2368.

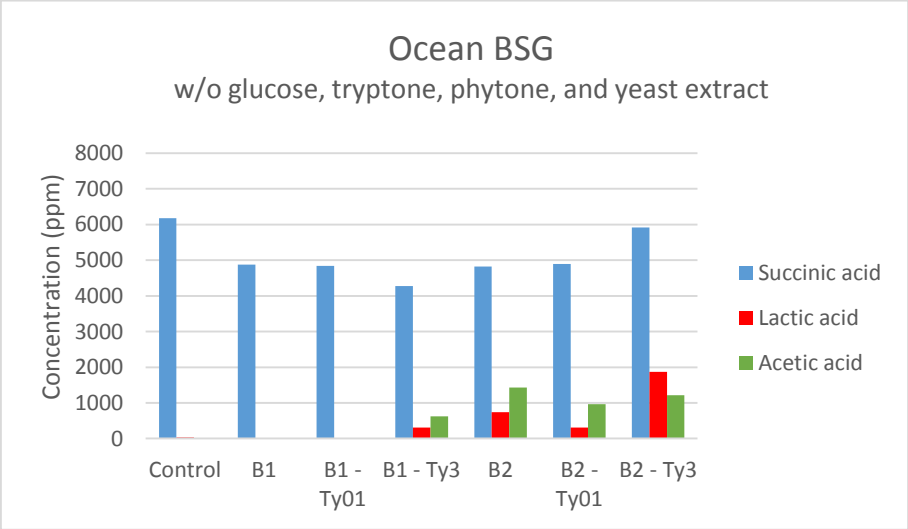


Figure 22. Organic acids identified in samples with Ocean BSG and TPY media witout glucose, tryptone, phytone and yeast extract.
Control – untreated Ocean BSG incubated without Bifidobacteria.
(B1) - untreated Ocean BSG incubated with *Bifidobacterium bifidum* B1906.
(B1 – TY01) – Ocean BSG fermented by *P. burtonii* TY01 and subsequently incubated with *Bifidobacterium bifidum* B1906.
(B1-TY3) - Ocean BSG fermented by *P. kudriavzevii* TY3 and subsequently incubated with *Bifidobacterium bifidum* B1906.
(B2) - unfermented Ocean BSG incubated with *Bifidobacterium breve* B2368.
(B2 – TY01) – Ocean BSG fermented by *P. burtonii* TY01 and subsequently incubated with *Bifidobacterium breve* B2368.
(B2-TY3) - Ocean BSG fermented by *P. kudriavzevii* TY3 and subsequently incubated with *Bifidobacterium breve* B2368.

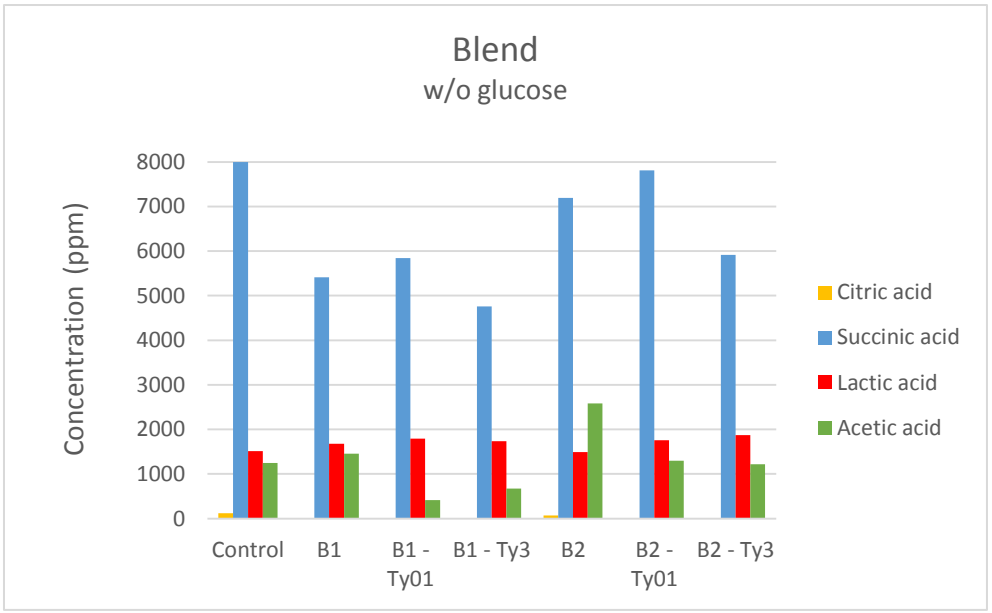


Figure 23. Organic acids identified in samples with Blend and TPY media witout glucose.
Control – untreated Blend incubated without Bifidobacteria.
(B1) - untreated Blend incubated with *Bifidobacterium bifidum* B1906.
(B1 – TY01) – Blend fermented by *P. burtonii* TY01 and subsequently incubated with *Bifidobacterium bifidum* B1906.
(B1-TY3) - Blend fermented by *P. kudriavzevii* TY3 and subsequently incubated with *Bifidobacterium bifidum* B1906.
(B2) - unfermented Blend incubated with *Bifidobacterium breve* B2368.
(B2 – TY01) – Blend fermented by *P. burtonii* TY01 and subsequently incubated with *Bifidobacterium breve* B2368.
(B2-TY3) - Blend fermented by *P. kudriavzevii* TY3 and subsequently incubated with *Bifidobacterium breve* B2368.

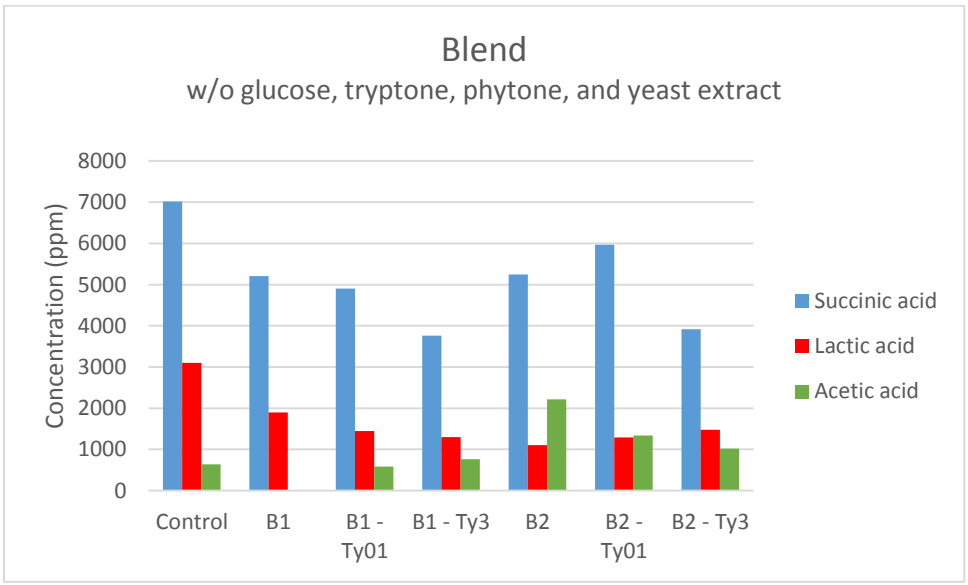


Figure 24. Organic acids identified in samples with Blend and TPY media witout glucose, tryptone, phytone and yeast extract.
Control – untreated Blend incubated without Bifidobacteria.
(B1) - untreated Blend incubated with *Bifidobacterium bifidum* B1906.
(B1 – TY01) – Blend fermented by *P. burtonii* TY01 and subsequently incubated with *Bifidobacterium bifidum* B1906.
(B1-TY3) - Blend fermented by *P. kudriavzevii* TY3 and subsequently incubated with *Bifidobacterium bifidum* B1906.
(B2) - unfermented Blend incubated with *Bifidobacterium breve* B2368.
(B2 – TY01) – Blend fermented by *P. burtonii* TY01 and subsequently incubated with *Bifidobacterium breve* B2368.
(B2-TY3) - Blend fermented by *P. kudriavzevii* TY3 and subsequently incubated with *Bifidobacterium breve* B2368.

