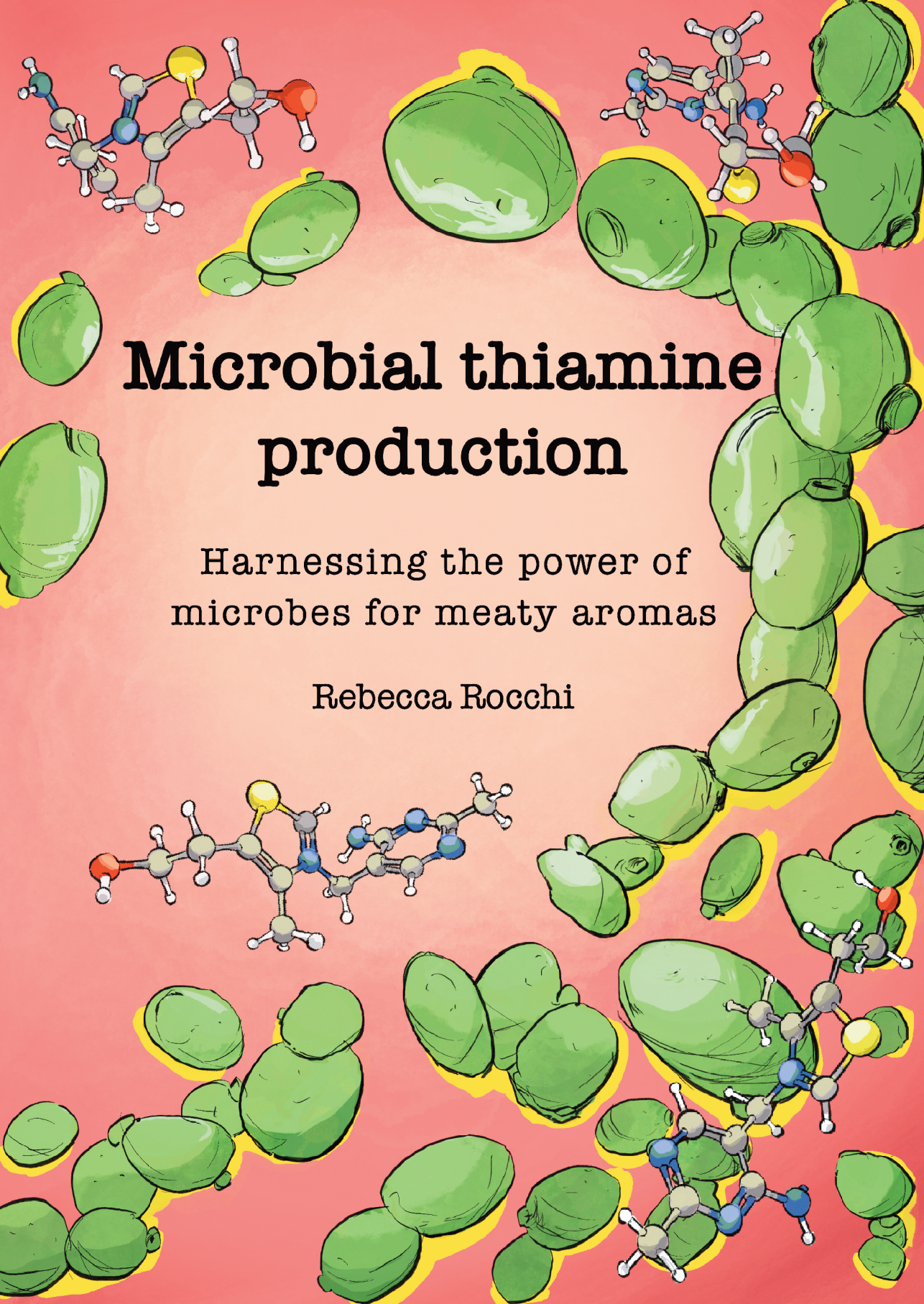

# Microbial thiamine production

## Harnessing the power of microbes for meaty aromas

Rebecca Rocchi

**Propositions**

1. Screening for natural variation in thiamine production is the most effective way to find thiamine-overproducing *Saccharomyces cerevisiae* strains in chemically-defined medium. (this thesis)

2. Enrichment of plant-based substrates with thiamine for flavour development using non-genetically modified *Saccharomyces cerevisiae* remains a challenge. (this thesis)

3. The research on the causes behind reaching a supercentenarian age is a result of human optimism.

4. Scientific collaboration drives high-quality research.

5. The definite article is wrongfully underused in scientific language.

6. Alcohol consumption is dangerously glamourised.

7. Labelling a person as an "angry feminist" reveals underlying misogyny.

Propositions belonging to the thesis, entitled
Microbial thiamine production - harnessing the power of microbes for meaty aromas

Rebecca Rocchi
Wageningen, 2 April 2024

# Microbial thiamine production

Harnessing the power of microbes for meaty aromas

**Rebecca Rocchi**

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This research was conducted under the auspices of the Graduate School VLAG Graduate School (Biobased, Biomolecular, Chemical, Food and Nutrition Sciences)

# Microbial thiamine production

## Harnessing the power of microbes for meaty aromas

**Rebecca Rocchi**

**Thesis**

submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr C. Kroeze,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 2 April 2024
at 1:30 p.m. in the Omnia Auditorium.

Rebecca Rocchi
Microbial thiamine production - Harnessing the power of microbes for meaty aromas, 180 pages.

PhD thesis, Wageningen University, Wageningen, The Netherlands (2024)
With references, with summary in English
DOI: https://doi.org/10.18174/647603

# Table of contents

# CHAPTER 1

## General introduction

Rebecca Rocchi

# 1

It's early June at lunchtime, the weather is perfect, the sun is shining, and the sound of chirping birds makes you feel that this can only be a great day to go for a walk and grab something to eat. Suddenly you smell something, *it must be the neighbours…. they are having a barbecue*, and instantly you realise you are craving something savoury, *some grilled portobello mushrooms! Maybe a pork chop? How about some grilled vegetarian sausages in a soft hot dog bun?*

Just as much as our sense of taste, our sense of smell is a powerful tool that awakes memories and stimulates our appetite, that's why flavour molecules are ingredients of key importance in food production.

## 1.1 General introduction

Thiamine is one of the most important precursors of meat aroma converted during cooking. This thesis presents the research work to investigate the potential of microbial vitamin $B_1$ (thiamine) production. Below we introduce the background of this research by presenting the current knowledge on the topic and explaining the research's relevance leading to this study's aim.

### 1.1.1 Meat consumption and the need for a protein transition

Since the last decades of the 20th century the world population has grown from approximately 3 billion people in 1961 to 7.9 billion in 2021.This increase in population, coupled with an increase in the income level (Figure 1A), has caused a rise in global meat production and consumption, which reached a historical maximum of 353 million tonnes in 2021 (Figure 1). The three main farmed animals for meat production are cattle, chickens, and pigs. While the cattle pro-capita production remained stable at around 9 kg from the 1970s till now, both the production of chicken and pig increased rapidly, going from 1.6 kg in 1961 to 15.3 kg in 2021, and from 1.6 to 15.2 kg, respectively. This overproduction of animal proteins is concerning for animal welfare, but also poses a massive burden on the environment. Indeed the greenhouse gas emissions produced by beef cattle, pork, and chicken increased by 59 %, 89 %, and 461 % from 1961 to 2010 (Caro et al., 2017). In general meat production leads to higher emissions of greenhouse gasses per energetic unit, compared to plant-based products, because of the loss of energy that occurs at each trophic level (Godfray et al., 2018). Moreover, livestock emit approximately 15 % of all anthropogenic emissions in $CO_2$ equivalents (Gerber et al., 2013), and livestock production accounts for the consumption of a third of all the freshwater used by human activities (Hoekstra et al., 2012). Because of the burden that

meat production poses on animal welfare and the environment, reducing global meatconsumption is paramount.

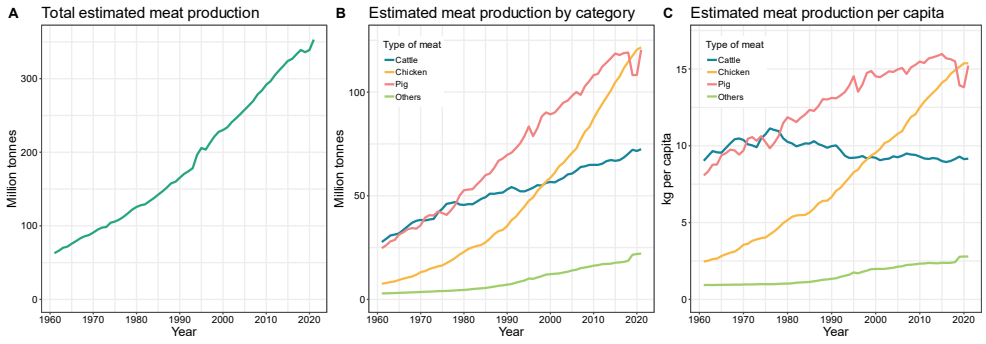


Figure 1- Data from 1961 to 2021 regarding (A) total estimated meat production, (B) estimated meat production divided by category (C), and estimated meat production pro capita.
Data retrieved in February 2023 from https://www.macrotrends.net/countries/WLD/world/population and https://www.fao.org/faostat/en/?#data. The increase in estimated meat production in the year 1995 is due to the inclusion of previously not available data on the production of turkey, rabbit, sheep, and pigeon meat.

### 1.1.2 The advent of meat replacers

A valuable strategy to reduce meat consumption is to substitute, partially or completely, the meat products consumed in our diet with plant-based meat-like alternatives that mimic the taste, texture, look, and nutritional values of their meat counterparts. Not only have the supermarket shelves been flooded by these foods, but so have restaurants. An example of these is the new range of vegan products launched in 2023 by McDonald's, based on pea proteins and co-developed by Beyond Meat (Vlietstra, 2023). These products claim to closely resemble meat in all aspects, from texture to taste to appearance, nutritional values, and flavour. One strategy to achieve such results is to incorporate functional ingredients to mimic meat qualities. An example is the Impossible burger, this meat replacer "bleeds" when raw, and turns brown upon cooking. This result is achieved by the addition of soy leghemoglobin, produced by genetically engineered *Pichia pastoris* (Voigt, 2020). Beyond delivering the desired colour change, the heme itself undergoes chemical reactions upon heating that produce meaty flavours (Devaere et al., 2022). Heme is only one of the numerous precursor molecules that contribute to the development of meat aromas upon cooking.

### 1.1.3 Vitamin $B_1$ as a meat aroma precursor

Protein, lipids, ribonucleotides, and thiamine are the main molecules involved in the complex chemical reactions that cause the formation of meat flavours during cooking.

The formation of the typical aromas derives mainly from the following processes: (i) the oxidation of lipids, (ii) the Maillard reaction, (iii) the Strecker degradation reaction of amino acids, (iv) the carbohydrate degradation reaction. Products of the Maillard reaction, can further react with lipid oxidation products or with the products of the Strecker degradation reaction (Flores, 2023). Finally (v) the thermal degradation of thiamine, also referred to as vitamin $B_{1,}$ also contributes to the development of meaty odours.

This vitamin is the precursor of many sulphur-containing compounds that have a potent meaty and roasted smell, and that are typical of roasted and cooked meat (Caplice et al., 1999; Li et al., 2022; Thomas et al., 2015). Thiamine is also known to confer umami and kokumi perceptions because of the formation of non-volatile compounds during its degradation (Brehm et al., 2019). Vitamin $B_1$ is composed of a thiazole moiety, linked by a methylene bridge to an amino-pyrimidine ring (Figure 2). The thiazole moiety of thiamine can undergo degradation in acidic environments, leading to the formation of 2-methyl-3-furanthiol, via 5-hydroxy-3-mercapto-2-pentanone (Cerny, 2015; Cerny et al., 2008). In mildly alkaline conditions, in which thiamine is less stable, the methylene bridge is broken, this leads to the formation of a pyrimidine derivative and sulfurol. Sulfurol itself has a weak odour, but other thiazole derivatives, which are formed at pH values higher than eight have a stronger smell. Such compounds are 2-methyl-3-furanthiol and 4,5-dihydro-2-methyl-3-furanthiol, 4-methyl-5-vinylthiazole, 4,5-dimethylthiazole, and 4-methylthiazole (Cerny, 2015; Guentert et al., 1990). A study conducted on solutions containing xylose, cysteine, and thiamine aiming at identifying the origin of the C-skeleton of the volatiles produced via thermal degradation, using [$^{13}C_5$]xylose, showed that thiamine itself is responsible for the formation of numerous compounds. These compounds were 3-mercapto-2-butanone, 4,5-dihydro-2-methyl-3(2 H)-furanone, 4,5-dihydro-2-methyl-3-furanthiol, and 4,5-dihydro-2-methyl-3(2 H)-thiophene, while 2-methyl-3-furanthiol and 3-mercapto-2-pentanone were originating both from thiamine and xylose when cysteine was present (Cerny, 2007). Since all these thiamine degradation products have a typical savoury, roasted, and meaty odour, thiamine itself is used in food production as a precursor of meat aroma either when producing meat flavours (Giacino,1968; Van Delft et al., 1978; Fraser et al., 2017), or directly in food products that will undergo a thermal treatment (Milani et al., 2022). Currently, thiamine is chemically synthesised, rather than produced via biological means, although many micro-organisms such as bacteria, moulds, and yeasts can produce it.

### 1.1.4 Food fermentation: trends and potential

Food fermentation is an ancient practice that relies on microbial life to induce desirable changes in a food substrate. These changes can affect the aroma, nutrient composition, texture, colour, and taste of a food product. Numerous fermented products are incorporated into the daily diet of people all over the world (Tamang et al., 2020). While bread, cheese, wine, and beer are prime examples of food products consumed in most Western diets, soy sauce, natto, tempeh and kimchi are common food products widely consumed in Asia. Injera pancakes, made with spontaneously fermented teff flour are a symbol of Ethiopian, Eritrean, and Sudan cuisine. This past summer many TikTok videos went viral showing how to make homemade tepache, a Mexican refreshing beverage obtained by spontaneous fermentation of pineapple peels. Nowadays, fermentation is becoming increasingly popular, and the interest towards fermented food products sees an increased trend (Figure 3), indicating a strong interest among consumers and higher acceptance.

During fermentation, the consumption of sugars leads to the production of organic acids and alcohol. This is the most classical example of how fermentation affects the chemical composition of foods. Plenty of volatile compounds are also developed during fermentation, for instance, yeasts are well known to produce numerous esters, and

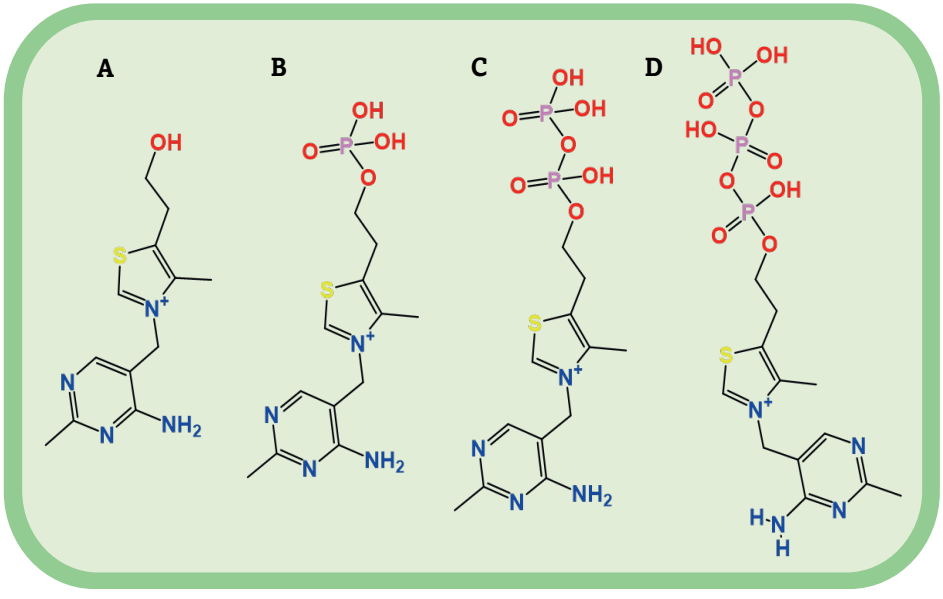


Figure 2- Molecular structure of thiamine (A), thiamine phosphate (B), thiamine pyrophosphate (C), and thiamine triphosphate (D).

lactones with pleasant fruity smells (Park et al., 2009; Romero-Guido et al., 2011). Moreover, there are plenty of examples of vitamin enrichment of foods via fermentation. An interesting example is the production of tempeh with a specific starter culture of *Rhizopus oryzae* and *Propionibacterium freudenreichii*, which leads to the production of almost 1 mg of vitamin $B_{12}$ in tempeh made with lupin beans, which is in the same order of magnitude of beef (Czerwonka et al., 2014). Another example of vitamin fortification of a food product via fermentation is natto. This sticky, slimy, product obtained by fermentation of soybeans with *Bacillus subtilis* contains a remarkably high content of vitamin $K_2$ that is produced during fermentation, making natto one of the richest sources of this vitamin (Kaneki et al., 2001).

### 1.1.5 *Saccharomyces cerevisiae*, an all-time favourite in biotechnology

*Saccharomyces cerevisiae* is a unicellular fungus and is one of the most exploited microorganisms used in food production and industrial fermentations. This mighty microbe earned the popular nickname of ”brewer’s yeast” or “baker’s yeast,” as it is the best friend of brewers and bakers all around the world. *S. cerevisiae* has a round, ovoid-like shape (as you might have noticed from the drawing of *S. cerevisiae* in this thesis’s cover), and has a diameter of about 5-10 µm. Its genome consists of approximately 6470 genes, for a total of 12 Mb, organised in 16 chromosomes ("*Saccharomyces cerevisiae*"). It was the first eukaryotic organism to be completely sequenced, after a collective, decade-long, worldwide effort (Goffeau et al., 1996). *S. cerevisiae* can grow in the absence of oxygen, in a broad range of temperatures (close to 0°C up to 45°C), at pH levels between 3 to 8 (Goddard, 2008; Serrano et al., 2006). For this reason, *S. cerevisiae* occupies numerous environmental niches, from fruits to soil, to seawater, to the guts of different animals (Goddard et al., 2015). *S. cerevisiae* has simple nutritional requirements, indeed chemically defined synthetic media (CDM) are often used to grow *S. cerevisiae* in laboratory settings. These media usually only contain salts, vitamins, small amounts of selected amino-acids, and inorganic nitrogen, such ammonium, as main nitrogen source (Perli, Moonen, et al., 2020). As *S. cerevisiae* can produce every amino acid, it is common to omit them from the CDM. The same applies to selected vitamins, although usually their omission causes a growth delay in *S. cerevisiae*, this effect, together with vitamin auxotrophy, is strain dependent.

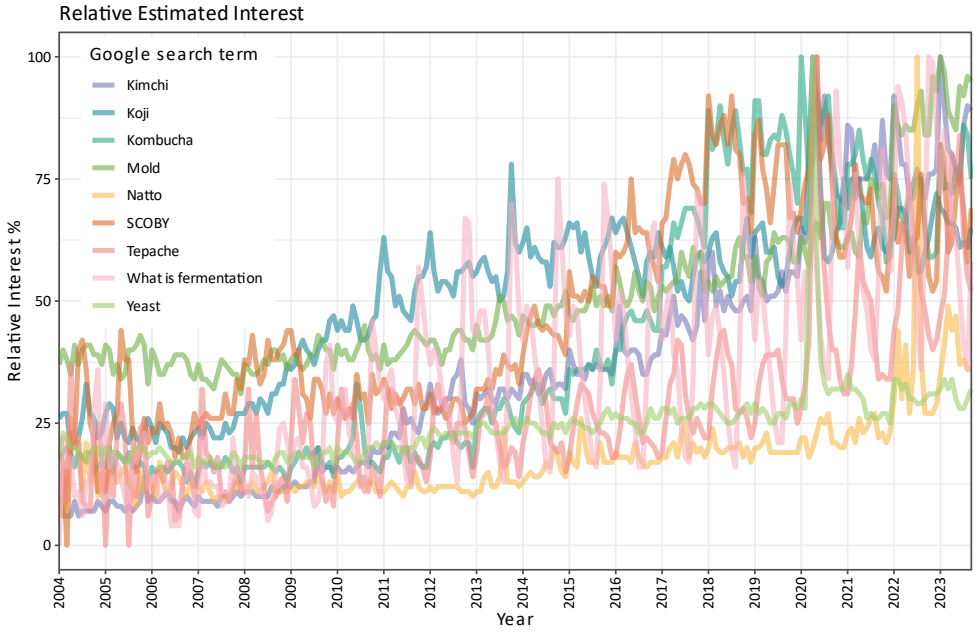


Figure 3- Relative estimated interest for different Google search terms of fermented food products. The y-axis represent search interest relative to the highest point of the graph for the region and period indicated. The value 100 indicates the highest search frequency for the term, 50 indicates half of the searches. Data retrieved from https://trends.google.com/trends/explore?geo=NL&hl=it in September 2023 regarding the search terms indicated on the legend.

### 1.1.6 Vitamin $B_1$ metabolism in *Saccharomyces cerevisiae*

Thiamine pyrophosphate (TPP) is the biologically active form of thiamine, it has a central role in the metabolism of *S. cerevisiae*. Its co-factor activity aids the cleavage of C-H and C-C bonds during enzymatic reactions (Figure 4, Table 1) (Frank et al., 2007; Hohmann et al., 1998; Perli, Wronska, et al., 2020). Starting from the metabolism of pyruvate via respiration or alcoholic fermentation, both pyruvate dehydrogenase and pyruvate decarboxylase are thiamine-dependent enzymes (Hohmann, 1991; Hohmann et al., 1990; Hohmann & Meacock, 1998; Muller et al., 1999; Pronk et al., 2002; Steensma et al., 1990). Moreover, the minor isoform of pyruvate decarboxylase Pdc6 can also decarboxylate 2-keto-3-methyl-valerate, indolepyruvate, and other 2-oxo acids, therefore contributing to the degradation of isoleucine, phenylalanine, tryptophan, and valine (Dickinson et al., 2003). Two molecules of pyruvate are converted to acetolactate by the TPP-dependent acetolactate synthase (Falco et al., 1985; Pohl et al., 2004), this is the first metabolic reaction towards the biosynthesis of isoleucine, valine, and consequently leucine. The enzyme 2-oxoglutarate dehydrogenase catalyses the

oxidative decarboxylation of α-ketoglutarate, starting from succinyl-CoA, in the tricarboxylic acid cycle (TCA) (Repetto et al., 1989). Transketolase, which is also TPP-dependent, has a bridging role between carbohydrate metabolism, and the biosynthesis of aromatic amino acids (Kochetov et al., 2014). Phenylpyruvate decarboxylase, which constitutes the first step of the Ehrlich pathway, is involved in the catabolism of aromatic, branched-chain amino acids, and methionine (Kneen et al., 2011). A putative 2-hydroxy acyl-CoA lyase, Pxp1, also shows striking structural similarities to thiamine pyrophosphate-binding proteins (Nötzel et al., 2016), therefore its function is possibly TPP-dependent. Finally, Thi3, a thiamine biosynthesis regulatory protein, forms a complex upstream the thiamine biosynthetic genes, with Thi2 and Pdc2. This complex induces transcription of the thiamine biosynthetic genes. When intracellular levels of TPP are high, Thi3 binds to TPP, therefore, the complex formed between Thi2,3 and Pdc2 is disrupted, and the transcription of THI genes is blocked.

Table 2- Thiamine pyrophosphate requiring proteins. Table adapted from Perli, Wronska, et al. (2020)

| Protein | Protein name |
|---|---|
| Pdc1 | Pyruvate decarboxylase isozyme 1 |
| Pdc5 | Pyruvate decarboxylase isozyme 2 |
| Pdc6 | Pyruvate decarboxylase isozyme 3 |
| Pda1 | Pyruvate dehydrogenase E1 component subunit alpha |
| Pdb1 | Pyruvate dehydrogenase E1 component subunit beta |
| Ilv2 | Acetolactate synthase catalytic subunit |
| Kgd1 | 2-oxoglutarate dehydrogenase |
| Aro10 | Phenylpyruvate decarboxylase |
| Tkl1 | Transketolase 1 |
| Tkl2 | Transketolase 2 |
| Pxp1 | Putative 2-hydroxyacyl-CoA lyase (?) |
| Thi3 | Thiamine metabolism regulatory protein |

In rich medium, where thiamine and nutrients are not limiting, both the sirtuins ($NAD^+$ dependent proteins with histone deacetylase activity) Hst1, and to a lesser extent Sir2, as well as high $NAD^+$ levels, repress the expression of certain thiamine biosynthetic genes, such as Thi4 (Li et al., 2010). This causes a repression at the basal level. But in thiamine limiting conditions, the Thi2, Thi3, and Pdc2 complex binds and start site of thiamine biosynthetic genes, and initiate transcription. This overrules the basal repressive effects of the sirtuins, that bind higher upstream, compared to Thi2, Thi3 and Pdc2. Thiamine regulation, therefore, is mainly under control of Thi2, Thi3, and Pdc2.

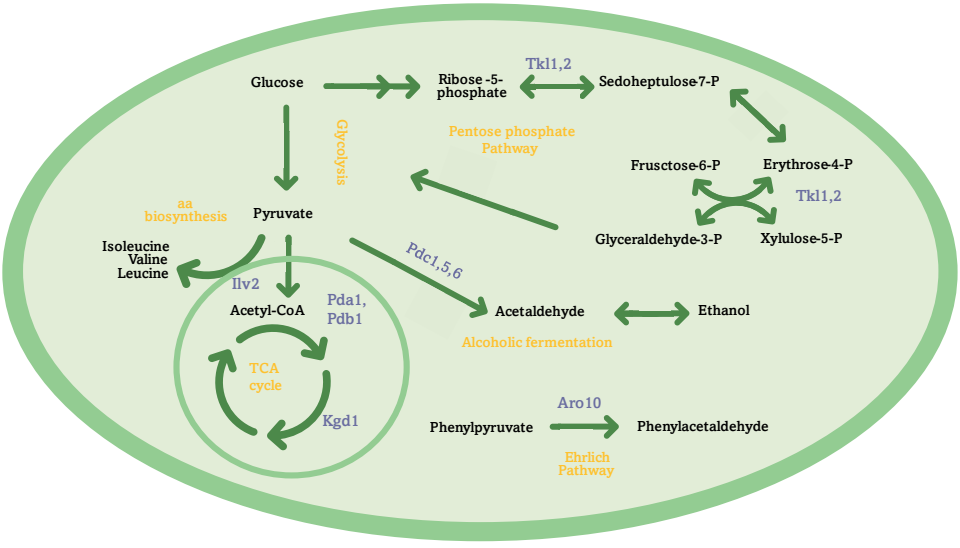


Figure 4- Simplified overview of the metabolic pathways (yellow) with TPP-dependent enzymes (purple). The mithocondrion is represented by a darker greencircle.

When thiamine is not present in the growth substrate, de novo biosynthesis is induced (Figure 5). The thiazole subunit (HET-P) of thiamine is produced from glycine, $NAD^+$, and Thi4 (Figure 2). Thi4 is a suicide-enzyme, meaning that it undergoes a single catalytic turnover, by donating a sulfur atom from a cysteine residue (Cys 205) (Chatterjee et al., 2011). The aminopyrimidine ring (HMP-P) is formed from pyridoxal-5- phosphate (vitamin $B_6$), and L-histidine (Coquille et al., 2012), which is donated by the active site of either Thi5, Thi11, Thi12 or Thi13. These are proteins with high similarity, that are located respectively in different chromosomes (Wightman et al., 2003). They have marginally different regulations giving different responses during nutrient limiting conditions (Wightman & Meacock, 2003). HMP-P is further phosphorylated by Thi20 or Thi21 to form HMP-PP (Haas et al., 2005). Thi20 and Thi21 are trifunctional proteins that are also involved in thiamine salvage (Onozuka et al., 2008). Indeed, Thi20 and 21 have thiaminase II activity, cleaving the methylene bridge between the two thiamine subunits, and HMP kinase activity (French et al., 2011; Haas et al., 2005). Once the HMP-PP and HET-P are produced, Thi6 forms a methylene bridge between the two molecules, and thiamine phosphate (TP) is formed. While most bacteria can directly phosphorylate TP to TPP (Begley et al., 1999), *S. cerevisiae* firstly removes the phosphate group of TP via an acid phosphatase (EC 3.1.3.2), to then forms the biologically active form TPP in a single step, carried out by Thi80 (Nosaka et al., 1993). Thiamine triphosphate (TTP) is a vitamer of thiamine that is known to

accumulate in *Escherichia coli* in response to amino acid starvation or hypoxia. Its presence and concentration in yeasts has been previously reported to be extremely low, its biological function is still unknown (Lakaye et al., 2004; Makarchikov et al., 2003). In the presence of thiamine in the growth substrate, *S. cerevisiae* does not synthesize thiamine de novo but uptakes it via the ATP-dependent transporter Thi10, also known as Thi7 (Figure 2) (Enjo et al., 1997). Thiamine phosphate esters are not substrates of Thi10. Therefore, a periplasmic acid phosphatase (Pho3), removes the phosphates groups to produce unphosphorylated thiamine (T) in the periplasmic space. In this way thiamine phosphate esters can also be salvaged from the growth medium.

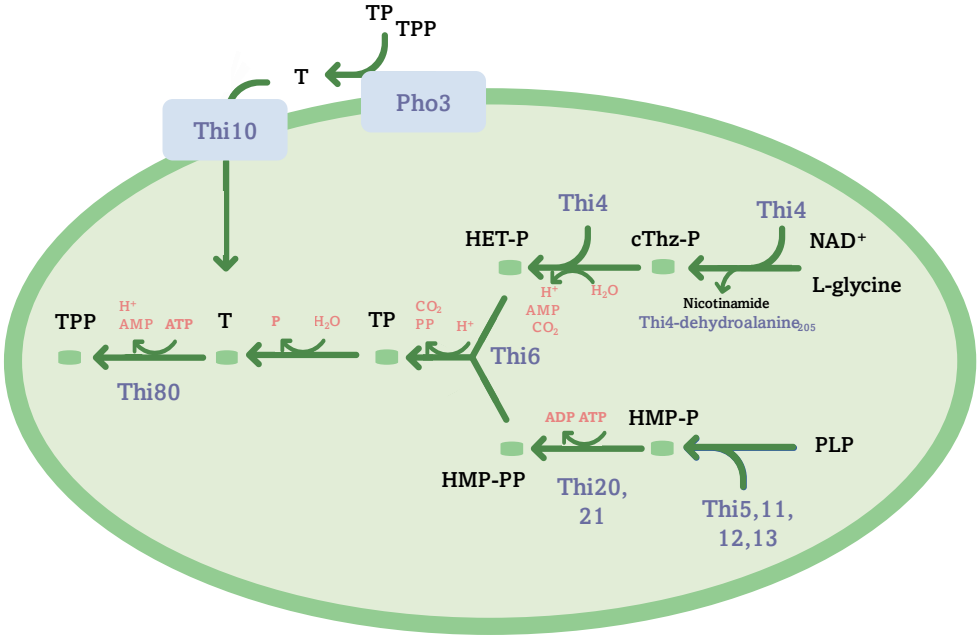


Figure 5- Schematic overview of thiamine transport and metabolism, thiamine biosynthetic or thiamine transport proteins are coloured in purple, Thi4, Thi5,11,12,13 are single-turnover enzymes.

## 1.2 Aim and outline of this thesis

This research focused on exploring the potential of fermentation and microbial production of vitamin $B_1$ to be applied as a novel, sustainable mean for the enrichment of plant-based substrates with meat-like flavours. The requirements of our framework stipulated that the strains created in this research could not generate genetically modified organisms (GMOs), in accordance with the definitions set in the Directive 2001/18/EC of the European Parliament and the Council, of March 12, 2001.

To explore the potential of micro-organisms, specifically *Saccharomyces cerevisiae*, and less extensively, *Bacillus subtilis*, we first developed a novel method to extract thiamine from fresh yeast biomass (**Chapter 2**). The extraction method relies on the use of beads for mechanical cell disruption. Furthermore, we developed a novel detection method, based on pre-column derivatization of thiamine and thiamine-phosphate esters to the fluorescent products thiochrome and phosphates. The fluorescent products are then quantified via HPLC. The combination of our extraction and detection methods is a great tool for the measurement of thiamine in biological samples, which are characterised by low concentrations of the vitamin and low purity of the extracts while allowing the distinct determination of each single vitamer.

Consequently, this sensitive and specific method was applied to screen the potential of thiamine production in 48 *S. cerevisiae* strains from different origins of isolation (**Chapter 3**). This screening carried was out in a simple chemically defined, amino acids free, and thiamine free medium, aiming at identifying natural thiamine overproducers. Moreover, this chapter also shows the effectiveness of adaptive laboratory evolution (ALE) in evolving thiamine overproducing strains, as in the screening we included 3 strains evolved for 300 generations in the thiamine-free medium (Dragosits et al., 2013).

We followed up this work by selecting from our screening work a high-thiamine producing, and a low-thiamine producing strain (referred to as wild-types, WT) and evolved them (adaptive laboratory evolution, ALE) in a thiamine-free medium for at least 370 generations to study the impact of adaptation on thiamine production and physiology. We screened the evolved variants for their thiamine-production phenotype and measured their growth rate as an indication of fitness. We also did whole genome sequencing of all the isolated variants to identify the mutations that occurred during evolution. We then selected two variants with the most interesting phenotype and analysed their proteome in thiamine-free medium in comparison to their relative WT (**Chapter 4**).

The last chapter (**Chapter 5**) serves as a proof of concept to show the significant impact that the selection of a specific strain-substrate combination has on the final quality of a food product, and how micro-organisms can effectively enrich food substrates in vitamins. To do so, we studied the production of natto, a protein and vitamin rich food product well known for its intense umami flavour. We investigated the effect of different strains and different substrates on natto quality attributes, with particular attention to the aroma formation and vitamin production during fermentation.

To conclude this research work we present a critical general discussion on the prospects of meat-flavour production via fermentation, focusing on its potentials, and limitations, and offering an outlook on the prospects of the use of fermentation as a tool to produce meat-like flavours (**Chapter 6**).

*Enjoy the reading!*

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# CHAPTER 2

## A simple, sensitive, and specific method for the extraction and determination of thiamine and thiamine phosphate esters in fresh yeast biomass

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Published in the Journal of Microbiological Methods, Volume 201, October 2022,

DOI: https://doi.org/10.1016/j.mimet.2022.106561

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## Abstract

Thiamine is an essential vitamin for most living organisms, of which yeasts are a rich nutritional source. In this study we developed a thiamine extraction and determination method to detect thiamine in fresh yeast biomass. The thiamine determination method combines the derivatization of thiamine to a highly fluorescent product, with chromatographic separation (HPLC) and fluorescence detection. The method specifically detects free thiamine (T), thiamine phosphate (TP), and thiamine pyrophosphate (TPP). It has a high sensitivity of 2 ng/ml for TPP and TP, and 1 ng/ml for T, excellent instrumental repeatability, and low day-to-day variation in retention time of the different phosphate forms. We demonstrated the robustness of the method by proving that the fluorescence signals of the derivatised samples are stable for at least 82 h after derivatization, and by showing that the final pH of the samples does not influence the fluorescent response. In addition, we developed and validated a thiamine extraction method consisting of beads beating the fresh yeast biomass in 0.1 M HCl using a lysing matrix composed of 0.1 mm silica spheres. The performance of this method was compared to extraction via heat treatment at 95°C for 30 min, and a combination of beads beating and heat treatment carried out in different order. We demonstrated that thiamine extraction via beads beating is the only method that prevents the biologically active form thiamine pyrophosphate to be degraded to thiamine phosphate, therefore, the extraction method developed and described in this study is preferred when the different thiamine vitamers need to be detected in their actual proportions. The combination of the extraction via beads beating, the conversion of all vitamers to the thiochrome derivatives, and the separation of these compounds on the HPLC with a fluorescence detector, yielded a sensitive, specific, repeatable, and robust method for extraction and determination of vitamin $B_1$ in fresh yeast biomass.

## 2.1 Introduction

Thiamine pyrophosphate (TPP) is the biologically active form of thiamine (vitamin $B_1$), it is an important co-factor of different enzymes that catalyse metabolic reactions occurring in living organisms. Yeasts are a well-known rich source of vitamin $B_1$ (Tylicki et al., 2017). They can salvage thiamine from the environment and synthesize it de novo (Fitzpatrick et al., 2014). In yeast species like *Saccharomyces cerevisiae*, thiamine pyrophosphate is known to be a co-factor of enzymes active in the pentose phosphate pathway, the citric acid cycle, and the metabolism of amino acids (Hohmann et al., 1998). Although TPP represents the major thiamine form detected in yeast cell extracts, vitamin $B_1$ is also found in its unphosphorylated form (T), and in the mono and tri-phosphorylated forms, namely thiamine phosphate (TP) and thiamine triphosphate (TTP), the latter being found in very low concentrations (Perli et al., 2020).

Thiamine is stable at low pH, but it easily degrades once exposed to alkaline pH (Voelker et al., 2021). For this reason, thiamine is often extracted through acid hydrolysis with either hydrochloric, sulfuric, or trichloroacetic acid (TCA) (Edwards et al., 2017). Moreover, as it is a co-factor, this vitamin has a non-selective protein-binding capacity (Edwards et al., 2017), therefore, to aid the acid hydrolysis, extraction is usually carried out at high temperatures with samples that are either heat-treated at around 80-100°C, or autoclaved to reach even higher temperatures. When thiamine extracts are heat-treated, degradation of thiamine diphosphate to thiamine monophosphate can occur (data shown in this paper), therefore, it is not possible to use heat treatment for extraction when the concentrations of all $B_1$ vitamers need to be determined specifically.

Following the extraction, vitamin $B_1$ can be quantified using different assays. Microbiological assays measure thiamine indirectly as a function of microbial growth. In general, these methods are very sensitive but laborious and time-consuming. Moreover, the most commonly used microbiological assays are not specific for the different vitamers of thiamine, although the use of specific auxotrophic strains can be used to detect specific phosphates forms and precursors (Strobbe et al., 2022). Standard HPLC-UV methods are used instead to measure thiamine directly, in addition these methods have high specificity but lower sensitivity. A sensitive and specific method is obtained by combining the derivatization of thiamine and thiamine phosphates to the highly fluorescent thiochrome derivatives, with liquid chromatographic separation (HPLC) of the phosphate esters and finally detection of fluorescence. Potentially such a method, in combination with an extraction procedure that preserves the thiamine phosphate forms from degradation, could be applied to yeast biomass extracts with low purity and low concentration of vitamin $B_1$ vitamers for their specific detection.