5. Discussion

5.1 Determination of β-glucan content

The average β-glucan content in the Ocean BSG was found to be 0.9%, which is slightly higher compared to the level in standard BSG material (0.5%).

The β-glucan content of the reference flour provided in the Megazyme assay kit was stated to be 4.1% by the manufacturer and the result obtained by using the assay was 4%. Comparing these values it can be concluded that the method is accurate. It should be noted that the reference flour provided with the kit does not reflect the normal levels in wheat flour.

The difference in preparation of the malt material between the two experimental runs (bigger sample and more extensive mixing in the second run) may explain the smaller standard deviation of the results from the second run compared to the first. Furthermore, this could indicate inhomogeneous distribution of β-glucan in the BSG material which is also to be expected. However, the result can be considered reproducible when comparing the values obtained from the two experimental runs.

It should be acknowledged that the statistical relevance of the results remains unknown due to the limited number of samples and replicates. However, the purpose of the analysis was only to get an indication of the β-glucan content in the BSG material.

5.2 Screening of extracellular β-glucan activity

Hanseniaspora guilermondi (?) and *Klyveromyces marxianus* (Lopes *et al.*, 2014) were included in this study based on their ability to produce β-glucanase. The two strains of *Saccharomyces cerevisiae* were used as negative controls based on their known inability to produce β-glucanase. The rest of the yeast strains were included out of curiosity as no information about β-glucanase activity was found.

The outcome of the screening on solid YPD media with 0.2% β-glucan distinct transparent zones could be observed around colonies where enzymes had degraded the β-glucan in the media. However, the same result was not obtained when the yeast strains were streaked directly on the screening plates. It is evident that the state of the cells has a significant impact on the production of β-glucanase enzyme.

The screening experiments done on solid YNB without glucose gave an unexpected result. All except two of the yeast strains (*P. guilermondi* TY09 and *H. guilermondi* TY14) were able to grow but none showed any enzyme activity. The hypothesis was that yeast with the ability to produce β-glucanase enzymes would be able to grow with β-glucan as the only available carbon source, and yeast without this ability would not survive. The unexpected result may be explained by the yeasts ability to store energy and nutrients from the pre-culture media. Another explanation could be that some of the yeast

strains were able to use succinate from the YNB buffer as carbon source. Furthermore, it was speculated that the presence of additional nutrients may be necessary for production of β-glucanase enzymes. However, the addition of yeast extract solution to the YNB media did not give rise to enzyme activity. The result of the screenings on YNB media without glucose may indicate that production of β-glucanase is glucose dependent. However, this was not investigated further.

5.3 Fermentation of the malt material by yeast

5.3.1 Background microbial levels

After an initial trial fermentation it was discovered that Blend, Cinnamon and Tipple were heavily contaminated, shown in figure 14. The less contaminated Ocean BSG had been stored as whole grains (not milled) at -20°C in a freezer. However, the malt had been milled to a fine flour which increases the accessibility of the nutrients to microorganisms and kept in a walk-in fridge together with other food items which increased the risk of contamination.

New samples (grains) of the three malt varieties were collected and stored at -20°C in a freezer. The contamination level of milled samples from the new malt showed low levels of microorganisms, shown in figure 14. Therefore, it was decided that all the following experiments would be based on the new malt materials.

5.3.2. Growth of yeast in the malt materials

All yeast strains included in the fermentation experiment were able to grow with the malt materials as the only nutrient source. Furthermore, the differences in cell concentrations and growth patterns between the different strains were relatively small. However, the BSG generated a lower increase in cell number compared to the malted barley.

The cell concentrations calculated from the CFU values should only be regarded as indications. The growth experiment was not repeated and the CFU method can be unreliable due to the many potential sources of error. The malt samples contained big particles that affected the accuracy of the pipetting. In addition, cells can aggregate with the particles in the sample and subsequently influence the CFU result. Another important point is the background microbial level in the malt material. Selective plates containing antibiotics against bacteria could have been used to ensure that only yeast could grow on the plates. However, this would not have prevented growth of other yeast species already present in the flour. An alternative approach was used instead; the samples were inoculated with a high number of cells in an attempt to outcompete and impede growth of contaminants.

5.4 Impact of the fermented malt material on probiotic bacteria

5.4.1 Growth of bifidobacteria in the malt materials

The presumed outcome of the growth test of bifidobacteria in the different malt-based media was that samples with fermented malt would generate a larger increase in cell numbers compared to samples with unfermented malt.

Cinnamon malt increased the cell density in several samples which had been fermented by yeast, for both strains of bacteria. However, the evaluation of bifidobacteria growth in the malt based-media was largely inconclusive. The reason behind the inconclusive results was most likely linked to errors made during the dilution of the samples for CFU determination. Futhermore, the background microbial level may have been a greater issue than what was expected during the design of the experiments. It was presumed that the anaerobic environment would inhibit growth of yeast and many species of bacteria that might have been present in the malt or BSG and inoculation with a high number of bifidobacteria was expected to impede growth of other species to a certain extent.

Examination using a phase-contrast microscope of randomly selected samples of CFUs from the growth analysis, showed that other species than bifidobacteria were present. Bifidobacteria are rod shaped, usually with one end split in two resembling a "y". However, some of the microorganisms encountered were round, cocci shaped, and growing in chains which is characteristic for the species *Staphylococcus*. Fortunately, none of the CFUs on the TPY containing cycloheximid was identified as yeast.

Bifidobacteria are anaerobic and relatively fastidious. Despite the efforts to remove oxygen from the liquid, the head space of the culture tubes and in the environment of the culture jar it is not certain that the conditions were strictly anaerobic. Furthermore, the previous fermentation with yeast may have generated metabolites such as ethanol which may have affected the growth and viability of the bifidobacteria. However, samples with fermented malt material showed similar growth or even increased cell density compared to samples with unfermented malt.

The results obtained from this growth study are most likely not reproducible due to the many possible sources of error associated with the method. The indications noted in the experiment should not be used to draw any conclusions. However, the positive effects of Cinnamon malt are of interest for further investigation.

5.4.2 Organic acid production

Concerning organic acid production by Bifidobacteria, butyric acid is regarded as the most beneficial SCFA. However, the method used to detect and quantify the organic acids was not developed to include butyric acid and may have needed extensive optimization to generate appropriate retention times and clear peaks. Furthermore, butyrate has a distinctly foul odour (reminding of vomit or rancid

butter) making it unpleasant to handle. Lactic acid is produced by Lactic Acid Bacteria (LAB) such as *Bifidobacterium* and *Lactobacillus* but not by yeast. Therefore, lactic acid was used as a marker for activity of the bifidobacteria.

It was hypothesized that a greater concentration of lactic acid would be observed in samples with fermented malt than in samples with the untreated malt and the control without added Bifidobacteria would not contain any lactic acid. The result of the organic acid profile in Cinnamon malt-based media confirms this hypothesis. The controls showed little or no lactic acid present and the lactic acid level was higher in the samples with fermented malt compared to untreated malt.

The result in Ocean BSG-based media did not show the same trends as Cinnamon malt. This may be explained by difference in nutritional content between BSG and malted barley. The Ocean BSG has not been characterized, other than the β-glucan content, but the content of protein and dietary fiber in standard BSG is higher compared to Cinnamon malt. In contrast, the average molecular weight of β-glucan and the overall content is lower in BSG. It is not possible to link the nutritional content of the malt materials to the production of organic acids based on the result of this study. However, this is of interest for future research. Furthermore, the lactic acid observed in the controls may be produced by the background microorganism. Therefore it would have been relevant to use an additional control with unfermented malt without added bifidobacteria and without being incubated with the rest of the samples.

High levels of lactic acid was found in all samples with Blend malt and the concentration in the control was as high as or higher than the other samples. This may be explained by the result of the high number of background microorganism found in Blend malt and it can be presumed that LABs present in the flour are responsible for the lactic acid detected in the control and possibly in the rest of the samples containing Blend malt.

5.5 Remarks and future research

The initial aim of this project was to characterize the β-glucan profile and study the differences in the profile caused by the degradation by yeast and possibly link this change to an improved prebiotic effect. However, during the start of the project it became evident that determination of the β-glucan composition is complex and the focus was shifted to an indirect approach where the impact the fermented material on organic acid and SCFA production and growth of bifidobacteria was studied. The cause of the observed effects cannot be explained by this study but is of interest for future research.

The goal of this project was to get an indication of how changes in the polysaccharide composition caused by fermentation of the barley malt materials may affect its potential prebiotic properties. Based on the findings in this project the following points could be of interest for future research.

- Repeat the experiments to confirm results and possibly use sterilized malt materials to avoid growth of unwanted bacteria that could affect the results.
- Evaluate factors affecting the β-glucanase production and enzyme activity.
- Determine the β-glucan composition to study the effects of fermentation by yeast and link the potential changes in β-glucan composition with the observed increase in growth of bifidobacteria and organic acid production.

6. Conclusion

The aim of this master thesis project was to find yeast strains with high potential to modify the polysaccharide composition in malted barley and investigate if the potential prebiotic properties of barley malt materials can be enhanced by fermentation with the selected yeast strains.

In total 6 yeast strains were found to degrade β-glucan and all these strains had the ability to grow with the malted barley or BSG as only source of nutrients. However, the effect of the fermentation on the polysaccharide composition was not investigated.

Based on degree of β-glucanase activity and highest increase in cell concentration in the test fermentation experiment, two strains (*P. burtonii* TY01 and *P. kudriavzevii* TY3) were chosen to be used in further experiments with bifidobaceria. Both strains reached higher final cell concentrations in malt than in BSG, which could be expected due to the differences in nutritional content between the materials.

Indications of increased growth of bifidobacteria in media based on Cinnamon malt fermented by *P. burtonii* TY01 and *P. kudriavzevii* TY3 was observed. No increase in yeast growth was seen in unfermented malt samples.

Higher concentrations of lactic- and acetic acid was found in Cinnamon malt fermented by yeast compared to the unfermented samples and controls. In addition, samples of BSG fermented by *P. kudriavzevii* TY3 generated lactic acid production. However, the organic acid concentrations in BSG were lower than in Cinnamon malt. This result may be a reflection of the difference in β-glucan content.

In summary, the result of this study indicates that fermentation of barley malt materials by β-glucanase producing yeast strains promotes growth of bifidobacteria and the production of organic acids.

There may be a link between the β-glucan content and the prebiotic properties of the malt materials. However, further investigation is needed to establish a correlation between the potential change in β-glucan profile after fermentation and the beneficial effects observed.

7. Acknowledgements

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To Alexa, Mads, Loïc and Alexandre, my partners in crime.

To A, my love.

Lastly but most importantly I want to thank my grandparents Gudrun and Sune, and my fairy godmothers Christina, Lisbeth and Elisabeth, for constantly providing me with love, support and inspiration throughout my life. I would never have gotten this far without you.

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Appendix I - Solutions and Media

I. General solutions and media

Sterile NaCl solution (0.9%, 1l)

9g NaCl

1l Distilled water

Dissolve NaCl in distilled water.

Autoclave

Succinate buffer (1l)

6g NaOH

9.9g Succinate

1l Distilled water

HCl solution

Dissolve NaOH and Succinate in distilled water.

Adjust pH to 5.5 with HCl solution.

Autoclave

YPD plates (1l)

10g Yeast extract

20g Peptone

20g Glucose

20g Agar

1l Distilled water

Dissolve yeast extract, peptone, glucose and agar in distilled water.

Autoclave

Shake flask gently to divide the agar evenly before pouring the liquid into petri dishes.

Let the plates cool down. Turn upside down as soon as the liquid has solidified. Keep plates at room temperature for 1-2 days before use or storage. Store in fridge upside down. Let fridge stored plates reach room temperature before use.

Liquid YPD (1l)

10g Yeast extract

20g Peptone

20g Glucose

1l Distilled water

Dissolve yeast extract, peptone, and glucose in distilled water.

Autoclave

Liquid YNB (100ml)

90ml Succinate buffer

2g glucose

10ml sterile YNB 10x stock

Dissolve glucose in succinate buffer.

Autoclave

Add 10ml sterile YNB 10x stock when the mixture has cooled down.

YNB 10x stock (100ml)

6.7g YNB

100ml MilliQ

Dissolve YNB in MilliQ water in a sterile flask.

Filter sterile using a 0.2µm sterile tip into sterile falcon tube(s).

II. Beta-glucan determination

Ethanol 50% (v/v)

500ml Ethanol (99.5%)

500ml MilliQ water

Measure ethanol in measure cylinder.

Add to 1l volumetric flask.

Add MilliQ.

Let sit.

Adjust volume to 1l.

Sodium hydroxide solution (200mM)

40g Sodium hydroxide (NaOH)

1l MilliQ water

Add 1l MilliQ water to a glass flask containing a magnetic stirrer.

Add 40g NaOH, little at a time.

Sodium phosphate buffer (20mM, pH 6.5)

3.12 g (NaH2PO4.2H2O)

1l MilliQ water

Approx 25ml NaOH solution

Add 900ml MilliQ water to a volumetric flask containing a magnetic stirrer.

Add 3.12 g NaH2PO4.2H2O

Adjust pH to 6.5 with NaOH

Adjust the volume to 1l and transfer content to a glass bottle.

Stable for 2 months at 4C

Sodium acetate buffer (50mM, pH 4.0)

2.9 ml glacial acetic acid

1l MilliQ

NaOH solution

0.2g Sodium azide

In a volumetric flask with a magnetic stirrer insert 900 ml MilliQ water.

Add 2.9 ml of glacial acetic acid.

Adjust pH to 4.0 with NaOH.

Add 0.2 g Sodium azide.

Adjust the volume to 1 l and transfer the content to a glass bottle.

Stable for 2 months at 4C.

Sodium acetate buffer (200mM, pH 4.0)

11.6 g glacial acetic acid

1l MilliQ water

NaOH solution

0.2g Sodium azide

In a volumetric flask with a magnetic stirrer insert 900 ml MilliQ water.

Add 11.6 ml of glacial acetic acid.

Adjust pH to 4.0 with NaOH.

Add 0.2 g Sodium azide.

Adjust the volume to 1 l and transfer the content to a glass bottle.

Stable for 2 months at 4°C.

Lichenase

Lichenase enzyme (Bottle 1 in the kit)

Sodium phospohate buffer (20mM)

Transfer the content of the lichenase enzyme bottle to a 20ml volumetric flask

Rinse the bottle with Sodium phosphate buffer (20mM) to the flask.

Adjust the volume to 20ml with buffer.

Divide the content into eppendorf tubes.

Stable for 2 years at -20°C

β-glucosidase

Beta-glucosidase enzyme (Bottle 2 in the kit)

Sodium acetate buffer (mM)

Transfer the content of the glucosidase enzyme bottle to a 20ml volumetric flask.

Rinse the bottle with Sodium acetate buffer (xxmM)

Adjust the volume to 20ml with buffer

Divide the content into eppendorf tubes.

Stable for 2 years at -20°C

GOPOD Reagent

(Bottle 3 in the kit)

1l MilliQ

(Bottle 4 in the kit)

Transfer the content of Bottle 3 in the kit to a 1l volumetric flask.

Rinse the bottle with MilliQ water.

Adjust the volume to 1l with buffer. Use immediately.

Transfer the content to a bottle covered with aluminium foil.

Dissolve the content of Bottle 4 in this mixture. Stable 3 months at 2-5°C.

Glucose Standards

Prepare 3 eppendorf tubes as follows:

(0.5mg glucose/ml) 0.5 ml Sodium acetate buffer (mM) + 0.5 ml glucose 1mg/ml (Bottle 5)

(0.25mg glucose/ml) 0.5 ml Sodium acetate buffer (mM) + 0.5 ml glucose 0.5 mg/ml (previous solution)

(0mg glucose/ml) 0.5 ml Sodium acetate buffer (mM) + 0.5 MilliQ water

III. Screening for enzyme activity

YPD plates with 0.2% beta-glucan (125ml)

1.25g Yeast extract

2.5g Peptone

2.5g Glucose

2.5g Agar

100ml Distilled water

0.25g beta-glucan

Dissolve yeast extract, peptone, glucose and agar in 75ml distilled water.