In this paper, we describe an optimised method for the extraction and quantification of thiamine, thiamine phosphate, and thiamine pyrophosphate in fresh yeast biomass. The extraction method is based on the use of silica beads for cell disruption in the presence of hydrochloric acid. The method for the determination of thiamine in the extracts is based on the pre-column derivatization of thiamine and phosphates esters to the fluorescent thiochrome products as described by Gerrits et al. (1997), using potassium hexacyanoferrate as an oxidizing reagent in alkaline conditions, followed by pH adjustment of the extracts. This derivatization method was combined with liquid chromatographic separation (reversed-phase HPLC) and fluorescence detection as described by Sander et al. (1991). By combining the extraction and determination procedures mentioned above, we developed a fast, reproducible, sensitive, and specific method for detection and quantification of thiamine and phosphorylated thiamine forms.

## 2.2 Materials and Methods

### 2.2.1 Materials and chemicals

Thiamine pyrophosphate, thiamine monophosphate chloride dihydrate, thiamine hydrochloride, hydrochloric acid (HCl), potassium hexacyanoferrate, and glycerol were purchased from Sigma Aldrich (St. Louis, MO, USA). Yeast nitrogen base was purchased from United States Biologicals (Salem, MA, USA). Pore size 0.2 µm sterile polyethersulfone filters for microbiological media sterilization, and pore size 0.2 µm sterile polyethersulfone filters with 13 mm diameter for filtration of yeast extract were purchased from Thermo Fischer Scientific (Walthman, MA, USA). Membrane filters with a pore size of 0.2 µm used for yeast biomass determination were purchased from Pall Corporation (Ann Arbor, MI, USA). Lysing matrix tubes and Fast Prep were purchased from MP Biomedicals (Irvine, CA, USA). HPLC vials, 0.3 ml TPX Crimp/Snap Vial 32 x 11.6 mm (clear), and Snap Ring Cap (blue) with X-slitted Septa Silicone/PTFE caps were purchased from BGB Analytik (Harderwijk, NL). Agar bacteriological was from OXOID (Wesel, Germany). Glucose was purchased from Merck Millipore (Burlington, MA, USA). Methanol was purchased from Actu-All Chemicals (Oss, NL). Dipotassium phosphate ($K_2HPO_4$) was purchased from VWR (Radnor, Pennsylvania, USA).

### 2.2.2 Yeast strains and media

The *Saccharomyces cerevisiae* 540 used in this study is property of the Food Microbiology Chairgroup culture collection at Wageningen University & Research and was isolated from rice wine in Vietnam. The yeast strain was preserved in 30 % (vol./vol.) glycerol and stored at - 80°C. The liquid growth thiamine free medium minimal medium (MM

medium) used in this study was composed of 20 g/l of glucose, 5 g/l ammonium sulphate, and 1.71 g/l of Yeast Nitrogen Base w/o ammonium sulphate and thiamine, and filter sterilised using a sterile 0.2 µm polyethersulfone filter (PES). For MM agar plates 20 g of glucose and 15 g of Agar Bacteriological were added to 800 ml of demineralised water and autoclaved at 121°C for 15 minutes, the solution was cooled to 50°C in a water bath and a solution containing 1.71 g of YNB in 200 ml of demineralised water was filter sterilised ad added to the mixture.



### 2.2.3 Yeast growth

Yeast culture preserved in glycerol and stored at - 80°C was streaked in MM plates and incubated for 72 h at 30°C. One colony was transferred to an Erlenmeyer flask containing MM medium and cultivated for 24 or 72 or 90 h at 30°C 180 rpm. The cell cultures were used for thiamine extraction. The specific incubation times are indicated in the figure's caption.

### 2.2.4 Determination of biomass

Cell dry weight (DW) measurement was used to establish yeast cell biomass. Membrane filters with a pore size of 0.2 µm were dried for at least 24 h and weighted, then used to filter a defined volume of yeast cell culture using a vacuum pump. After filtration of the yeast culture the filters were dried at 80°C for 24 or 48 h and weighted using an analytical balance to determine the DW.

### 2.2.5 Extraction of thiamine

In this study we first optimized a thiamine extraction method based on the mechanical cell disruption via beads beating. The influence of the lysing matrix composition on the extraction yield and vitamer percentage was tested using 3 different beads beating matrices from MP Biomedicals: (i) lysing matrix B composed of 0.1 mm silica spheres, (ii) lysing matrix C composed of 1 mm silica spheres and (iii) lysing matrix E composed of 1.4 mm ceramic spheres, 0.1 mm silica spheres, and one 4 mm glass bead. Subsequently, we tested the influence of the number of beads beating rounds on the total extraction yield and vitamer distribution percentage, ranging from a minimum of 3 rounds, to a maximum of 12. Afterwards, we compared the optimised thiamine extraction method via beads beating to hot acid hydrolysis, and a combination of these carried out in different orders.
Aliquots of yeast cultures were centrifuged at 10,000 x g for 10 minutes, the supernatant was discarded, and yeast pellets were resuspended in the same amount of 0.1 M HCl used for extraction. For the beads beating extraction 1.5 ml of liquid yeast suspension

was pipetted into the lysing matrix tubes and extracted with Fast Prep 5.5 m/s for 1 minute followed by three other rounds separated by 1-minute intervals. For the hot acid hydrolysis extraction, the yeast suspension was heat-treated at 95°C for 30 minutes and then cooled on ice until it reached room temperature. The combined extraction via a beads beating and hot acid hydrolysis was carried out by performing the two methods described above together. Extracts obtained by beads beating were centrifuged at 10,000 x g for 5 minutes and filtered through a sterile 13 mm 0.2 µm PES filter, then stored at 4°C before being derivatised. To assess the degradation of TPP to TP during hot acid hydrolysis, the cells were first extracted by beads beating, then the extracts were centrifuged at 10,000 x g for 5 minutes, and filtered through a 0.2 µm PES filter, to be then heat treated at 95°C for 30 minutes, subsequently cooled down in ice until they reached room temperature, and finally filtered again as described above. Samples were stored at 4°C before the derivatization.
In this manuscript we refer to beads beating as BB, to hot hydrolysis as HH, to the process of beads beating followed by hot hydrolysis as BB+HH, to the process of hot hydrolysis followed by beads beating as HH+BB. We finally refer to the samples and process treated via beads beating, followed by filtering, and then hot hydrolysis as BB+F+H.

### 2.2.6 Preparation of standard solutions and derivatization reagents

Stock solutions and standard solutions of T, TP, and TPP were prepared freshly before use by dissolving the vitamin in 0.1 M HCl, and subsequently diluting the solution in 0.1 M HCl to reach an appropriate concentration. HPLC grade methanol was used for derivatization. The oxidation reagent was freshly prepared before use, it was composed of a solution of 12.1 mM $K_3Fe(CN)_6$ in 3.35 M NaOH. After derivatization, a 1.4 M solution of H3PO4 was used to adjust the pH of the samples before injection.

### 2.2.7 Derivatization of samples and standard solutions

Thiamine derivatization and HPLC determination was carried out within 24 h from the extraction. The derivatization procedure was executed as described by Gerrits, Eidhof (1997). The extract (1 ml) was pipetted in a 10 ml polystyrene tube, after which, 100 µl of methanol was added and mixed, and 200 µl of oxidation reagent was added and mixed, to reach a pH above 12. After 30 seconds of derivatization, 250 µl of 1.4 M phosphoric acid solution was added, and the content of the tubes was mixed again. The final pH of the prepared samples and standards was 6.9 +/- 0.2. Finally, 200 µl of the derivatized sample was pipetted in the HPLC vial.

### 2.2.8 HPLC- fluorescence detection set-up

The derivatized samples were separated using a 50 mm x 4.6 mm Cortecs C-18-2,7 µm column, with a pre-column Cortecs C-18 VanGuard cartridge, 3.9 mm x 5 mm, 2.7 µm. The detection was performed by a fluorescence detector, UV-Waters 2575 FLR (Waters Corporation, Milford, MA, USA), with an excitation wavelength of 365 nm and emission of 450 nm. The limit of detection (LOD) was determined using a signal/noise ratio of 3. The limit of quantification (LOQ) was determined by setting a 500 millivolts lower area rejection limit. Samples were stored in the HPLC autosampler compartment at 5°C. The chromatographic conditions used in this study have been previously reported by Sander et al. (1991) and are briefly described below, with a modification of the injection volume. A volume of 5 µl of derivatised samples was injected. Two mobile phases were used for the analysis, mobile phase A was a solution of 25 mM $K_2HPO_4$ (4,35 g/l) with a final pH of 8.4, mobile phase B was HPLC-grade methanol, with a flow rate of 0.5 ml/minute, details of the gradient elution percentage are described in Figure 1. The total run time was 16 minutes per sample, with integration after 1.5 minutes.

### 2.2.9 Data analysis

Data were stored using Excel software v.16.0 (Microsoft Corporation, Redmond, WA, USA), data analysis and figures were done using Rstudio software v. 4.0.2 (RStudio®, Boston, MA, USA). Chromatograms were analysed using Chromeperfect v. 6.0.18 (Denville, NJ, USA). Data were visually checked, and statistical significance was assessed via ANOVA, followed by Tuckey post-hoc test.

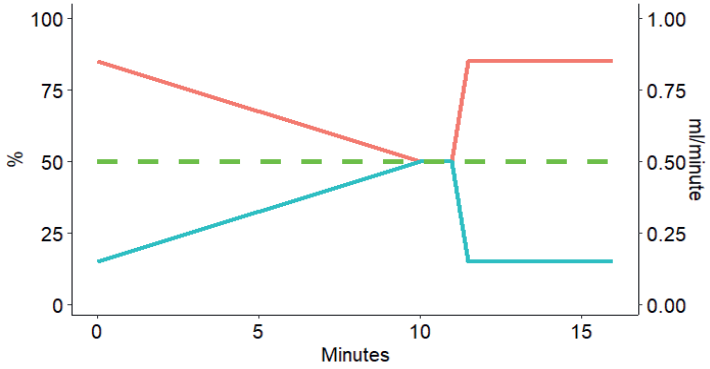



Figure 1- Gradient elution percentage of mobile phases A (red) and B (blue), and flow rate (dashed green line) as ml/min.

## 2.3 Results

### 2.3.1 Chromatograms, linearity, LOD and LOQ

The derivatization of thiamine to thiochrome was executed in this study as reported by Gerrits et al. (1997) which gave adequate chromatographic separation of the vitamers and a high fluorescence response (Figure 2). The retention times for TPP, TP, and T were 1.94, 2.79, and 5.91 min respectively in the derivatised mixed standard solution, and 1.92, 2.75, and 5.90 respectively in the yeast extract. Minor day to day variation in

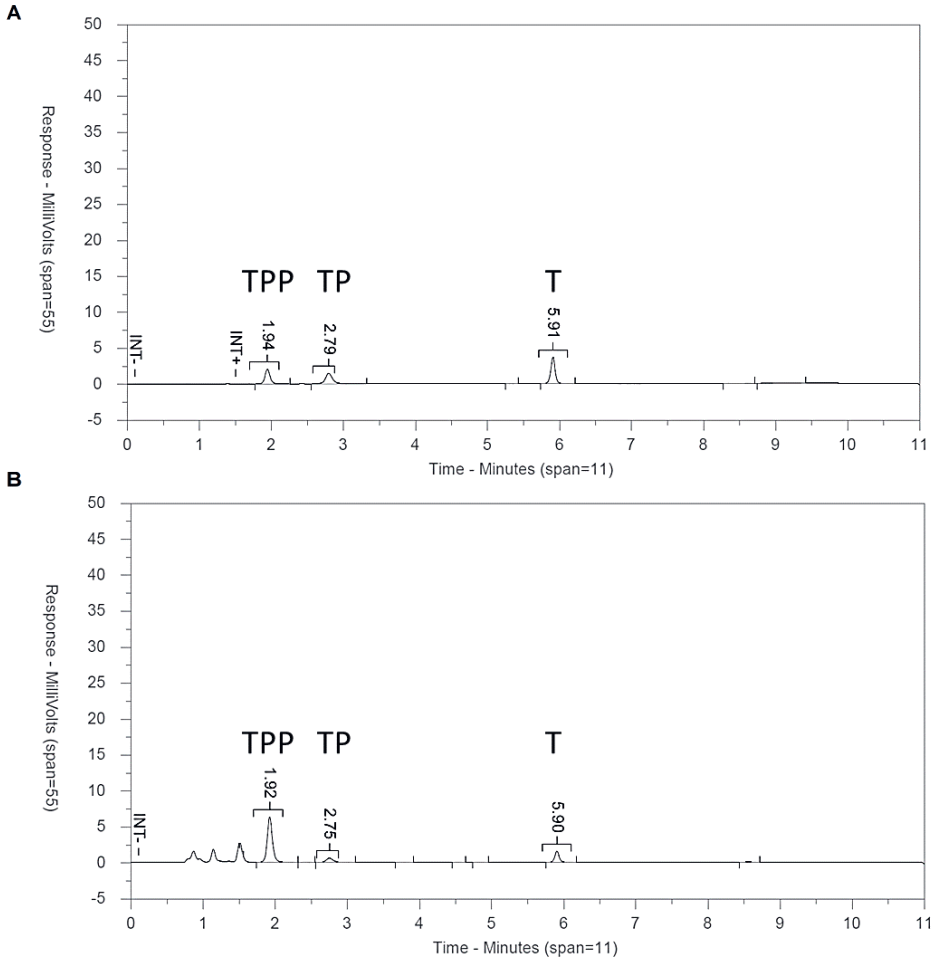



**Figure 2-** Chromatogram of the standard solution (A) and yeast extract sample (B). The fluorescence response of the different thiamine forms is given in millivolts and the respective retention times are presented in min.

retention time occurred (Table 1). Peak areas are plotted against the vitamer concentrations and linearity was proven by linear least-square regression (Figure 3A) with an R value above 0.99 for all the vitamers. The range of linearity was tested by a 10 fold serial dilution (Figure 3B), starting from 6.38, 5.84, and 5.87 µmol/l, of TPP, TP, and T respectively. Reaching a concentration just above the detection limit of 6.38, 5.84, and 5.87 nmol/l respectively. The LOD was calculated by establishing a 500 millivolts lower area rejection limit, corresponding to approximately 1.4 ng/ml for TPP and TP, and 0.6 ng/ml for T (3.29 mol/l TPP, 4.05 nmol/l TP, 2.26 nmol/l T). Since T gave a double fluorescent response compared to the other derivatives, the LOQ was calculated to be 1 ng/ml for T, and 2 ng/ml for both TPP and TP (4.7 nmol/l for TPP, 5.8 nmol/l for TP, and 3.8 mol/l T).

### 2.3.2 Robustness and repeatability

To assess the robustness of the method we checked the stability of the derivatised samples, and the influence of the pH adjustment after the derivatization on the fluorescence response. The first experiment aimed at assessing a possible loss of fluorescence in the derivatised samples over time when the samples are stored at 5°C. As the run time is 16 min per sample, when many samples are analysed, several hours pass between the derivatisation and the injection. Single vitamer standards and mixed standard solution were derivatised in triplicates and injected right after derivatization, after 36, 58, and 82 h. No significant change in peak area occurred over time (*p*-value >0.1) for all the tested vitamers and time points, indicating no loss of fluorescence for the various samples over time. The relative standard deviation (RSD) calculated as a percentage was below 1.6 % for every sample tested (Table 1). To investigate the influence of the pH of the samples before injection on the fluorescent response, an increasing amount of 1.4 M phosphoric acid was added to the mixed standard solutions in triplicates. Peak areas of the standard solutions were normalised by the amount of phosphoric acid added, and compared to the reference amount of phosphoric acid needed to reach a final pH of 6.9, the preferred pH indicated by Gerrits et al. (1997). No significant difference in corrected peak area was found between samples with different pH before injection (*p*-value>0.1) (Figure 3D). Instrumental repeatability, extraction method reproducibility, and day to day variation of retention time were checked to validate the method. The instrumental reproducibility was checked by injecting the same sample 10 times (Table 1), for all the vitamers the RSD was lower than 1.5 %. The reproducibility of the method was checked by performing BB extraction of the same yeast culture 10 times, the RSD for each vitamer was found to be below 4.1 % for all vitamers. The day to day variation of retention time for the different vitamers in the standard solutions was below 2.4 %, indicated as RSD, for a total of 8 separate analyses.

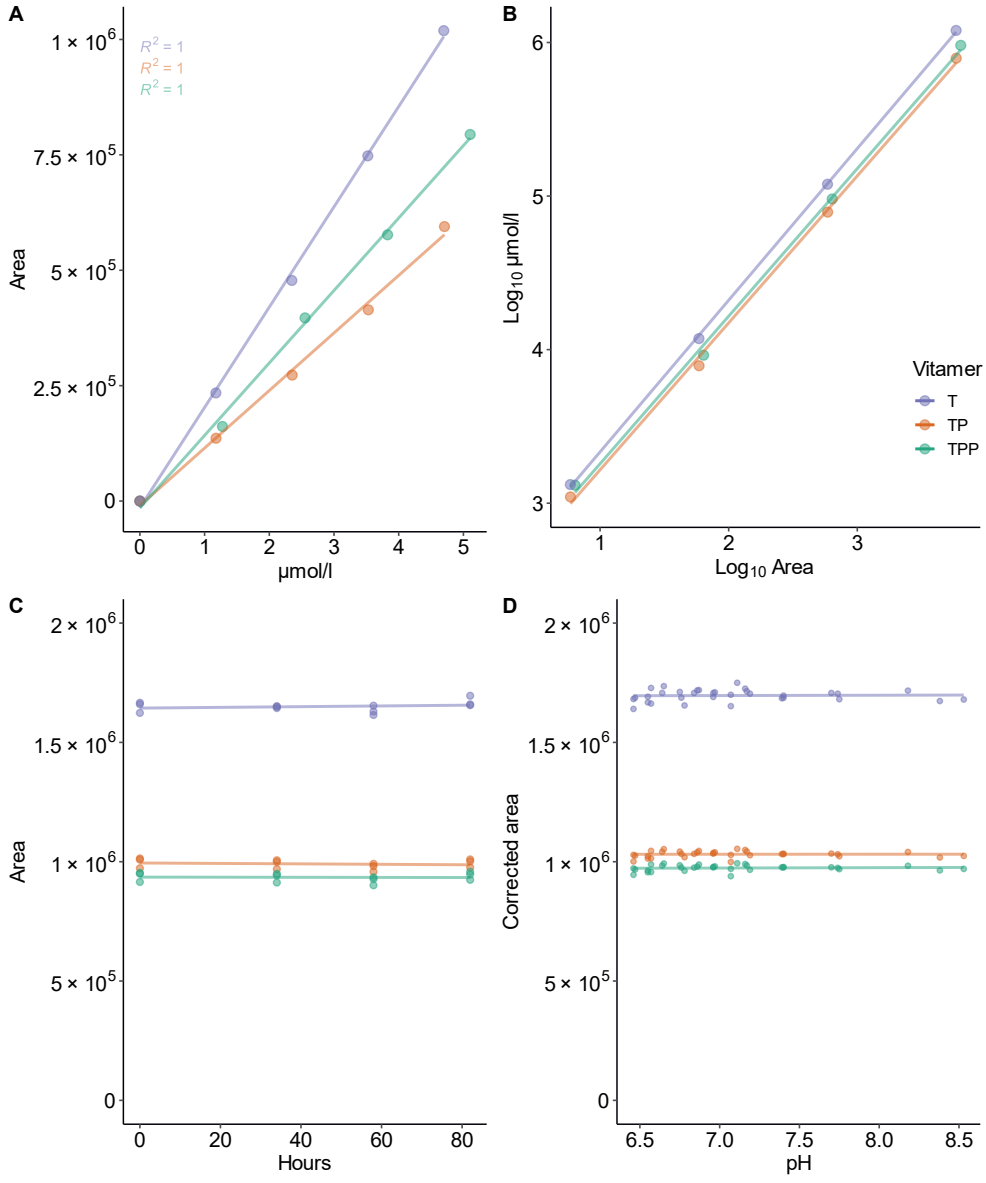



Figure 3 - T is depicted in purple, TP is depicted in orange, and TPP is depicted in green. Peak area as a function of vitamer concentration in the analysed single standard solution of each vitamer, the linearity was proved by linear least squares regression (A). log10 peak area as a function of log10 vitamer concentration in the analysed single standard solutions, the range of linearity was tested for different concentrations of TPP, TP, and T, the analysis was carried out in triplicates (B). Peak area as a function of time, samples were analysed at t=0, after 36, 58, and 82 h, the analysis was carried out in triplicates (C). Corrected peak areas as a function of the pH of the derivatised samples treated with an increased amount of 1.4 M phosphoric acid, the analysis was carried out in triplicates (D).

The instrumental and method reproducibility, and low day-to-day retention time variation of the different vitamers, indicate that this method is highly robust, repeatable, and reproducible.

Table 1- RSD % ± standard deviation of the area of the derivatised mixed standard solution and single standard solutions after derivatization, and after 36, 58, and 82 h for TPP, TP, and T. Instrumental repeatability, method repeatability, and day-to-day retention time variation expressed as RSD % for TPP, TP, and T, number of samples in brackets

| | | RSD % | | |
|---|---|---|---|---|
| | | TPP | TP | T |
| RSD % of the area after derivatization, and at 36, 58, and 82h | Mixed standard solution | 1.04 ±0.01 | 0.98 ±0.17 | 1.33 ±0.42 |
| | Single standard solutions | 1.52 ±0.11 | 1.58 ±0.30 | 1.40 ±0.28 |
| Repeatability | Instrumental repeatability ($n$=10) | 1.19 | 1.23 | 1.43 |
| | Method repeatability ($n$=10) | 2.95 | 4.07 | 1.66 |
| | Retention time variation ($n$=8) | 2.32 | 2.29 | 0.8 |

### 2.3.3 Optimization of beads beating extraction

To optimize the extraction of thiamine using BB two parameters were considered in this study: the type of lysing matrix and the number of subsequent beating rounds. Three different lysing matrices were tested to extract thiamine from the same yeast batch. Matrix B is routinely used to lyse bacteria, fungal tissues, and spores, matrix C is designed lysis of yeasts and fungi, and matrix E is generally applied for mixed samples. The total thiamine extraction yield and respective vitamer proportions were determined using the different lysing matrices (Figure 4A and 4B). Lysing matrix B gave a significantly higher extraction yield of 54.1 nmol/g, compared to the matrices C and E which yielded 46.9 and 22.7 nmol/g ($p$-value<0.05) respectively. No significant differences in vitamer proportions were found between lysing matrix B and C for TPP, TP and T ($p$-value>0.1). TPP accounted for approximately 69 % of the total thiamine in both matrices. In matrix B, TP accounted for 8.2 % of the total thiamine, while T accounted for 22.5 %. In matrix C, TP accounted for 7 %, and T accounted for 23.8 % of the total thiamine. Lysing matrix E yielded significantly less total thiamine, and no TPP was detected in the extracts, T accounted for 66.8 % of the thiamine and TP for 33.2 %. Therefore, lysing matrix B was chosen as the preferred matrix for thiamine extraction. To further optimize the BB extraction, on the same yeast culture we increased the number of rounds from 3 up to 12 using lysing matrix B. No significant differences were found between the BB rounds and the total thiamine yield ($p$-value>0.1), also the vitamer distribution percentage did not differ significantly when the beating rounds were progressively increased from 3 to 12 ($p$-value>0.1) (Figure 4C and 4D). Therefore, 3 beating rounds were considered sufficient to extract thiamine.

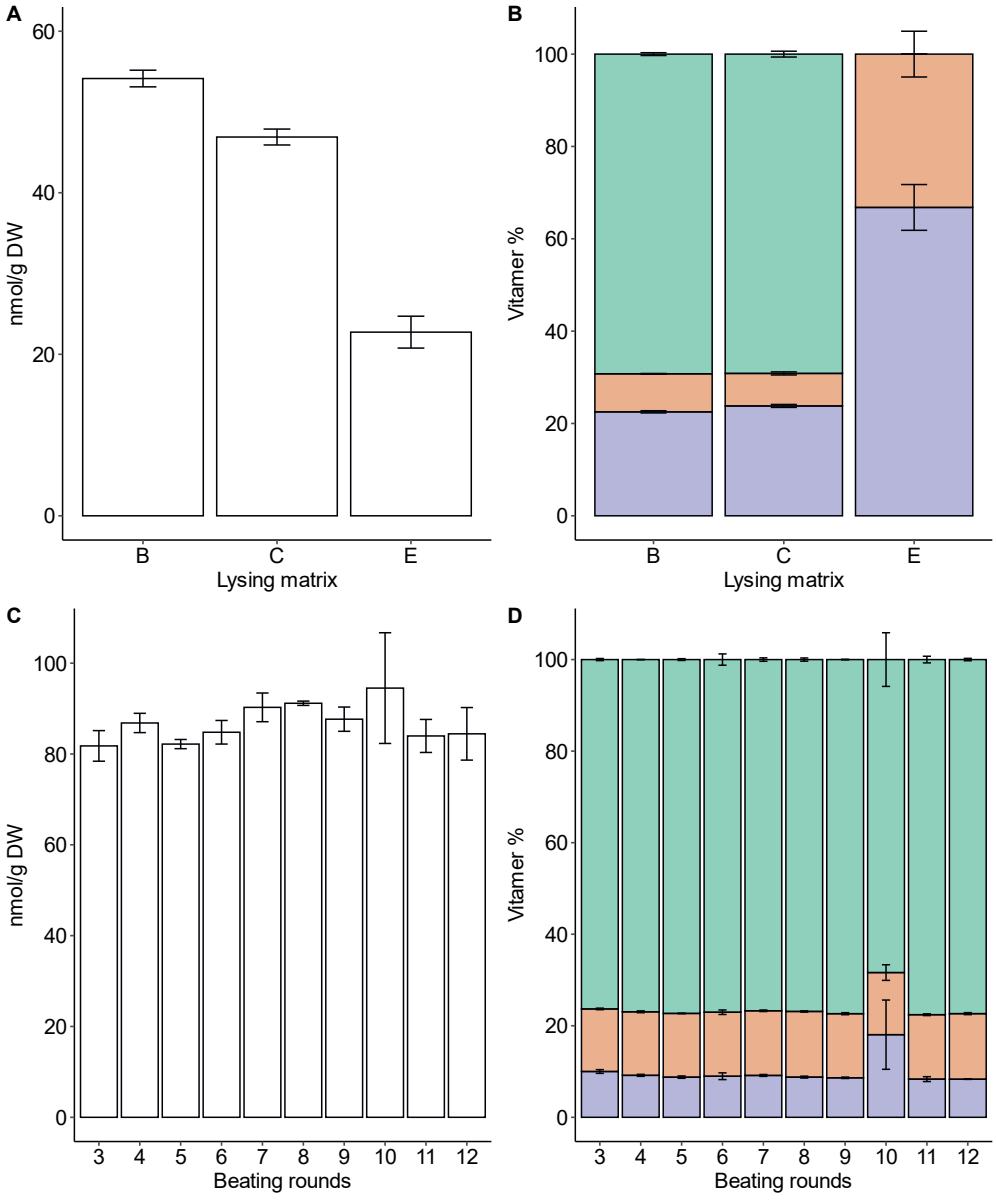



Figure 4- T is depicted in purple, TP is depicted in orange, and TPP is depicted in green. Total thiamine yield in nmol/g DW (A) and vitamer distribution percentage of thiamine (B) in yeast extract. Yeast biomass was harvested from *S. cerevisiae* cultures grown in MM for 90 h at 180 rpm and extracted in triplicate using lysing matrix B, C, and E. Total thiamine yield in nmol/g DW (C) and vitamer distribution percentage of thiamine (D), fresh yeast biomass was harvested from *S. cerevisiae* cultures grown in MM for 24 h at 180 rpm and extracted in duplicate using lysing matrix B, BB round tested were increased from 3 to 12.

### 2.3.4 Comparing beads beating extraction to hot acid hydrolysis

Extraction of vitamin $B_1$ via hot acid hydrolysis has been largely used for the thiamine determination in foods and yeast samples (Yamanaka et al., 1996; Haj-Ahmad et al., 1992); Schweingruber et al., 1991; Yamanaka et al., 1994). In this study, we compared the extraction of vitamin $B_1$ via BB, HH, and a combination of these two treatments carried out in different orders, to assess the effect on the total thiamine extraction yield and the vitamer distribution. Vitamin $B_1$ extraction via BB had the lowest yield compared to all the other methods ($p$-value<0.1), with an average total of 55.7 nmol/g DW (Figure 5A). The highest total thiamine extraction was achieved by BB+HH, yielding on average 71.8 nmol/g DW. HH alone yielded on average 71.2 nmol/ g DW, and HH+BB yielded on average 68.1 nmol/g DW. The vitamer ratios also differed among the samples (Figure 5B), with T staying virtually unchanged in all samples, accounting for approximately 25 % of the total yield, and TPP and TP as the remaining 75 % approximately. TPP proportionally decreased as TP increased in all the heat-

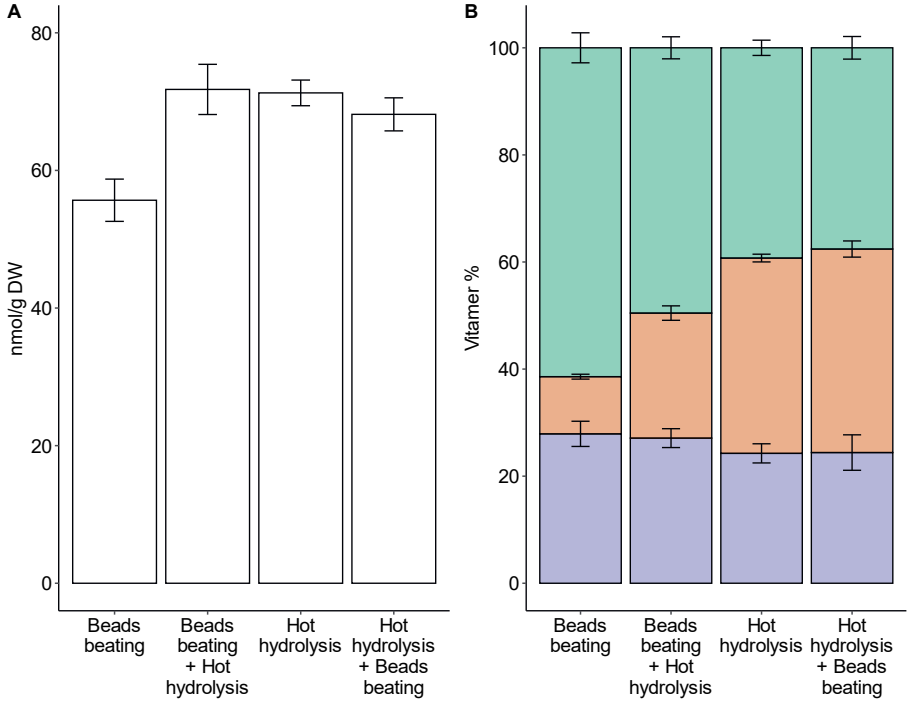



Figure 5- T is depicted in purple, TP is depicted in orange, and TPP is depicted in green. Total thiamine yield in nmol/g DW (A) and vitamer distribution percentage of thiamine (B) in yeast extract. Fresh yeast biomass was harvested from three different *S. cerevisiae* cultures grown in MM for 72 h at 180 rpm and extracted in triplicate, yeast biomass was lysed using lysing matrix B and 3 BB rounds of 1 minute, heat treated extract were treated for 30 min at 95 °C, these treatments were tested both individually and combined in a different order.

treated samples. In the samples treated via BB only, TPP accounted for 61.5 % of the total thiamine while TP for 10.7 % on average. In the BB+HH samples TPP accounted for 49.5 % and TP for 23.5 %. The decrease in TPP and increase in TP compared to the BB sample was even more remarkable in the samples that were only treated via HH. In these samples TPP represented roughly 39.3 % of the total thiamine and TP 36.5 %. A similar vitamer proportion was found in the sample treated by HH+BB, in which TPP represented on average 37.6 % of the total yield and TP accounted for 38 %.

### 2.3.5 Degradation of thiamine pyrophosphate during hot acid hydrolysis

To confirm possible degradation of TPP to TP during hot acid hydrolysis, cells that were first extracted by only BB, then extracts were centrifuged and filtered to remove the biomass, they were then heat treated at 95°C for 30 min (BB+F+HH). The total thiamine yield and vitamer distribution was compared to the BB samples. We found a significant difference ($p<0.05$) in the BB+F+HH samples, with a total average yield of 66.9 nmol/g DW, compared to 55.7 nmol/g DW of the BB samples (see Figure 6A). The vitamer distribution percentage also changed in the BB+F+HH samples. The T content remained roughly unchanged, representing on average 27.9 % of the total thiamine in the BB samples, and 26.1 % in the BB+F+HH samples. However, the TPP accounted for 28.3 % of the total thiamine detected in the BB+F+HH samples, while TPP accounted on average for 61.4 % of the total thiamine pool in the BB sample. Consequently, TP increased in the BB+F+HH samples reaching 45.6 % of the total thiamine yield. These results indicate that a partial degradation of TPP to TP occurs during heat treatment. To further confirm the degradation of TPP to TP, a defined mixed solution of TPP, TP, and T was heat-treated at 95°C for 30 min, similar results were obtained in this case. The total thiamine yield did not differ significantly before and after heat treatment. Instead TPP, which first accounted for 34 % of the total thiamine, represented only 19.5 % of the total thiamine in the heat treated samples. TP, which accounted for 39.7 % of the total thiamine in the untreated sample, increased above 53 % after heat treatment. This confirms that heat-treating thiamine extracts leads to a 1:1 conversion of TPP to TMP, while T remains unchanged, thereby leading to a non-specific detection of vitamin $B_1$ vitamers.

## 2.4 Discussion

HPLC-UV detection methods are often used for thiamine quantification. These methods detect thiamine directly, and are usually applied to products with relatively high concentrations of thiamine, for instance in fortified foods. HPLC-UV methods are not sufficiently sensitive when products contain low amounts of vitamin $B_1$. Another pitfall

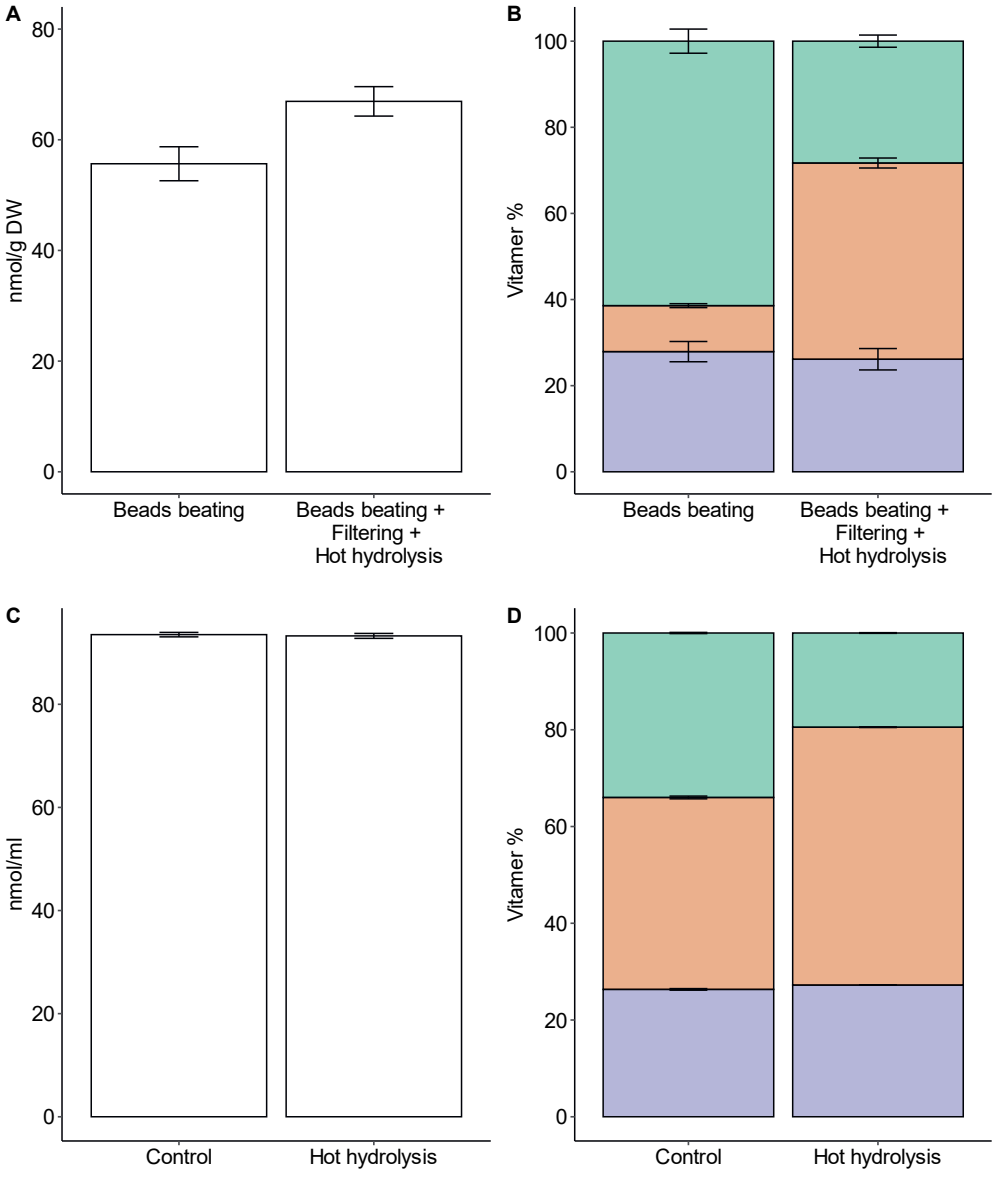



Figure 6- T is depicted in purple, TP is depicted in orange, and TPP is depicted in green. Total thiamine yield in nmol/g DW (A) and vitamer distribution percentage of thiamine (B) in fresh yeast extract. Fresh yeast biomass was harvested from three different *S. cerevisiae* cultures grown in MM for 72 h at 180 rpm and extracted in triplicate, yeast biomass was lysed using lysing matrix B and 3 BB rounds of 1 minute, afterwards, extracts were filtered. The filtered extract was used as a control ("Beads beating") or heat-treated for 30 minutes at 95 °C and filtered again before derivatization ("Beads beating + Filtering + Boiling"). Total thiamine as nmol/ml (C) and vitamer distribution percentage of thiamine (D) in the mixed standard solution. A mixed standard solution of TPP, TP, and T was used as control, the same solution was heat-treated for 30 minutes at 95 °C ("Hot hydrolysis"), then filtered again, and samples were analysed in duplicate.

of these methods is their low specificity when the analysed matrix contains UV absorbing impurities. This will produce chromatograms in which thiamine peaks are overlapping with the UV-detectable impurities, resulting in difficult thiamine quantification. In this study, we propose the derivatization of thiamine to thiochrome to increase both specificity and sensitivity of detection. The pre-column derivatization of thiamine to thiochrome can be both done manually or automatically. Manual pre-column derivatization, although laborious, is easier and cheaper to set up compared to an automated pre-column derivatization system. During derivatization methanol was used as it is reported to increase fluorescence (Bubeshko et al., 2011), potassium hexacyanoferrate was used as an oxidation reagent because of its lower toxicity compared to other reagents such as cyanogen bromide or mercuric chloride (Edwards et al., 2017). The purpose of adding phosphoric acid is to quench the derivatization reaction, and at the same time neutralizes the extracts. The latter prevents wearing and damaging of the pre-column, column, and pipes. An important consideration to have an accurate thiamine determination is to minimize phosphate ester degradation. This can be achieved by working with freshly prepared thiamine standard solutions and by monitoring the possible thiamine phosphates esters degradation by injecting them both in combination and separately. We tested the method for the detection of TTP in 0.1 M HCl, the method is able to detected this thiamine vitamer (Supplementary material 1).