Dissolve 0.25g of beta-glucan in 25ml distilled water in a separate bottle.

Autoclave the YPD and the glucan solutions (if mixed before autoclaving unwanted reactions between the proteins in the YPD and the gulcan may happen).

Add the glucan solution to the YPD media under sterile contidions. Gently shake to distribute agar and glucan evenly before pouring into petri dishes.

YNB plates containing 0.2% beta-glucan (125ml)

112.5 ml Succinate buffer

2.5g Agar

0.25g Beta-glucan

12.5 YNB 10x stock solution

Dissolve agar and glucan in succinate buffer.

Autoclave

Add 12.5ml YNB 10x stock solution. Gently shake to distribute agar and YNB stock evenly before pouring into petri dishes.

YE stock solution (2ml)

1g Yeast extract

2 ml MilliQ water

Dissolve yeast extract in MilliQ water in a sterile falcon tube and vortex. Filter sterile into sterile falcon tube.

IV. Impact on probiotic bacteria

TPY (1l)

10g Tryptone (Sigma-Aldrich)

5g Phytone (BD)

15g Glucose

2.5g Yeast extract (Scharlau)

1ml Tween 80 (dissolved in hot water)

0.5g Cysteine

1.5g K2HPO4

0.5g MgCl*6H2O

1000 ml Distilled water

(20g Agar)

(100-150µg/ml cycloheximid)

Dissolve tryptone, phytone, glucose, yeast extract, cysteine, K2HPO4, MgCl.6H2O (and agar if solid media is desired) in 900ml distilled water.

Add 1 ml Tween 80 to an E-flask containing 100ml of distilled water. Microwave at 750W until solution is boiling/has turned milky white. Mix with the TPY solution.

Cycloheximid can be added to avoid yeast from growing on the plates. Note that it is highly toxic and should be handled with appropriate care and equipment; gloves, in fume hood etc.

Autoclave.

If agar was added, shake flask gently before pouring the liquid into petri dishes.

TPY 3x stock w/o G (without glucose, 100ml)

3g Tryptone

1.5g Phytone

0.75g Yeast extract

0.3ml Tween 80 (dissolved in hot water)

0.15g Cysteine

0.45g K2HPO4

0.15g MgCl.6H2O

100 ml Distilled water

TPY 3x stock w/o G,T,P,YE (without glucose, tryptone, phytone, yeast extract, 100ml)

0.3ml Tween 80 (dissolved in hot water)

0.15g Cysteine

0.45g K2HPO4

0.15g MgCl.6H2O

100 ml Distilled water

MRS (Man, Rogosa and Sharpe) Ingredients g/l

10.0 Peptone

8.0 Meat extract

4.0 Yeast extract

20.0 D(+)-Glucose

2.0 Dipotassium hydrogen phosphate

5.0 Sodium acetate trihydrate

2.0 Triammonium citrate

0.2 Magnesium sulfate heptahydrate

0.05 Manganous sulfate tetrahydrate

+ 0,05% of cysteine

Skim Milk Ingredients g/100 ml

10.0 Skim Milk

0.3 Yeast extract

3.0 Lactose

100ml

Appendix II - Lab proceedings

I. β-glucan determination

- Mill the freeze-dried samples or of known moisture content.
- Turn on the water bath with magnetic stirrer at **50°C**, and the boiling water bath (90 °C).
- Add **200 mg** or **100 mg** of sample (for malt or flour, respectively) in duplicates to a glass centrifuge tube (50 mL capacity). Do the same with β-glucan control. Tap the tube to ensure that the entire sample falls to the bottom of the tube.
- Turn on the boiling water bath.

Enzymatic degradation of β-glucan:

- Take the enzymes out of the freezer. Place the magnetic stirrers in each tube.
- Add **0.2 mL of ethanol 50 %** under stirring.
- Add **4 mL Na-phosphate buffer** and stirr. On mixing, immediately place the tube in a boiling water bath and incubate for **60 sec**. Vigorously stir the mixture in the magnetic stirrer, incubate at 100°C for a further **2 min**, and stirr again.
- Incubate the tube at **50°C** and allow equilibrating for **5 min**.
- Add **0.2 mL Lichenase** and seal with parafilm. Incubate for **1 h** at **50°C** stirring continuously.
- Add **2 mL** or **5 mL** (for malt or flour, respectively) of **Na-acetate buffer** and stir. Remove the magnets and allow the tubes to equilibrate to room temperature (5min). Centrifuge at 1000 *g*, 10 min.
- Carefully and accurately dispense aliquots of **0.1** mL from each tube into the bottom of three test tubes (12 mL capacity).
- Add **0.1 mL β-glucosidase** to two of these tubes. To the third (the reaction blank), add **0.1 mL acetate buffer**. Incubate all the tubes at **50°C** for **10 min.**
- Prepare three tubes for glucose standards. In each tube add **0.2 mL** of different concentration of glucose (as previously prepared in g) Glucose Standards).
- Add **3.0 mL GOPOD** reagent to each tube and incubate for further **30 min** at **50°C**. Turn on the spectrophotometer.
- Remove the tubes from the water bath, allow the samples to equilibrate at room temperature for **5 min** and measure the absorbance **(510 nm)** within 1h.

Note: Instead of vortex use magnetic stirrer.

II. β-glucanase enzyme activity screen

Day 1 - Preculture

Add 5ml of liquid YPD or YNB media (depending on which solid media the screening plates are made of) to culture tubes.

Pick a colony from YPD plates with each yeast strain to be used in the experiment and inoculate in the liquid media.

Incubate for 24h at 30°C.

Day 2

Centrifuge the preculture tubes at 4000min-1 for 5 min.

Discard the supernatant.

Resuspend the pellet in 5ml sterile NaCl solution (0.9%).

Create a dilution series for each strain. (10x, 100x, 1000x, 1000x)

Add a 10µl droplets of each dilution to a screening plate containing 0.2% beta-glucan (4 droplets from 4 different strains for each plate).

Incubate at 30.5°C for 48h.

Day 4

Remove the cells by alternately scraping with a loop and washing with water.

Stain by flooding plates with Congo Red (0.02%) and incubate for 15 min.

Discard the dye and view result. Clear zones around colonies indicate enzyme activity.

(Congo Red is toxic and endocrine disruptive. The dying should subsequently be done in fume hood, using gloves and any waste should be collected …)

Validation

The experiment can be done by picking a colony and streak directly on the screening plate, instead of liquid pre-culture and adding droplets onto the plates.

III. Fermentation of malt material

Day 1 - Preculture

Add 5ml of liquid YPD media to culture tubes.

Pick a colony from YPD plates with each yeast strain to be used in the experiment and inoculate in the liquid YPD media.

Incubate for 24h at 30C.

Day 2 - Fermentation

Add malt flour (2%, 80g if liquid volume will be 4ml) to sterile culture tubes marked with malt variety and yeast strain, number the tubes if there are several of the same kind.

(This step can be done in advance if the tubes are kept in the freezer until use, to save time).

Centrifuge the preculture tubes at 4000min-1 for 5 min.

Discard the supernatant.

Wash the cells by resuspending the pellet in 5ml sterile NaCl solution (0.9%). Vortex before centrifuging again at 4000min-1 for 5 min.

Discard supernatant and resuspend in 5ml succinate buffer and vortex.

Measure OD of the cell solution. (Dilute the cell solution in 2 steps 10x and 100x. Use NaCl solution (0.9%) as blank. Reliable measurements should be between Abs 0.1-0.7.)

Calculate the amount of cell solution needed to reach a final OD of 1 in the malt samples.

Vof cell solution to transfer to malt solution=(OD in malt samples*Vmalt samples)/OD in cell solution

Add 4ml of succinate buffer to each malt sample.

Transfer appropriate amount of cell solution to each malt sample.

(If needed, take samples for CFU. Make a dilution series and add 10µl droplets of the 1000 and 10000 dilutions of each sample in 4 replicates to a YPD plate. Incubate CFU plates at 30C for approximately 24h, some strains may need a bit longer, before counting.)

Incubate for 48h at 30C.

(Day 3 - if needed, take samples for CFU.)

Day 4

(If needed, take samples for CFU.)

Remove samples from incubator after 48h and freeze immediately.

Store samples in freezer until use in further experiments.

IV. Impact of the fermented malt on probiotic Bifidobacteria.

Subculture

Day 1

Inoculate freeze dried bacteria or bacteria frozen in skim milk solution in liquid MRS or TYP media.

Incubate anaerobically in jar with anaerocult for 24h at 37C (36.5C).

Day 2

Switch media from MRS to TPY or simply switch to new TPY media.

- Vortex the glass tubes containing the Bifidobacteria and pour content into sterile falcon tubes.
- Centrifuge at 5000min-1 for 15min.
- Discard supernatant.
- Resuspend the pellet by transferring 1ml of TPY from the sterile TPY glass tubes to the tubes containing the bacterial pellet. Mix and add the bacteria to the TPY glass tubes.

Incubate anaerobically in jar with anaerocult for 48h at 37C (36.5C).

Growth of Bifidobacteria, fatty acid production and beta-glucan profiling

Day 4

- Thaw the fermented malt samples.
- Add 4ml of each sample to a sterile glass tube (marked with yeast strain, malt variety, type of media and Bifido strain).
- Weigh malt flour (2% = 80mg) in sterile glass tubes.
- Add 4ml of succinate buffer to the malt flour samples. These samples will be used as control.
- Add 2 ml of TYP 3x stock media *(w/o G or w/o GTPYE) to all the samples*.
- Transfer 1ml of each sample to Eppendorf tubes and freeze for HPLC analysis (and beta-glucan profile).
- Measure the OD of the Bifidobacteria.
- Add appropriate amounts of bacteria to reach a final OD of 0.5 in the samples.
- Make a dilution series for CFU analysis on TPY plates.

(Add 100µl of sample to 900ml of sterile NaCl solution (0.9%) and dilute in steps. The dilutions for the start values should be 10 to 10^4. Add 10µl droplets of the 10^3 and 10^4 dilution of each sample in duplicates on the TYP plates. Thus, a plate will have 4 columns with replicates of the 10^3 and 10^4 dilutions of the samples containing media w/o G and w/o GTP of the same yeast strain(or no yeast strain for controls), Bifido strain(or no yeast strain for some of the controls), and malt variety.)

- Put the tubes in vacuum approximately 1h to remove the oxygen in the liquid before incubation.

Incubate the samples anaerobically in jar with anaerocult for 48h at 37C (36.5C).

Incubate the CFU plates anaerobically in jar with anaerocult for 24h? at 37C (36.5C).

Day 5

Count colonies on the CFU plates.

Day 6

Add 1ml of each sample to eppendorf tubes and freeze for HPLC analysis (just supernatant, so it needs to be centrifuged first?).

Make a dilution series for CFU analysis on TPY plates.

(The dilutions for the final values should be 10, to 10^6).

Add 10µl droplets of the 10^4, 10^5, and 10^6 dilutions on the TYP plates. Thus, a plate will have 4 columns with dilutions of the samples containing media w/o G and w/o GTPYE of the same yeast strain, Bifido strain, and malt variety.

Incubate the CFU plates anaerobically in jar with anaerocult for 24h? at 37C (36.5C).

Day 7

Count colonies on the CFU plates.

V. Analysis of organic acids – HPLC

Thaw the samples and centrifuge the eppendorf tubes at 12000g? for 15 min.

Dilute the samples 5 times by transferring 100µl of the supernatant to a HPLC vial and add 900µl distilled water.

An external standard containing 100ppm of citric-, tartaric-, malic-, succinic-, lactic-, acetic- and propionic acid was used to enable identification and quantification of the peaks.

Appendix III – Additional results and figures

I. Determination of β-glucan content

Table 6. Result of Megazyme Mixed-linkage β-glucan determination. Samples; C – control, S – sample, 1-2 first run 3-4 second experimental run, a & b - replicates taken from same sample number (before addition of β-glucosidase). A; absorbance of glucose originating from the β-glucan (blank subtracted from the sample), W; weight of sample, X – volume factor, β-glucan calculated as percent of total weight, Std – standard deviation in percent (first column std between replicates of same sample, second column std between all samples in the experimental run, third column std between all samples).

Sample	**ΔA** (abs^{510nm})	**W** (mg)	**X**	**β-glucan** (%)	**Std** (%)		
C1a	0.5076	103.6	8.46	4.2	1.82	1.33	3.96
C1b	0.4947			4.1			
C2a	0.5132	102.8		4.3	1.80		
C2b	0.5003			4.2			
C3a	0.5238	102.1		4.4	1.42	1.31	
C3b	0.5344			4.5			
C4a	0.5584	101.5		4.7	5.97		
C4b	0.5132			4.3			
S1a	0.3337	200.3	5.76	0.86	2.15	6.57	9.82
S1b	0.3237			0.84			
S2a	0.3126	201.2		0.81	5.52		
S2b	0.2891			0.75			
S3a	0.3974	208.1		0.98	0.45	1.36	
S3b	0.3949			0.97			
S4a	0.3815	206.7		0.94	1.65		
S4b	0.3905			0.97			

II. Fermentation of the malt material by yeast

Background microbial levels

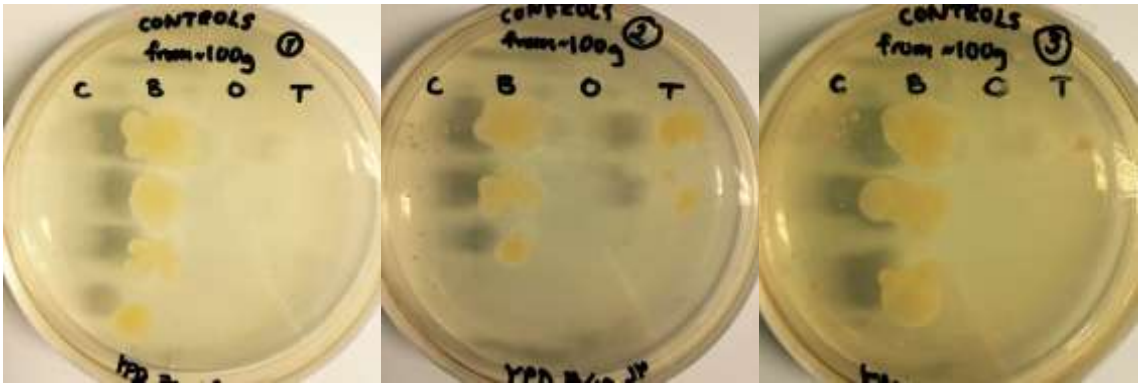

Figure 25. Backgrund microbial level assessment. Triplicates of result on YPD media. C – cinnamon malt, B – Blend, O – Ocean BSG, T – Tipple malt.

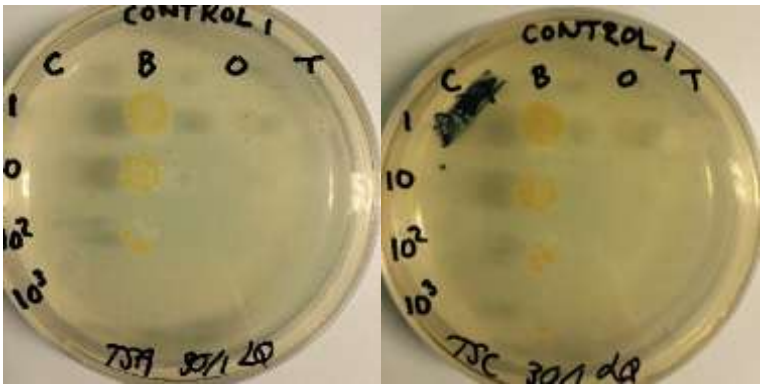

Figure 26. Backgrund microbial level assessment. Result on TSA and TSC media. C – cinnamon malt, B – Blend, O – Ocean BSG, T – Tipple malt.