Studies on thiamine extraction using acid hydrolysis reported low thiamine recovery due to the formation of impurities and precipitates when using sulfuric acid and TCA. Therefore in this study hydrochloric acid was the preferred solvent for thiamine extraction, as it has been extensively used for extracting thiamine from yeast biomass (Edwards et al., 2017). The different extraction methods tested not only showed different thiamine yields, but also different vitamer proportions in each sample. When hot acid hydrolysis was used to extract thiamine from the yeast samples TP increased in proportion compared to BB alone, while T stayed roughly the same in all samples. This decrease in TPP and increase in TP could be due to higher amounts of TP being extracted through hot acid hydrolysis, and/or to the conversion of TPP into TP. As was confirmed by the follow-up experiments, total extraction yield increased when samples were heat-treated after BB, independently of the use of filtered or unfiltered samples before heat-treatment. This indicates that the binding of thiamine to proteins plays a role in the extraction, and consequently this would advocate for the use of heat during the extraction (Chandra-Hioe et al., 2015). However, heat treatment also causes TPP degradation to TP, but not to T, as proven by the boiling of the standard solutions (Figure 6C and 6D). This heat-induced conversion of TPP into TP should not be neglected for studies that require a specific determination of the vitamers. Therefore,

hot hydrolysis alone, or a combination of hot hydrolysis and BB could lead to determination of an incorrect vitamer proportion and thus biologically irrelevant data. This is even more important for studies aimed at better understanding of thiamine biosynthesis and metabolism in yeasts. Furthermore, it is also possible to accelerate protein degradation by using a proteases. This requires an additional step of incubation of the extracts with the enzyme, followed up by enzymatic inactivation, possibly via heat treatment (thereby risking the degradation of TPP to TP). The use of an enzymatic step could therefore potentially increase the thiamine recovery, but lengthen the extraction procedure.

During BB extraction using lysing matrix E no TPP was found in the extract after derivatization, the reason behind this is still unknown. We assume this could be due to some interaction between the TPP and components found only in lysing matrix E, such as ceramic beads and a glass sphere, in addition to the silica beads that are also present in matrix B and C.
The biological state of the yeast and the growth phase determine the vitamer proportions and the intracellular total thiamine. Total thiamine is highest during exponential growth to then decreases during the stationary phase. The vitamer proportions also changes during yeast growth (Kowalska et al., 2012), therefore, the variations in the thiamine yield and the vitamer composition that were observed in this study between the different experiments are due to the incubation time of the yeast culture before extraction.

## 2.5 Conclusions

In this study we combined two previously known methods for thiamine detection. The thiochrome derivatization method was combined with reversed phase-HPLC coupled to fluorescence detection, to create a new reproducible thiamine-vitamer detection and quantification method of which we proved the robustness, reproducibility, and repeatability. In conjunction to the detection and quantification method, we developed and optimised a thiamine extraction method using beads-beating with silica beads. We proved that the commonly used heat-treatment of fresh yeast biomass for extracting thiamine leads to the degradation of TPP to TP, resulting in a non-selective determination of the thiamine vitamers. Although this mechanical cell disruption extraction method yields lower amounts of thiamine, it can still be used to measure and determine the different thiamine phosphate forms with increased specificity. This new finding is particularly useful for all studies that aim to determining the thiamine proportions in fresh yeast biomass specifically.

## Funding statement

This project is financially supported by the Dutch Topsector Agri & Food under contract TKI AF18081. Within the Topsector, private industry, knowledge institutes and the government are working together on innovations for safe and healthy food for 9 billion people in a resilient world.



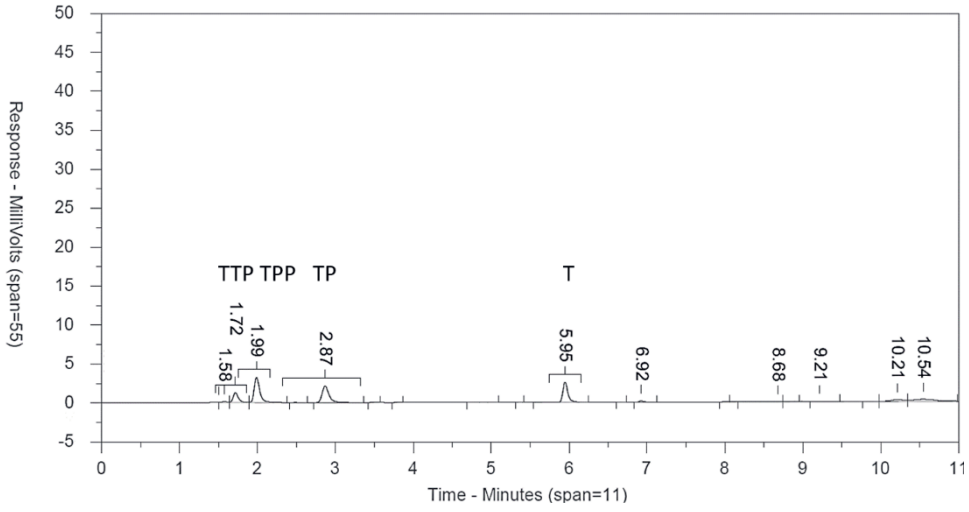
## Acknowledgements

Judith Wolkers-Rooijackers is acknowledged for technical support.

## Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## 2.6 Supplementary material




**Supplementary 1-** Chromatogram of the standard solution containing TTP, TPP, TP, and T. The fluorescence response of the different thiamine forms is given in millivolts and the respective retention times are presented in min.

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# CHAPTER 3

## Strain diversity in *Saccharomyces cerevisiae* thiamine production capacity

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Published in Yeast, Volume 40, Issue 12, December 2023, Pages 628-639

DOI: https://doi.org/10.1002/yea.3906

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## Abstract

Vitamin $B_1$, also known as thiamine, is an important vitamin that besides its role in human health is converted to meat aromas upon exposure to high temperatures. Therefore, it is relevant for the production of vegan meat-like flavours. In this study, we investigated 48 *Saccharomyces cerevisiae* strains for their thiamine production capacity by measuring the intracellular and extracellular vitamins produced in the thiamine-free minimal medium after 72 h of growth. We found approximately an 8.2-fold difference in overall thiamine yield between the highest and lowest-producing strains. While the highest thiamine yield was 254.6 nmol/l, the highest thiamine-specific productivity was 160.9 nmol/g DW. To assess whether extracellular thiamine was due to leakage caused by cell damage, we monitored membrane permeabilization using propidium iodide (PI) staining and flow cytometry. We found a good correlation between the percentage of extracellular thiamine and PI-stained cells (Spearman's $\rho$=0.85). Finally, we compared *S. cerevisiae* CEN.PK113-7D (WT) to 3 strains evolved in a thiamine-free medium for their thiamine production capacity. On average, we saw an increase in the amount of thiamine produced. One of the evolved strains had a 49 % increase in intracellular thiamine-specific productivity and a biomass increase of 20 % compared with the WT. This led to a total increase in thiamine yield of 60 % in this strain, reaching 208 nmol/l. This study demonstrated that it is possible to achieve thiamine overproduction in *S. cerevisiae* via strain selection and adaptive laboratory evolution.

## 3.1 Introduction

*Saccharomyces cerevisiae* is a yeast that has been employed, and therefore domesticated, for food and beverage production since ancient times. Nowadays, it is often used to produce numerous compounds for industrial applications (Kavšček et al., 2015). A vitamin produced by *S. cerevisiae* that is of particular interest is vitamin $B_1$, also known as thiamine. This vitamin comprises two moieties: a thiazole ring joined by a methylene bridge to an aminopyrimidine ring. Thiamine (T) is found in *S. cerevisiae* in different phosphorylated forms: thiamine phosphate (TP), thiamine pyrophosphate (TPP), and thiamine triphosphate (TTP) (Perli, Wronska, et al., 2020). TPP is the biologically active form of this vitamin, and it is an important co-factor in *S. cerevisiae* for the dissipation of pyruvate. This process occurs either via pyruvate decarboxylase (Arjunan et al., 1996), pyruvate dehydrogenase (Robinson et al., 1993), or acetolactate synthase that catalyses the first step into the synthesis of leucine, isoleucine, and valine (Murashchenko et al., 2016). While the degradation of these three amino acids is catalysed by the TPP-dependent branched chain α-keto acid dehydrogenase (Labuschagne et al., 2021). Thiamine is also a co-factor of 2-oxoglutarate dehydrogenase, which is part of the Krebs cycle (Hohmann et al., 1998), and of transketolase, involved in the pentose-phosphate pathway. Another TPP-dependent enzyme is phenylpyruvate decarboxylase, which catalyses the first step in the Ehrlich pathway by producing phenylacetaldehyde via decarboxylation of phenylpyruvate (Kneen et al., 2011). For this reason, *S. cerevisiae* needs vitamin $B_1$ to grow, whether it is salvaged from the environment or produced de novo.

The de novo biosynthesis of thiamine in *S. cerevisiae* starts from the formation of the two precursors: 4-amino-5-hydroxymethyl-2-methylpyrimidine diphosphate (HMP-PP) and 5-(2-hydroxyethyl)-4-methyl thiazole phosphate (HET-P), which both require single-turnover enzymes that act as co-substrates to be produced (Chatterjee et al., 2011; Coquille et al., 2012). Therefore, the de novo biosynthesis of thiamine is an indispensable yet extremely costly biological process. First, 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P) is synthesised from pyridoxal-5-phosphate and histidine, which is donated from the active site of the single turnover enzyme HMP-P synthase, this enzyme is encoded by four genes (*THI5*, *THI11*, *THI12*, and *THI13*), showing a genetic redundancy for this specific precursor (Hohmann & Meacock, 1998; Wightman et al., 2003). Afterward, HMP-P is phosphorylated again by HMP-PP kinase (encoded by *THI20* and *THI21*), which donates the phosphate group from ATP. *THI20* also has a thiaminase II activity (Haas et al., 2005; Sudarsan et al., 2005); therefore, it is involved in the salvage pathway of thiamine. Indeed, the N-terminus of the Thi20

protein is homologous to *Bacillus subtilis* YjbV, and to *Escherichia coli* ThiD (Sudarsan et al., 2005), both catalyse the phosphorylation of HMP and HMP-P. Thi20 is also homologous to the C-terminus of *B. subtilis* TenA that is responsible for the cleavage of thiamine to form HMP (4-amino-2-methyl-5-hydroxymethylpyrimidine) and -5-(2-hydroxyethyl)-4-methyl thiazole (HET). The other moiety of thiamine, HET-P, is produced by a single-turnover enzyme encoded by *THI4* (Chatterjee et al., 2011). This enzyme uses glycine and nicotinamide adenine dinucleotide ($NAD^+$) as substrates and donates a sulphur atom from a cysteine residue. Once the two moieties are produced, thiamine-phosphate phosphorylase, encoded by *THI6*, binds them together, forming a methylene bridge resulting in the formation of TP. Thi6 is a bifunctional protein that can also phosphorylate 5-(2-hydroxyethyl)-4-methyl thiazole to form HET-P. Bacteria can directly convert TP to its active form TPP. Instead, *S. cerevisiae* first releases the phosphate group producing free thiamine; this reaction is catalysed by two generic phosphatases (encoded by *PHO11*, *PHO8*). Then, T is converted to TPP via pyrophosphorylation by a thiamine pyrophosphokinase Thi80. TPP can be further phosphorylated to form TTP, but its biological function in *S. cerevisiae* remains unknown.

Thiamine salvage from the environment is preferred to de novo biosynthesis because of its high production costs (Labuschagne & Divol, 2021). Two proteins are responsible for thiamine uptake: a periplasmic thiamine-repressible acid phosphatase (encoded by *PHO3*) (Nosaka, 1990) and an active transporter, encoded by *THI10* (Enjo et al., 1997; Labuschagne & Divol, 2021). The Pho3 protein is not directly involved in thiamine transport but releases the phosphate groups from TP and TPP; afterward, the thiamine transporter Thi10 uptakes selectively free thiamine to be then phosphorylated. The *THI10* encoded thiamine transporter is an active transport system capable of accumulating high amounts of thiamine intracellularly, up to 10000 fold the extracellular concentration, at the expense of ATP (Ruml et al., 1988). Other proteins, namely Thi71, Thi72, and Nrt1, are also capable of transporting thiamine, but with low affinity and low capacity, therefore considered to have a minor role in thiamine transport, especially at low exogenous thiamine concentrations (Ceschin et al., 2014; Mojzita et al., 2006).

Vitamin $B_1$ is also fundamental for human health because of its activity in many important metabolic reactions. These reactions are involved in the pentose-phosphate pathway, the metabolism of pyruvate and α-ketoacids, and the oxidation of branched-chain fatty acids (Brown, 2014). A severe deficiency of vitamin $B_1$ causes Beriberi syndrome and eventually death (Wilson, 2020). Moreover, thiamine is a precursor of many flavour compounds perceived as meaty and roasted (Thomas et al., 2014). Upon exposure to heat, free unphosphorylated thiamine degrades to form a diverse array of

potent aroma compounds, such as 2-methyl-3-furanthiol, 2-methyl-3-(methyldithio)furan, and bis(2-methyl-3-furyl) disulphides (Güntert et al., 1992). All these molecules contribute to the aroma of cooked meat (Kale et al., 2022; Thomas et al., 2014). Due to a growing market of vegan meat-like food products, and the well-established role of thiamine in forming meat-like flavours, it is of particular interest to study the strain variation in the thiamine production capacity of *S. cerevisiae*.

In this study, we screened 48 thiamine prototrophic *S. cerevisiae* strains for their ability to produce thiamine in a chemically defined media free of amino acids and thiamine and containing ammonium sulphate as the only nitrogen source. We measured intracellular and extracellular thiamine concentration and its release via cell lysis with a newly developed extraction and determination method with specificity for each vitamer (Rocchi et al., 2022). Finally, we compared three strains evolved in a thiamine-free medium by Perli, Moonen, et al. (2020) to the corresponding wild type to assess whether adaptive laboratory evolution is a successful strategy for the development and isolation of high thiamine-producing *S. cerevisiae* strains.



## 3.2 Materials and Methods

### 3.2.1 Yeast strains growth

In this study, we screened 48 *S. cerevisiae* strains originating from various sources (Supplementary material 2). Among these strains, we included the wild-type strain CEN.PK113.-7D, along with three evolved strains (IMS0747, IMS0748, and IMS0749) derived from it through adaptive laboratory evolution in a thiamine-free-medium for 300 generations (Perli, Moonen, et al., 2020). Stock cultures were preserved in 30 % v/v glycerol and stored at -80°C, streaked on chemically defined thiamine-free solid agar medium (CDA; see below) and incubated at 30°C for 72 h. To prepare the pre-inoculum, one colony was picked to inoculate 10 ml of chemically defined thiamine-free liquid medium (CDM; see below) and incubated at 30°C for 24 h. Afterward, 1 ml of pre-inoculum was used to inoculate 100 ml of CDM in a sterile 250 ml Erlenmeyer flask, which was then incubated at 30°C for 72 h while shaking at 180 rpm.

### 3.2.2 Media

In this study, we used a chemically defined thiamine-free solid agar medium (CDA) composed of 20 g/l glucose (Sigma-Aldrich), 15 g/l bacteriological agar (Oxoid), 5 g/l ammonium sulphate (AS; Sigma-Aldrich), and 1.71 g/l yeast nitrogen base without ammonium sulphate, amino acid, and thiamine (YNB; U.S. Biological). The 20 g of glucose and 15 g of agar were dissolved in demineralised water (DW), reaching a mass of 800g, and autoclaved at 121°C for 15 min, then 1.71 g of YNB and 5 g of ammonium sulphate were mixed with 200g of water and filtered sterilized into the agar and glucose mixture using a 0.2 µm Minisart® syringe filter (Sartorius). The chemically defined thiamine-free medium liquid medium (CDM) was prepared by mixing 20 g glucose, 5 g AS, and 1.71 g YNB with DW for each 1000 g medium. It was filtered through a 0.2 µm filter using a Nalgene Rapid-Flow vacuum filter unit (VWR) into a sterile bottle and stored at 4°C until use.



### 3.2.3 Cell dry weight determination

Cell dry weight was determined as described by Rocchi et al. (2022). Yeast cell cultures (20 ml) were filtered via a vacuum filtration unit through a membrane filter with a pore size of 0.2 µm which was previously kept at 80°C for 48 h and pre-weighed; we used DW to rinse the filters. The wet membranes were dehydrated again at 80°C for 48 h before measuring the cell biomass.

### 3.2.4 Intracellular thiamine extraction

As reported by Rocchi et al. (2022), thiamine extraction was carried out with only minor changes. Briefly, 10 ml of yeast culture was centrifuged at 10,000 x g for 10 min, and the supernatant was removed and filtered with a 0.2 µm sterile polyethersulfone filter and stored at 4°C before derivatization and measurement of the extracellular thiamine. The remaining yeast pellet was resuspended in 5 ml of 0.1 M HCl. Cells were lysed via bead beating, 1.5 ml of liquid yeast suspension was pipetted in lysing matrix tube B from MP Biomedicals (Irvine, CA, USA), with two technical replicates for each strain tested, and extracted with Fast Prep in three rounds of 5.5 m/s for 1 min with 1 min intervals each. Afterward, the extracts were centrifuged at 10,000 x g for 5 min, filtered through a sterile 13 mm 0.2 µm PES filter, and stored at 4°C before derivatisation and measurement. Two independent experiments were carried out for each strain, using fresh media, newly streaked plates, and pre-inoculums. The intracellular and extracellular vitamin concentration was measured in technical duplicates for each experiment.

### 3.2.5 Thiamine measurement

Thiamine was measured as reported by Rocchi et al. (2022), except that the pH of the mobile phase A was set to 7 instead of 8.4. Thiamine solutions for producing a standard curve were freshly prepared by dissolving the different vitamers in 0.1 M hydrochloric acid (HCl) and diluted in 0.1M HCl to reach an appropriate concentration. The reagent used to oxidise thiamine to thiochrome was prepared right before use by dissolving potassium ferricyanide ($K_3Fe(CN)_6$) in 3.35 M NaOH to reach a concentration of 12.1 mM. Thiamine derivatization and HPLC determination were carried out within 24 h after the extraction. To derivatize the samples, 1 ml of extract was pipetted in a 10 ml polystyrene tube, to which 100 µl of methanol was added and mixed, followed by oxidation of thiamine to thiochrome via the addition of 200 µl of the oxidation reagent. After allowing the derivatization for approximately 30 s, the pH of the derivatised sample was adjusted by adding 250 µl of 1.4 M $H_3PO_4$, and subsequently, the sample was mixed. The final pH of the derivatised samples and standards was approximately 6.9 +/− 0.2. Afterward, 200 µl of the derivatised sample was transferred to an HPLC vial for measurement.



Table 1- Description of gradient elution of the two mobile phases via HPLC

| Time | Mobile phase | |
|---|---|---|
| | A | B |
| 0-10 min | 85 % | 15 % |
| 10-11.5 min | 50 % | 50 % |
| 11.5-16 min | 85 % | 15 % |
| 0 min ->15 min | analysis time | |
| 15 min -> 15.5 min | purge time | |
| 15.5 -> 20 min | equilibration time | |

The derivatised samples were stored in the HPLC autosampler compartment during the analysis and kept at 5°C. To analyse the samples, 5 µl of derivatised samples were injected. The separation of the derivatised samples was executed using a 50 mm × 4.6 mm Cortecs C-18-2,7 µm column with a Cortecs C-18 VanGuard pre-column cartridge (3.9 mm × 5 mm, 2.7 µm). The fluorescent detection was performed by a UV-Waters 2575 FLR detector (Waters Corporation), with an excitation of 365 nm and an emission of 450 nm. The detection limit was set by a signal-to-noise ratio of 3, and the quantification limit was determined by setting a 500 mV lower area rejection. The two mobile phases used for the analysis were constituted of a solution of 25 mM $K_2HPO_4$ (mobile phase A), with a final pH of 7, and HPLC-grade methanol (mobile phase B), the mobile phases had a flow rate of 0.5 ml/min with gradient elution (Table 1). The total

run time of analysis was 16 min/sample, with 1.5 min before integration. The limit of detection of the method is 3.3 nmol/l TPP, 4.1 nmol/l TP, 2.3 nmol/l T, corresponding to 1.4 ng/ml for both TPP and TP and 0.6 ng/ml for T.

### 3.2.6 Flow cytometry

To assess cellular membrane damage, 1 ml of cell culture samples were stained using 1 µl of 2 mM propidium iodide (PI) and 1 µl of 0.33 mM SYTO9. Both dyes were dissolved in dimethyl sulfoxide before addition to the cells, vortexed after addition and incubated in the dark for 15 minutes. Both stains bind to nucleic acids but differ in their uptake. Whereas SYTO 9 will cross intact and compromised cellular membranes staining all cells green, PI will only permeate the membrane-compromised cells, resulting in a red colour. The stained samples were analysed using a FACS Aria III flow cytometer (BD Biosciences), collecting data from 100,000 cells per measurement. We used a 488-nm laser for measuring FSC, SSC (488/10 filter) and SYTO9 (502 LP with 530/30 filter). A 561-nm laser was used to determine the PI stain values, applying a 600 LP with a 610/20 filter set. The data were preselected using FSC, SSC and SYTO9 signals, removing background noise and enabling the gating of yeast populations. As a membrane compromised control, we used 1 ml of cell culture heated at 70°C for 15 minutes. To validate SYTO 9 staining (viability control), we used a fresh overnight culture of strain 1252, which exhibited low levels of cell damage as observed in our preliminary experiment. All the samples were collected and analysed from two independent experiments.

### 3.2.7 Data and statistical analysis

The data generated in this study were stored as xlsx files using Excel v 16.0, and data analysis and graphical representation were carried out with RStudio v 4.2.1. We employed the software Chromperfect v 6.0.18 to analyse the chromatographic data. Flow cytometry data was analysed using FlowJO v10 to determine the percentage of PI-stained cells. To assess the correlation between extracellular thiamine and cell lysis, we carried out a Shapiro-Wilk test to check for the normality of the data and established the correlation via Spearman's rank correlation coefficient. We used an analysis of variance and Tukey post hoc test to assess statistical significance, with an $\alpha$ set at 0.05.

### 3.2.8 Glossary

This study refers to the total amount of thiamine, including all the vitamers, as "thiamine." When we refer to a specific vitamer, we use the corresponding abbreviation: "T" (unphosphorylated thiamine), "TP" (thiamine phosphate), "TPP" (thiamine

pyrophosphate), and "TTP" (thiamine triphosphate). When we talk about the thiamine yield for biomass produced (nmol/g DW), we refer to it as "specific productivity", while we use the term "yield" (nmol/ l) to refer to the production of thiamine in a litre of liquid culture. Finally, when we refer to the specific proportion of the different vitamers compared to the total amount of thiamine, we do it using the term "pool" or "proportion" (this is either a percentage or a fold-change).

## 3.3 Results

### 3.3.1 Thiamine production is strain dependent

Since thiamine production is growth stage dependent, the 48 *S. cerevisiae* strains were grown for 72 h in CDM, reaching a stationary growth phase to properly compare their capability of producing thiamine. We extracted and measured thiamine from the fresh yeast biomass and took samples of the cell-free supernatant of the yeast cultures (Figure 1 A). The highest thiamine specific productivity, considering both the intracellular and extracellular thiamine pool, was found in strain CBS 3012, which produced 160.9 nmol/g DW. Strain CBS 2888 was found to be the second highest thiamine producer,

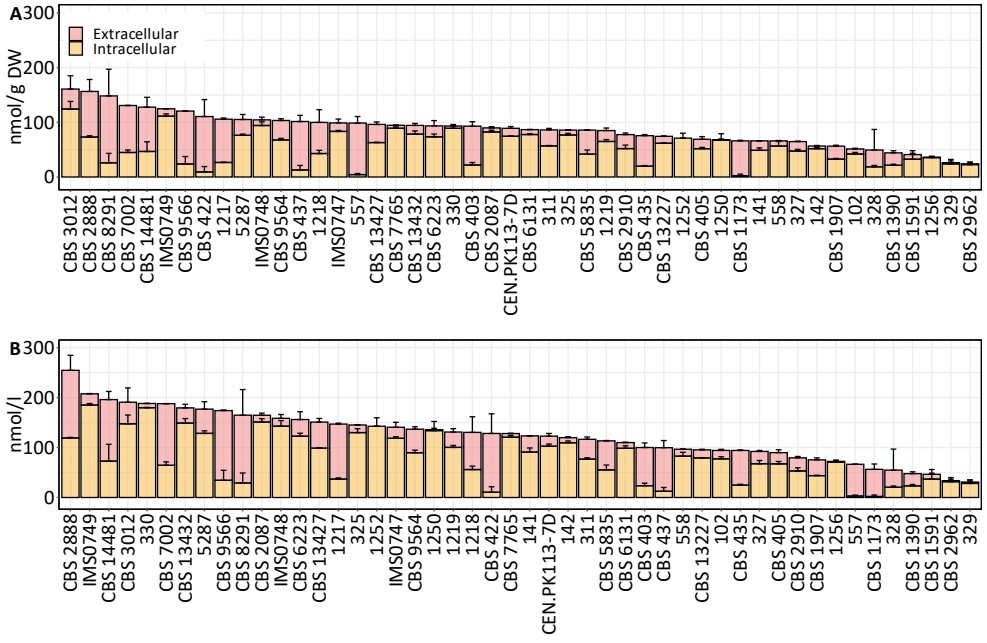


Figure 1- Total thiamine specific productivity (A) and yield (B) in 48 strains *of Saccharomyces cerevisiae* after 72 h of growth in the thiamine-free medium at 30°C and 180 rpm. Error bars represent the standard deviation of two independent experiments. The X-axis indicates the strain name. Total extracellular thiamine is coloured in pink, while total intracellular thiamine is in yellow.

which produced a total of 156.5 nmol/g DW of thiamine. The two lowest thiamine producers were CBS 2962 and 329, with 24.1 and 26.2 nmol/g DW respectively, whereas the overall average thiamine specific productivity was 86.6 nmol/g DW. The average thiamine yield of all tested strains was 124 for nmol/l of thiamine (Figure 1 B). Strain CBS 2888 yielded 254.6 nmol/l, more than twice the average value, while the second highest yielding strain IMS0749, produced 207.6 nmol/l. The lowest thiamine yield was measured in strain 329, with only 30.9 nmol/l, well below the average value and about 8.2 times lower compared to the highest strain, CBS 2888.

Most of the thiamine was found intracellularly, representing, on average, 64.6 % of the combined thiamine pool (Figure 5 C). However, for specific strains like 557 and CBS 1173, 95.4 % and 96 %, respectively, all the thiamine was found in the supernatant. Remarkably, no thiamine was detected in the supernatant of strain 1252.

There was a large variation between different strains for the concentration of intracellular thiamine, both in production (nmol/g DW) and yield (nmol/l). The highest overall thiamine producer, strain CBS 3012, had 124.4 nmol/g DW of intracellular thiamine, which is over 60-fold higher compared to the lowest intracellular thiamine found in strain CBS 1173, which had only 2.2 nmol/g DW (Figure 2 A). On average, the

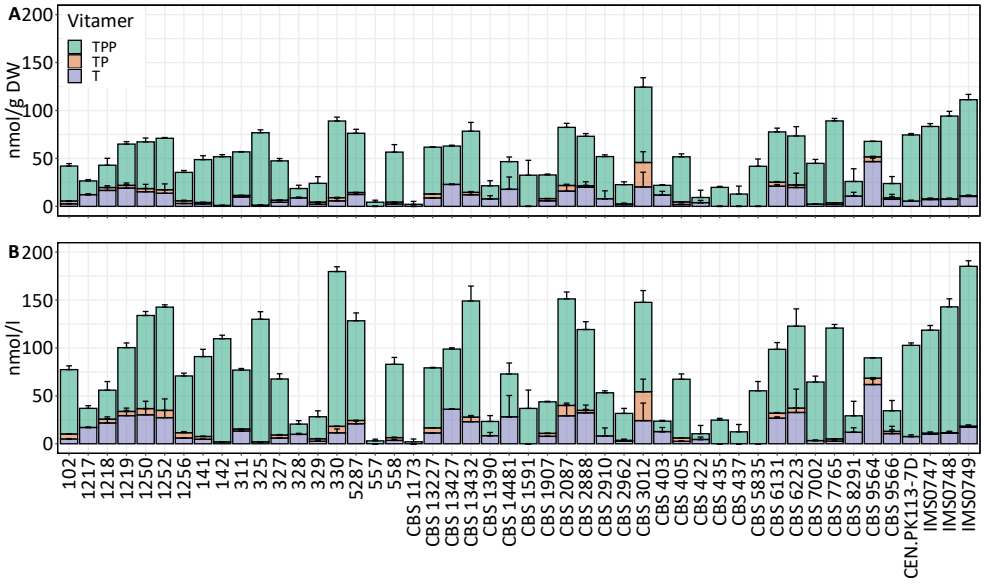


Figure 2- Intracellular thiamine specific concentration (A) and productivity (B) in 48 *Saccharomyces cerevisiae* strains after 72 h of growth in the thiamine-free medium at 30°C and 180 rpm. Error bars represent the standard deviation of two independent experiments. The X-axis indicates the strain name. T is coloured in purple, TP in orange, and TPP in green.

yeasts produced about 52.2 nmol/g DW of intracellular thiamine. Concerning the overall intracellular yield (Figure 2 B), strain IMS0749 yielded a remarkably high amount of 185.2 nmol/l of thiamine, which is very close to the second highest strain, 330, which yielded 179.7 nmol/l. CBS 1173 was still the lowest, with 2.1 nmol/l of thiamine. In terms of intracellular yield, there is a 90-fold difference between the highest and lowest strains, with an average yield of 80.2 nmol/l across all the strains.

### 3.3.2 Extracellular thiamine is released via cell lysis

Extracellular thiamine was detected in the supernatant of all the tested strains except for strain 1252. Other strains, such as 1256 and 1250, showed thiamine levels barely above the detection limit (see section 2.5) of 0.7 for T and 1 nmol/g DW for TP and TPP in total (Figure 3 A). While the highest level of thiamine found in the supernatant was for strain CBS 8291, which has a specific productivity of 122.3 nmol/g DW, which was considerably higher than the average of 34.1 nmol/g DW, strain CBS 9566 had the highest total extracellular thiamine yield (139.6 nmol/l) (Figure 3 B). Due to the expensive nature of this metabolite, and its ATP-dependent active transporter structure, we hypothesised that thiamine is released by *S. cerevisiae* via cell lysis. To test this, we stained the cultures with SYTO9 and propidium iodide to check for damage to the cell membrane. The presence of thiamine in the supernatant of damaged cell cultures and

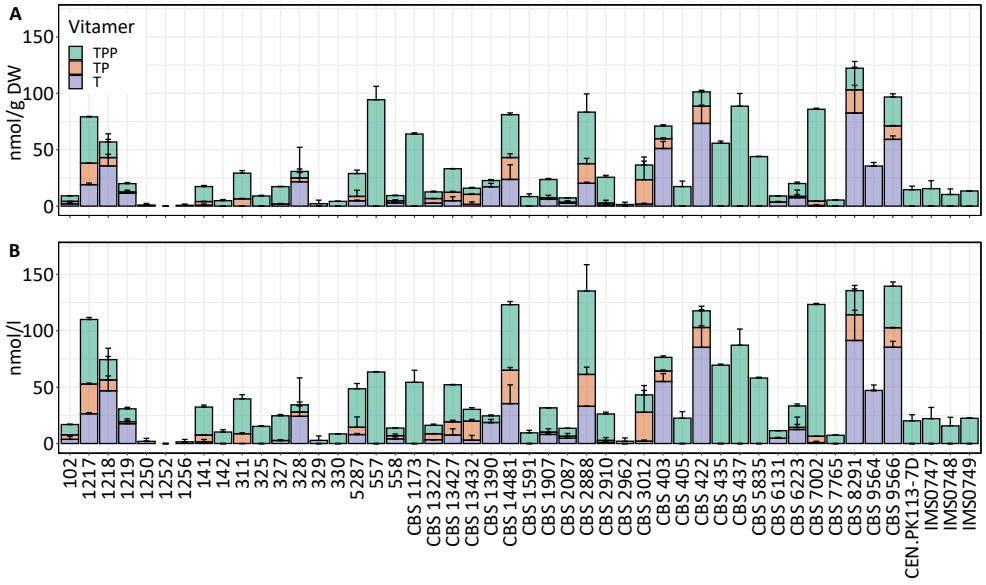


Figure 3- Extracellular thiamine specific concentration (A) and productivity (B) in 48 *Saccharomyces cerevisiae* strains after 72 h of growth in the thiamine-free medium at 30°C and 180 rpm. Error bars represent the standard deviation of two independent experiments. The X-axis indicates the strain name. T is coloured in purple, TP in orange, and TPP in green.

the absence of thiamine in the supernatant of an undamaged cell culture would prove that thiamine is released by leakage from the damaged cell. We found a positive correlation between the percentage of extracellular thiamine and the percentage of PI-stained cells (Spearman's ρ =0.85) (Figure 4), with few outliers, that deviated from the generally observed trend, such as strain 328, CBS 3012, and CBS 2962. These outliers showed relatively high cell damage but a low percentage of extracellular thiamine. Moreover, we observed a high degree of cell damage across all the strains, with 14 strains showing PI staining in more than 90 % of the cells. Additionally, no strain showed the release of extracellular thiamine without signs of cell damage. These observations are consistent with the supposed release of thiamine through cell damage and lysis.



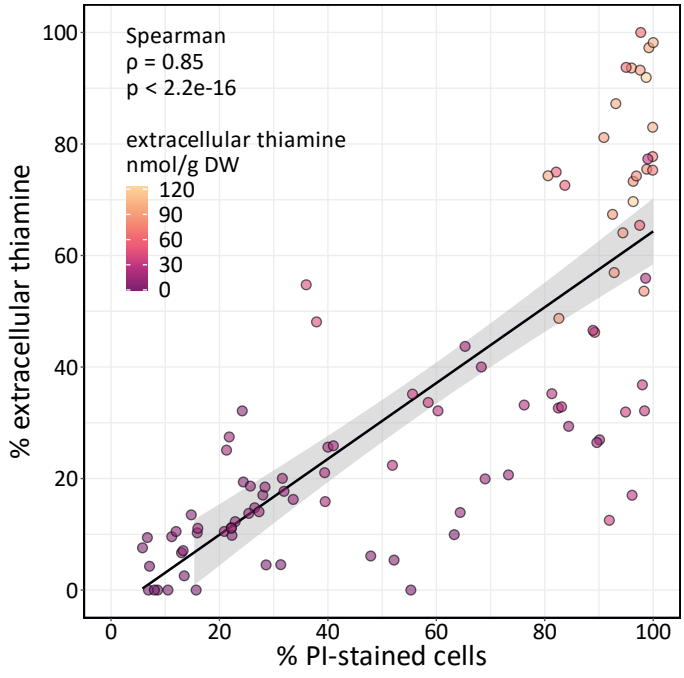


Figure 4- Correlation between the percentage of extracellular thiamine (y-axis) and the percentage of PI-stained cells (x-axis). Each point represents a unique observation. The correlation between the two variables was calculated via Spearman's rank correlation coefficient. The colour indicates the concentration of extracellular thiamine, with high amounts coloured yellow and low amounts in purple.

### 3.3.3 Thiamine pyrophosphate is the most abundant vitamer

The vitamer with the highest intracellular concentration detected was TPP, followed by T and TP (Fig. 5A); no TTP was found in any of the extracts or supernatants. TPP was present in all the tested strains. It was the only detectable intracellular vitamer in six strains and accounted for, on average, 83.5 % of the total thiamine vitamer pool (Figure

5 A). The lowest intracellular TPP proportions were found in strain CBS 9564 and strain CBS 422, accounting for 32.7% and 37.8 % of the total thiamine, respectively. TPP comprised at least 58.6 % of the total vitamer pool in all the other strains. T was detected in 42 out of the 48 strains, displaying a large variation in the distribution of this vitamer. It constituted between 1 % for strain 325 and 41.4 % for strain CBS 403 of the total intracellular thiamine pools, except for CBS 422 and CBS 9564, in which it constituted 62.2 % and 59.1 %, respectively, the former strain having a large standard deviation in the vitamer distribution. TP was not detected in 19 of the tested strains, and in the other strains, it represented between 0.3 % for strain IMS0748 and 8.2 % for strain CBS 9564, with an average of 2.7 % of the entire thiamine pools. The only exception was the highest TP-containing strain, strain CBS 3012, for which TP constituted about 18.9 % of the total thiamine. When examining the extracellular vitamer distribution in the supernatant (Figure 5 B), TPP still appeared to be, on average, the most abundant vitamer detected. TPP constituted 66.1 % of the total extracellular thiamine pool, followed by T with 24.2 % and TP with only 9.7 %. The variation in the vitamer proportion of the extracellular total thiamine pool seemed higher than the total intracellular thiamine pool, but for most strains, the vitamer proportion percentage resembled the intracellular one. This further provides evidence that thiamine leaks out of the yeast cell because of cell damage rather than through active transport, in which case it would, more likely, be vitamer specific. The only exceptions to this trend were strains 1250 and 1256, having a very low concentration of extracellular thiamine, mostly consisting of the T vitamer. In contrast, TPP was the most abundant intracellular vitamer.



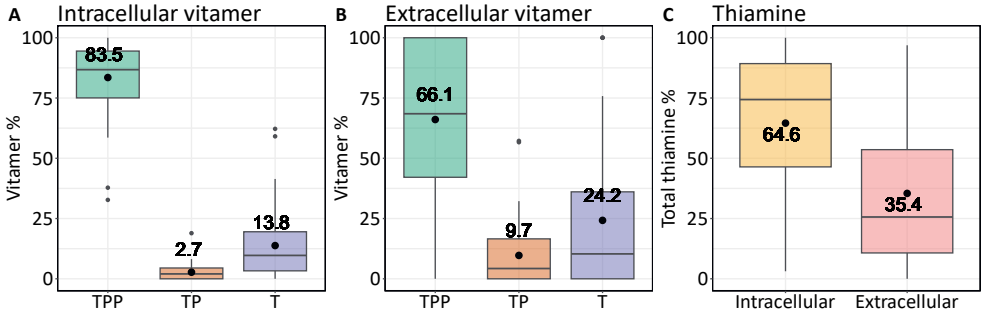


Figure 5- Intracellular (A) and extracellular (B) vitamer distribution percentage and thiamine distribution (C) among all the 48 strains. The black dot represents the average, while the number represents the percentage value in 48 *S. cerevisiae* strains after 72 h of growth in the thiamine-free medium at 30°C and 180 rpm. T is coloured in purple, TP in orange, and TPP in green. Total extracellular thiamine is coloured in pink, while total intracellular thiamine is in yellow.