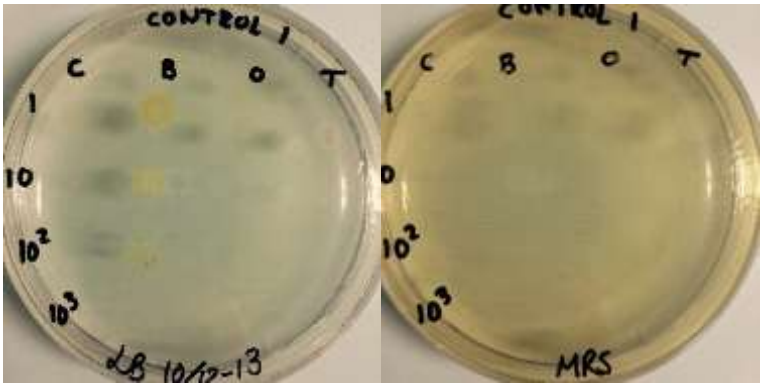

Figure 27. Backgrund microbial level assessment. Result on LB and MRS media. C – cinnamon malt, B – Blend, O – Ocean BSG, T – Tipple malt.

Test fermentation of malt by yeast

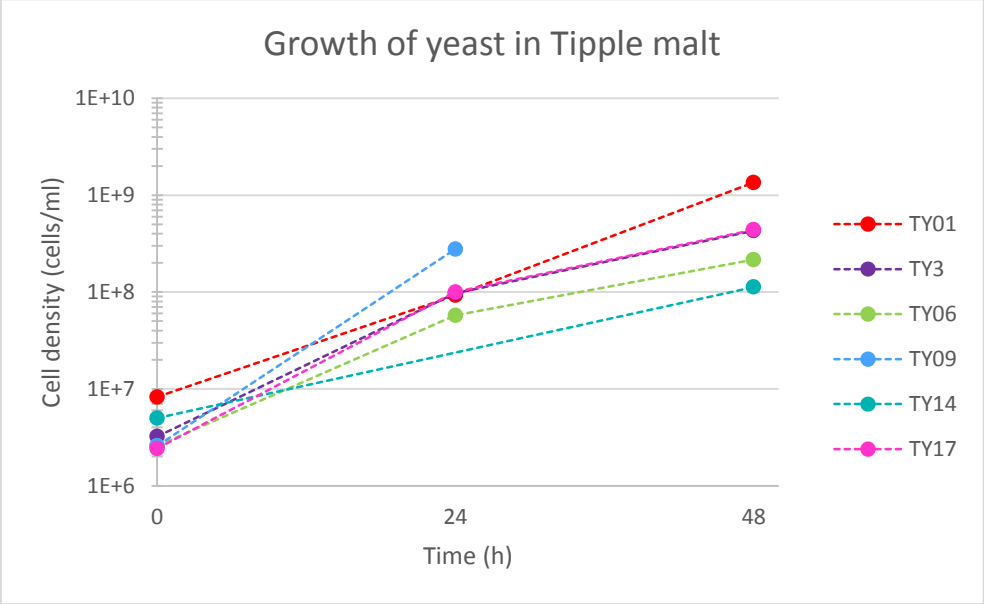

Figure 28. Growth of the different yeast strains in Tipple malt (2%). The cell density is based on CFU on YPD plates, with samples collected at time points 0h, 24h and 48h after incubation. The yeast strain were *P. burtonii* TY01 (red), *P. kudriavzevii* TY3 (dark purple), *P. anomala* TY06 (light green), *P. guilermondi* TY09 (light blue), *H. guildermondi* TY14 (turquoise), *K. marxianus* TY17 (magenta).

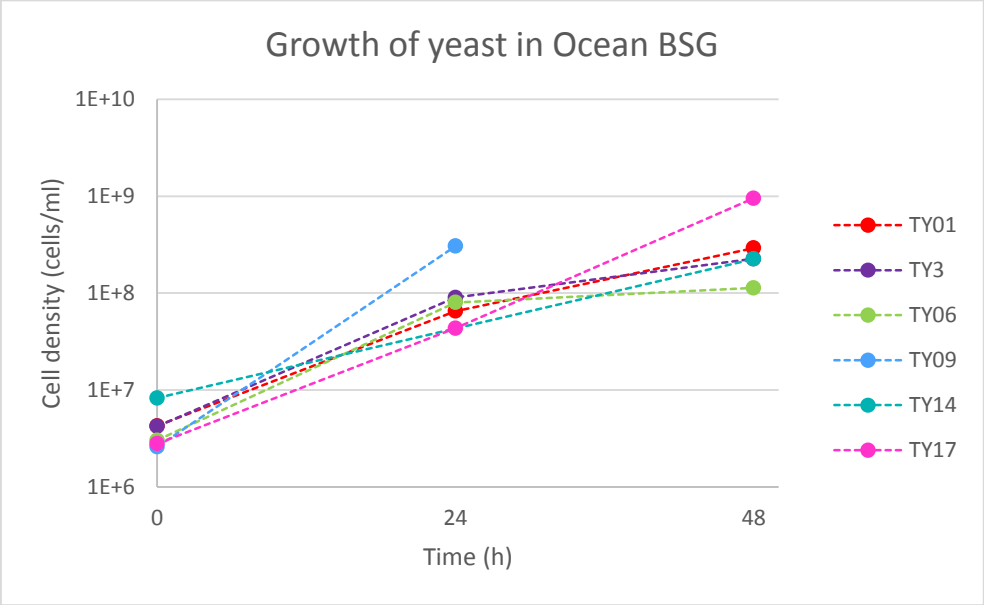

Figure 29. Growth of the different yeast strains in Ocean BSG (2%). The cell density is based on CFU on YPD plates, with samples collected at time points 0h, 24h and 48h after incubation. The yeast strain were *P. burtonii* TY01 (red), *P. kudriavzevii* TY3 (dark purple), *P. anomala* TY06 (light green), *P. guilermondi* TY09 (light blue), *H. guildermondi* TY14 (turquoise), *K. marxianus* TY17 (magenta).

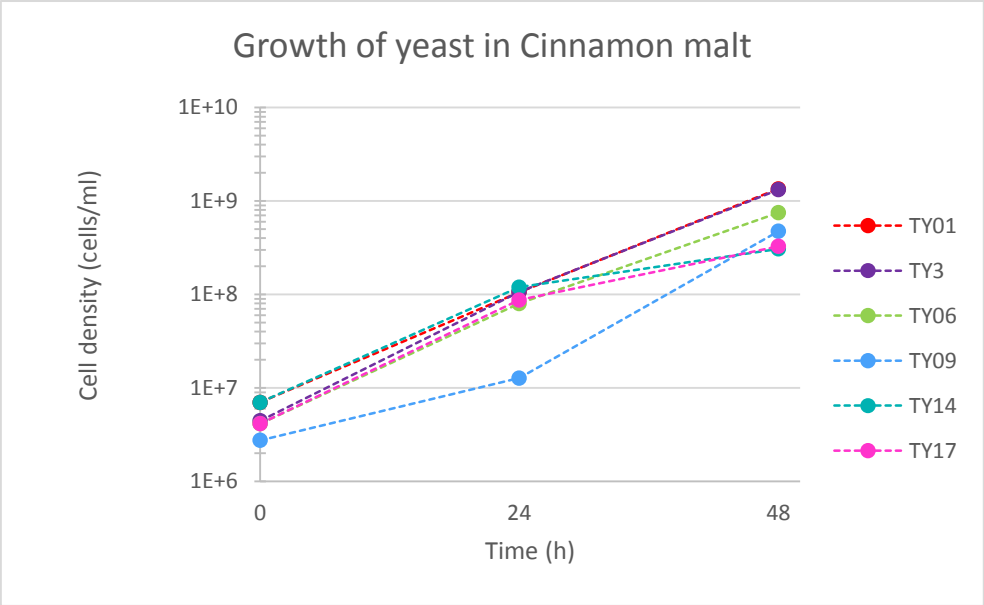

Figure 30. Growth of the different yeast strains in Cinnamon malt (2%). The cell density is based on CFU on YPD plates, with samples collected at time points 0h, 24h and 48h after incubation. The yeast strain were *P. burtonii* TY01 (red), *P. kudriavzevii* TY3 (dark purple), *P. anomala* TY06 (light green), *P. guilermondi* TY09 (light blue), *H. guildermondi* TY14 (turquoise), *K. marxianus* TY17 (magenta).

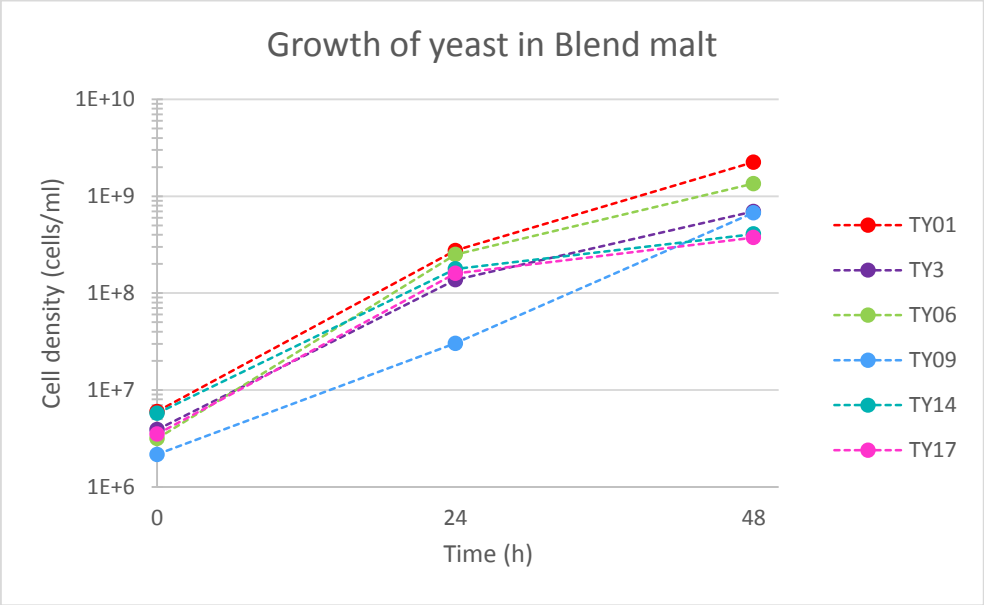

Figure 31. Growth of the different yeast strains in Blend (2%). The cell density is based on CFU on YPD plates, with samples collected at time points 0h, 24h and 48h after incubation. The yeast strain were *P. burtonii* TY01 (red), *P. kudriavzevii* TY3 (dark purple), *P. anomala* TY06 (light green), *P. guilermondi* TY09 (light blue), *H. guildermondi* TY14 (turquoise), *K. marxianus* TY17 (magenta).

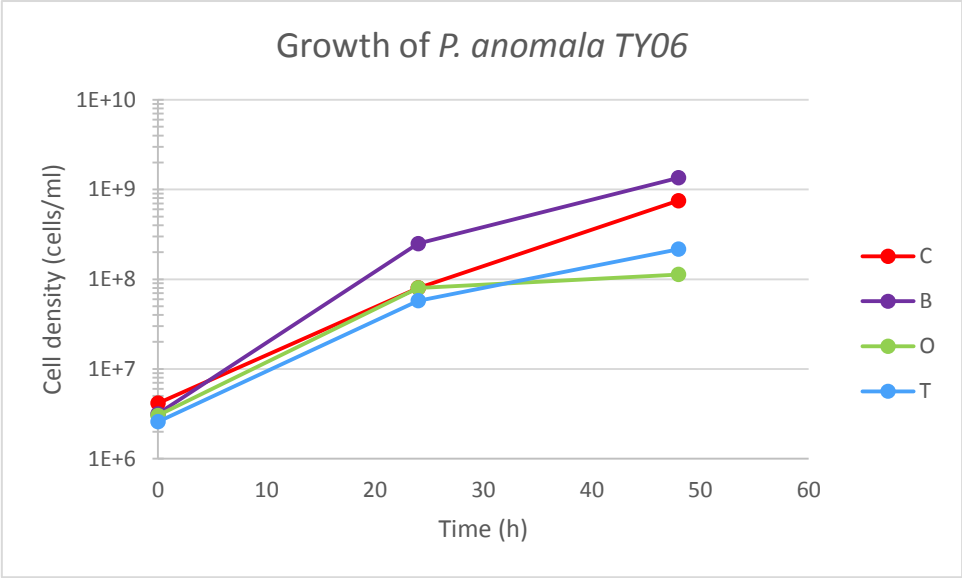

Figure 32. Growth of *P. anomala* TY06 in the different malt solutions (2%). The values of the cell densities are based on the number of CFU on solid YPD media at the start, after 24h and at the end of the incubation time.

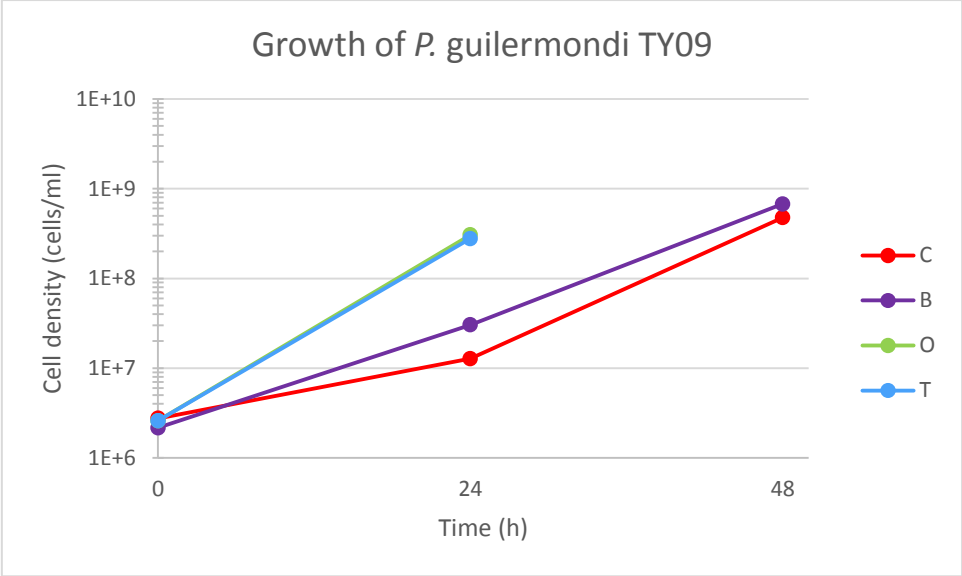

Figure 33. Growth of *P. guilermondi* TY09 in the different malt solutions (2%). The values of the cell densities are based on the number of CFU on solid YPD media at the start, after 24h and at the end of the incubation time.

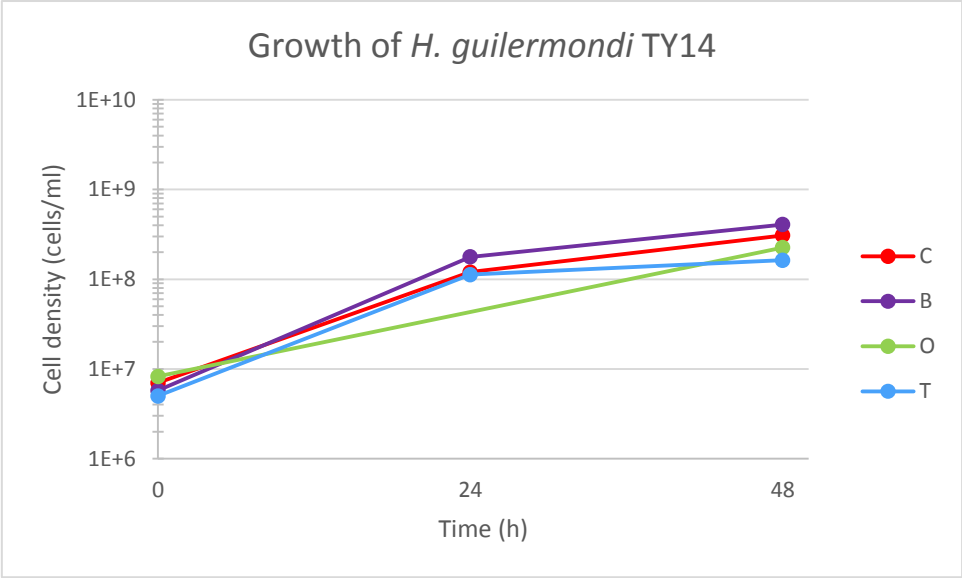

Figure 34. Growth of *H. guilermondi* TY14 in the different malt solutions (2%). The values of the cell densities are based on the number of CFU on solid YPD media at the start, after 24h and at the end of the incubation time.