### 3.3.4 Adaptive laboratory evolution can influence thiamine productivity

Adaptive laboratory evolution using a thiamine-free medium was previously carried out by Perli, Moonen, et al. (2020) on strain CEN.PK113-7D (WT) for 300 generations. This yielded various mutants selected for having growth rates comparable to the WT strain grown in a thiamine-rich medium. These selected mutants were IMS0747, IMS0748, and IMS0749. We included these evolved strains in this screening to assess whether adaptive laboratory evolution led to elevated thiamine productivity and increased yield. Overall, we observed that, on average, the evolved strains had higher thiamine productivity, yield, and biomass formation than the WT. However, not all these differences were statistically significant (Figure 6) ($\alpha$=0.05). Concerning the intracellular concentration, strain IMS0749 had the highest increase in total thiamine at 49 % compared to the WT, followed by strain IMS0748 with an increase of 26 %, and IMS0747 with only a slight 12 % increase (Figure 6 A). Yet, for the latter strain, this increase was not statistically significant ($p$-value>0.05).



The biomass production after 72 h of growth for the WT was 1.38 g DW/l (Figure 6 E); strain IMS0747, IMS0748, and IMS0749 produced 1.42, 1.52, and 1.66 g DW/l, respectively. The only significant increase in biomass was for strain IMS0749 ($p$-value<0.05). When coupling this increase in biomass with the intracellular thiamine productivity, it was observed that the intracellular thiamine yield increased significantly for IMS0748, and IMS0749, with the latter strain having an overall increase of 80 %. Comparing this strain to all the ones screened during this study, it had the second highest specific intracellular thiamine productivity of 111.3 nmol/g DW and the highest intracellular thiamine yield of 185.2 nmol/l. The sum of intracellular and extracellular thiamine productivity increased significantly for strain IMS0749 by 40 % compared to the WT (Figure 6C). This increase was not significantly different from the other evolved *S. cerevisiae* strains, which increased only by 11 % and 17 %. Regarding the total thiamine yield (Figure 6D), CEN.PK113-7D produced 123 nmol/l of thiamine, while the evolved variant IMS0749 showed a significant 60 % increase in thiamine, reaching 208 nmol/l. IMS0747 and IMS0748 increased, but not significantly, by 15 % and 29 % respectively. Adaptive laboratory evolution did not significantly impact the intracellular or extracellular vitamer distribution (Supplementary material 4), extracellular thiamine concentration (Figure 3 A and B), nor the extent of cell lysis which appeared to decrease only slightly (Figure 7B).

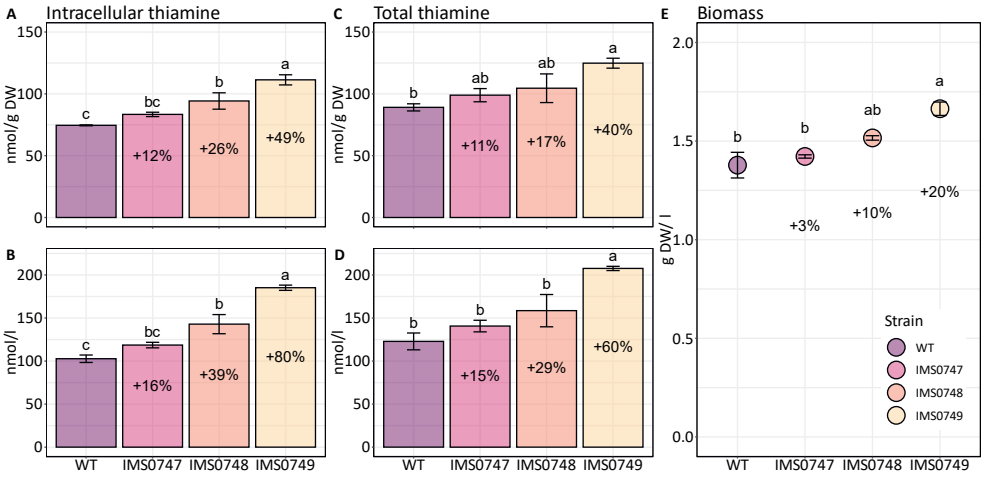


Figure 6- Intracellular thiamine specific productivity (A), yield (B), total thiamine specific productivity (C), yield (D), and biomass formation (E), in strain CEN.PK113-7D (WT) and evolved strains. Different letters indicate a significant difference ($p$-value<0.5) calculated with analysis of variance and a Tukey post-hoc test. The percentage represents the increase of a specific value compared with the WT. X-axis indicates the strain name. Different colours are used to represent each strain.

## 3.4 Discussion

This study aimed at assessing strain-to-strain variations in thiamine production capabilities in *S. cerevisiae* with specificity for each of the vitamers detected in the cell extracts and supernatants. For this purpose, we selected 48 strains and grew them in a thiamine-free chemically defined minimal medium, which contained glucose and ammonium sulphate as the main carbon and nitrogen sources, respectively. Afterwards, we measured the intracellular thiamine concentration and the thiamine in the supernatant. We also stained the cells with PI and SYTO9 and analysed them using flow cytometry to assess possible cell damage. This study constitutes the first systematic screening of a large number of *S. cerevisiae* strains originating from a variety of niches for thiamine production (with each vitamer specifically detected), coupled with the assessment of cell permeability. We chose this growth medium because it is simple, cheap, and chemically defined; therefore, there are no batch-to-batch variations in the composition which could affect growth or thiamine productivity. The medium also lacked amino acids to select prototroph strains for all amino acids and therefore did not require medium supplementation. The latter is relevant because any supplementation affects thiamine production and potential costs. In this study, we correlated the presence of extracellular thiamine in the tested strains with the degree of cell membrane integrity, thereby showing the role played by cell damage due to aging and low pH in the growth medium in the release of thiamine. Cell damage was expected, as

ammonium sulphate causes a fast decrease in pH of this unbuffered medium (Prins et al., 2021).

The strains used in this study were either of unknown origin or were isolated in Asia, Europe, Africa, and even Antarctica. The isolation substrates ranged from fruit to alcoholic beverages, fermented food products, fish, seawater, and human microbiota (Supplementary material 2). We selected a broad diversity of strains hoping to find differences in phenotype regarding thiamine production and ecological niche. However, we could not find any correlation between the source of isolation and the phenotype of the yeasts in terms of biomass, cell permeability, and extracellular and intracellular thiamine productivity (Supplementary material 1, Supplementary material 3).



Thiamine triphosphate (TTP) is a thiamine vitamer with an unknown biological role in *S. cerevisiae*. TTP was found to accumulate in low amounts in yeast cells (Makarchikov et al., 2003). However, in this study, we could not detect TTP in the cell extracts, although our detection method can detect it. Therefore, in our samples, TTP was either absent or present in concentrations below the detection limit (see section 2.5). Either way, the presence of this vitamer is assumed to be negligible when quantifying the total thiamine produced, also because of the low concentrations in which it is usually detected (Makarchikov et al., 2003).

We measured cell damage and lysis using PI-staining. Strain 328, CBS 3012, and CBS 2962 were outliers since they showed high staining but a very low percentage of extracellular thiamine. This can be explained by the fact that the PI-staining signal does not indicate the extent of the membrane damage but only the occurrence of it (Davey et al., 2011). Therefore, it is possible that the mentioned strains had only minimal damage on a big percentage of the entire cell population, resulting in minimal thiamine leakage in the growth medium.

The presence of TPP as the most abundant vitamer agrees with the existing literature (Labuschagne & Divol, 2021; Nishimura et al., 1997). It is reasonable that the active form of the vitamin is the most abundantly present since T and TP are intermediates of TPP biosynthesis and are not known to have any biological activity. We identified some outliers regarding intracellular vitamin composition (Supplementary material 4), for instance, strain CBS 3012 and CBS 9564. Both strains had a lower proportion of TPP and were relatively higher in TP and T, and strain CBS 3012 had the highest thiamine productivity overall. Especially the vitamer distribution in strain CBS 9564 is particularly odd. The elevated levels of T, a direct precursor of TPP, could suggest a possible reduced capability of converting T to its active form. Nishimura et al. (1991) isolated a

naturally resistant mutant on a medium containing the thiamine antagonist oxythiamine that displayed reduced thiamine pyrophosphokinase activity only when in a thiamine-rich medium. Once grown on a thiamine-free medium, this mutant had standard vitamer proportion (high TPP and low TP and T) and T concentration. Therefore, it is likely that the outliers in our study that display such a phenotype in the thiamine-free medium could have either a downregulation or a mutation, directly or indirectly affecting *THI80* and resulting in a lower efficacy in converting T to TPP. Strains with this phenotype are particularly interesting because of their potential biotechnological application as thiamine producers. Indeed, the thermal degradation of TPP does not produce certain meat-like flavours, such as 2-methyl-3-furanthiol and 2-furfuryl-thiol (Grosch et al., 1992), which are typically formed during the thermal degradation of T. Therefore, a strain that produces more T in the free form would develop meat flavours more easily upon exposure to heat since thermal treatment of TPP is effective in converting it to TP, but not to T (Rocchi et al., 2022). This trait is especially useful for the in-situ fortification of a food product via fermentation to convert thiamine to a meat flavour.



All the tested strains were thiamine prototrophic since they were grown for at least three sequential transfers (from cryovials to plates, to pre-inoculum, and to shake-flasks) in thiamine-free medium to avoid carry-over during this screening. The biomass produced ranged from 0.68 g DW/l and 0.85 g DW/l for strain 557 and CBS1173, respectively, and 2.12 g DW/l for strain 142 (Figure 7 A). We searched for a correlation between the amount of intracellular thiamine produced (per g DW) and the dry weight (Supplementary material 3), but we found only a mild positive correlation between the two ($\rho$=0.56). We also found no correlation between the total thiamine produced (per g DW) and the amount of dry weight. In general, the evolved strains showed a trend in increased thiamine concentration, productivity, and biomass, although this increase was not always significant for all the evolved strains. Indeed, IMS0749 was the only strain for which this trend was consistently significant. The genomes of CEN.PK113-7D and the evolved strains have been previously sequenced by Perli, Moonen, et al. (2020), and none of the mutations found in the evolved strains had a direct link with thiamine metabolism. Therefore, it is not possible to establish a causal relationship to higher thiamine production. However, a segmental amplification on chromosome VII from nucleotide 802,500 to 837,000 of 34 kb was observed in strain IMS0749. This amplification led to double copy numbers of the genes in that region, which included *THI4*, the single turnover enzyme responsible for the formation of HET-P. Overall, the amplification of the chromosomal segment harbouring *THI4* may explain the increased thiamine productivity.

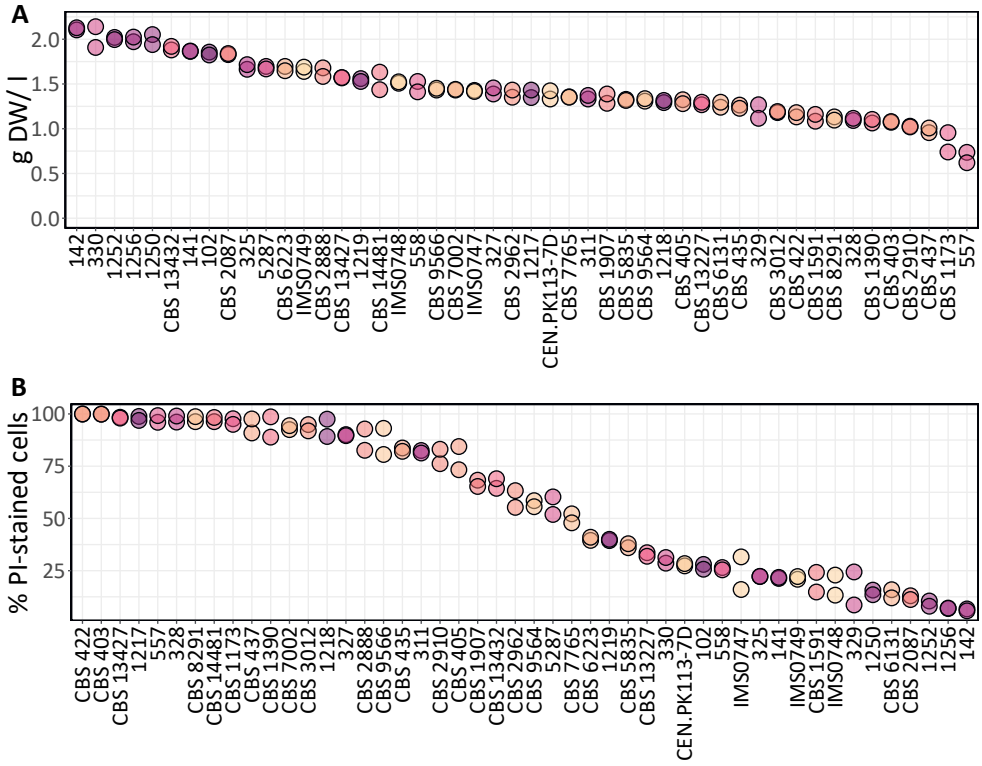


Figure 7- Biomass formation (A) and percentage of PI-stained cells (B) in 48 *Saccharomyces cerevisiae* strains after 72h of growth in thiamine-free medium at 30°C and 180 rpm. Each circle represents an independent experiment. The X-axis indicates the strain name. Different colours are used to represent each strain.

The highest thiamine concentration was found in strain CBS 3012, which produced 160.9 nmol/g DW; this amount corresponds to 42.7 µg/g DW. While the highest total productivity achieved was 254.6 nmol/l (CBS 2888), corresponding to about 67.6 µg/l. Thiamine addition to a substrate has been reported to increase the formation of meaty odours (Dreher et al., 2003) and the overall aroma intensity in a dose-dependent way. For instance, Thomas et al. (2015) added between 8 and 739 mg/kg of thiamine to ham and found that between 8.6 and 56 mg/kg, there was the steepest increase in aromatic score, with 99.5 mg/kg already yielding an intense flavour after cooking. In our case, CBS 2888 and CBS 3012 produced less than the 100 ppm (or 100 mg/kg) required to produce a meat flavour. Still, the amount of thiamine naturally found in pork meat ranges from 5.5 to 8 mg/kg, and around 1.1 mg/kg for beef (Dawson et al., 1988; Driskell et al., 1998), which is far below the 42.7 mg/kg DW we found in our yeasts' biomass. A possible limitation in using *S. cerevisiae* strains for the in-situ fortification is

the presence of vitamin $B_1$ in the substrate. Protein-rich legumes, commonly used as substitutes to meat or substrate to produce meat replacers, have a thiamine concentration varying between 9 mg/kg for dried soybeans, or 7.5 mg/kg for beans, to 5.2 mg/kg in lentils (Lebiedzińska et al., 2006; Vidal-Valverde et al., 2002). This could be a problem since thiamine is synthetized de novo only when absent from the growth substrate. However, this problem could be overcome by isolating thiamine-transporter mutants, for instance, via using thiamine's toxic analogues (Hohmann & Meacock, 1998).

Strain CEN.PK113-7D produced, on average, 123 nmol/l of thiamine, not falling far from the average amount of 124 nmol/l produced by the tested strains. It is possible to isolate a natural thiamine overproducer without genetic engineering by using adaptive evolution. This is because a variant of this strain, which had undergone evolution, produced 60 % more thiamine compared to the parental strain, making it the second highest producer. This is particularly important since such a strain would find an application as a starter culture for a fermented product. Strain CBS 2888 also has an interesting phenotype for the in-situ fortification of food products because it tends to autolyse. On average, this strain showed signs of cell damage in 87.7 % of the cells, releasing thiamine in the supernatant. Yeast autolysis could cause the release of thiamine in the growth substrate, making the vitamin more easily accessible to heat degradation and aiding the formation of meat-like flavours.



## 3.5 Conclusion

In this study, we demonstrated a large variation in thiamine production capacity among *S. cerevisiae* strains of different origins. By accurate selection, it is possible to identify strains with high natural thiamine productivity and yield. We also showed that the active form of this vitamin, TPP, is the most abundant intracellular vitamer across most of the tested strains. In this study, extracellular thiamine release is correlated with cell lysis, explaining the presence of the vitamin in the culture medium after growth. Finally, we demonstrated that it is possible to significantly boost the overall thiamine productivity by carrying out ALE in a thiamine-free medium, increasing thiamine yield compared with the parental strain. The knowledge gained in this study can potentially contribute to the development, via strain selection and/or adaptive laboratory evolution, of *S. cerevisiae* strains that produce elevated amounts of thiamine while growing in a simple minimal medium. The developed strains could be employed for a fermentation-based production of thiamine and for the in-situ enrichment of vitamin $B_1$ in different food products. With this study, we are a step closer to producing precursors for meat-like flavours using fermentation with non-genetically modified organisms.

## Conflict of interest statement

The authors declare no conflict of interest.

## Acknowledgements

We would like to acknowledge Kees van Kekem for helping with the HPLC analysis of thiamine. We would also like to thank Jean-Marc Daran from Delft University for kindly providing the strains IMS0747, IMS0748, and IMS0749 to be included in this screening. Finally, we would like to acknowledge the efforts of Jacqueline Berghout in providing managerial support for this TKI project. This research is part of the project B-Twelve Insight, which is co-financed by the Top Consortium for Knowledge and Innovation Agri & Food by the Dutch Ministry of Economic Affairs. The project is registered under contract number TKI AF18081.

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# CHAPTER 4

## Understanding differences in thiamine production via adaptive laboratory evolution of two *Saccharomyces cerevisiae* strains

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## Abstract

Vitamin $B_1$ has a central role in the metabolism of carbohydrates and amino acids in *Saccharomyces cerevisiae*. To understand the link between fitness and thiamine production, we conducted adaptive laboratory evolution (ALE) on two thiamine producing *S. cerevisiae* strains. These strains had opposite thiamine production phenotypes: the high-producing, fast-growing CBS 3012, and the low-producing slow-growing CBS 1390. We evolved them via sequential propagation in a chemically defined thiamine-free glucose medium, containing ammonium sulphate as nitrogen source, reaching at least 370 generations. Afterwards, we isolated variants from both ancestors and characterised their phenotype in terms of maximum growth rate ($\mu_{max}$), biomass, and thiamine production. The variants evolved from the low-producing strain reached significant increase in $\mu_{max}$. The biomass formation increased by 30 % on average, and thiamine production went up from 16 nmol/l to a maximum of 125 nmol/l. The variants isolated from the high thiamine-producing ancestor did not have a significant increase in $\mu_{max}$ after evolution, and thiamine yield decreased by more than half, going from 222 nmol/l to around 100 nmol/l, but had an increase in biomass formation of about 10 %. The thiamine yields of the evolved variants (EVs) from both WTs showed convergence towards similar thiamine yields in this specific medium. The whole genome sequencing of all the evolved isolates and ancestor strains showed no mutations in genes encoding for thiamine biosynthetic or thiamine-dependent proteins. We analysed the proteome of each wild type of strain and compared it with each other, and to the proteomes of a selected EV. All thiamine biosynthetic enzymes were estimated to account for 6 % to 7 % of all proteins expressed in the exponential growth phase, and between 3.6 % to 3.9 % in the stationary phase. The high fraction of the proteome dedicated to thiamine productivity shows the high costs of the de novo biosynthesis of thiamine.

## 4.1 Introduction

Vitamin $B_1$ (thiamine) is an important molecule that acts as a co-factor in decarboxylation reactions and transferase-type reactions. In both cases, thiamine-dependent enzymes break carbonyl-adjacent C-H and C-C bonds Frank et al. (2007). In *Saccharomyces cerevisiae*, such reactions are involved in the central carbon metabolism (transketolase, pyruvate decarboxylase, pyruvate dehydrogenase, and α-ketoglutarate dehydrogenase), and the biosynthesis and catabolism of amino acids (phenylpyruvate decarboxylase, acetolactate synthase, branched-chain α-ketoacid dehydrogenase, pyruvate decarboxylase)(Hohmann et al., 1998). Therefore, the growth of *S. cerevisiae* is strictly dependent on thiamine pyrophosphate, the biologically active form of thiamine.

*S. cerevisiae* can synthesize thiamine de novo, starting from the synthesis of the thiazole and pyrimidine subunits (Figure 1). This biosynthetic process is metabolically expensive, as both the thiazole and pyrimidine subunits are produced from two enzymes, that by acting as co-substrates, undergo a single catalytic turnover (Chatterjee et al., 2011; Coquille et al., 2012; Wightman et al., 2003). The two moieties are united by a methylene bridge, to form thiamine phosphate (TP), which then loses its phosphate group to become thiamine (T) (Nosaka et al., 1994). The biologically active form thiamine pyrophosphate (TPP) is derived from the pyrophosphorylation of free thiamine (Nosaka et al., 1993). *S. cerevisiae* will preferentially take up thiamine when present in the environment. Uptake is preceded by removal of the phosphates, via a periplasmic acid phosphatase, followed by the free thiamine using an active transport system (Enjo et al., 1997; Singleton, 1997). Because of the metabolically expensive nature of this metabolite, *S. cerevisiae* tends to grow slower when vitamin $B_1$ is absent in the growth medium (Perli et al., 2020).

Adaptive laboratory evolution (ALE) is the experimental procedure of evolving an organism towards having a specific trait, by carefully applying selective pressure by designing specific growth conditions. This approach consists of sequentially propagating a microbial culture, for a prolonged period for spontaneous mutations to occur, and for the one that gives increased fitness in this environment to become dominant. ALE has been employed in laboratory settings to selectively adapt cells to have desired phenotypical traits, improved fitness in specific cultivation conditions, and to better understand the biological mechanisms behind such adaptations (Dragosits et al., 2013).

In our previous work (Rocchi et al., 2023) we screened 48 strains of *S. cerevisiae* for their thiamine production capacity, along with the specific thiamine yields and thiamine

production of three *S. cerevisiae* strains that were evolved from CEN.PK113-7D for 300 generations in thiamine-free chemically defined medium (Perli et al., 2020). We observed a large difference in thiamine production between different strains. Moreover, all the three variants evolved in thiamine-free medium showed an increase in thiamine yield up to 60 %, and a higher growth rate in the thiamine-free medium compared to the WT. These findings raised questions regarding the trade-offs between thiamine production and fitness.

With the aim of answering these questions, we selected two strains with an opposite phenotype in terms of thiamine production capacity. Both strains show a higher growth rate in the presence of thiamine (Rocchi et al., 2023). We sequentially transferred the cultures across three separate evolution lines, in thiamine-free medium for both the high and low producing strain, reaching approximately 370 generations. Afterwards we characterised the phenotype of the evolved isolates by measuring their thiamine yield and their maximum growth rate in thiamine-free and thiamine-rich medium. We then sequenced the whole genome of the variants and WTs to identify the mutations that emerged via evolution. Finally, we compared the proteome of the evolved variants (EVs) to their ancestral WT strains, and the proteomes of two WT strains to understand the difference in metabolism leading to different thiamine-production phenotypes.



## 4.2 Materials and Methods

### 4.2.1 Yeast strains and media

Chemically-defined thiamine-free liquid medium (CDM) and agar (CDA) were prepared as described by Rocchi et al. (2023). CDM and CDA used in this study contained 20 g/l of glucose, 5 g/l of ammonium sulfate, 1.71 g/l of yeast nitrogen base (YNB) without ammonium sulfate, amino acids, and thiamine (U.S. Biological). CDA also contained 1.5 % of agar. Chemically defined thiamine-rich liquid medium (CDM-T) and solid medium (CDA-T) were prepared by adding a solution of thiamine chloride to the thiamine-free medium, reaching a final concentration of 0.4 mg/l.

### 4.2.2 Adaptive Laboratory Evolution

*S. cerevisiae* CBS 1390 and CBS 3012 were selected to be evolved in the thiamine-free medium due to their difference in thiamine productivity in CDM. Each strain underwent evolution in three separate lines by propagating 0.1 % of the culture every 24 h to a fresh batch of 50 ml CDM in a 100 ml Erlenmeyer flask. The flasks were incubated at 180 rpm and 30°C. To monitor adaptation, the $OD_{600}$ of the cultures was measured before each propagation to estimate the generation times based on the number of $OD_{600}$

doublings before transfer. We estimated that each evolution line evolved to reach at least 370 generations.

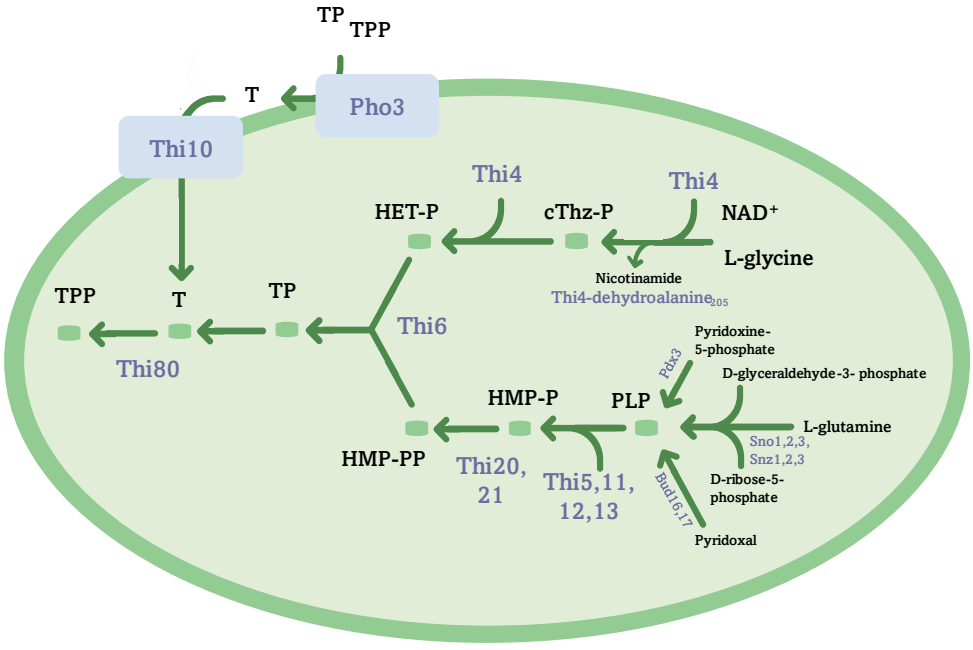



Figure 1- Schematic overview of thiamine transport and de novo biosynthesis. Thiamine biosynthetic enzymes and transporters are purple. Thi4, Thi5,11,12,13 are single turnover enzymes. Pyridoxal phosphate (PLP), the active form of vitamin $B_6$, is synthesized from L-glutamine, D-ribose-3-phosphate, D-glyceraldehyde-3-phosphate by Snz1,2,3 or Sno1,2,3, or via pyridoxal by Bud16,17, or via Pdx3 from pyrozidine-5-phosphate. This molecule reacts together with Thi5, which donates and histidine residue, (or one of the isoforms Thi11,12,13) to form a reaction intermediate 4-Amino-2-methyl-5-(phosphooxymethyl)pyrimidine (HMP-P) that is then converted by Thi20,21 to 4-Amino-5-hydroxymethyl-2-methylpyrimidine diphosphate (HMP-PP). $NAD^+$ and glycine react together with Thi4, which itself donates a sulphur atom from a cysteine residue, to form adenylated thiazole (cThz-P), which is converted again to 5-(2-hydroxyethyl)-4-methyl thiazole phosphate (HET-P). Thi6 binds together HET-P and HMP-PP to form thiamine phosphate (TP), which loses a phosphate group to become thiamine (T) (this reaction is catalysed by a generic phosphatase), and then its converted to the biologically active form TPP by thiamine pyrophosphokinase (Thi80). Thiamine phosphate esters are first hydrolysed by a periplasmic acid phosphatase (Pho3), the free thiamine formed is then transported by Thi10 (also known as Thi7), an active transport system, inside the cell, before being converted again to its active form.

### 4.2.3 Cell dry weight determination

Cell dry weight was determined as described by Rocchi et al. (2022). In brief, membrane filters with a pore size of 0.2 µm were pre-dried at 80°C for at least 48 h and weighed. A vacuum filtration unit was used to filter the yeast culture, the wet membranes were then dehydrated again at 80°C for at least 48 h before weighing.

### 4.2.4 Intracellular thiamine extraction

Thiamine extraction was carried out as reported by Rocchi et al. (2022). An aliquot of yeast culture was first centrifuged at 10,000 x g for 10 minutes. The resulting supernatant was then removed, passed through a 0.2 µm sterile polyethersulfone filter, and kept at 4°C until further processing for thiamine analysis. The leftover yeast pellet was resuspended in double the initial aliquot volume of 0.1 M hydrochloric acid. To lyse the cells, we put a 1.5 ml of yeast suspension in lysing matrix tube B (MP Biomedicals). Each yeast strain was extracted in technical duplicates. The lysis was performed using the Fast Prep system, applying three cycles of bead beating at 5.5 m/s for 1 minute each, with 1-minute intervals. Following this, the extracts were centrifuged again at 10,000 x g for 5 minutes, filtered using a sterile 13 mm, 0.2 µm PES filter, and stored at 4°C prior to derivatization. Three biological replicates for each strain were used to measure intracellular and extracellular vitamin concentrations, the thiamine extraction and measurement were conducted in technical duplicates.

### 4.2.5 Thiamine measurement



Thiamine was quantified as reported by Rocchi et al. (2022), however, we changed the pH of the mobile phase A to 7.0, and the injection volume was increased from 5 µl to 20 µl, to increase the sensitivity of detection. The new limit of detection changed to approximately 0.8 nmol/l TPP, 1 nmol/l TP, 0.6 nmol/l T, which corresponds to approximately 0.4 ng/ml for TPP and TP, and 0.2 ng/ml for T.

### 4.2.6 Maximum growth rate determination

To determine the maximum growth rate ($\mu_{max}$) of the yeast cultures in thiamine-free and thiamine-rich media, yeast strains preserved in glycerol at -80°C were streaked on CDM plates and incubated for 72 h at 30°C. Afterwards, one single colony was suspended in 0.5 ml of thiamine-free CDM, 0.2 ml of this cell suspension were used to inoculate each 10 ml of thiamine-free and thiamine-rich CDM. This pre-culture was incubated for 24 h at 30°C. After growth, we made a $10^{-2}$ dilution of the overnight cultures in the respective media. To estimate the $\mu_{max}$, we made four consecutive 2-fold dilutions of the $10^{-2}$ diluted cultures in a 100-well bioscreen plate, with a final media volume of 0.4 ml for each well. This plate was incubated shaking at 30°C, and $OD_{600}$ measurement were taken every 5 minutes, the measurement was stopped when all the cultures in each well were fully grown. The $10^{-2}$ dilution used to inoculate the 100-wells plates was further diluted to $10^{-4}$ in peptone physiological saline and 50 µl of this culture were spread-plated on the corresponding thiamine-free or thiamine-rich medium and incubated for 72 h at 30°C. µmax was calculated as the negative inverse slope of the time to reach an $OD_{600}$ of 0.2

against the natural logarithm of the initial cell concentration. The $\mu_{max}$ was estimated as the average of at least three-independent experiments.

### 4.2.7 Proteomics analysis

#### 4.2.7.1 Sample preparation

Cultures in early exponential growth (OD~0.6, 8 ml of liquid culture) and in early stationary phase (20h after the first sampling, and with an OD~5, 2 ml of liquid culture), were pelleted at 17,000 x g for 3 minutes at -5°C and washed with freshly thawed sterile water, pelleted again, and frozen in liquid nitrogen, and stored at -80°C until extraction. Cells were suspended in 200 µl SDS lysis buffer (10 % SDS, 100 mM triethylammonium bicarbonate, TEAB pH 7.55), and lysed via bead beating (7 rounds of 1 minute each at maximum speed), afterwards samples were prepared for proteomics analysis according to the S-Trap™ Micro Spin Column Digestion Protocol (ProtiFi, Huntington NY, U.S.A.). Briefly, protein disulphide bonds were reduced by adding TCEP (tris (2-carboxyethyl) phosphine) reaching a final concentration of 10 mM. Iodoacetamide was added to a final concentration of 40 mM to alkylate the cysteines. An aliquot of approximately 100 µg (estimated from initial cell density before lysis) was taken, phosphoric acid was added to 1.2% final and six volumes of 90 % methanol with 0.1M TEAB (triethylammonium bicarbonate was added to generate a protein precipitate suspension. This was loaded on the S-trap mini spin column and subsequently filtered and washed three times with 90 % methanol with 0.1M TEAB. Trypsin was added at 1µg per 100 µg of proteins for overnight digestion incubation at 37°C. Peptides were eluted with 50 mM TEAB, and subsequently with 50 % and 84 % acetonitrile, 0.1 % formic acid. Eluates were dried by vacuum centrifugation and dissolved in 50 µl 2 % acetonitrile, 0.1% formic acid.

#### 4.2.7.2 LC-MS analysis

Protein digests were analysed by LC-MS using a M-class UPLC system (Waters) connected to a Qexactive PLUS (ThermoFisher Life sciences). Peptides were loaded onto a trap column (Symmetry C18 5um beads 300um * 50 mm column) and subsequently eluted onto a Phenomenex Kinetex 2.6um XB-C18 300um * 150 mm column at a flow rate of 3 µl per minute at column temperature of 40°C. Gradient elution was from 4 % to 35 % buffer B (acetonitrile in 0.1% formic acid) during 100 minutes, followed by a gradient to 45 % B during 10 minutes, 5 minutes to 85 % B, 3 minutes at 85 % B, and 5 minutes back to 2 % B, with final stabilisation during 7 minutes. MS data collection was performed in data dependent (DDA) mode with MS1 resolution 70000 in profile mode, AGC target $1 \cdot 10^6$, maxIT 50ms and top10 MSMS precursor ion fragmentation with 2Da isolation window at resolution 17500 in centroid mode, AGC

target $1 \cdot 10^5$, maxIT 100 ms, preferred peptide selection, and a 30 sec dynamic exclusion list.

#### 4.2.7.3 Data processing

The raw data were processed by Fragpipe workflow manager (V 19.0) with MSfragger verison 3.8 (Kong et al., 2017; Nesvizhskii et al., 2003; Teo et al., 2021), IonQuant v1.9.8 (Yu et al., 2021), and Philosopher v5.0 (da Veiga Leprevost et al., 2020). The database search was against the *Saccharomyces cerevisiae* reference proteome in UNiProt UP000002311_for ATCC204508. The search settings were trypsin as enzyme with max 2 missed cleavages, carbamidomethyl as fixed modification for cysteine and oxidation of methionine and acetylation at the N-terminus as variable modifications.

The peptide and the protein table obtained from MSFragger were processed using the online software FragPipe-Analyst (http://fragpipe-analyst.nesvilab.org/, Nesvizhskii (2022)). The statistical analysis was based on the combined_protein.tsv file generated via MSFragger. The software first removed contaminant proteins, and proteins that were not identified/quantified consistantly in the same condition. Then, the MaxLFQ intensity values were converted to $\log_2$ scale, and the samples were grouped by conditions. Finally, the missing values were imputed using the 'Missing not At Random' (MNAR) method, which uses random draws from a left-shifted Gaussian distribution of 1.8 standard deviation apart with a width of 0.3. For the differential expression analysis, the protein-wise linear models were combined with empirical Bayes statistics. To generate a list of differentially expressed proteins for each pair-wise comparison the "limma" package from R Bioconductor was used. We applied a cut-off of the adjusted *p*-value of 0.05 (Benjamini-Hochberg method) along with a $|\log_2$ fold change$|$ of 1 to determine proteins that are significantly differentially regulated in each comparison group. KEGG pathway enrichment analysis was carried out on the online app ShinyGO v0.77 (http://bioinformatics.sdstate.edu/go/, Ge et al. (2020)). The differentially expressed proteins between comparison groups were uploaded, KEGG pathway database was used for background gene counts. The minimal pathway size was set to 2, and the maximum to 2000. We removed the redundancy and choose a maximum FDR cut-off of 0.05.

### 4.2.8 Whole genome sequencing

#### 4.2.8.1 DNA extraction

Genomic DNA for sequencing was extracted using the Wizard Genomic DNA extraction and purification kit according to the manufacturer's instructions. The DNA concentrations were measured using the NanoPhotometer N6 (Implen). The short-

reads library preparation and whole genome sequencing was carried out using Illumina NovaSeq 6000 S4 PE150 XP based paired-end sequencing by Eurofins with 2 x 150 bp paired-end read mode, with a minimum 70x average coverage.

#### 4.2.8.2 DNA sequencing

For long-read WGS, whole genome amplification, fragmentation and loading was performed according to protocol WAL_9070_v109_revT provided by Oxford Nanopore, with slight modifications. In short, the genome DNA was replicated using the REPLI-g mini kit (Qiagen) after which it was fragmented with T7 Endonuclease I (NEB) and this reaction was stopped using Protease K (NEB). The resulting DNA fragments were phosphorylated and repaired using the NEBNext Ultra II end repair/dA tailing module (NEB) and adapters were ligated to the fragments using the SQK-LSK109 Ligation sequencing kit (Oxford Nanopore) in combination with Quick T4 DNA Ligase (NEB). Between steps the samples were cleaned up using Agencourt AMPure XP beads (Beckman Coulter). Prepared DNA libraries were loaded on a Minion R9.4.1 flow cell installed in a MinION Mk1B (Oxford Nanopore) and data acquisition was performed using MinKNOW v23.07.8 with a minimal read length of 1000 bp. Basecalling was done with dorado v0.4.2 using the dna_r9.4.1_e8_sup@v3.6 model.



### 4.2.9 Bioinformatic analysis

#### 4.2.9.1 Genome assembly

For both ancestors the genome assemblies and annotations were performed using the same pipeline. To limit the length and input coverage of the nanopore reads, an initial filtering step was performed, only keeping reads larger than 25 kb. This resulted in a coverage of raw reads between 30 and 40x coverage. The Flye v2.9.2 assembler was used to assemble the genome (--genome-size 12mb) (Lin et al., 2016). Afterwards, this initial genome was polished, also using Flye. Further smaller scale polishing was achieved using the short read libraries of the ancestors in three rounds of Pilon polishing v1.25 (Li, 2013). Reads were mapped with bwa-mem2 v2.2.1 (Walker et al., 2014), and then filtered for quality (-q 20) and sorted using Samtools v1.9 (Li et al., 2009). The resulting bam files were then used as input to Pilon, which resulted in a final assembly.