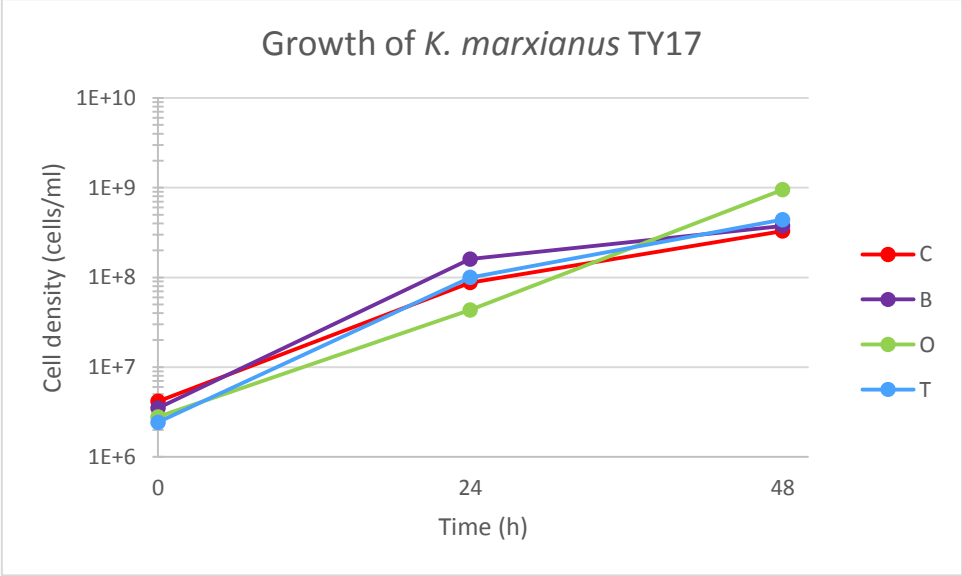

Figure 35. Growth of *K. marxianus* TY17 in the different malt solutions (2%). The values of the cell densities are based on the number of CFU on solid YPD media at the start, after 24h and at the end of the incubation time.

IV. Impact of the fermented malt material on Bifidobacteria

Growth and viability of Bifidobacteria

Table 7. Result from cell density determination by CFU for growth of Bifidobacteria in malt-based media. The samples with increased cell density after incubation are written in bold letters.

Sample (bacteria-yeast-malt-media)	**Initial cell density** (cells/ml)	**Final cell density** (cells/ml)
B1-Ty1-O-G	-	$4.25*10^6$
B1-Ty1-O-GTPYE	-	$2.25*10^5$
B1-Ty1-B-G	$3*10^7$	$3.6*10^7$
B1-Ty1-B-GTPYE	$1.6*10^7$	$3.6*10^7$
B1-Ty1-C-G	$9*10^6$	$3.2*10^8$
B1-Ty1-C-GTPYE	-	$1.6*10^8$
B1-TY3-O-G	-	$1.4*10^7$
B1-TY3-O-GTPYE	-	$1.5*10^6$
B1-TY3-B-G	$3.2*10^7$	$2*10^7$
B1-TY3-B-GTPYE	$9*10^6$	$2.9*10^7$
B1-TY3-C-G	$4*10^6$	$2.3*10^8$
B1-TY3-C-GTPYE	$3*10^6$	$9*10^7$
B1-(-)-O-G	-	-
B1-(-)-O-GTPYE	-	-
B1-(-)-B-G	-	-
B1-(-)-B-GTPYE	-	-
B1-(-)-C-G	-	-
B1-(-)-C-GTPYE	-	-
B2-Ty1-O-G	-	-
B2-Ty1-O-GTPYE	-	-
B2-Ty1-B-G	$2*10^7$	$3*10^7$
B2-Ty1-B-GTPYE	$2.4*10^7$	$1.8*10^7$
B2-Ty1-C-G	$5*10^7$	$1.6*10^7$
B2-Ty1-C-GTPYE	$2.1*10^7$	$2.75*10^8$
B2-TY3-O-G	-	-
B2-TY3-O-GTPYE	-	-
B2-TY3-B-G	$3.3*10^7$	$1.25*10^8$
B2-TY3-B-GTPYE	$1.3*10^7$	$1.8*10^6$
B2-TY3-C-G	$3.8*10^7$	$2.05*10^8$
B2-TY3-C-GTPYE	$5*10^6$	$2.65*10^6$
B2-(-)-O-G	$2.5*10^7$	-
B2-(-)-O-GTPYE	$4.8*10^7$	-
B2-(-)-B-G	$4.5*10^7$	$1.3*10^6$
B2-(-)-B-GTPYE	$4.1*10^7$	$1.2*10^6$
B2-(-)-C-G	$2.4*10^7$	$6.5*10^7$
B2-(-)-C-GTPYE	$4*10^7$	-

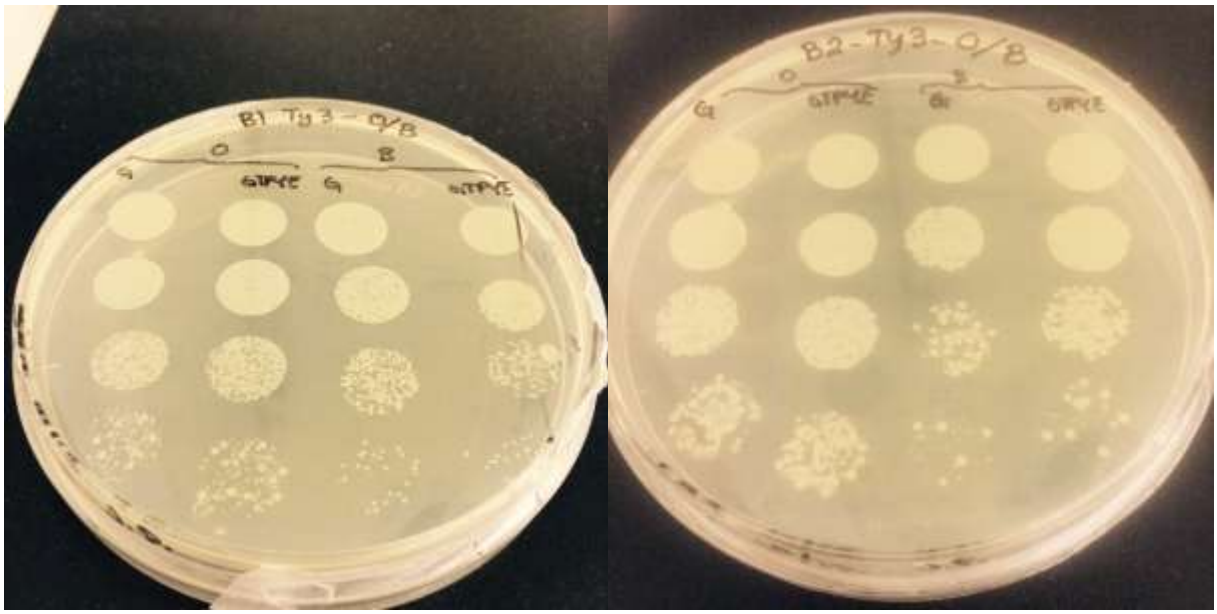

Figure 36. Example of CFU for *Bifidobacterium*. Growth of *Bifidobacterium bifidum* B1 and *Bifidobacterium breve* B2 in media mixed with 2% malt material, Ocean BSG or Blend, fermented with the yeast Pichia kudriavzevii TY3.