#### 4.2.9.2 Genome annotation

The genome was annotated using a combination of Augustus v2.5.5 (Stanke et al., 2006) to identify gene positions and YGAP online tool (Proux-Wéra et al., 2012) to establish the identity of these genes. To perform the Augustus annotation Blat v36 (Kent, 2002) was used to map RNA sequences of reference R64 yeast transcriptome (R64-4-1) on the assembled genome (-minIdentity=0.92 -t=dna -q=RNA). The resulting file was

converted to an Augustus hintsfile ('hints.gff') using blat2hints.pl, and then a gff file with full annotations was produced using the Augustus main program (--species= saccharomyces_cerevisiae_S288C --extrinsicCfgFile=extrinsic.E.cfg --hintsfile=hints.gff). From this annotation only gene, exon, cds start and stop positions were retained. This gff file was then used to format an assembly specific annotation library used by snpEff v4.3 (Cingolani et al., 2012) to determine the variant position and effect.

#### 4.2.9.3 Repeat masking

For repeat masking the snpable pipeline of the seqibility toolkit (https://lh3lh3.users.sourceforge.net/snpable.shtml) was used to identify repeat regions in the genome. The assembly was split into 35 bp kmers (splitfa -35 | split -l 20000000) into separate fastq files. These were map using bwa aln and samse v0.7.15 (Walker et al., 2014) which were further processed using gen_raw_mask.pl, gen_mask (-l 35 -r 0.5) and apply_mask_s to produce a fasta file in which the lowercase letters indicate repeat regions. Then the lowercase nucleotides in the genome were identified and coordinates were transformed to bed file format using a custom script.



#### 4.2.9.4 Mapping and variant calling

Raw reads were mapped on the assembled genomes using bwa-mem2 (Li, 2013), after which alingments were filtered for quality (-q 20), sorted and turned into bam format with samtools (Li et al., 2009). Then picard tools v2.26.10 (Broad, 2023) was used to mark and remove duplicates. Freebayes was used to call variants (freebayes-parralel, -F 0.3, v0.9.21) (Garrison et al., 2012) after which snpEff was used to annotate the variants (Cingolani et al., 2012). Then variants were filtered using a custom script in RStudio (v4.0.2). The vcf was read using vcfR (Knaus et al., 2017), with which the reference and alternative read depths were determined using extract.gt function. Variants were considered if they had a minimal and maximum mean (over all samples) coverage of 20 and 300 respectively. Because the CBS 1390 ancestor was a haploid, while the CBS 3012 ancestor was expected to be diploid, variants were differentially filtered. For CBS 1390, variants were allowed to only have one sample with alternative read frequency between 0.2 and 0.8. For CBS 3012, samples were called homozygotes if the alternative read frequency was below 0.01 or above 0.99, while they were called heterozygous if this frequency was between 0.3 and 0.7. Variants were omitted if all individuals, including the ancestor, were heterozygous (as effectively they are similar) and if variants had frequencies between 0.01 and 0.3, or 0.7 and 0.99 in alternative read frequency.

#### 4.2.9.5 Structural variation analysis

We used a previously developed custom pipeline to identify structural variants (Grum-Grzhimaylo et al., 2021). From the final bam files, we identified which reads had a split or reversed layout, or otherwise had larger or smaller than expected insert length. This was done by selecting reads with cigar annotation “I”, “D”, “S” or “H” and flags 65, 67, 81, 97, 113, 115, 129, 131, 145, 161, 177 and 179 and collected these reads into a new bam file with potential structural variants. Then coverage information per 100 bp windows is obtained from all reads and the structural variant reads, using bedtools (v2.27.1) (Quinlan et al., 2010) makewindows and bedtools coverage. Then we calculated the probability that the frequencies were equal between an evolved sample and the ancestor. These were then ranked and the mostly differentiated variants are visually inspected in IGV (v2.8.0) (Robinson et al., 2011).

### 4.2.10 Data and statistical analysis

The data generated during this study were saved as xlsx files using Excel v16.0. We conducted data analysis and created graphical representations using RStudio (v4.2.1). For the analysis of chromatographic data, we utilized Chromperfect (v6.0.18) software. We conducted a Shapiro-Wilk test to examine the data's normality. In case of normally distributed data, we assessed statistical significance via analysis of variance and conducted a Tukey post-hoc test, with an $\alpha$ level set at 0.05. When data did not meet the requirements of normal distribution, we did a Kruskal-Wallis test, followed by a Dunn’s test, the $\alpha$ level set at 0.05.

## 4.3 Results

### 4.3.1 Phenotype characterization of the evolved isolates

We conducted adaptive laboratory evolution for at least 370 generations in thiamine-free medium, with three separate evolution lines for each wild-type. Afterwards, from each line we isolated single colonies, referred to as evolved variants (EVs). We compared them to the ancestral WT strain in terms of maximum growth rate ($\mu_{max}$) in both thiamine-free and thiamine-rich medium, biomass yield and total thiamine yield in thiamine-free medium (Figure 2). The first number in the EVs identification code, followed by an underscore "_" indicates the evolution line from which the specific EV was isolated (for instance 3_9 means evolution line 3, isolate number 9).

For the slow-growing and low thiamine-producing strain CBS 1390, the $\mu_{max}$ in thiamine-free medium significantly increased upon sequential propagation for all EVs ($p<0.05$). The growth rate increased from 0.22 $h^{-1}$ to above 0.3 $h^{-1}$ for all tested strains, with six out of ten strains having a growth rate above the $\mu_{max}$ of the WT in thiamine-rich medium (which was 0.33). Similarly, in thiamine-rich medium, eight out of ten variants had a significantly higher $\mu_{max}$ compared to the wild-type in the same condition.

This increased maximum growth rate was, on the contrary, not observed in the EVs of CBS 3012. Only one variant, CBS 3012 2_18 showed an increase in $\mu_{max}$ in thiamine-free medium compared to the WT (from 0.31 to 0.38), whereas four out of the thirteen isolated variants had a decreased $\mu_{max}$. In thiamine-rich medium, five of the variants had higher growth rate compared to the WT, which had a $\mu_{max}$ of 0.45. Finally, variant CBS 3012 2_18, which already showed increased fitness in thiamine-free medium, had the highest $\mu_{max}$ overall of 0.52.

The biomass yield after 48 h of growth significantly increased for all the CBS 1390 and CBS 3012 variants, except for CBS 1390 2_19, and CBS 3012 2_1, 2_17 and CBS 3012 3_16. While the ancestor strain CBS 1390 produced on average 0.98 g/l, the EVs showed a noteworthy increase in yield by 30 %, producing on average 1.32 g/l. The high thiamine producing ancestor CBS 3012 produced on average 1 g/l, while the EVs isolated after evolution produced on average 1.1 g/l, which is a modest increase of only 10 %.

All the EVs evolved from CBS 1390 had an increase in thiamine yield (intended as nmol of thiamine per litre of culture medium) after 48 h of growth compared to the WT, which initially yielded only 16.4 nmol/l of thiamine. The highest increase in thiamine yield was reached in the EV CBS 1390 2_9, which produced about 124.9 nmol/l of thiamine. The

other isolated EVs produced between 51.9 and 77.9 nmol/l of thiamine. Among these EVs, CBS 1390 2_19 stands out as most of the thiamine produced was found extracellularly. The not-significantly higher biomass yield of the latter EV, and the presence of mostly extracellular thiamine, indicates a high degree of cell lysis. The intracellular vitamer distribution of the isolated EVs is mostly unchanged compared to the WT, except for strain 2_9 which shows a reduction in TPP and a proportional increase in T.

Strain CBS 3012, when grown for 48 h in CDM yielded 222 nmol/l of thiamine, while all the EVs had decreased thiamine yields by more than half, with an average yield of 100 nmol/l, and no significant differences between the different isolates. There were only slight differences in the proportion between extracellular and intracellular thiamine. In the WT intracellular thiamine accounted for 87.5 % of the total, whereas the evolved isolates had on average approximately 95 % of intracellular thiamine. Concerning the intracellular vitamer distribution there was no difference between the WT and the EVs, besides for a minor increase in TPP and proportional increase in T, with TP left unchanged.



### 4.3.2 Whole genome sequencing

We sequenced the genome of both the WTs and EVs to identify the mutations that occurred during ALE. For the two WTs and EVs we used Illumina short-read whole genome sequencing technique. Additionally, for the two WTs we also sequenced using nanopore long-read. In this section we only report non-synonymous single nucleotide polymorphism (SNPs) and structural variations affecting known annotated genes. We excluded the discussion of the observed mutations in unannotated regions of the genome, or non-coding sequences.

#### 4.3.2.1 Genetic variations in the CBS 1390 variants

We detected at least two mutations in each EV isolated from the haploid WT CBS 1390 (Table 1), none of these were in genes encoding thiamine biosynthetic, or thiamine-dependent proteins. Moreover, we detected two deletions, but no gene duplication from our structural variation analysis (Table 2).

*GDS1* is the only gene affected by mutations in the majority of the EVs we isolated except for CBS 1390 2_9 and 2_19. Therefore, this gene is the main target of evolution in these conditions. The protein Gds1 is indirectly involved in promoting the transcription of ribosomal proteins by interacting and modulating the activity of the NuA4 histone acetyltransferase complex. Gds1's structural features also suggest a role in transcription regulation via DNA binding (Joo et al., 2022).

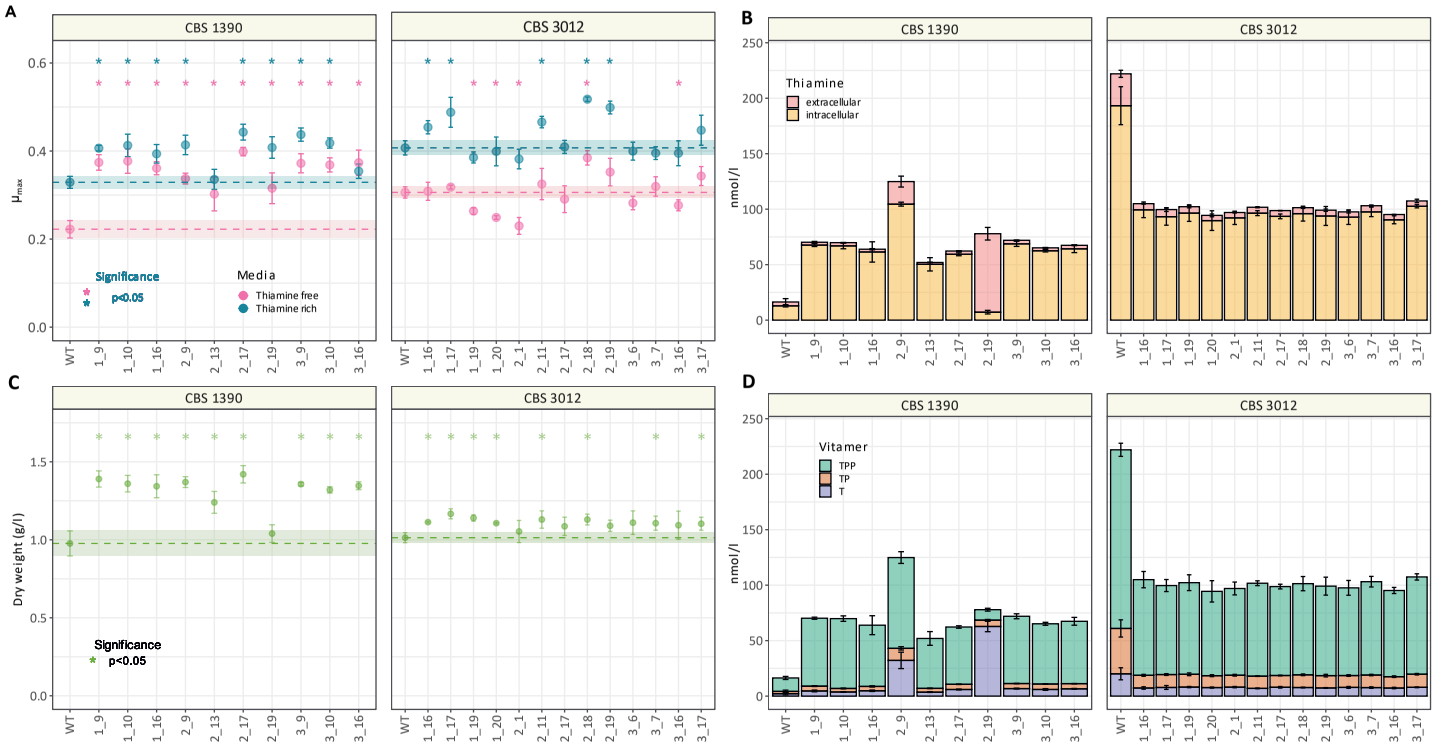



Figure 2- μmax (A) of *S. cerevisiae* and EVs in thiamine-rich (blue) and thiamine-free medium (pink). Biomass yield (B). The sum of extracellular (pink) and intracellular (yellow) thiamine specific yield (C), and vitamer specific distribution of intracellular and extracellular thiamine (D) after 48 h of growth in thiamine-free medium at 30°C and 180 rpm, in both wild type *S. cerevisiae* and EVs. The data is represented on the X-axis by the name of the strain or variant. Total extracellular thiamine is shown in pink and total intracellular thiamine is shown in yellow. T is shown in purple, TP in orange, and TPP in green. The asterisk (*) represents statistical significance compared to the WT ($p<0.05$) calculated with t-test. The standard deviation of three independent experiments is represented by the error bars.

The EVs CBS 1390 2_9 and 2_19, in which *GDS1* is not mutated shared a mutation in *UME6*. This gene encodes a transcriptional repressor protein that regulates the transcription of early meiotic genes, which are repressed during mitotic growth, by recruiting histone deacetylase complexes (Kadosh et al., 1997; Rundlett et al., 1998; Vershon et al., 2000). It also plays a role in controlling the induction of autophagy (Bartholomew et al., 2012), and is involved in transcriptional regulation of *CAR1* (arginase), *CAR2* (ornithine transaminase), *FOX3* (peroxisomal oxoacyl thiolase), and *PHR1* (DNA photolyase) (Einerhand et al., 1995; Mathieu et al., 1997; Sebastian et al., 1991; Strich et al., 1994; Sweet et al., 1997).

In the EV CBS 1390 2_9, which is the highest thiamine producer, we found two missense variants in *AMS1*, *MAS1*, and a deletion affecting *NPL3*. These mutations were not shared with CBS 1390 2_19, therefore are candidate to increase thiamine production. *AMS1* is a vacuole alpha-mannosidase primarily involved in autophagy via the degradation of fructooligosaccharides, and vacuolar protein sorting (Chantret et al., 2003; Umekawa et al., 2016). *MAS1* encodes for a mitochondrial protein of the mitochondrial processing protease complex (West et al., 1992; Witte et al., 1988). *NPL3*, which was affected by a deletion of 70 bp from position 200080 to 200150 (Table 5), encodes for a RNA-binding protein involved in pre-mRNA splicing (Kress et al., 2008), and RNA transport from nucleus to cytoplasm (Windgassen et al., 2004), thereby impacting gene expression. We cannot link any of these mutations directly to the improved thiamine yield phenotype, therefore we conducted proteomic analysis on this EV and the ancestor strain CBS 1390.

#### 4.3.2.2 Genetic variations in the CBS 3012 variants

In the EVs generated from the diploid WT CBS 3012 we did not detect any mutation in genes encoding thiamine-biosynthetic or dependent proteins. We also did not detect any mutation shared across multiple EVs (Table 3). We detected at least one mutation in all EVs except in EV CBS 3012 1_19 and 3_6 in which no mutations were found. In EV 2_17 we detected two mutations, in DNA regions that are not annotated. We found structural variations in three EVs compared, affecting the genes *HPF1*, and *HXT11* (Table 4).

Table 1– List of mutations affecting the annotated genes detected in the variants isolated from CBS 1390. The coverage is indicated in the WT CBS 1390, and in the variants only when the mutations are detected

| Contig or Scaffold | Position | Reference | Alternative | Effect | Amino acid change in protein | Systematic Name | Gene Name | Variant and coverage CBS 1390 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | WT | 1_9 | 1_10 | 1_16 | 2_9 | 2_13 | 2_17 | 2_19 | 3_9 | 3_10 | 3_16 |
| Contig 14 | 299704 | CTGTT | CT | disruptive inframe deletion | p.Gln143del | YCL066W | *HMLA LPHA1* | 164 | | 137 | | | | | | | | |
| Contig 9 | 223931 | T | A | missense variant | p.Glu403Asp | YBR180W | *DTR1* | 157 | | | | | | | | | 233 | |
| Contig 9 | 350094 | G | C | missense variant | p.Cys334Ser | YBR112C | *CYC8* | 156 | | | | | | 128 | | | | |
| Contig 7 | 634074 | C | T | stop gained | p.Gln8* | YDR207C | *UME6* | 129 | | | | 153 | | | 172 | | | |
| Contig 5 | 123271 | A | C | missense variant | p.Leu365Val | YGR234W | *YHB1* | 143 | | | | | | | 181 | | | |
| Contig 5 | 853413 | G | T | missense variant | p.Ser3Tyr | YGL156W | *AMS1* | 161 | | | | 131 | | | | | | |
| Contig 16 | 56926 | G | T | missense variant | p.Gln162Lys | YHR187W | *IKI1* | 169 | | | | | | | | 148 | | |
| Contig 3 | 59920 | C | T | missense variant | p.Val428Ile | YKL203C | *TOR2* | 169 | | | | | | | | | | 122 |
| Scaffold 39 | 273854 | G | A | missense variant | p.Met217Ile | YLR081W | *GAL2* | 142 | | 131 | | | | | | | | |
| Scaffold 39 | 448134 | G | C | missense variant | p.Ala298Gly | YLR163C | *MAS1* | 150 | | | | 127 | | | | | | |
| Contig 4 | 984087 | T | A | stop gained | p.Leu62* | YOR355W | *GDS1* | 154 | 164 | 128 | 133 | | | | | | | |
| Contig 4 | 984317 | A | G | missense variant | p.Lys139Glu | YOR355W | *GDS1* | 168 | | | | | | | | | 272 | |
| Contig 4 | 984381 | C | T | missense variant | p.Ser160Leu | YOR355W | *GDS1* | 161 | | | | | 171 | 146 | | | | |
| Contig 4 | 984413 | GTT | GT | frameshift variant | p.Ile174fs | YOR355W | *GDS1* | 159 | | | | | | | | 145 | | 169 |
| Contig 13 | 98355 | C | G | missense variant | p.Gln809Glu | YPL231W | *FAS2* | 169 | | | | | 161 | | | | | |
| Contig 13 | 650952 | GAAAAA AAC | GAAAAA AAAC | frameshift variant | p.Asn902fs | YPR055W | *SEC8* | 130 | | | | | | | | | | 150 |

Table 2- Structural variations detected in the variants of CBS 1390 compared to the WT, with indicated the length of deletion in bp when detected

| Contig | Position of variation | Type of variation | Affected genes | Variant and bp length of structural variations | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1_9 | 1_10 | 1_16 | 2_9 | 2_13 | 2_17 | 2_19 | 3_9 | 3_10 | 3_16 |
| Contig 7 | 360600 to 363900 | Deletion | *HXT3* YDR344 Complete *HXT6* | 3300 | | 3300 | | | | | | | |
| Contig 7 | 200080 to 200150 | Deletion | Partial *NPL3* Partial | | | | 70 | | | | | | |

### 4.3.3 Proteome analysis

We analysed the proteome of the two WT's and one selected mutant for each WT in exponential growth and stationary phase, while also measuring thiamine and biomass production. For CBS 1390 we selected the EV 2_9, since it had highest thiamine yield, and most mutations compared to the ancestor strain. For CBS 3012 we selected the EV 2_17, which showed unchanged maximum growth rate and biomass, but lower thiamine yield.

In total we identified 2880 unique proteins, derived from the detection of 25975 peptides. After filtering out contaminant proteins, and proteins which were not constantly detected in the same biological conditions, we selected 1895 unique proteins. To identify significant changes in the proteome we set an α level for the adjusted *p*-value to 0.05. Biological changes in protein abundance a fold change between the two conditions of at least one $|\log_2$ fold change$|$ (indicating a doubling), were considered to be significant. Due to the high similarity of the proteins Thi5, Thi11, Thi12, and Thi13, we could not distinguish them based on the amino acid sequence of the detected peptides. Therefore, we selected Thi11 to be the preferred name for this gene family in this context, that was quantified all together. Moreover, we refer to the thiamine transporter Thi10 (YLR237W) with its alias Thi7, as this was the gene name selected by the data analysis software

Table 3– List of mutations affecting the annotated genes detected in the variants isolated from CBS 3012. The coverage is indicated in the WT CBS 3012, and in the variants only when the mutations are detected

| Contig or scaffold | Position | Reference | Alternative | Effect | Amino acid change in protein | Systematic Name | Gene Name | Variant and coverage CBS 3012 | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | WT | 1_16 | 1_17 | 1_19 | 1_20 | 2_1 | 2_11 | 2_17 | 2_18 | 2_19 | 3_6 | 3_7 | 3_16 | 3_17 |
| contig 11 | 230261 | T | A | missense variant | p.Asn168Lys | YIL048W | *NEO1* | 127 | | | | | | | | | | | 202 | | |
| contig 11 | 374736 | C | G | missense variant | p.Gly155Arg | YIR027C | *DAL1* | 151 | | | | 167 | | | | | | | | | |
| contig 11 | 380841 | C | T | missense variant | p.Arg465His | YIR031C | *DAL7* | 136 | | | | | | | | | | | 202 | | |
| contig 14 | 16830 | A | G | missense variant | p.Asn101Asp | YHR043C | *DOG2* | 168 | | | | | 133 | | | | | | | | |
| contig 14 | 148461 | C | G | missense variant | p.Asp270His | YHL025W | *SNF6* | 134 | | | | | | 223 | | | | | | | |
| contig 20 | 4709 | C | T | missense variant | p.Arg391Lys | YGR166W | *TRS65* | 224 | | | | | | | | 225 | | | | | |
| contig 20 | 706100 | C | T | missense variant | p.Glu140Lys | YGL208W | *SIP2* | 220 | | | | | | | | | 174 | | | | |
| contig 29 | 926872 | C | T | missense variant | p.Ala3Val | YDL190C | *UFD2* | 151 | | | | | | | | | | | | | 100 |
| contig 31 | 200102 | C | T | upstream gene variant | | YGR271W | *SLH1* | 227 | | | | | | | | | | | | | 109 |
| contig 37 | 660348 | G | A | missense variant | p.Ala647Val | YNL279W | *PRM1* | 133 | | 125 | | | | | | | | | | | |
| contig 38 | 108832 | C | A | missense variant | p.Val68Phe | YAL034C | *FUN19* | 149 | | | | | | | | | | | | | 107 |
| contig 38 | 170131 | G | A | missense variant | p.Glu192Lys | YOR308C | *SNU66* | 140 | | | | | | 217 | | | | | | | |
| contig 51 | 304710 | T | A | missense variant | p.Asn361Ile | YDR371W | *CTS2* | 148 | | | | | | | | | | | 172 | | |

Table 4- Structural variations detected in the variants of CBS 1390 compared to the WT, with indicated the length of deletion (del) in bp when detected

| Contig | Position of variation | Type of variation | Affected genes | Variant and bp length of structural variations | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1_16 | 1_17 | 1_19 | 1_20 | 2_1 | 2_11 | 2_17 | 2_18 | 2_19 | 3_6 | 3_7 | 3_16 | 3_17 |
| Contig 38 | 1018250 to 1020250 | Deletion | *HPF1* partial | 2000 | | | | | | | | | | | | |
| Contig 38 | 1017750 to 1021250 | Deletion | *HPF1* complete | | | | | | | | 3500 | | | 3500 | | |

All the tested strains had a relatively similar proteome profile in exponential growth (Figure 4A), with each WT and respective EV showing closer similarity to each other. In stationary phase, each WT grouped together with its respective EV, but deviated from the others. This mirrors differences in intracellular and extracellular thiamine concentrations and yields (Figure 3), which are similar in exponential growth among all the strains but deviate significantly during stationary phase. We found that for all the strains, except for the high thiamine producing WT CBS 3012, thiamine concentration per grams of biomass decreases in stationary phase compared to exponential. This coincides with the change in relative abundance of Thi4, the thiamine thiazole-synthase single-turnover enzyme, decreasing 4-fold in stationary phase compared to exponential, in all strains with the only exception of CBS 3012, for which it decreased only 2-fold (Table 5). Thi11 is significantly above the doubling threshold, in the exponential growth compared to stationary phase only for CBS 3012. Pdc1 is high in the exponential growth phase and lower in the stationary phase. Tkl2, which is not detected in any sample in exponential phase, is detected in CBS 1390 and EV in stationary phase. Levels of Thi6, Thi20, and Thi21 remained relatively constant. Thi80, thiamine pyrophosphokinase, is about 2-fold lower during exponential growth compared to stationary phase in all strains. Because of the different phenotypes concerning thiamine production, we wanted to determine the differences in the proteome fraction allocated to thiamine biosynthesis among these strains.

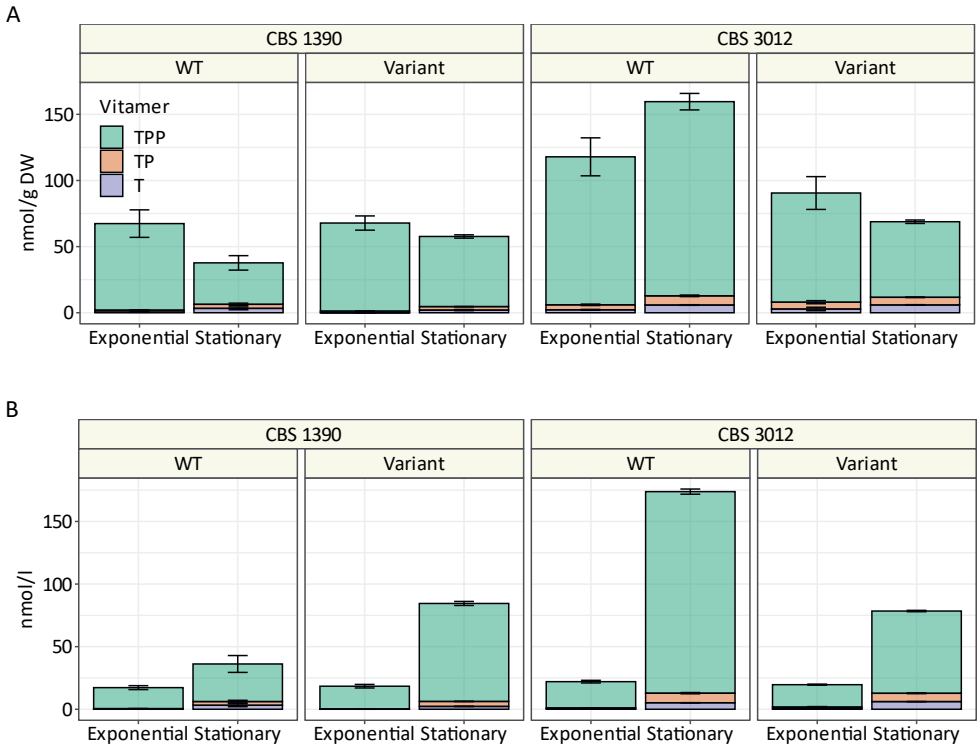
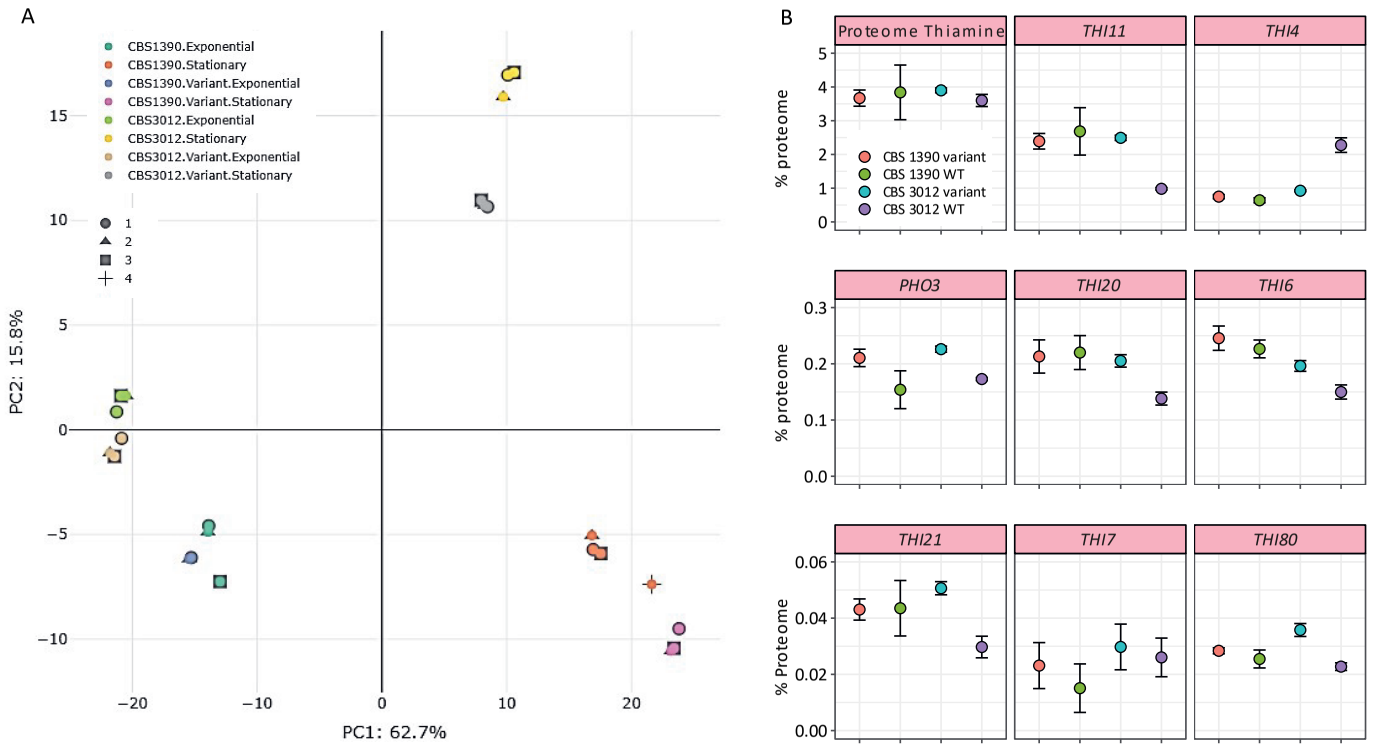



Figure 3- Total thiamine concentration (sum of extracellular and intracellular thiamine) in exponential and stationary phases in the WTs and selected variants. T is in purple, TP in orange, and TPP in green. The error bars represent the standard deviation of three biological replicates.

Figure 4- PCA plot of the proteome of all the tested strains using not imputed data (A), shape indicates the biological replicate, the colour indicates the strain and the sampling point. Only the full observed proteins are included in the PCA. Generated with FragPipe-analyst (http://fragpipe-analyst.nesvilab.org/). (B) proteome percentage, calculated as the ratio between the MaxLFQ intensity of the specific thiamine biosynthetic enzymes, and the total MaxLFQ intensity of the specific sample. The error bar indicates the standard deviation of biological replicates from independent experiments.



#### 4.3.3.1 Proteome fractions of the thiamine-dependent enzymes

To have an estimation of the proteome fraction allocated to thiamine production, we calculated the proteome fraction of each protein by dividing the MaxLFQ intensity of that protein by the sum of the MaxLFQ intensities of every protein detected in that sample. During exponential growth, we estimated that the thiamine biosynthetic or transport proteins represented between 6 % and 7 % of the proteome, which is a remarkable amount. In stationary phase the proportion of proteome allocated to thiamine biosynthesis and transport enzymes decreased overall to reach between 3.6 % and 3.9 % (Table 7, Figure 4B), with no significant differences among the strains. From these results it is evident that most of the thiamine is synthetised during exponential growth compared to stationary phase. Indeed, the proteome fractions allocated to thiamine productivity is much higher during exponential growth compared to stationary phase in all tested strains. Thi11 and Thi4 were among the top ten of most abundant proteins in the whole proteome. In all strains, except for CBS 3012, Thi11 was estimated to represented approximately 2.5 % of the whole proteome fraction in stationary phase, for CBS 3012 it was approximately 1 %. Thi4 had the opposite trend, here it accounted for approximately 2.5 % of the entire proteome in CBS 3012, and around 1 % for all the other strains. Thi6, Thi20, Thi21, and Thi80 proteome in fractions are proportionally lower in CBS 3012 compared to all the other strains (Figure 4B). Pho3, the constitutive thiamine acid phosphatase, had a higher proteome fraction in the EVs compared to the WTs, representing over 0.2 % of the proteome fraction in both EVs.

#### 4.3.3.2 Proteomic differences and KEGG pathway enrichment analysis

We found no significant differences in thiamine-biosynthetic enzymes between CBS 1390 and the EV in stationary, or exponential phase (Figure 5 A, Table 6). Concerning thiamine-dependent enzymes, Pdc6 had an increase of 1.1-fold change in the EV in exponential growth, reaching 1.98 in stationary phase compared to the corresponding WT. KEGG pathway enrichment analysis on the differentially expressed proteins in stationary phase (Figure 6 A, Supplementary material 2) shows that in the WT the pathway for vitamin $B_6$ biosynthesis via Snz1 and Snz3, and pentose phosphate pathway are enriched. Both the metabolism of vitamin $B_6$ and the pentose phosphate pathway are strictly linked to the one of thiamine (Rodríguez-Navarro et al., 2002). The pentose phosphate pathway, which involves the thiamine-dependent enzymes Tkl1 and its paralog Tkl2 (Kowalska et al., 2012), supplies D-ribose-5-phosphate, which is converted by Snz1,2,3 or Sno1,2,3 to PLP (a form of vitamin $B_6$), a thiamine precursor (Paxhia et al., 2019). In the EV, only the KEGG pathway “nucleocytoplasmic transport” showed a high fold enrichment.

CBS 3012 in stationary phase, when compared to the EV, had higher abundance of Thi4 (1.32-fold change), and a proportional decrease in Thi11 (also 1.32 fold change) (Figure 5B, Table 6, Supplementary material 2). All the other thiamine biosynthetic enzymes are significantly higher in the EV, but below the doubling threshold. Concerning thiamine-dependent enzymes, this strain, during both exponential growth and stationary phase had double Pdc1 compared to the EV. KEGG enrichment analysis on the differentially expressed proteins between CBS 3012 and EV in stationary phase (Figure 6B) shows no specific pathway enriched for the EV, but strong enrichment for CBS 3012 of N-glycan biosynthesis via Alg12 and Alg2, and starch and glucose metabolism through the upregulation of Gsk2 and Fsk1 (Supplementary material 3).

Comparing the two WTs in stationary phase, we found opposite trends for Thi4 and Thi11. While Thi4 is higher in CBS 3012, Thi11 is higher in CBS 1390 (Figure 5C, Figure 4B). All the other thiamine biosynthetic enzymes were significantly higher in CBS 1390. We found numerous KEGG pathways enriched in CBS 3012 and CBS 1390. For instance, CBS 3012 had upregulated folate biosynthesis, purine metabolism, and O-glycan biosynthesis. CBS 1390 had 26 KEGG pathways enriched (Supplementary material 3, Figure 6), with the highest fold enrichment in the galactose metabolism pathway (Gal10, Gal1, Gal3, Emi2, Hxk1, Ima1, Suc2, Ugp1, Pgm2). The upregulations of these genes point at the conversion of glucose to UDP-galactose. This process starts from Hxk1 that converts glucose to form alpha-D-glucose-6-phosphate, followed by Pgm2 that forms D-glucose-1-phosphate, which is then converted to UDP-glucose via Ugp1, which is transformed to UDP-galactose (Gal10) (Kanehisa et al., 2015). UDP-galactose plays a role in cell structuring and cell-wall integrity, as UDP-galactose units are fundamental building blocks for the biosynthesis of glycoproteins and polysaccharides. Moreover, we found correlations between the enriched KEGG pathways in CBS 1390 and thiamine metabolism. In CBS 1390 we found enrichment of the metabolism of tryptophan via the proteins Bna1, Bna5. This is interesting, as Bna1 and Bna5, catalyse the biosynthesis of NAD via the degradation of tryptophan and in that context, it is good to realize that $NAD^+$ is a precursor of thiamine. The pentose phosphate pathway was enriched via upregulation of Tkl2, which uses thiamine as co-factor. This pathway supplies D-ribose-5-phosphate to form PLP, which is a precursor of thiamine. Pdx3, which is upregulated (KEGG pathway: biosynthesis of cofactors), produces PLP from pyridoxine-5-phosphate, which is the precursor of the HMP-P thiamine moiety.

Table 5- The fold change and adjusted p-values of the proteins involved in thiamine biosynthesis, or the thiamine dependent enzymes, for the pairwise comparisons between each strain at exponential versus stationary phase. Adjusted *p*-value was calculated via student t-test. A positive fold change value indicates higher abundance in exponential phase compared to stationary, while a negative fold change indicates higher abundance in stationary phase

| Protein | Description | CBS 1390 Exponential vs Stationary | | CBS 1390 Variant Exponential vs Stationary | | CBS 3012 Exponential vs Stationary | | CBS 3012 Variant Exponential vs Stationary | |
|---|---|---|---|---|---|---|---|---|---|
| | | $Log_2$ fold change | $-Log_{10}$ Adjusted *p*-value | $Log_2$ fold change | $-Log_{10}$ Adjusted *p*-value | $Log_2$ fold change | $-Log_{10}$ Adjusted *p*-value | $Log_2$ fold change | $-Log_{10}$ Adjusted *p*-value |
| Ilv2 | Acetolactate synthase catalytic subunit, mitochondrial | 0.55 | 3.66 | 1.05 | 5.97 | 0.54 | 3.31 | 0.44 | 2.48 |
| Kgd1 | 2-oxoglutarate dehydrogenase, mitochondrial | -0.36 | 2.72 | -0.65 | 4.42 | 0.01 | 0.03 | -0.14 | 0.61 |
| Pda1 | Pyruvate dehydrogenase E1 component subunit alpha, mitochondrial | -0.25 | 1.5 | 0.07 | 0.22 | -0.32 | 1.91 | -0.15 | 0.59 |
| Pdb1 | Pyruvate dehydrogenase E1 component subunit beta, mitochondrial | -0.22 | 1.32 | -0.19 | 0.86 | -0.37 | 2.5 | -0.4 | 2.76 |
| Pdc1 | Pyruvate decarboxylase isozyme 1 | 1.65 | 11.07 | 1.56 | 9.75 | 1.05 | 8.04 | 1.04 | 7.88 |
| Pdc5 | Pyruvate decarboxylase isozyme 2 | 0.29 | 2.23 | 0.49 | 3.47 | -0.07 | 0.28 | 0.22 | 1.32 |
| Pdc6 | Pyruvate decarboxylase isozyme 3 | -1.62 | 2.24 | -2.51 | 3.1 | -0.59 | 0.48 | -1.24 | 1.35 |
| Tkl1 | Transketolase 1 | 0.15 | 1.07 | -0.13 | 0.71 | -0.29 | 2.39 | -0.11 | 0.61 |
| Tkl2 | Transketolase 2 | -5.75 | 8.19 | -5.32 | 6.67 | -0.35 | 0.24 | -0.7 | 0.57 |
| Pho3 | Constitutive acid phosphatase | -0.66 | 2.73 | -1.17 | 4.32 | 0.33 | 0.95 | 0.39 | 1.15 |
| Thi11 | 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate synthase *THI11* | 0.45 | 1.92 | 0.82 | 3.31 | 1.55 | 7.07 | 0.94 | 4.38 |
| Thi20 | Hydroxymethylpyrimidine/phosphomethylpyrimidine kinase *THI20* | -0.27 | 1.28 | -0.33 | 1.33 | -0.09 | 0.26 | -0.26 | 1.04 |
| Thi21 | Hydroxymethylpyrimidine/phosphomethylpyrimidine kinase *THI21* | 0.64 | 3.26 | 0.33 | 1.04 | 0.36 | 1.36 | 0.43 | 1.72 |
| Thi4 | Thiamine thiazole synthase | 1.99 | 10.69 | 1.94 | 9.51 | 1.08 | 6.72 | 1.76 | 9.4 |
| Thi6 | Thiamine biosynthetic bifunctional enzyme | -0.36 | 2.92 | -0.19 | 0.92 | -0.24 | 1.48 | -0.24 | 1.48 |
| Thi7 | Thiamine transporter | 1.72 | 1.98 | 0.69 | 0.44 | -0.42 | 0.26 | 0.53 | 0.32 |
| Thi80 | Thiamine pyrophosphokinase | -0.77 | 6.53 | -0.93 | 6.58 | -0.75 | 6.01 | -0.88 | 6.81 |

Table 6- The fold change and adjusted p-values of the proteins involved in thiamine biosynthesis, or the thiamine dependent enzymes, for the pairwise comparisons between each WT and relative variant at exponential or stationary phase. Adjusted *p*-value was calculated via student t-test. A positive fold change value indicates higher abundance in WT compared to the variant, while a negative fold change indicates higher abundance in the variant compared to the WT

| Gene | Description | CBS 1390 vs Variant Exponential | | CBS 1390 vs Variant Stationary | | CBS 3012 vs Variant Exponential | | CBS 3012 vs Variant Stationary | |
|---|---|---|---|---|---|---|---|---|---|
| | | $Log_2$ fold change | $-Log_{10}$ Adjusted *p*-value | $Log_2$ fold change | $-LOG_{10}$ Adjusted *p*-value | $Log_2$ fold change | $-Log_{10}$ Adjusted *p*-value | $Log_2$ fold change | $-Log_{10}$ Adjusted *p*-value |
| Ilv2 | Acetolactate synthase catalytic subunit, mitochondrial | -0.13 | 0.11 | 0.36 | 1.78 | -0.04 | 0.01 | -0.15 | 0.31 |
| Kgd1 | 2-oxoglutarate dehydrogenase, mitochondrial | 0.08 | 0.05 | -0.21 | 1.02 | -0.03 | 0.01 | -0.18 | 0.58 |
| Pda1 | Pyruvate dehydrogenase E1 component subunit alpha, mitochondrial | 0.01 | 0 | 0.33 | 1.82 | -0.28 | 0.89 | -0.11 | 0.22 |
| Pdb1 | Pyruvate dehydrogenase E1 component subunit beta, mitochondrial | 0.14 | 0.21 | 0.17 | 0.73 | -0.27 | 0.91 | -0.3 | 1.35 |
| Pdc1 | Pyruvate decarboxylase isozyme 1 | 0.25 | 0.80 | 0.15 | 0.73 | 1.00 | 6.17 | 0.98 | 6.47 |
| Pdc5 | Pyruvate decarboxylase isozyme 2 | 0.01 | 0 | 0.21 | 1.15 | -0.28 | 1.15 | 0.01 | 0.01 |
| Pdc6 | Pyruvate decarboxylase isozyme 3 | -1.1 | 0.50 | -1.98 | 2.4 | 1.06 | 0.53 | 0.4 | 0.15 |
| Tkl1 | Transketolase 1 | 0.02 | 0 | -0.26 | 1.92 | -0.06 | 0.07 | 0.12 | 0.4 |
| Tkl2 | Transketolase 2 | -0.05 | 0 | 0.38 | 0.23 | 0.26 | 0.03 | -0.09 | 0.01 |
| Pho3 | Constitutive acid phosphatase | 0.09 | 0 | -0.42 | 1.18 | -0.42 | 0.67 | -0.37 | 0.7 |
| Thi11 | 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate synthase *THI11* | -0.19 | 0.12 | 0.19 | 0.44 | -0.71 | 2.29 | -1.32 | 5.08 |
| Thi20 | Hydroxymethylpyrimidine/phosphomethylpyrimidine kinase *THI20* | 0.16 | 0.15 | 0.1 | 0.25 | -0.39 | 1.16 | -0.55 | 2.34 |
| Thi21 | Hydroxymethylpyrimidine/phosphomethylpyrimidine kinase *THI21* | 0.35 | 0.61 | 0.04 | 0.07 | -0.83 | 3.01 | -0.76 | 2.86 |
| Thi4 | Thiamine thiazole synthase | -0.12 | 0.08 | -0.17 | 0.6 | 0.64 | 2.99 | 1.32 | 6.65 |
| Thi6 | Thiamine biosynthetic bifunctional enzyme | -0.24 | 0.69 | -0.06 | 0.2 | -0.37 | 1.82 | -0.37 | 2 |
| Thi7 | Thiamine transporter | 0.1 | 0 | -0.93 | 0.64 | -1.13 | 0.46 | -0.18 | 0.03 |
| Thi80 | Thiamine pyrophosphokinase | 0.05 | 0.01 | -0.11 | 0.44 | -0.51 | 2.99 | -0.63 | 4.12 |

Table 7- Percentage of proteome dedicated to thiamine biosynthesis or uptake, with standard deviation

| Strain | Growth phase | Percentage of the proteome | |
|---|---|---|---|
| CBS 1390 | exponential growth | 6.05 % | ±0.55 |
| | stationary phase | 3.84 % | ±0.81 |
| CBS 1390 variant | exponential growth | 6.78 % | ±0.25 |
| | stationary phase | 3.67 % | ±0.24 |
| CBS 3012 | exponential growth | 6.67 % | ±0.20 |
| | stationary phase | 3.60 % | ±0.18 |
| CBS 3012 variant | exponential growth | 7.07 % | ±0.30 |
| | stationary phase | 3.90 % | ±0.07 |

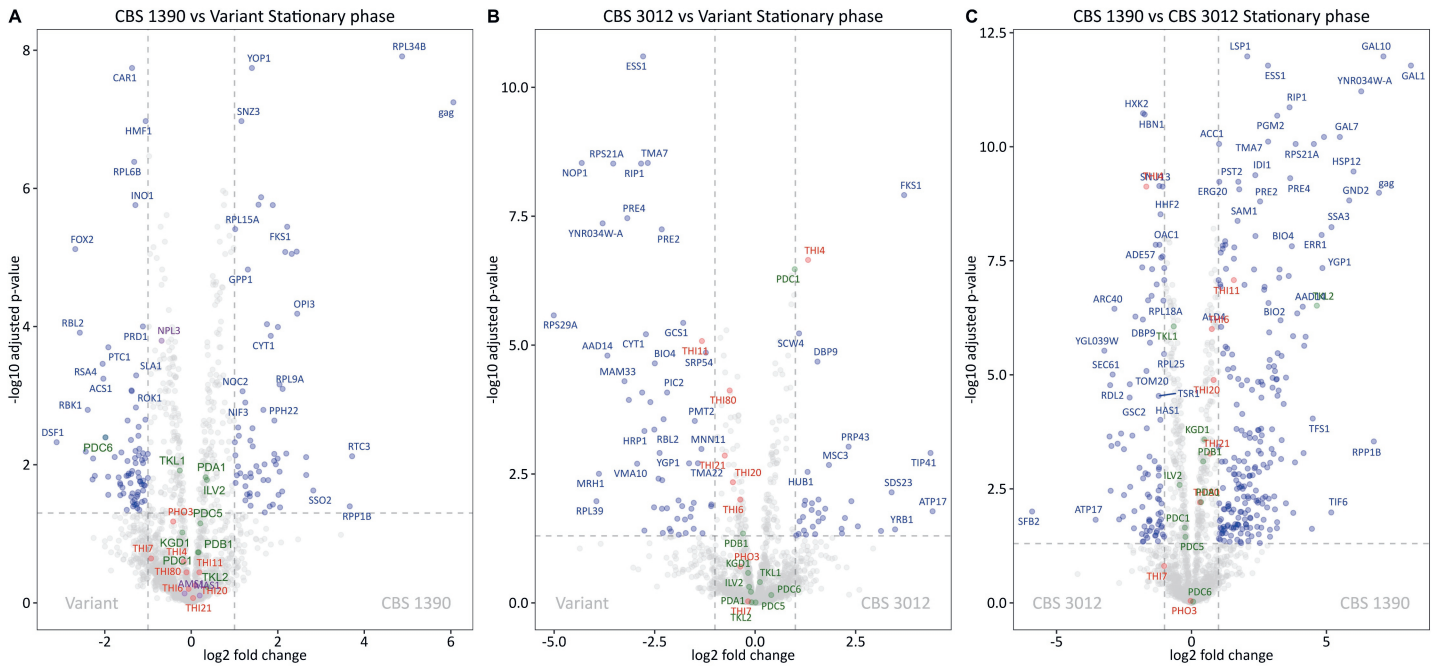



Figure 5- Volcano plots of the differentially expressed proteins. Proteins differenctially expressed are coloured in blue, proteins involved in thiamine transport or biosynthesis are coloured in red, protein in which we found mutations are purple, proteins that use thiamine diphosphate as cofactor are coloured in green. All the other proteins are grey. The α-level for the adjusted *p*-value was set to 0.05.

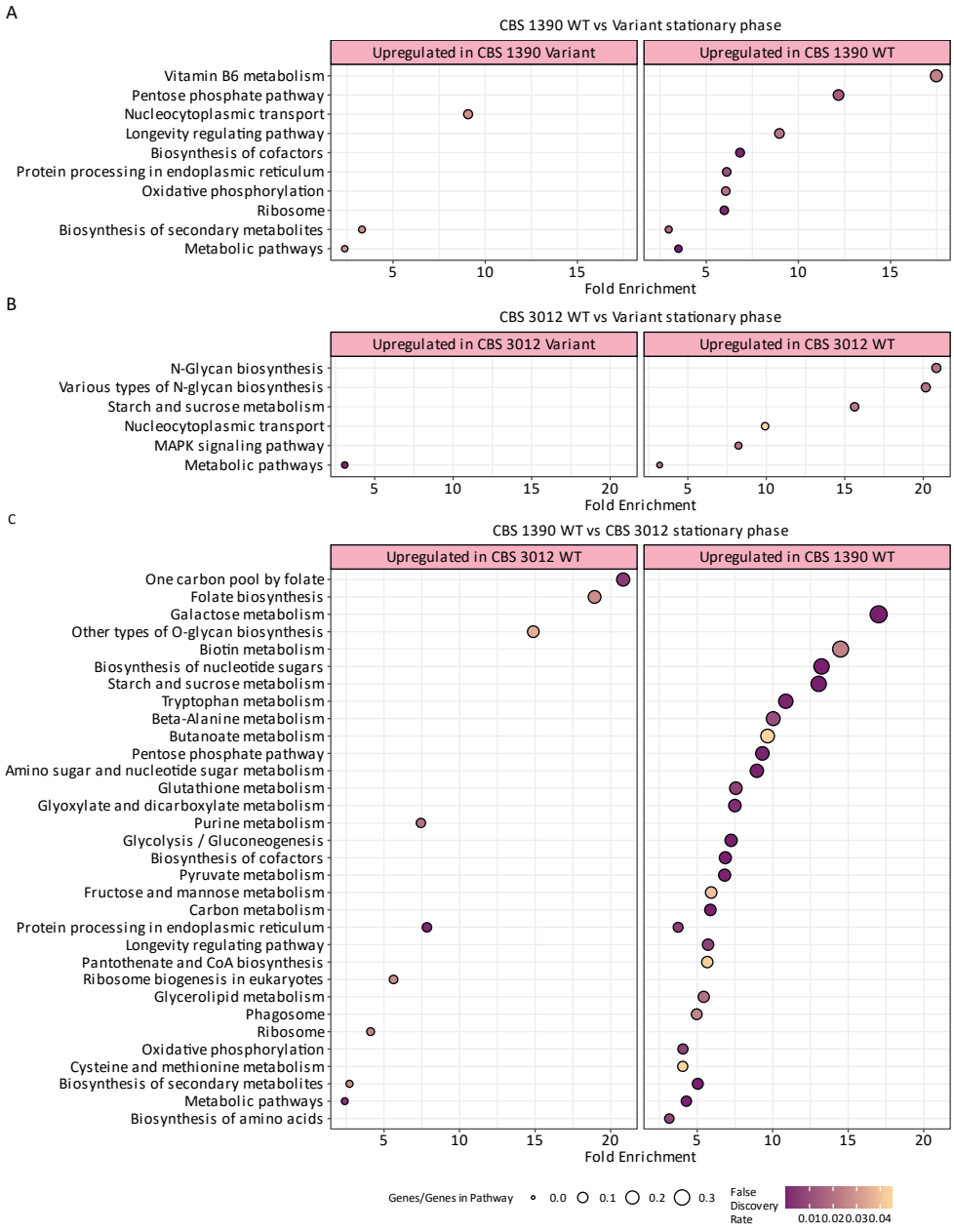



Figure 6- KEGG pathway enrichment analysis of the differentially expressed proteins, results were obtained via the online software ShinyGO v 0.77. On the x-axis the fold enrichment, defined as the percentage of genes in the list belonging to a pathway, divided by the corresponding percentage in the background, on the y-axis the enriched KEGG pathway, the size of the dots represents the ratio between the number of genes and the total number of genes in the specific pathway. The false discovery rate is calculated based on nominal *p*-value from the hypergeometric test.

## 4.4 Discussion

We evolved two wild type strains of *S. cerevisiae*, a high thiamine-producer, and a low thiamine-producer in thiamine-free medium. While the amount of thiamine produced by the evolved variants (EVs) isolated from CBS 1390 (low thiamine producer) increased significantly, thiamine production halved after evolution for strain CBS 3012 (the high thiamine producer). This led to a convergence towards a similar level of thiamine produced by all the EVs isolated from both ancestral WTs.

### 4.4.1 CBS 1390 and variants

No genes encoding for thiamine biosynthetic of thiamine-dependent proteins were found to be mutated in the EVs originating from CBS 1390. Since the phenotype of these EVs is significantly different from the WT, the relatively small number of mutations found in each EV has a big impact on fitness in terms of biomass production, maximum growth rate, but also indirectly on thiamine yield. The genes *GDS1* and *UME6* had mutations shared across multiple evolution lines or multiple isolates. The proteins derived from these genes have a broad influence on metabolism since they are involved in transcriptional regulation and post-translational protein modification (Bartholomew et al., 2012; Joo & Buratowski, 2022; Kadosh & Struhl, 1997; Steber et al., 1995; Strich et al., 1994; Sweet et al., 1997; Vershon & Pierce, 2000). Interestingly we found that in the EV CBS 1390 2_9 (highest thiamine producer among the EV from CBS 1390), in which we detected a mutation in *UME6*, in stationary phase the proteins Car1 and Car2 are upregulated compared to the WT (Figure 5A, Supplementary material 3). These two proteins are subjected to transcriptional regulation by *UME6* (Messenguy et al., 2000). They both participate in the catabolism of arginine, Car1 degrades arginine to ornithine, Car2 is an ornithine aminotransferase. They are strongly expressed during growth on arginine and during nitrogen starvation. Our results are in accordance to Strich et al. (1994), who showed that mutations in *UME6* cause a derepression of *CAR1* and *CAR2* even in medium containing ammonia as N-source, comparable to the level of expression of these genes in the WT under nitrogen starvation.

The EVs 2_9 and 2_19 share the same mutation in *UME6* and have very similar growth rate in thiamine-free and thiamine rich medium (Figure 2A) but different phenotypes in terms of thiamine production. While CBS 1390 2_9, had the highest thiamine yield, CBS 1390 2_19 had a similar thiamine yield compared to all the other EVs but a high degree of cell lysis, indicated by the low biomass and the presence of thiamine mostly extracellularly. The high degree of cell lysis could be caused the mutation in the gene *CYC8* in this strain. This gene encodes a transcriptional co-repressor (Smith et al., 2000).

Mutations in *CYC8* have been reported to have a broad effect on the phenotype, and null mutants have been shown to have a decreased chronological lifespan (Marek et al., 2013), abnormal cell size, flocculation, altered morphology (Lee et al., 2023), and inability to respire (Dimmer et al., 2002). Such a strain has potential application since autolysis could help making thiamine more easily accessible for thermal degradation and conversion to meaty aromas, compared to yeast strains that produce thiamine only intracellularly.

Strain CBS 1390 has limited fitness in the thiamine-free medium in terms of growth rate and biomass production. Adaptive laboratory evolution led to an increase of about 30 % in biomass yield and a 1.5 to 2-fold increase in growth rate, depending on the specific EV. Although thiamine productivity increased for this strain significantly after evolution, the proteomics analysis of EV 2_9 (the highest thiamine-yielding EV) showed that neither in exponential growth nor stationary phase, the thiamine-dependent and thiamine biosynthetic enzymes were significantly different to the ones of the WT. The only exception was Pdc6, that was higher in the EV compared to the WT in stationary phase (Supplementary material 1). Pdc6 is a minor isoform of pyruvate decarboxylase (Pdc1, Pdc5, Pdc6), this isoform can also decarboxylate other 2-oxo acids, contributing to the degradation of valine, isoleucine, tryptophan, and phenylalanine (Dickinson et al., 2003). Pdc6 is highly expressed during aerobic growth on non-fermentable C-sources, during growth on ethanol, or in conditions of sulphur limitation, as Pdc6 contains less sulphur-containing amino acids compared to Pdc1 and Pdc5 (Hohmann, 1991). Nevertheless, Pdc6 was detected in extremely low amounts compared to Pdc1 and Pdc5 (Supplementary material 1).

The KEGG pathway enrichment analysis done for CBS 1390 and the corresponding EV showed that the metabolism of vitamin $B_6$ was enriched in the WT via the upregulation of Snz1 and Snz3. This is relevant as vitamin $B_6$ is a precursor of the aminopyrimidine moiety of thiamine (Rodríguez-Navarro et al., 2002). Our data are in agreement with Paxhia and Downs (2019), showing that *SNZ1* alone synthesises pyridoxal 5′-phosphate when thiamine is present in the growth medium. But the expression of either *SNZ2* or *SNZ3* is indispensable when thiamine is absent.

### 4.4.2 CBS 3012 and variants

CBS 3012 evolved to produce half the amount of thiamine. We did not find any shared SNP among the isolated EVs originating from CBS 3012, but they all gave similar yields in biomass and thiamine. Therefore, the mechanism that led to a reduction in thiamine productivity is unlikely related to any of the identified single genetic mutations.

Moreover, the mutations we found were identified in just one allele of the gene in question. It is important to note that in many cases, a single allele mutation may not be sufficient to induce a notable phenotypic change. Therefore, while the discovery of these mutations is significant, their impact on the organism's phenotype may be limited due to the presence of one unaltered, functional allele, and needs to be further investigated.

For most of the isolated EVs, we did not detect a significant increase in the growth rate in thiamine-free or thiamine rich medium, but only a marginal increase in biomass production of about 10 %. This indicates that in most cases, thiamine "overproduction" by the WT, was not causing a decreased fitness by decreasing the growth rate but could have limited biomass formation. In CBS 3012 we found significant differences in the relative abundance of thiamine biosynthetic enzymes compared to the EV 2_17. Thi4 doubled in stationary phase in the WT compared to the EV, whereas the EV had doubled relative abundance of Thi11. Thi80, Thi21, Thi20, and Thi6 were also significantly more abundant in the EV. The thiamine-dependent enzyme with the biggest difference in relative abundance between WT and EV in stationary phase was found to be the major form of pyruvate-decarboxylase, Pdc1. Pdc1 is strongly expressed in actively fermenting yeast cells. When thiamine is limiting, it is expressed together with Pdc5, which is strongly induced during thiamine limitation (Seeboth et al., 1990). Their expression is subjected to autoregulation (Eberhardt et al., 1999), and requires the transcription factor Pdc2 (Mojzita et al., 2006), that also regulates thiamine biosynthesis. Nonetheless, Pdc5 was still more strongly expressed compared to Pdc1 (Supplementary material 1A).

The KEGG pathway enrichment analysis showed that Gsc2 and Fks1 were upregulated in the WT CBS 3012 compared to the EV. Gsc2 is the catalytic subunit of 1,3-beta-glucan synthase complex. This polysaccharide is the main structural component of the cell wall (Klis et al., 2002). Gsc2 has a paralog, Fks1. Both catalyse the transfer of the glucose moiety of UDP-glucose to the 1,3-beta-glucan chain (Douglas et al., 1994), but Gsc2 seems to be induced under starvation, or during sporulation, while Fks1 is primarily expressed during growth (Mazur et al., 1995).

### 4.4.3 The two WTs in comparison

CBS 1390 and CBS 3012 have very different phenotypes in term of thiamine production and $\mu_{max}$. We looked their proteome during exponential growth and stationary phase to find insights that would explain their differences in thiamine production. We found many more pathways enriched in CBS 1390 compared to CBS 3012 in stationary phase.

Some of these pathways were linked to the two thiamine precursors (Supplementary material 3). The enrichment in the pentose phosphate pathway via Tkl2, Sol2, Nqm1, Sol4, Gnd2, and Pgm2 is linked to thiamine metabolism since this pathway supplies D-ribose-5-phosphate, that together with L-glutamine and D-glyceraldehyde-3-phosphate form PLP, the active form of vitamin $B_6$, precursor of HMP-P via Snz1,2,3 or Sno1,2,3. Moreover, the upregulation of Pdx3 indicates a possible increase in flux from pyridoxine-5-phosphate to PLP as well (Loubbardi et al., 1995). The upregulation of tryptophan metabolism, via Bna1 and Bna5, together with Ald3 Ald4, and Ctt1 is also link to thiamine de novo biosynthesis. This is because Bna1 and Bna5 are essential for the de novo synthesis of NAD (nicotinamide adenine dinucleotide), from tryptophan via the formation of nicotinic acid mononucleotide as intermediate (Bedalov et al., 2003; Kucharczyk et al., 1998). $NAD^+$ and L-glycine, together with a cysteine from Thi4, form the HET-P moiety of thiamine (Figure 1). Interestingly, their expression is under the regulation of Hst1, that has been proved to play a role in thiamine regulation in rich medium, such as YPD (Li et al., 2010). We also looked at the proteins that were upregulated in both stationary and exponential phase in the high producing strains CBS 3012 compared to CBS 1390. We found one interesting overexpressed protein: YGR017W. This protein is a pyridoxamine-phosphate oxidase-related protein (PNPO-RP), that Marbaix et al. (2019) reported not to be active in converting pyridoxine-5-phosphate to PLP, but to be responsible for the oxidation of the damaged form nicotinamide adenine dinucleotide to $NAD(P)^+$. In *S. cerevisiae* its deletion causes an accumulation of 4,5,6-$NAD(P)H_3$,which inhibits several dehydrogenases (Marbaix et al., 2019). These results show the complexity of the metabolic network involved in the biosynthesis of thiamine and its precursors, and that the upregulation of proteins related to thiamine precursors does not lead, in this case, to higher thiamine yields.

Comparing the two WTs to their respective variants, Fks1 was the only significantly higher protein in both WTs during stationary phase. Whether this is linked to differences in growth rate and/or biomass remains to be elucidated. However, it is likely that the two EV are more efficient in using ammonium sulphate as N-source, compared to the respective WTs, which are directing glucose towards storage and cell-wall synthesis, a sign of nitrogen-limitation.

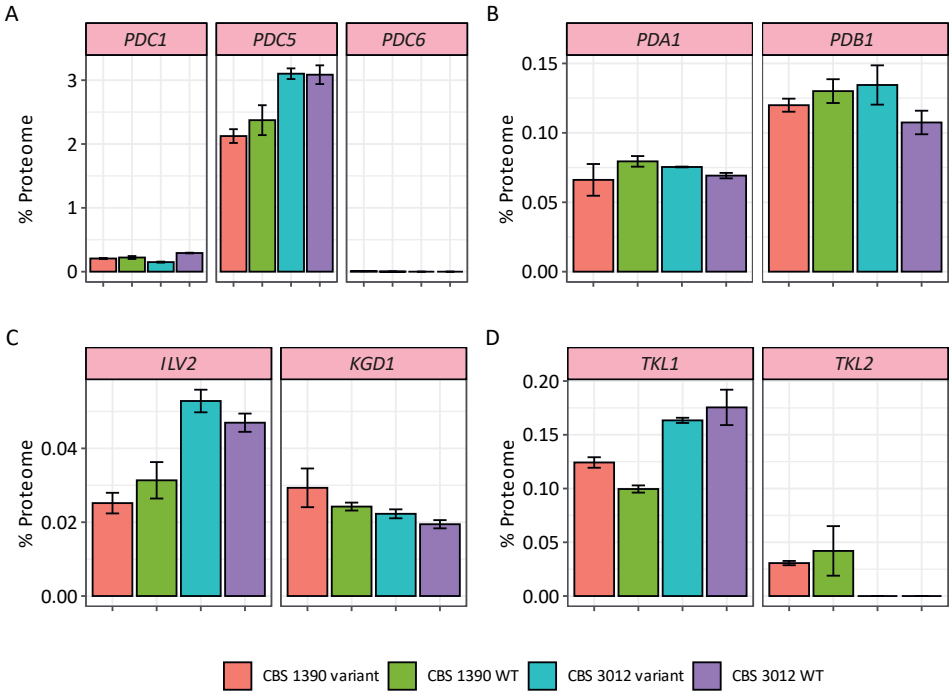
In this study, the total proteome fractions allocated to thiamine productivity and transport (Figure 4B, Table 7) were not significantly different for all the tested strains in both exponential and stationary phase. Still, we found large differences in the thiamine pools between the two WTs. The high thiamine producer CBS 3012 had lower relative abundance of all the thiamine biosynthetic proteins compared to the other strains, but

higher Thi4 proportion. This is not surprising, in fact the main limiting step in thiamine production is the formation of the two moieties, rather than all the other reactions that are catalysed by enzymes that undergo multiple catalytic turnovers.

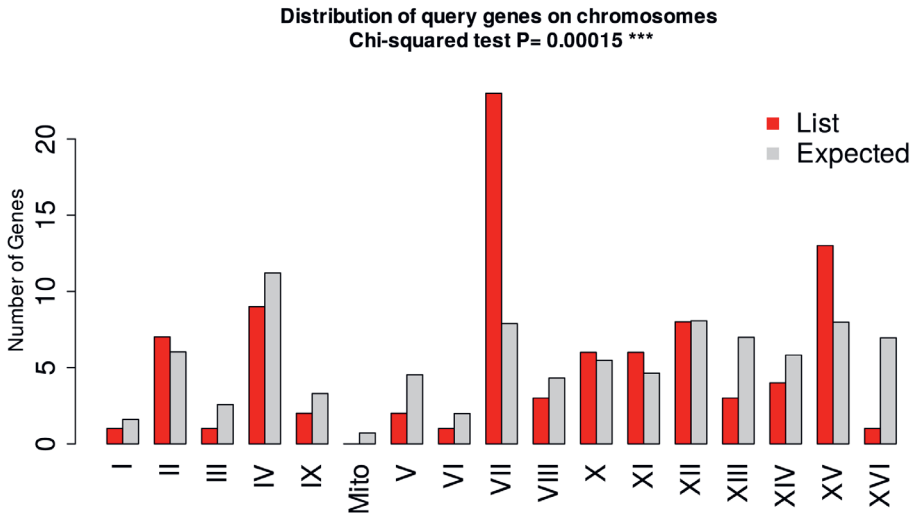
## 4.5 Conclusion

Evolution led to an increase in biomass of the EVs originated from both strains. Thiamine concentrations, on the other hand, increased in the low-producing strain, and decreased in the high producing one, leading to a convergence in the thiamine yield produced by all the EVs. This further sheds a light on the fine balance between thiamine production and fitness, especially in chemically-defined minimal medium, in which there are no amino-acids supplied, and thiamine production may be limited by the significant protein costs of biosynthesis. Therefore, we can conclude that in a strain with low fitness in minimal medium lacking thiamine, increased fitness achieved during evolution causes an increase in thiamine productivity. However, ALE is not successful in evolving strains to produce higher thiamine when they already produce elevated amounts of it. The fact that only in the high producer strain CBS 3012 thiamine concentration (as thiamine per grams of dry weight) is increased in stationary phase compared to exponential, could suggest that in this strain the transcription of thiamine biosynthetic genes is less sensitive to the intracellular levels of thiamine diphosphate. This could be the key to achieve thiamine overproduction in *S. cerevisiae*.

## 4.6 Supplementary materials




Supplmentary material 1- Proteome percentage, calculated as the ratio between the MaxLFQ intensity of thiamine-dependent enzymes, and the total MaxLFQ intensity of the specific sample. The error bar indicates the standard deviation of biological replicates from three independent experiments.




Supplmentary material 2- Location of genes encoding for proteins upregulated in stationary phase in CBS 3012 compared to CBS 1390. This image shows a significantly large amount of genes located in chromosome VII of *S. cerevisiae* S288C, where also Thi4 is located. Image obtained via ShinyGO 0.77 (http://bioinformatics.sdstate.edu/go/).

Supplementary material 3- KEGG pathway enrichment analysis results of the differentially expressed proteins in stationary phase. The comparison was between each WT and the corresponding variant, and between the two WTs.

| Comparison | Upregulated in | Enrichment FDR | Number of Genes | Genes in Pathway | Fold enrichment | Pathway name | Proteins |
|---|---|---|---|---|---|---|---|
| CBS 1390 vs Variant | Variant | 2.9E-02 | 4 | 63 | 9.1 | Nucleocytoplasmic transport | Gle2 Nup85 Nmd5 Nup2 |
| | | 2.9E-02 | 8 | 345 | 3.3 | Biosynthesis of secondary metabolites | Acs1 Hem1 Pdc6 Ino1 Met1 Sdh2 Car2 Car1 |
| | | 2.9E-02 | 13 | 781 | 2.4 | Metabolic pathways | Pmt2 Acs1 Rbk1 Hem1 Rnr1 Pdc6 Ino1 Fox2 Met1 Cox17 Sdh2 Car2 Car1 |
| | WT | 2.4E-02 | 2 | 13 | 17.5 | Vitamin $B_6$ metabolism | Snz3 Snz1 |
| | | 1.4E-02 | 3 | 28 | 12.2 | Pentose phosphate pathway | Sol2 Sol4 Rpe1 |
| | | 2.1E-02 | 3 | 38 | 9.0 | Longevity regulating pathway | Ssa3 Ssa4 Snf4 |
| | | 3.1E-04 | 8 | 133 | 6.8 | Biosynthesis of cofactors | Pdx3 Ycp4 Snz3 Bio2 Gsh1 Ura4 Snz1 Ura10 |
| | | 1.2E-02 | 5 | 93 | 6.1 | Protein processing in endoplasmic reticulum | Ssa3 Hsp26 Ubc4 Fes1 Ssa4 |
| | | 2.1E-02 | 4 | 75 | 6.1 | Oxidative phosphorylation | Cox2 Atp16 Cyt1 Pma2 |
| | | 5.5E-04 | 8 | 152 | 6.0 | Ribosome | Rpp1a Rpp1b Rpl9a Rpl34b Rpl16a Rpl15a Rpl37a Rps29 |
| | | 9.6E-08 | 24 | 781 | 3.5 | Metabolic pathways | Cox2 Pdx3 Ycp4 Sol2 Atp16 Lys4 Gly1 Snz3 Erg26 Cho2 Sol4 Bio2 Gsh1 Rpe1 Ham1 Opi3 Fks1 Ura4 Snz1 Ura10 Gre2 Cyt1 Pma2 |
| | | 1.7E-02 | 9 | 345 | 3.0 | Biosynthesis of secondary metabolites | Ycp4 Sol2 Lys4 Gly1 Erg26 Cho2 Sol4 Rpe1 Opi3 |
| CBS 3012 vs Variant | Variant | 1.1E-03 | 14 | 781 | 3.1 | Metabolic pathways | Pmt2 Pdx3 Sol2 Lys4 Rip1 Gnd2 Erg11 Vma10 Nit1 Mnn11 Thi11 Bio4 Cyt1 Hsp33 |
| | WT | 2.1E-02 | 2 | 30 | 20.8 | N-Glycan biosynthesis | Alg13 Alg2 |
| | | 2.1E-02 | 2 | 31 | 20.2 | Various types of N-glycan biosynthesis | Alg13 Alg2 |
| | | 2.2E-02 | 2 | 40 | 15.6 | Starch and sucrose metabolism | Gsc2 Fks1 |
| | | 4.5E-02 | 2 | 63 | 9.9 | Nucleocytoplasmic transport | Seh1 Nup85 |
| | | 2.1E-02 | 3 | 114 | 8.2 | MAPK signalling pathway | Ptc1 Gsc2 Fks1 |
| | | 2.1E-02 | 8 | 781 | 3.2 | Metabolic pathways | Gal7 Atp17 Alg13 Alg2 Gsc2 Thi4 Fks1 Hem15 |

*Table continued on the next page*

Table continued

| Comparison | Upregulated in | Enrichment FDR | Number of Genes | Genes in Pathway | Fold enrichment | Pathway name | Proteins |
|---|---|---|---|---|---|---|---|
| CBS 1390 vs CBS 3012 | CBS 3012 | 4.9E-03 | 3 | 15 | 20.8 | One carbon pool by folate | Gcv1 Ade8 Mtd1 |
| | | 2.8E-02 | 2 | 11 | 18.9 | Folate biosynthesis | Fol2 Fol1 |
| | | 3.6E-02 | 2 | 14 | 14.9 | Other types of O-glycan biosynthesis | Pmt1 Ktr1 |
| | | 1.0E-03 | 7 | 93 | 7.8 | Protein processing in endoplasmic reticulum | Ssh1 Emp47 Kar2 Sec61 Ero1 Sfb2 Sil1 |
| | | 2.0E-02 | 4 | 56 | 7.4 | Purine metabolism | Ade8 Ade5,7 Imd2 Prs5 |
| | | 2.8E-02 | 4 | 74 | 5.6 | Ribosome biogenesis in eukaryotes | Snu13 Utp10 Utp13 Nog1 |
| | | 2.6E-02 | 6 | 152 | 4.1 | Ribosome | Rpl32 Rpl30 Rpl24b Rpl34b Rlp7 Rpl25 |
| | | 2.8E-02 | 9 | 345 | 2.7 | Biosynthesis of secondary metabolites | Gcv1 Ade8 Ade5,7 Hxk2 Ser2 Imd2 Ste24 Erg3 Prs5 |
| | | 3.7E-03 | 18 | 781 | 2.4 | Metabolic pathways | Pmt1 Gcv1 Atp17 Ade8 Ade5,7 Hxk2 Gsc2 Thi4 Ser2 Mes1 Fol2 Imd2 Lac1 Mtd1 Erg3 Fol1 Prs5 |
| | CBS 1390 | 1.1E-08 | 9 | 23 | 17.0 | Galactose metabolism | Gal10 Gal1 Gal3 Emi2 Hxk1 Ima1 Suc2 Ugp1 Pgm2 |
| | | 2.4E-02 | 2 | 6 | 14.5 | Biotin metabolism | Bio2 Bio4 |
| | | 5.1E-06 | 7 | 23 | 13.2 | Biosynthesis of nucleotide sugars | Gal10 Gal1 Gal3 Emi2 Hxk1 Ugp1 Pgm2 |
| | | 6.9E-10 | 12 | 40 | 13.0 | Starch and sucrose metabolism | Tps1 Nth1 Emi2 Glc3 Hxk1 Ima1 Suc2 Ugp1 Tsl1 Pgm2 Tps3 Gph1 |
| | | 4.0E-04 | 5 | 20 | 10.9 | Tryptophan metabolism | Ctt1 Bna1 Bna5 Ald3 Ald4 |
| | | 1.0E-02 | 3 | 13 | 10.0 | Beta-Alanine metabolism | Ald3 Gad1 Ald4 |
| | | 4.5E-02 | 2 | 9 | 9.7 | Butanoate metabolism | Uga2 Gad1 |
| | | 2.0E-04 | 6 | 28 | 9.3 | Pentose phosphate pathway | Tkl2 Sol2 Nqm1 Sol4 Gnd2 Pgm2 |
| | | 7.0E-05 | 7 | 34 | 9.0 | Amino sugar and nucleotide sugar metabolism | Gal10 Gal1 Gal3 Emi2 Hxk1 Ugp1 Pgm2 |
| | | 6.8E-03 | 4 | 23 | 7.6 | Glutathione metabolism | Gnd2 Gtt1 Gsh1 Gsh2 |
| | | 2.4E-03 | 5 | 29 | 7.5 | Glyoxylate and dicarboxylate metabolism | Agx1 Ctt1 Tda10 Dal7 Aco1 |
| | | 2.5E-05 | 9 | 54 | 7.2 | Glycolysis / Gluconeogenesis | Gal10 Gpm2 Tpi1 Emi2 Hxk1 Pgm2 Ald3 Pyk2 Ald4 |
| | | 2.6E-11 | 21 | 133 | 6.9 | Biosynthesis of cofactors | Pdx3 Fap7 Pst2 Snz3 Cab4 Bio2 Gsh1 Bna1 Thi11 Ugp1 Sam1 Bna5 Ura4 Coq5 Ald3 Ura10 Bio4 Gsh2 Met7 Pyk2 Ald4 |

*Table continued on the next page*

Table continued

| Comparison | Upregulated in | Enrichment FDR | Number of Genes | Genes in Pathway | Fold enrichment | Pathway name | Proteins |
|---|---|---|---|---|---|---|---|
| | | 1.2E-04 | 8 | 51 | 6.8 | Pyruvate metabolism | Hsp31 Dal7 Cyb2 Ald3 Acc1 Pyk2 Ald4 Hsp33 |
| | | 4.0E-02 | 3 | 22 | 5.9 | Fructose and mannose metabolism | Tpi1 Emi2 Hxk1 |
| | | 2.6E-07 | 15 | 111 | 5.9 | Carbon metabolism | Tkl2 Sol2 Gpm2 Tpi1 Emi2 Agx1 Hxk1 Nqm1 Ctt1 Tda10 Sol4 Gnd2 Dal7 Aco1 Pyk2 |
| | | 6.8E-03 | 5 | 38 | 5.7 | Longevity regulating pathway | Ssa3 Ssa4 Ctt1 Sod2 Hsp104 |
| | | 4.3E-02 | 3 | 23 | 5.7 | Pantothenate and CoA biosynthesis | Cab4 Ald3 Ald4 |
| | | 2.0E-02 | 4 | 32 | 5.4 | Glycerolipid metabolism | Tda10 Ald3 Gcy1 Ald4 |
| | | 1.9E-17 | 40 | 345 | 5.0 | Biosynthesis of secondary metabolites | Bdh1 Gal10 Tkl2 Tps1 Sol2 Gpm2 Fap7 Nth1 Pst2 Tpi1 Lys4 Emi2 Glc3 Agx1 Hxk1 Nqm1 Ctt1 Tda10 Sol4 Gnd2 Gut2 Suc2 Dal7 Arg3 Erg20 Ugp1 Sam1 Aco1 Tsl1 Coq5 Pgm2 Ald3 Erg2 Gad1 Tps3 Acc1 Pyk2 Ald4 Idi1 Gph1 |
| CBS 1390 vs CBS 3012 | CBS 1390 | 2.5E-02 | 4 | 35 | 5.0 | Phagosome | Vma8 Vma10 Vma6 Ypt7 |
| | | 2.5E-34 | 77 | 781 | 4.3 | Metabolic pathways | Prx1 Uga2 Gal7 Gal10 Gal1 Etr1 Pdx3 Ysa1 Tkl2 Tps1 Sol2 Atp16 Gpm2 Fap7 Nth1 Gal3 Pst2 Tpi1 Lys4 Emi2 Hsp31 Glc3 Rip1 Vma8 Agx1 Snz3 Hxk1 Ole1 Nqm1 Ctt1 Xks1 Tda10 Sol4 Gnd2 Cab4 Bio2 Ima1 Vma10 Suc2 Dal7 Dal3 Gtt1 Arg3 Gsh1 Erg20 Bna1 Thi11 Ugp1 Sam1 Bna5 Aco1 Ura4 Vma6 Cyb2 Tsl1 Coq5 Adi1 Pgm2 Ald3 Erg2 Gad1 Tps3 Ura10 Acc1 Bio4 Gsh2 Cyt1 Gcy1 Met7 Pyk2 Ald4 Hsp33 Pma2 Idi1 Gph1 Gdb1 |
| | | 6.8E-03 | 7 | 75 | 4.1 | Oxidative phosphorylation | Atp16 Rip1 Vma8 Vma10 Vma6 Cyt1 Pma2 |
| | | 4.5E-02 | 4 | 43 | 4.0 | Cysteine and methionine metabolism | Gsh1 Sam1 Adi1 Gsh2 |
| | | 6.0E-03 | 8 | 93 | 3.7 | Protein processing in endoplasmic reticulum | Ssa3 Hsp26 Ubc4 Hsp42 Rad23 Ssa4 Dsk2 Hrt1 |
| | | 7.8E-03 | 9 | 124 | 3.2 | Biosynthesis of amino acids | Tkl2 Gpm2 Tpi1 Lys4 Nqm1 Arg3 Sam1 Aco1 Pyk2 |

Supplementary material 4- Genes mutated in the variants evolved from CBS 1380, information retrieved from https://yeastmine.yeastgenome.org/yeastmine/bag.do and https://pantherdb.org/list/list.do?numPerPage=20000&save=yes&searchModType=numperpage&listType=1

| Primary identifier | Secondary identifier | Symbol | Name | Length | Panther protein name |
|---|---|---|---|---|---|
| S000002752 | YDR344C | | | 444 | |
| S000002751 | YDR343C | *HXT6* | HeXose Transporter | 1713 | High-affinity hexose transporter |
| S000002753 | YDR345C | *HXT3* | HeXose Transporter | 1704 | Low-affinity glucose transporter |
| S000002840 | YDR432W | *NPL3* | Nuclear Protein Localization | 1245 | Nucleolar protein 3 |
| S000003124 | YGL156W | *AMS1* | Alpha-MannoSidase | 3252 | |
| S000000316 | YBR112C | *CYC8* | CYtochrome C | 2901 | General transcriptional corepressor |
| S000000384 | YBR180W | *DTR1* | DiTyRosine | 1719 | Dityrosine transporter 1 |
| S000006152 | YPL231W | *FAS2* | Fatty Acid Synthetase | 5664 | |
| S000004071 | YLR081W | *GAL2* | GALactose metabolism | 1725 | Galactose transporter |
| S000005882 | YOR355W | *GDS1* | | 1569 | |
| S000000571 | YCL066W | *HMLALPHA1* | HoMothallism Left | 528 | |
| S000001230 | YHR187W | *IKI1* | Insensitive to KIller toxin | 930 | |
| S000004153 | YLR163C | *MAS1* | Mitochondrial ASsembly | 1389 | Mitochondrial-processing peptidase subunit beta |
| S000006259 | YPR055W | *SEC8* | SECretory | 3198 | Exocyst complex component |
| S000001686 | YKL203C | *TOR2* | Target Of Rapamycin | 7425 | Serine_threonine-protein kinase |
| S000002615 | YDR207C | *UME6* | Unscheduled Meiotic gene Expression | 2511 | Transcriptional regulatory protein |
| S000003466 | YGR234W | *YHB1* | Yeast flavoHemogloBin | 1200 | Flavohemoprotein |

Supplementary material 5- Genes mutated in the variants evolved from CBS 3012, information retrieved from https://yeastmine.yeastgenome.org/yeastmine/bag.do and https://pantherdb.org/list/list.do?numPerPage=20000&save=yes&searchModType=numperpage&listType=1.

| Primary identifier | Secondary identifier | Symbol | Name | Length | Panther protein name |
|---|---|---|---|---|---|
| S000001470 | YIR031C | *DAL7* | Degradation of Allantoin | 1665 | Malate synthase 2 |
| S000003503 | YGR271W | *SLH1* | SKI2-Like Helicase | 5904 | RQC trigger complex helicase |
| S000003176 | YGL208W | *SIP2* | SNF1-Interacting Protein | 1248 | Carbon catabolite-derepressing protein kinase |
| S000002349 | YDL190C | *UFD2* | Ubiquitin Fusion Degradation | 2886 | E4 ubiquitin-protein ligase UFD2 |
| S000001466 | YIR027C | *DAL1* | Degradation of Allantoin | 1383 | Allantoinase |
| S000003398 | YGR166W | *TRS65* | TRapp Subunit | 1683 | |
| S000001017 | YHL025W | *SNF6* | Sucrose NonFermenting | 999 | |
| S000002134 | YAL034C | *FUN19* | Function Unknown Now | 1242 | SWIRM domain-containing protein |
| S000005835 | YOR308C | *SNU66* | Small NUclear ribonucleoprotein associated | 1764 | 66 kDa U4_U6.U5 small nuclear ribonucleoprotein component |
| S000005223 | YNL279W | *PRM1* | Pheromone-Regulated Membrane protein | 1986 | Phosphoribomutase |
| S000001085 | YHR043C | *DOG2* | DeOxyGlucose | 741 | 2-deoxyglucose-6-phosphate phosphatase 2 |
| S000002779 | YDR371W | *CTS2* | ChiTinaSe | 1536 | Sporulation-specific chitinase 2 |
| S000001310 | YIL048W | *NEO1* | NEOmycin-resistance | 3456 | Probable phospholipid-transporting ATPase |

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# CHAPTER 5

## Development of novel natto using legumes produced in Europe

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Published in Heliyon, Volume 10, Issue 5, March 2024

DOI: https://doi.org/10.1016/j.heliyon.2024.e26849

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## Abstract

Natto is a traditional Japanese fermented food consisting of cooked soybeans fermented with *Bacillus subtilis* var. natto. We assessed three different *B. subtilis* strains and investigated their impact on product quality aspects, such as microbial quality, textural quality (poly-γ-glutamate strand formation), free amino acids (FAA), and volatile organic compounds (VOCs), but also the vitamin $K_1$, $K_2$ and $B_1$ content, and presence of nattokinase. Using Bayesian contrast analysis, we conclude that the quality attributes were influenced by both the substrate and strain used, without significant differences in bacterial growth between strain or substrate. Overall, all the tested European legumes, except for brown beans, are adequate substrates to produce natto, with comparable or higher qualities compared to the traditional soy. Out of all the tested legumes, red lentils were the most optimal fermentation substrate. They were fermented most consistently, with high concentrations of vitamin $K_2$, VOCs, FAA.

## 5.1 Introduction

Itobiki-natto, also known as natto, is a traditional Japanese fermented food. It is produced by washing, soaking, and cooking soybeans before inoculation with *Bacillus subtilis* var. natto. The fermentation is followed by a maturation step before the product is ready for consumption. Fermentation is the most critical part of the natto production process, since it has a great impact on the final product characteristics such as texture, appearance, flavour, and nutritional values (Hosoi et al., 2008).

Natto has a typical sticky-slimy texture given by the γ-polyglutamic acid (PGA) strands produced by *B. subtilis* during fermentation and maturation (Ho et al., 2006). These strands are a biopolymer constituted by variable proportions of D-form and L-form glutamic acid linked by γ-glutamyl bonds. In addition, soy is a rich source of proteins, and during fermentation the proteolytic activity of *B. subtilis* releases ammonia and free amino acids, which contribute to the smell and taste of the product, whilst increasing the overall digestibility (Nguyen et al., 2020). Free amino acids (FAA) contribute directly to taste and flavour perception of natto since most amino acids are either sweet, bitter, or umami in taste. Furthermore, FAA are precursors of other metabolites contributing to the quality of natto. Glutamic acid for instance is the building block of γ-PGA, giving natto its slimy texture (Li et al., 2021). Additionally, they are also precursors of many secondary metabolites (SM) characterizing the aroma profile of natto, such as pyrazines and ketones (Kada et al., 2008; Kłosowski et al., 2021; Larroche et al., 1999; Liu, Song, et al., 2018; MacLeod et al., 1988). Ammonia is the major SM contributing to natto aroma, followed by 2,5-dimethylpyrazine and acetoin, arising from conversions catalysed by *B. subtilis* (Fisher et al., 1991; Larroche et al., 1999; Liu, Su, et al., 2018). Pyrazines and especially 2,5-dimethylpyrazine are potent molecules with a high flavour dilution and deliver a nutty or baked nut-like smell (Liu, Song, et al., 2018). Acetoin itself has a high odour threshold but is easily oxidized to diacetyl (2,3-butanedione), which has a buttery aroma. Minor common contributions to natto aroma are beany flavours, such as benzaldehyde and 2-pentylfuran originating from legumes (Dajanta et al., 2011; Liu, Song, et al., 2018).

An important vitamin present in natto is thiamine (vitamin $B_1$), this vitamin is both of plant and microbial origin. *B. subtilis* can synthesize thiamine de novo as well as salvage it from the environment (Begley et al., 1999). Thiamine vitamers include the free unphosphorylated from, as well as various phosphorylated ones. The free unphosphorylated thiamine is a precursor of flavour compounds, such as 2-methyl-3-furanthiol, 2-methyl-3-methyldithiofuran, and bis(2-methyl-3-furyl)disulphide, that derive from the thermal degradation that occurs during cooking, these compounds have

a strong meaty and roasted aroma (Tang et al., 2013; Thomas et al., 2015). Whereas its phosphorylated form, thiamine pyrophosphate is biologically active as a co-factor of many enzymes with an important role in human metabolic functions, indeed a severe deficiency causes beriberi syndrome, that can lead to death (Wilson, 2020).

An increase in natto consumption made with legumes produced in Europe is not only favourable for a sustainability stance, but also for the health-promoting effects that have been attributed to natto consumption. Examples are anti-hypertensive (Okamoto et al., 1995) anti-thrombotic (Masada, 2004), and anti-diabetic (Araki et al., 2020) activities as well as reduction in cardiovascular mortality (Nagata et al., 2017). These health benefits are attributed to bioactive compounds in natto such as vitamin $K_2$, and nattokinase (Beulens et al., 2013; Chen et al., 2018; Tsukamoto, Ichise, et al., 2000a).

In natto we find two variants vitamin K: phylloquinone (vitamin $K_1$), and menaquinone (vitamin $K_2$). Both are important in human health, since they contribute to biological activities related to haemostasis, calcium, and bone metabolism (Beulens et al., 2013; Bolton-Smith et al., 2007; Schurgers et al., 2006). Phylloquinone is of plant origin, therefore is already present in soy, while menaquinone - specifically MK-7 - is produced by *B. subtilis* during fermentation. Natto has significantly higher amounts of menaquinone compared to most plant-based foods, and it is one of the richest sources of this vitamin (Kaneki et al., 2001). Another compound present in natto that is produced via fermentation is an enzyme of the subtilisin family known as nattokinase. Nattokinase is a trypsin-like serine protease made up of a single polypeptide chain composed of 275 amino acids (Nakamura et al., 1982; Sumi et al., 1987). It is stable under acidic conditions and can enter the blood stream intact by intestinal absorption through the epithelial cells or through tight junctions (Ero et al., 2013; Fujita et al., 1995). In animal and human trials nattokinase has shown potent fibrinolytic (Fujita et al., 1995; Sumi et al., 1987), anti-hypertensive (Fujita et al., 1993; Jensen et al., 2016; Kim et al., 2008), anti-atherosclerotic (Ren et al., 2017; Suzuki et al., 2003), lipid-lowering (Duan et al., 1956; Ren et al., 2017), anti-platelet/anti-coagulant (Jang et al., 2013) and neuroprotective actions (Fadl et al., 2013; Ji et al., 2014). Fibrinolytic activity of nattokinase has been observed to degrade blood clots in vivo and in vitro. It works through direct degradation of fibrin-to-fibrin degradation products, or through indirectly activating plasminogen to plasmin, which in turn degrades fibrin (Nakamura et al., 1982; Sumi et al., 1987). These properties make natto and nattokinase relevant in the prevention and treatment of cardiovascular diseases (Weng et al., 2010).

Soy, the traditional substrate for natto, is a sub-optimal crop to promote for large consumption in Europe, since it is an allergen, it is not native to Europe, and the European Union is heavily reliant on its import. Therefore, natto production with European legumes can increase food security and reduce reliance on import. For this reason, to further improve the sustainability of natto as an alternative protein source, it is relevant to study the use of legumes cultivated in Europe as alternative fermentation substrates (European commission, 2022; Pedersen et al., 2008). In this study we selected five legumes that are cultivated in Europe: brown beans, green peas, and lupin, which are commonly produced in Central and Northern Europe; and red lentils, and chickpea, which are mostly produced in Southern Europe (Alandia et al., 2020; Toker et al., 2010). The rising interest in fermented products, and natto's nutritional profile, show potential for further development of the European market of natto. Therefore, this study aimed to develop a novel natto product made with the previously mentioned European legumes. To study the impact of the choice of strain on the final product we assessed three different *B. subtilis* strains on these substrates, and the natto obtained were compared to those produced using soy as the reference substrate. The comparison was done on the microbial growth and alkalinization, the production of γ-polyglutamic acid strands, the release of free amino acids, the content of vitamin $B_1$, $K_1$, and $K_2$, and the amount of nattokinase produced.

## 5.2 Materials and Methods

### 5.2.1 Legumes and bacterial strains

Brown beans (*Phaseolus vulgaris*), chickpeas (*Cicer arietinum)*, green peas (*Pisum Sativum)*, split dehulled lupins (*Lupinus albus)*, red lentils (*Lens culinaris)* and soybeans (*Glycine max)* were purchased from DO-IT Organic (Barneveld, Netherlands). They were stored sealed at room temperature until use. Three *Bacillus subtilis* strains were used for this study, two wild-type strains isolated from natto, namely 1.8 and 2.8, and DSM 1092 strain, purchased from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany). Strain 1.8 was isolated from natto from Takano Foods (Japan), and 2.8 was isolated from natto by NATTODAN (The Netherlands). In this paper we refer to strain DSM 1092 as 92. Strains were stored at -80°C in 30 % glycerol.

### 5.2.2 Inoculum preparation

A cell suspension of *B. subtilis* var. natto was prepared from a glycerol stock on nutrient agar plates (Thermo Fisher, Manchester, UK) and incubated at 30°C for 15 h, and afterwards at 4°C for 48 h. For each strain, one colony was taken from that plate and

put into a 50 ml Erlenmeyer with 25 ml nutrient broth (nutrient agar composition omitted of agar) and incubated at 30°C at 180 rpm for about 22 h. 20 ml of overnight culture were washed twice in 20 ml of PBS 2 ml of PBS. This cell suspension had a concentration of about 8 $\log_{10}$ CFU/ml.

### 5.2.3 Natto production

Natto was prepared according to the method of Wei et al. (2001), with some adaptions. In brief, legumes (except green peas and red lentils) were washed and soaked for 16 hours at 25°C with bean:water ratio 1:3. Legumes were cooked in a Presto 23-quart pressure canner and cooker (National Presto Industries Inc., Eau Claire, WI, USA) at 41 kilopascals (kPa) at 110 °C. The soybeans, lupin and chickpeas were cooked for 30 minutes, the brown beans and green peas were cooked for 22 minutes, and the red lentils for 15 minutes. Legumes were dried in open air on a metal mesh (cooling tray) for 2.5 hours. 200 g of legume was manually mixed in a bag with 0.2 ml of inoculum (with about 8 $\log_{10}$ CFU/ml). Each sample was put in a sterile fermentation container of 14×9x3.5 cm with holes of 1 mm diameter 3 cm apart. Samples were fermented at 37°C, static, under constant conditions for 24 hours, followed by a maturation step at 4°C for 24 hours.

### 5.2.4 Total viable count

To estimate the viable plate count of *B. subtilis* in natto, 10 gof natto together with 90 ml PBS were added to a stomacher bag and homogenised in a stomacher at 230 rpm for 1 minute. The samples were appropriately diluted in PBS and a 0.1 ml was spread plated on a nutrient agar plate and incubated at 37°C for about 16 h. Colonies present on the overnight plate were counted for total viable count enumeration.



### 5.2.5 pH measurement

The pH of the samples was measured before and after fermentation according to the following procedure. Ten grams of cooked legumes or natto were weighed in a 50 ml Greiner tube with 20 ml demineralised water. Then the samples were homogenised using an ULTRA-TURRAX® T 25 (IKA-Werke GmbH, Germany) for about 30 seconds at 16.000 rpm, until a thick slurry was formed, the pH was measured using a pH meter (MeterLab PHM240, Denmark).

### 5.2.6 Polyglutamic acid analysis

To analyse γ-polyglutamic acid formation, the samples were vigorously mixed with a sterile spoon for 10 seconds and pictures were taken of all the sample. The pictures were visually examined and to each sample a value between 0 and 3 was assigned. A

value of 0 indicates no formation of strands, a value of 3 indicates that a high amount of thick strands was formed.

### 5.2.7 Sample preparation

To prepare the natto samples for analysis of free amino acids, thiamine, and vitamin $K_1$ and $K_2$ and nattokinase, 10 grams of each sample was frozen in liquid nitrogen, after evaporation of nitrogen the frozen samples were transferred into a coffee grinder (Krups F203, Solingen, Germany) and ground up for 20 seconds obtaining a fine powder. The homogenized samples were by -20°C until further analysis.

### 5.2.8 Thiamine extraction and determination

An aliquot of the ground-up frozen natto, 2.5 g, was placed in a tube and diluted 10 times with 22.5 ml 0.1 M HCl, and extracted for 30 minutes in a water bath at 95°C. Afterwards, 1 ml of the extract was diluted with 9 ml 0.1 M HCl and filtered using a 0.2-micrometre filter and stored at -20°C before derivatization and HPLC analysis as described by Rocchi and co-workers (2022), with the only modification of the pH of the mobile phase A, which was set to 7.

### 5.2.9 Vitamin $K_1$ and $K_2$ extraction and determination

The method of Tsukamoto, Ichise and co-workers (2000a) was adapted as described below for the extraction of vitamin $K_1$ and $K_2$. An aliquote of 2.5 g of ground-up frozen natto was placed in a tube and diluted 10 times with 22.5 ml of demineralised water. As an internal standard 200 µl of 5.2 mg/l MK-4 was added to each sample, along with 5 ml of isopropanol. The solution was mixed for 10 minutes at 220 rpm, and 6 ml hexane (EMSURE®, Sigma-Aldrich) was added, followed by a second mixing step for 10 minutes at 220 rpm. The phase separation was accelerated with centrifugation for 10 minutes at 3000 g. The upper phase was collected and put in a glass bottle. This extraction with 6 ml hexane was repeated another time, and the second upper phase was added to the first. Finally, the hexane was evaporated using a nitrogen flow for about 45 minutes. The glass vials containing the extracted $K_2$ were rinsed with 0.5 ml of isopropanol (EMSURE®, Sigma-Aldrich). The dissolved $K_2$ was put in a dark glass vial and stored at -20°C until analysis. The samples were analysed using liquid chromatography coupled with mass spectrometry (LC-MS). Vitamin $K_2$ determination was done as described by Liu and co-workers (2019), with the following modifications. The volume of injected sample was 35 µl, the mobile phase A was demineralised water and mobile phase B was acetonitrile (EMSURE®, Sigma-Aldrich). Standard standard solution of MK-4 (Sigma-

Aldrich), MK-7 (Sigma-Aldrich), and vitamin $K_1$ (Sigma-Aldrich) in the concentration range from 1 ng/ml to 3 µg/ml were analysed to obtain the calibration curves.

### 5.2.10 Determination fibrinolytic activity

Nattokinase concentration was determined as a function of fibrinolytic activity in natto, unfermented legumes, and overnight cultures of *B. subtilis* strains on nutrient broth. Sterile nutrient broth Plasminogen (Sigma-Aldrich, St. Louis, Missouri, USA) and commercial nattokinase (Brand: Doctor's Best Inc; obtained from Superfoodsonline.nl, Groningen, The Netherlands; manufactured in Tustin, California, USA; 10 and 100 FU/ml; additional ingredients: maltodextrin, Hypromellose, magnesium stearate) were used as negative and positive control, respectively. Fibrin gels were made in 12-wells plate (Ø23 mm) by successive addition of 1 ml fibrinogen (plasminogen depleted, from human plasma; Sigma-Aldrich; 5 mg/ml), 0.1 ml Tris-HCl buffer (50 mM, pH 7.0), 0.1 ml thrombin (Sigma-Aldrich; 20 U/ml) and 0.78 ml agarose solution (1 %). Plates were left to solidify. Natto and legume samples were prepared by suspending 1 g of sample in 5 ml Tris-HCl buffer. Samples were stored overnight at 4°C and incubated 30 min at 37°C in shaking water bath for 30 min, directly before measurement. Overnight cultures were grown on nutrient broth for 24 h at 37°C at 160 rpm and subsequently OD was measured. All samples were centrifuged (6400 x g, 5 min) and 3 µl of the supernatant was applied in the centre of the gel. Plates were incubated overnight at 37°C and diameter of clear zone was measured. Results. Commercial nattokinase (2000 fibrinolytic units/capsule) was used as a standard.



### 5.2.11 Free amino acid content determination

Free amino acid profile was determined by Ultra-High Pressure Liquid Chromatography (UPLC) using AccQ-Tag Ultra Derivatization Kit (Waters, Millford, MA, USA). The procedure of Scott et al. (2021) was followed with some adaptions. The adaption being that 1 g of ground-up frozen natto was suspended in 9 g of demi water.

### 5.2.12 Volatile organic compound analysis

Volatile organic compounds (VOC) present in natto were analysed using Gas chromatography-mass spectrometry (GC-MS) following the procedure of Scott et al. (2021). Only adaption was that samples were prepared by putting about 1.1 g of each in 5 ml GC-MS glass vials, capped and stored at -20°C until analysis. The peak identification was done by comparing the mass spectra with the profiles in the NIST (National Institute of Standards and Technology) main library, and the peak areas were

determined via the MS quantitation peak, considering the highest m/z peak for each compound.

### 5.2.13 Statistical analysis and graphical representation

The effect of product and strain on the quality attributes measured was evaluated by Bayesian linear contrast analysis (Allen, 2017).

For the contrast analysis, the strain and product effects on each quality attribute (y) was described as a multilevel linear model, as shown in Equation (1). Accordingly, the quality attribute follows a normal distribution with expected value, $\mu$, and unknown variance $\sigma$.

$$y \sim \text{Normal}(\mu, \sigma) \quad (1)$$

The strain and product affects are defined as linear perturbations on $\mu$. As shown in Equation 2, the product effect is quantified by the deviation, $\varepsilon_p$, with respect to the grand mean $a_0$. Then, the strain-effect for each product is modelled as a nested perturbation $\varepsilon_{s|p}$. Both perturbations are assumed to follow a normal distribution with mean zero and unknown variances $\sigma$p and $\sigma$s.

$$\mu \leftarrow a_0 + \varepsilon_p + \varepsilon_{s|p} \quad (2)$$

$$\varepsilon_p \sim \text{Normal}(0, \sigma_p)$$

$$\varepsilon_{s|p} \sim \text{Normal}(0, \sigma_s)$$

All the calculations were done in R version 4.2.1 [R Core Team (2022)].The models were fitted using the no-U-turn Hamiltonian Monte Carlo sampler included in Stan (Carpenter et al., 2017) using the brms package (Bürkner, 2017). The convergence of the model fit was evaluated by visualising the trace plots of the Markov chains and, as well as ensuring the R-hat index was lower than 1.01, resulting in four independent chains with 6,000 iterations and 3,000 warmup iterations.

The contrast for each product, i, ($C_{p[i]}$) was defined as the difference with respect to the grand mean as shown in Equation (3). It was calculated directly from the Monte Carlo iterations, determining the significance of the product effect based on the quantiles of the posterior distribution.

$$C_{p[i]} = a_0 - \varepsilon_{p[i]} \quad (3)$$

The contrast for each strain, j, conditional to each product, i, ($C_{s[j]|p[i]}$) was defined as the difference with respect to control (uninoculated) conditions as shown in Equation (4).

Again, the posterior of the contrast was calculated directly from the Monte Carlo iterations.

$$C_{s[j]|p[i]} = (a_0 + \varepsilon_{p[i]} + \varepsilon_{s[control]|p[i]}) - (a_0 + \varepsilon_{p[i]} + \varepsilon_{s[j]|p[i]}) \quad (4)$$

Data were stored using Excel software v.16.0 (Microsoft Corporation, Redmond, WA, USA), data analysis and figures were done using R version 4.2.1.

HPLC-MS data were processed using MassLynx software (Waters). The peak areas of the samples were adjusted based on the extraction yield on the MK-4 internal standard. HPLC-Fluorescence chromatogram data was processed using Chromperfect v6.0.18 (Denville, NJ, USA). Data generated by GC-MS were analysed in Chromeleon v7.3.1 (Thermo Scientific, MA, USA). We did statistical analysis and generated figures using RStudio v4.2.0.

## 5.3 Results

### 5.3.1 Microbial activity

The growth of *B. subtilis* on the different legumes affects the pH and the development of the typical texture of natto, which are indicators of a successful fermentation. Samples inoculated with strain 2.8 had on average 4.6 $\log_{10}$ CFU/g at the onset of fermentation, while strain 1.8 and 92 had respectively 5.2 and 5.6 $\log_{10}$ CFU/g (Figure 1A). Bacterial growth occurred in all the samples, with no significant difference between different strains or substrates at the end of the maturation process. The highest viable bacterial counts were found in all the soybean samples and in chickpea natto inoculated with strain 92, with about 8.5 $\log_{10}$ CFU/g. The lowest cell counts were found on lupin inoculated with strain 2.8, where the average microbial concentration was 7.6 $\log_{10}$ CFU/g. In general, the choice of substrate had more influence on the final bacterial cell count compared to the choice of strain, but overall, the total microbial concentration was comparable across all the natto products.

The unfermented legume with lowest pH was lupin, with a value of 6.0, while the other legumes had an initial pH between 6.5 for brown beans and 6.7 for soybeans (Figure 1B). After maturation, there was no significant difference in pH between fermented and unfermented substrates in both soybeans and brown beans. In these two substrates the pH reached a value of 6.9 with strain 2.8 and 92 in soybeans, and with strain 1.8 and 92 in brown beans. Green peas 1.8 had a significantly higher pH, compared to the control, while green peas 2.8 and 92 did not. In chickpea natto, the only sample that did not increase significantly compared to the control was strain 92. All the fermented lupin and

red lentils samples were significantly higher compared to the control condition. Overall, the highest alkalinization was observed in red lentils, with an average pH of 8.1 for both strain 1.8 and 92, compared to the control that had an initial pH of 6.5 in this case both the choice of strain and substrate impacted the outcome of the alkalinization process.

Overall, the natto with the highest amount of strands formed was chickpeas, and the lowest were brown beans and red lentils (Figure 1C). The highest γ-polyglutamate (PGA) producing strain was strain 2.8, while the lowest was strain 92, which produced no strands at all in brown beans and red lentils, and few strands in all the other substrates, except for chickpeas.

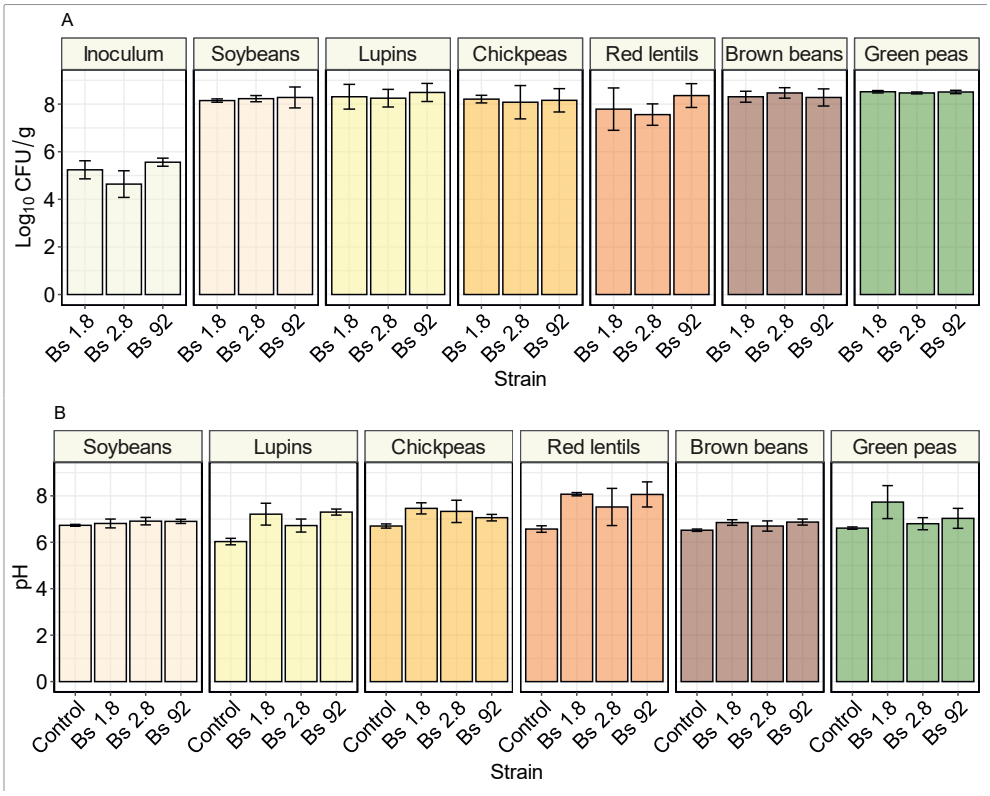


Figure 1- Total viable count of inoculum and fermented natto samples after fermentation and maturation, on nutrient agar (A). pH of control and fermented natto samples after fermentation and maturation on nutrient agar (B). The error bar represents the standard deviation of the means of three biological replicates.

### 5.3.2 Vitamin $B_1$ content

Thiamine was detected in all the unfermented samples (Figure 2A), and its concentration did not significantly change for any of the legumes during fermentation and after maturation. Overall, lupin had a lower concentration of thiamine compared to the other substrates assessed in this study. Because the fermentation did not significantly change the amount of thiamine detected, the choice of *B. subtilis* var. natto strain did not impact the overall presence of thiamine. Green peas had the highest amount of thiamine found in the unfermented substrates, on average 273 µg/100 g, and lupin had the lowest, with 129 µg/100 g.

While the total thiamine concentration did not change in all the fermented substrates, the distribution of the vitamers did. The relative contribution of unphosphorylated thiamine decreases during the fermentation while the relative pools of phosphorylated and diphosphorylated increased (Figure 2B), except for soy fermented with strain 2.8 and 92. Especially in red lentils, the free form of thiamine went from representing over 93 % of the total thiamine to 69 %, 58 %, and 57 % in samples 1.8, 2.8 and 92. Soy fermented with strain 92 was the only substrate for which there was an increase in proportion of thiamine, where free thiamine went from 40 to 61 %. Instead, for soy fermented with strain 1.8, thiamine decreased while thiamine phosphate and pyrophosphate increased. Soy fermented with strain 2.8 showed virtually unchanged proportions of the different vitamers. This change in the different vitamers pool before and after fermentation is a sign of microbial activity, since only thiamine pyrophosphate is biologically active, and the heat treatment of the substrate is expected to inactivate all the plant derived enzymes.



Table 1- Table indicating the average value (n=3) of the string formed in the natto samples, assessed viavisual observation

| Strain | Soybeans | Lupins | Chickpeas | Red lentils | Brown beans | Green peas | Strain average |
|---|---|---|---|---|---|---|---|
| Bs 1.8 | 2 | 2 | 2 | 1 | 2 | 1 | 1.7 |
| Bs 2.8 | 2 | 2 | 3 | 2 | 1 | 2 | 2.0 |
| Bs 92 | 1 | 1 | 2 | 0 | 0 | 1 | 0.8 |
| Legume average | 1.7 | 1.7 | 2.3 | 1.0 | 1.0 | 1.3 | |

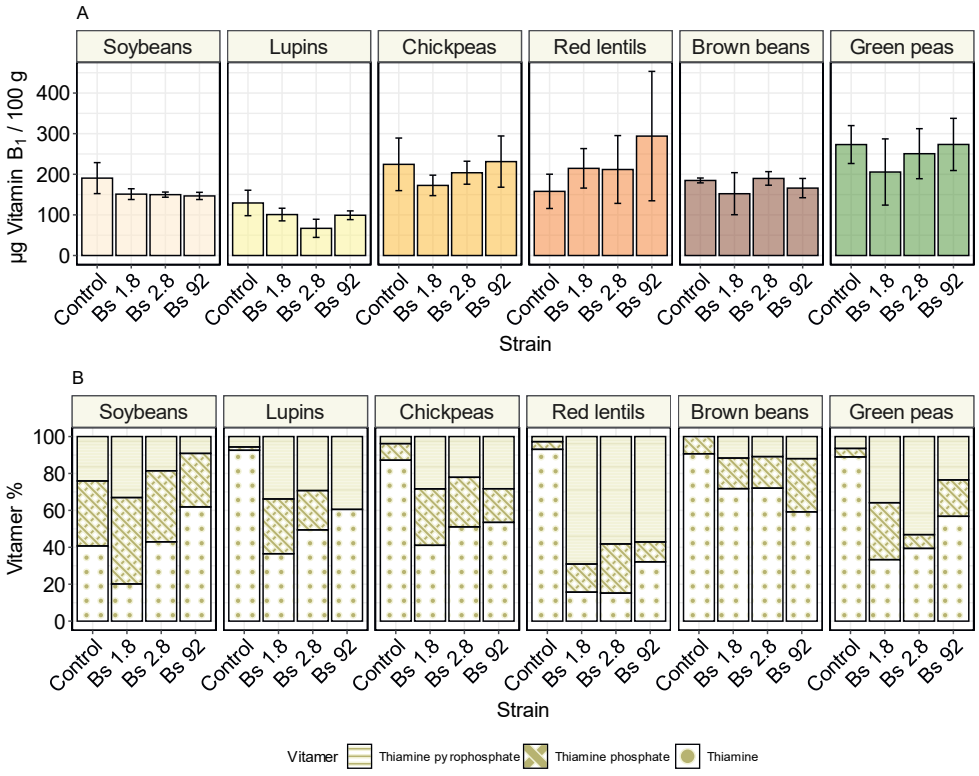


Figure 2- Total thiamine content of control and fermented natto samples after fermentation and maturation (A). Vitamin $B_1$ vitamer distribution percentage of control and fermented natto samples (B). The error bar represents the standard deviation of the means of three biological replicates.



### 5.3.3 Vitamin $K_1$ and $K_2$ content

Vitamin $K_1$ and $K_2$ are known to be present in natto and have a well-established positive influence on human health. Each one of these vitamers is specific to the organism that produces it. Phylloquinone (vitamin $K_1$) is exclusively of plant origin, vitamin $K_2$ can be both of animal and bacterial origin. Menaquinone 7 (MK-7) is produced during fermentation by *B. subtilis,* and it is exclusively of microbial origin. In this study we used the animal derived menaquinone 4 (MK-4), as internal standard to determine the efficiency of extraction of MK-7 and vitamin $K_1$. Phylloquinone was found in all the fermented and unfermented samples, and its concentration was unaffected by fermentation and maturation (Figure 3A). Soy had significantly higher concentration of phylloquinone compared to all the other substrates, with on average 19.6 µg/100 g. The lowest concentration of phylloquinone was found in lupin, with 6.1 µg/100 g on average, this amount was significantly lower compared to all other substrates. In chickpeas and

brown beans, we found respectively 7.3 and 8.9 µg/100 g of phylloquinone, while in green peas and red lentils had 12 µg/100 g and 10.8 µg/100 g of phylloquinone, respectively.

As expected, no MK-7 was found in any of the unfermented substrates (Figure 3B). During fermentation and maturation MK-7 increased significantly in all the samples. Strain 92 consistently produced more MK-7 on average compared to all the other strains. Whereas substrate-wise, red lentils had on average higher MK-7 concentrations, with respectively 690, 1283, and 1647 µg/100 g for strain 1.8, 2.8, and 92. The highest amount of MK-7 was found in chickpeas fermented with strain 92, with on average 2002 µg/100 g. The lowest average amount of MK-7 was produced in brown beans, inoculated with strain 1.8, in which only 78.9 µg/100 g was found. Overall, both the choice of strain and substrate impacted the production of vitamin $K_2$ and strain 92 consistently yielded more menaquinone.

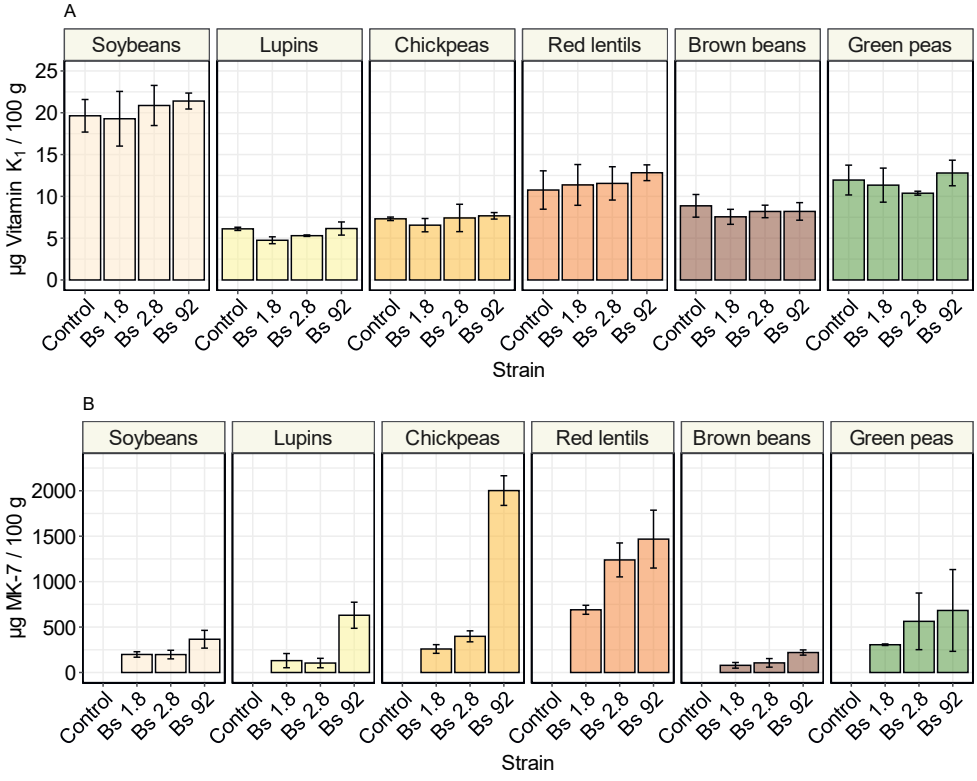


Figure 3- Vitamin $K_1$ content in fermented natto samples, after fermentation and maturation, on nutrient agar (A). Vitamin $K_2$ content of natto samples after fermentation and maturation (B). The error bars represent the standard deviation of the means of three biological replicates.

### 5.3.4 Fibrinolytic activity

In this study we detected and quantified fibrinolytic activity in the different natto samples, and supernatants of overnight cultures by monitoring the diameter of the clearance zone in fibrin plates upon the addition of sample extracts (Figure 4). Sterile nutrient broth and commercially available nattokinase were used as negative and positive control, respectively, and for calibration. The fibrinolytic activity detected varied depending on the strain and substrate used for natto production. Overall, nattokinase production was influenced more by strain than substrate (based on Bayesian contrast analysis). No clear zone was observed in unfermented legumes. Commercial nattokinase supplement diluted to match 10, 100, 500, and 1000 nattokinase units produced respectively 5.5, 15.0, 19.0, and 23 mm clear zones. Fibrinolytic activity was only observed in fermented natto samples, with a clear zone from 0.3 to 11.0 mm, corresponding to 5 to 350 nattokinase units/g. Only fermented brown beans did not show any fibrinolytic activity. The lowest nattokinase producing strain was strain 92. The highest nattokinase producing legume was soy, on average. In soy, fermented with strain 1.8 and 2.8 88 units/g and 347 units/g were detected, respectively. This was the only instance that the same strain produced more nattokinase in a legume, compared to nutrient broth. The lowest detectible nattokinase activity (7 units/ g) was observed in lupin natto fermented with strain 2.8.

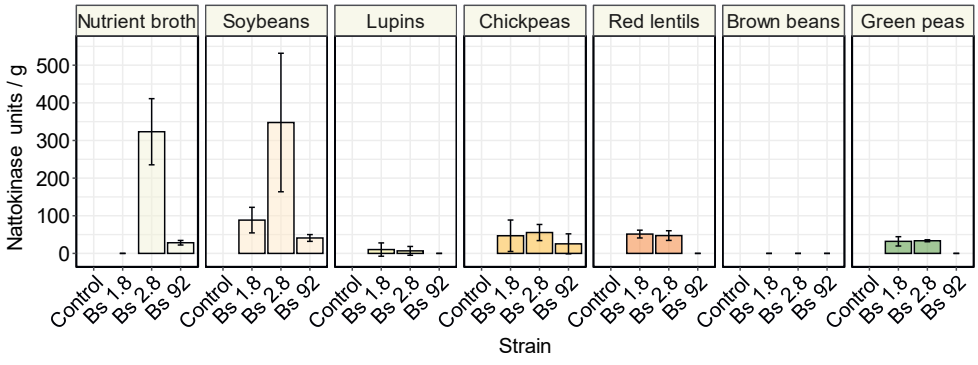


Figure 4- Nattokinase content of nutrient broth, control, and fermentation natto samples after fermentation and maturation. The error bars represent the standard deviation for the means of three biological replicates.

### 5.3.5 Free amino acids and ammonia content

During fermentation, proteolytic activity of *B. subtilis* leads to the release of free amino acids (FAAs) (Chantawannakul et al., 2002). The pool-size of the free amino acids depends on rate of protein hydrolysis, and the rate of amino acid uptake by the fermenting microorganisms. The pool-size of the FAAs is a predictor of natto quality, as well as an indicator of successful fermentation, since the FAAs are precursors of many volatile organic compounds, and other relevant metabolites. FAA content in unfermented legumes was relatively low, around 0.8 ± 0.2 mmol/100 g, but fermentation resulted in an increase in free amino acid pools. Brown beans were the exception. In this fermentation the total free amino acids were reduced. Unfermented brown beans contained 0.7 ± 0.05 mmol/100 g, while brown beans fermented with strain 1.8 and strain 2.8 contained 0.24 ± 0.05 and 0.32 ± 0.04 mmol/100 g, respectively. The highest increase in FAA occurred in red lentils, for which strain 1.8 and strain 2.8 resulted in approximately a 10-fold increase, going from 0.9 mmol/100 g to between 10.5 and 10.7 mmol/100 g, respectively. Strain 92 had a lower, but still substantial increase of up to 6 mmol/100 g. Apart from brown beans, the lowest increase in FAA occurred in natto from soybeans in which the amount of FAA increased 4-fold at best in strain 2.8, from 0.5 mmol/100 g to 0.2 mmol/100 g. In general, after maturation green peas, lupins, and red lentils had significantly higher free amino acid pools as compared to the control. Whereas for the other legumes this increase was not significantly higher, except for chickpeas fermented with strain 2.8. Overall, the production of free amino acids was impacted the most by the choice of legume, rather than the strain that was used for fermentation. Furthermore, fermentations with strain 1.8, 2.8 and 92, respectively, resulted in similar FAA profiles (Figure 6). Within samples, production of free glutamic acid, leucine and lysine was particularly high (Figure 6). This trend in free amino acid profile was observed in all strains for lupins, chickpeas, red lentils, and green peas.

Ammonia concentration was highest in red lentils fermented with strain 2.8 and strain 1.8, with respectively 1.1 ± 0.5 and 1.2 ± 0.6 mmol/100 g (Figure 5). Following fermented red lentils, ammonia concentration was highest in descending order in fermented lupins, green peas, chickpeas, soy, and brown beans. Ammonia production positively correlated with the glutamate and the total free amino acid content. These legumes similarly contained the highest concentrations of ammonia of all the substrates (Figure 5).

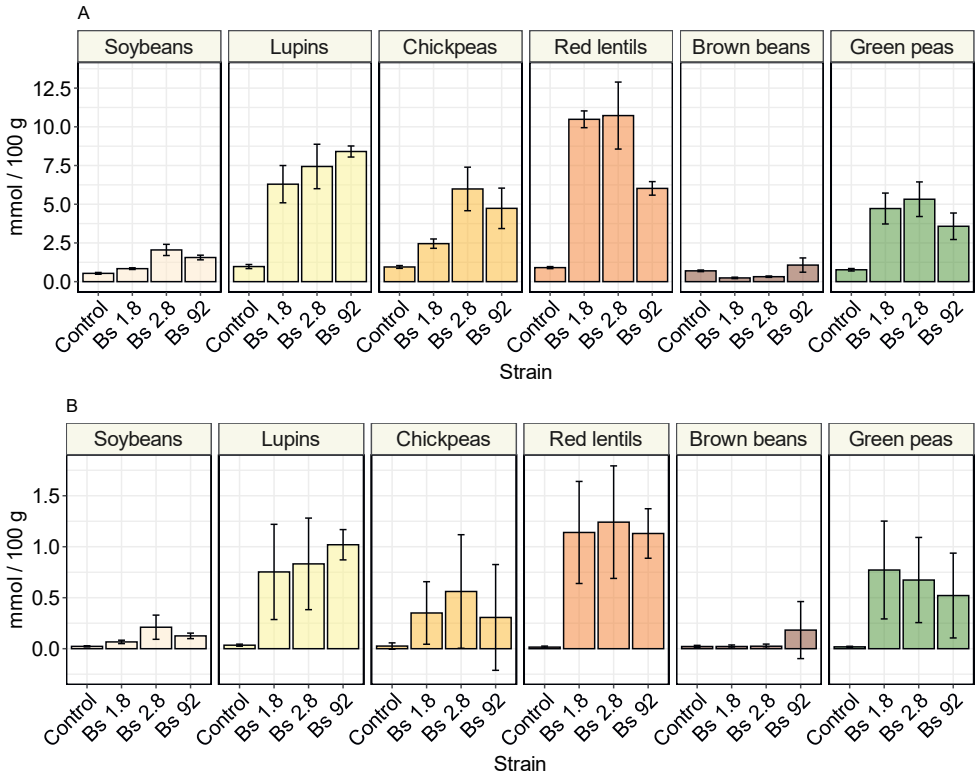


Figure 5- Total concentration of free amino acids in control and fermented natto samples (A). Total ammonia concentration in control and fermented natto samples, after fermentation and maturation (B). The error bars represent the standard deviation of the means of three biological replicate.

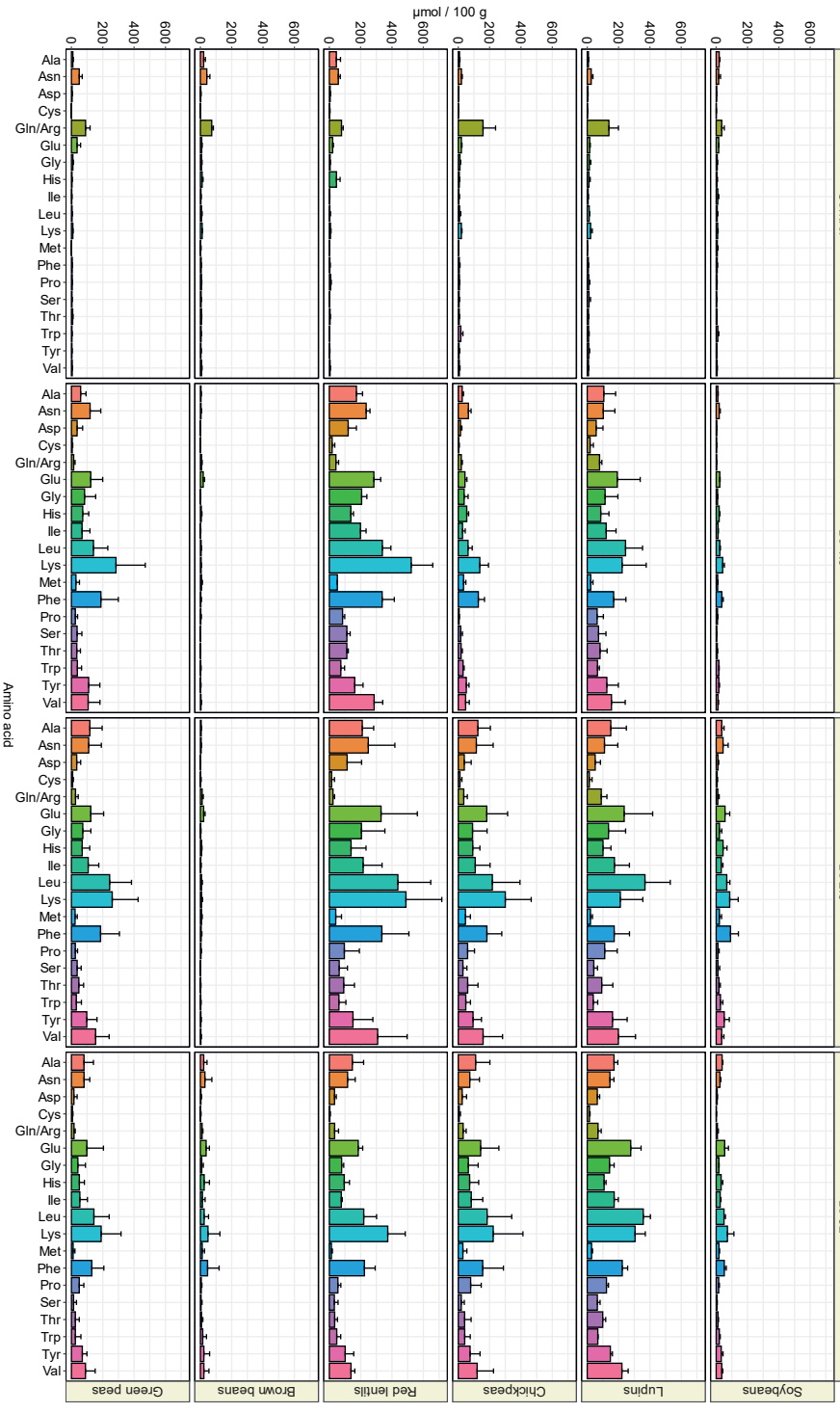


Figure 6- Full amino acid profile of control and fermented natto samples. Each different colour represents a single amino acids, except for glutamine and arginine which are not detected non -specifically with the HPLC method. The error bar represents the standard deviation of the means of three biological replicates.

### 5.3.6 Volatile aroma profile

Commercial natto has a distinct aroma profile (Chen et al., 2022; Huang et al., 2012). Therefore, it is an important parameter in determining natto quality. Volatile organic compounds (VOCs) were determined using GC-MS, and in total 28 compounds were detected and identified (Figure 7). Boiled unfermented legumes were used as control and contained low amounts of VOCs. None of the strains fermenting brown beans produced a significant increase in total VOC. However, green peas and chickpeas fermented by all strains produced significantly more VOCs compared to their unfermented counterparts. The highest increase in VOCs was observed in green peas fermented with strain 2.8. The effect of strain on VOC production was dependent on the legume. In lupin natto only fermentation with strain 92 and 1.8 increased the total amount of VOCs, while for red lentils a significant increase in VOC was obtained with strain 1.8 and 2.8, but not with strain 92. Generally, the type of legume had a larger effect on the VOC profile compared to the strain. VOC profile of fermented legumes with low total VOC area were characterized by high proportions of acetone (pungent odour), 2-penthyl-furan (beany odour), and aldehydes such as benzaldehydes (almond-like odour) and butanal (pungent odour). Ratios of the different compounds within the VOC profile were similar in fermented lupins, chickpeas, red lentils, and green peas (characterized by high total VOC area). Their VOC profile was largely composed of pyrazines, 2,5-dimethyl-pyrazine and to a lower extent trimethyl-pyrazine and 3-ethyl-2,5-dimethyl-pyrazine. VOC profile of fermented soybeans and brown beans, characterized by lower total VOC area, were made up of ketones, mainly acetoin.

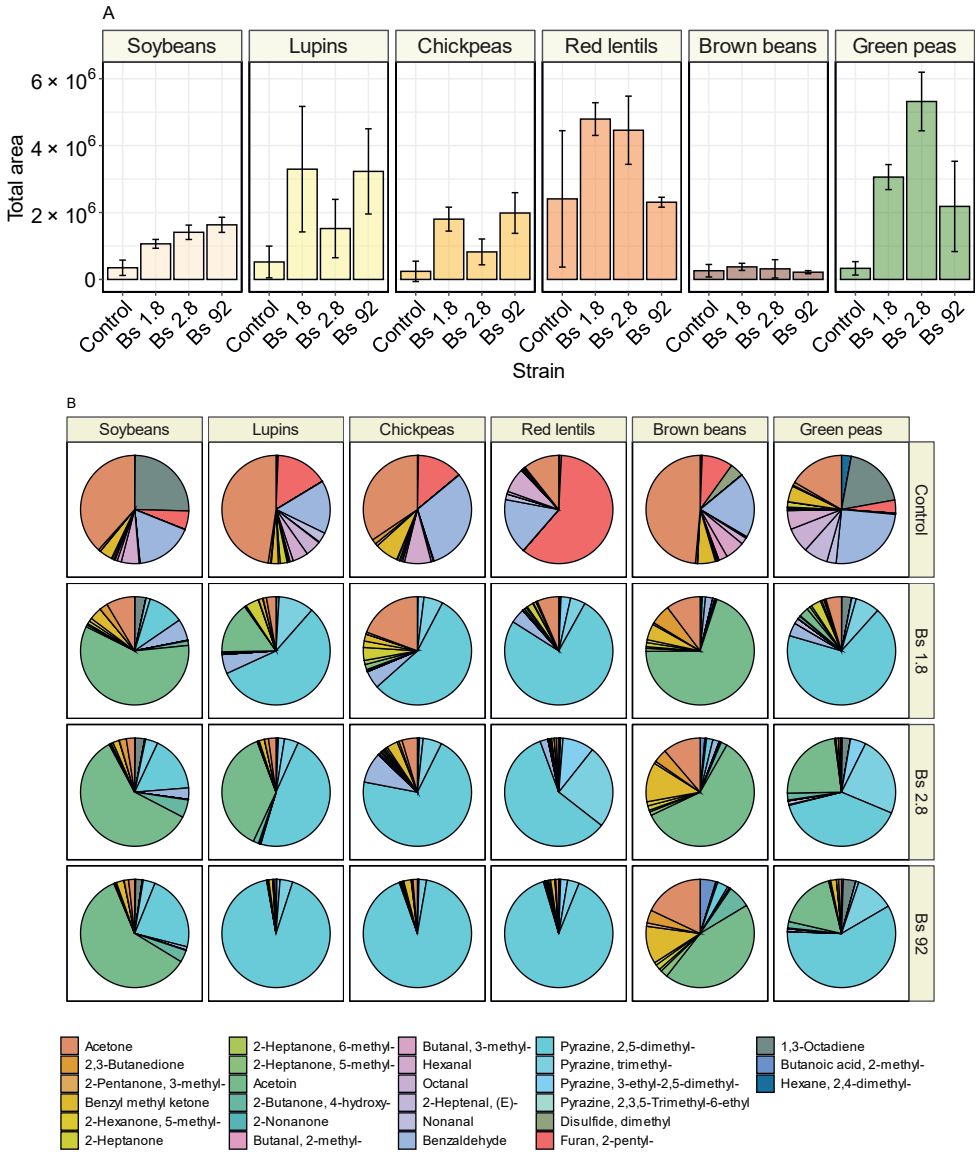


Figure 7- Total concentration of VOC of control and fermented natto samples after fermentation and maturation (A). The error bar represents the standard deviation of the means of three biological replicates. Distribution of VOC of control and fermented natto samples (B), each colour indicates a different compound. Ketones are depicted in orange and green, aldehydes in pink and purples, and pyrazines in turquoise.

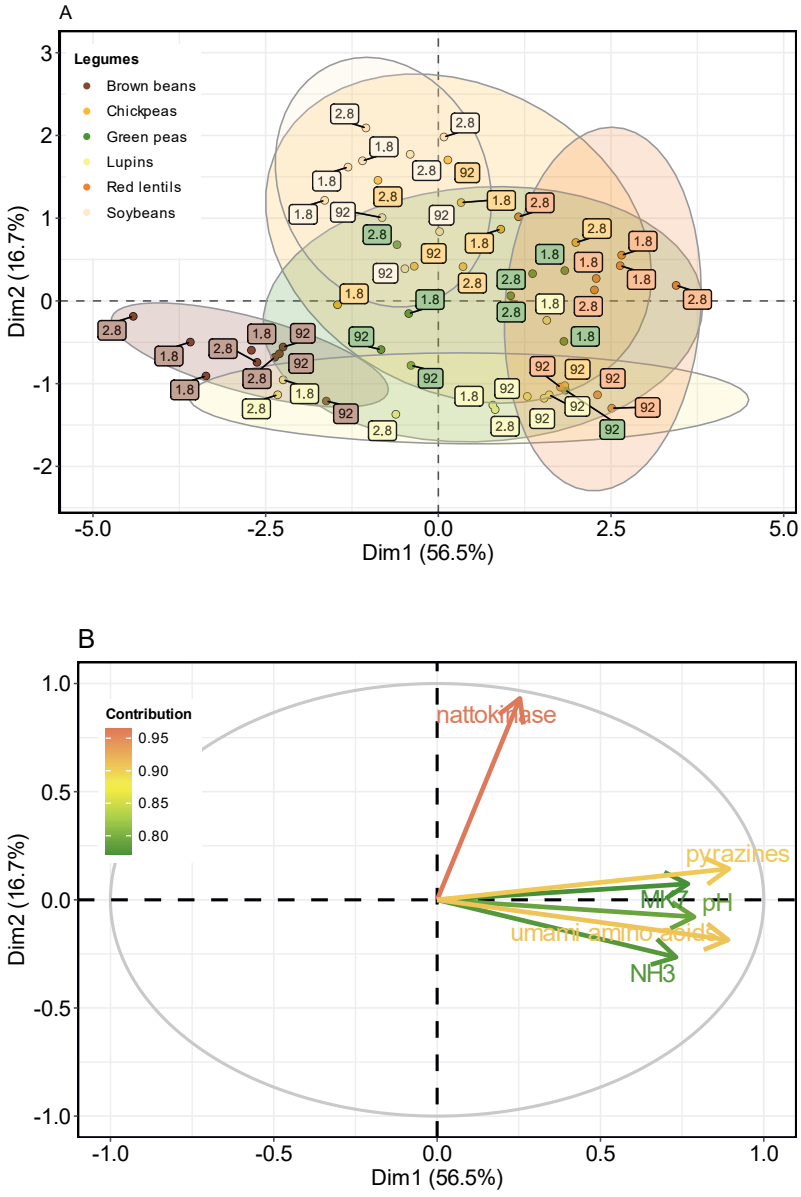


Figure 8- Principal component analysis (PCA) plot showing the separation of legume samples based on their metabolic profiles. Individuals plot of the first two principal components, Dim1 (56.5 %), and Dim2 (16.7 %), with each point representing a sample coloured by legume. Ellipses indicate a 95 % confidence interval around each group (A). Loadings plot showing the contribution of each metabolite to the principal components, with each loading vector coloured by its magnitude (B). The metabolites with the largest contributions are labelled.

## 5.4 Discussion

Overall, all the selected substrates, besides for brown beans, were considered appropriate for the development of a novel natto. All fermented brown beans developed a worst texture and were low in vitamin content as well as in FAA and VOCs. It is possible that the presence of the hull in brown beans caused a lower accessibility of the nutrients to the bacteria, which were not able to develop as many secondary metabolites, resulting in a suboptimal product.

We observed that the strain as well the substrate affected the development of the PGA strands, despite the observation that the difference in microbial growth between samples was not significant. Substrates fermented with strain 2.8 seemed to develop more PGA, especially when compared to products made with strain 92. The latter fermented product that did not show the development of abundant strands, besides for the chickpea sample, which seems to be the substrate that on average showed more PGA. These conclusions are however based on visual observation. To have a more quantitative assessment of PGA formation, the viscosity of natto by rheological analysis could be considered.

Natto was inoculated with approximately 5 $\log_{10}$ CFU/g of *B. subtilis* in all the tested substrates and the bacterial cell count after maturation reached values around of at least 7.6 $\log_{10}$ CFU/g. The difference in growth after maturation was not significant between the tested strains and substrates. This indicates that all the substrates and strains assessed are adequate to ensure sufficient microbial growth, also when compared to natto made from soybeans as reference. Compared to other studies, the bacterial cell counts found in our natto variants is at the lower end. Wei (2001) reported viable cell counts in the final product between 8.6 and 9.8 $\log_{10}$ CFU/g. These higher microbial cell counts could be attributed to the 10 to 100-fold higher inoculation that was used, the longer steaming times of the substrates and finally the strain differences.

Concerning overall alkalinization strain 2.8 was the only one that did not deliver a significant increase in pH during fermentation on any of the substrates, and at the same time it was also the greatest PGA producer, especially in chickpeas natto, and reached at least 8 $\log_{10}$ CFU/g in all the substrates, besides for lupin. Pradhananga (2019) reported that their natto made from soybeans had a final pH of about 7.6 after fermentation, while Wei (2001) reported that natto their had a pH between 7 and 8.1 after 20 h of fermentation, depending on the cooking time and strain used. Our findings are in line with these reported in literature; indeed, all the samples had a pH ranging from 6.7 and 8.1.

Phylloquinone (vitamin $K_1$) is a vitamin of plant origin, indeed it was found in all our fermented and unfermented samples. Fermentation did not affect the content of this vitamin which stayed unchanged. Soybeans had the overall highest vitamin $K_1$ concentration of approximately 20 µg/100 g of product. It has previously been reported that dried soybeans contain approximately 37 µg/100 g of phylloquinone (Booth et al., 1993). In the other legumes we measured a lower content of vitamin $K_1$ compared to soy. Dried chickpeas and dried lentils have been reported to contain 31 µg/100 g, and 34 µg/100 g of phylloquinone (Kim et al., 2022), which is higher than our values. This difference in vitamin $K_1$ compared to literature is likely due to water absorption during soaking and cooking.

Menaquinone is an essential co-factor in many metabolic reactions of *B. subtilis*. This microorganism has all the genes that encode for the enzymes of the menaquinone biosynthesis pathway. This vitamin is constituted by a naphthoquinone unit with isoprenyl sidechains of various lengths. The length of the isoprenyl side-chain is indicated by *n* in MK-*n* (Lenaz et al., 2013). Natto is notoriously rich in vitamin $K_2$, indeed it is one of the richest sources of this vitamin among food products. While animals produce exclusively MK-4, each bacterial species produces different menaquinones with different length of the sidechains. *B. subtilis* produces exclusively MK-7. Therefore, in this study MK-4, which is produced only in animal tissue, was used as an internal standard to establish the extraction yield of the microbially produced MK-7. Commercial natto contains approximately 775 µg/100 g (Tsukamoto, Ichise, et al., 2000a) to 865 µg/100 g of MK-7 (Tsukamoto, Ichise, et al., 2000b), while the highest content of MK-7 in soybeans natto, produced in this study with strain 92, was at best approximately half the commercial reported values. However, natto made with red lentils had either comparable amount of MK-7, or double compared to the commercial references here reported. The highest amount of MK-7 was found in chickpeas natto produced with strain 92, in which vitamin $K_2$ was almost three times higher than the reported values for standard natto. In this specific sample, the vitamin was even higher than the amount found in a natto obtained with a *B. subtilis* mutant overproducing vitamin $K_2$. This mutant was obtained by analogue resistance to intermediates of the shikimate pathway, and UV exposure (Tsukamoto et al., 2001), in which the amount of MK-7 was 1719 µg/100 g. These results indicate that the production of MK-7 is both strain and substrate dependent, and that these factors have significant interaction. Consequently, by selecting the appropriate strain and substrate, it is possible to have a natto with equal or even higher content of vitamin $K_2$ compared to the one currently available in the market. The relevance of this finding is enhanced by the well-establish

health benefits of vitamin $K_1$ and $K_2$, which are proven to contribute to bone and vascular health in humans (Fusaro et al., 2020).

Fibrinolytic activity was quantified by the clear zone on fibrin plates (5.0 - 23.0 mm) as an indicator for the presence of nattokinase (10 - 1000 FU/ml). Chickpeas, red lentils, and soybeans fermented with strain 1.8 and 2.8 showed fibrinolytic activity producing a clear zone between 3.7 and 11.0 mm. Therefore, fibrinolytic activity of these fermented samples is likely to fall in the range of 7 - 350 FU/g. These results are in a similar range compared to earlier work on soybean natto, which observed 130 FU/g (Lan et al., 2020). Significantly higher nattokinase concentrations were observed in soybeans fermented with strains 1.8 and 2.8 compared to the average. Soybeans fermented with strain 2.8 contained the highest concentration (347 FU/g). Studies on metabolic pathways of *B. subtilis* have found a positive correlation between the addition to the growth medium of soy peptone, water, or ethanolic extracts of soy as nitrogen sources, and nattokinase production (Liu et al., 2005; Man et al., 2019). In our study, a high nattokinase concentration of 323 FU/g, was observed in nutrient broth inoculated with strain 2.8, which contains meat extract, yeast extract, and peptone. Different studies observed that these are excellent nitrogen sources for high nattokinase production by *B. subtilis* (Liu et al., 2005; Man et al., 2019; Suwanmanon et al., 2014). Additionally, Man and co-workers (2019) observed that glycine addition showed a positive effect on nattokinase production, while addition of glutamate, asparagine, arginine, and serine decreased nattokinase production. In our results, fermented soybeans showed lower concentrations of all these amino acids, compared to lupins, chickpeas, and red lentils. The combination of the results by Man and co-workers (2019) and our finding is a strong indication for the presence of a negative feedback mechanism between increasing concentrations of these amino acids, and the productivity of nattokinase. Therefore, the lower FAA content in fermented soy could play a role in the high nattokinase concentrations observed.

Fungi, plants, and bacteria produce thiamine. Its deficiency in humans can lead to severe metabolic disfunctions, and death (Wilson, 2020). Thiamine is also well known for its role in development of meat flavour during cooking, indeed upon heat-degradation the thiazole subunit forms many sulphur containing compound that strongly contribute to the aroma of cooked meat (Thomas et al., 2015). In *B. subtilis*, thiamine plays a role in many biological processes, such as the central carbon metabolism, the metabolism of amino acids, and even vitamin $K_2$ production (Dawson et al., 2010). Thiamine biosynthesis in *B. subtilis* is strictly regulated by riboswitches, present in the three single genes and two operons encoding for enzymes involved in

thiamine biosynthesis, transport, and salvage. The riboswitches located upstream of the open reading frames (ORFs) bind with the target ligand, thiamine pyrophosphate, and forms intrinsic transcription terminators, and therefore repress the production of thiamine (Sudarsan et al., 2005). Since the de novo biosynthesis of thiamine is an energy expensive process for *B. subtilis*, its uptake is preferred when the molecule is present in the fermentation substrate. In this case the unchanged thiamine concentration before and after fermentation is expected since *B. subtilis* is not expected to produce thiamine when it is present already. Accordingly, the only effect that the microbial activity had on thiamine, was the observed conversion of thiamine to the biologically active form thiamine pyrophosphate, which increased in proportion in all the fermented legumes, exception for soy fermented with strain 2.8 and 92. The amount of thiamine found in cooked and fermented substrate is in the same order of magnitude compared to the reported literature values (5 - 9 mg/kg )(Lebiedzińska et al., 2006; Vidal-Valverde et al., 2002). But still too low in order to deliver a meaty flavour upon heat exposure (Dreher et al., 2003).

*B. subtilis* is well known for its capacity of secreting extracellular proteases (Degering et al., 2010; Nguyen & Nguyen, 2020). These enzymes are responsible for the breakdown of proteins and release of free amino acids which drives the subsequent release of ammonia in natto. Free amino acids are an important parameter in understanding extracellular protein degradation and subsequent production of secondary metabolites (Wu, 2009). Fermentation driven by *B. subtilis* leads to release of free amino acids followed by an increase in the extracellular pool size of amino acids in lupins, chickpeas, red lentils. This means that the uptake of released amino acids by *B. subtilis* is slower than the rate of release of amino acids driven by extracellular protein hydrolysis. Similar free amino acid profiles were observed for all strains in fermented lupins, chickpeas, red lentils, and green peas (Figure 6), indicating that similar proteolytic enzymes are excreted, and the same amino acids are utilized independently of the strain used. These similar profiles suggest that identical protein catabolism pathways were utilized in all strains. Net consumption of FAA during fermentation was only observed in glutamine/arginine. A result that can be explained by the fact that these amino acids are the preferential nitrogen sources for *B. subtilis* (Fisher et al., 2001). Secondly, γ-PGA production has been observed to result in consumption of glutamine, among others (Hong et al., 2019; Li et al., 2021). A larger increase in total FAA was observed in fermented lupins, chickpeas, red lentils, and green peas, was higher compared to the control soybeans. This was a sign of high proteolytic activity, which is an indication of a superior ability of *B. subtilis* to metabolize proteins in these substrates.

Ammonia is an integral part of the aroma of natto (Kada et al., 2008). Its acceptance by consumers differs based on cultural background and familiarity with the product (Nishinari et al., 2018). High ammonia concentrations were found in samples with a high pH. A correlation which can be explained by the high $pK_a$ of ammonia, which is 9.24 (Perrin, 1982). These results are in accordance with findings from Kada an co-workers (2008), who observed a positive correlation between contents of free L-glutamate, L-alanine, L-glutamine, L-arginine and L-aspartate and ammonia production in *B. subtilis* var. natto fermentation of soybeans. In this study they observed that ammonia content of natto decreased by half when glutamate dehydrogenase was inactivated, indicating that this step is one of the major ammonia-releasing reactions in natto fermentation. Furthermore, ammonia is a precursor in the formation of pyrazines (Fayek et al., 2021; Shu, 1999). High ammonia concentrations were observed in samples with high total FAA, these compounds were released in similar pathways, which explains their co-production (Kegg, 2022). This positive correlation was observed in our results, as well, with higher concentrations of ammonia found in red lentils, lupins, and green peas (Figure 5B). These legumes similarly contained highest concentrations of ammonia of all substrates (Figure 5B).

Natto is a very aromatic product (Hosoi et al., 2003). Major contributors to natto aroma are ketones and pyrazines (Dajanta et al., 2011; Liu, Su, et al., 2018). The main ketone observed was acetoin (3-hydroxybutanone). Acetoin has a high odour threshold but is readily oxidized to diacetyl (2,3-butanedione), with an odour reminiscent of butter or cream (Mottram, 1994). These compounds were present in the highest concentration in fermented soybeans and brown beans (Figure 7). Secondly, acetoin can be non-enzymatically transformed to 2,3,5,6-tetramethylpyrazine (Nawrath et al., 2010). Pyrazines are heterocyclic aromatic compounds, with a high flavour dilution value, and an odour reminiscent of roasted nuts (Liu, Su, et al., 2018). Chen (2022) observed that these non-enzymatic reactions in the formation of pyrazines occur later in the fermentation process, therefore, a high contribution of pyrazine indicate a mature fermented product. Pyrazines were the major contributor to the VOC profile of fermented lupins, chickpeas, red lentils, and green peas. Similarly, to fermented legumes, pyrazines were also a major factor in aroma profile of roasted soybeans (Cai et al., 2021). 2,5-dimethylpyrazine was present in the highest amount. L-threonine serves as a substrate for 2,5-dimethylpyrazine formation (Kłosowski et al., 2021; Larroche et al., 1999). This explains why free L-threonine concentrations remain low in fermentations with high pyrazine formation (Figure 6, Figure 7B, Figure 8).

Principle component analysis (PCA) was used to examine parameters affected by fermentation of our data (Figure 8). Samples were grouped by legume type and plotted in Dim1 and Dim2 (Figure 8A). Dim1 represented 56.5 % of the variability, while Dim2 represented 16.7 %. Loading vectors were coloured by magnitude of contribution (Figure 8B). All variables were well represented in these two dimensions. Samples clustered well by legume type, but much overlap was observed, especially among soybeans, lupins, green peas, and red lentils (Figure 8A). Clustering by strain was less clearly observed, indicating that the effect of legume choice is larger than that of strain. However, this is also indicative of the large biological variation among samples. All parameters were highly positively correlated, except for nattokinase. Since high values for all parameters included in this PCA are desired natto characteristics, samples positively correlating with these parameters are the most promising for natto production. Red lentils fermented with strain 1.8 and strain 2.8 most consistently correlate with all parameters (Figure 8A & B). Therefore, we can conclude that red lentils can be a promising alternative to soybeans, for natto production, regarding both product quality and nutritional aspects.

## 5.5 Conclusions

Brown beans were the only substrate that led to a final product which had lower vitamin content, aroma compounds, and PGA, compared to soybeans natto. In particular, the low free amino acid content and VOCs indicated a slower, thereby incomplete fermentation. While traditional soybean natto contained highest vitamin $K_1$, and fibrinolytic activity, red lentils, green peas, chickpeas, and lupins outperformed soybeans as substrate for natto in terms of alkalinization, free amino acid content (FAA), ammonia content, total volatile organic compounds (VOCs), proportion of pyrazines, and vitamin $K_2$ content. Natto made with green peas and inoculated with strain 2.8 had highest VOCs compounds. Fermented red lentils also contained highest vitamin $K_2$ and $B_1$ content, with menaquinone being two-fold higher compared to commercially available natto. While natto made with chickpeas inoculated with strain 92 had the highest vitamin $K_2$ content of 2 mg/100 g of product, three-fold higher compared to commercially available natto. This study shows that the production of natto using alternative legumes to soy is possible, but that the overall quality, regarding the texture, aroma, and vitamin content is determined by the interaction between the strain and legume.

## Acknowledgements

We would like to thank Judith Wolkers-Rooijackers (Wageningen University & Research, NL) for helping with the GC-MS analysis, Eric van Bennekom (Wageningen University & Research, NL) for his kind help in the determination of Vitamin $K_1$ and $K_2$. We would also like to thank Yue Liu (Wageningen University & Research, NL) for providing the *Bacillus subtilis* strains. Lastly, we would like to acknowledge Kees van Kekem for assisting with the analysis of thiamine.

## Funding statement

This research is part of the project B-Twelve Insight, which is co-financed by Top Consortium for Knowledge and Innovation Agri & Food by the Dutch Ministry of Economic Affairs. The project is registered under contract number TKI AF18081.

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# CHAPTER 6

## General discussion

Rebecca Rocchi

*Finally! Lunch is served…Ahhhh the smell of a burger… so delicious, but a much more complex matter than what I initially thought!*

Since this PhD dissertation represents my efforts to earn the degree of Doctor of Philosophy, please allow me to guide you through a small "philosophical detour" to grasp the beauty, complexity, and potential of food fermentation. This will be the prologue to my general discussion on the potential and challenges of this research. I find it useful to draw a parallel between the potential of food fermentation and the Gestalt psychological theory.

In the early years of the twentieth century, a new psychological school of thought, defined as structuralism, was developed. It theorised that the human mind is the sum of simple basic components (such as sensation, mental images, feelings, and past experiences), which combined, form complex mental structures. Therefore, a human mind could be formed, analysed, and understood by simply dissecting the role of each psychological experience that constitutes it.

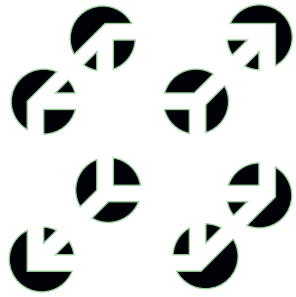

Figure 1- Our mind creates the perception of one cube and 8 black circles, this is much more than the sum of the black shapes that compose this image. Image created by Bernard Ladenthin, CC0, via Wikimedia Commons.

This way of thinking was considered reductive by Gestalt psychology, which on the contrary, showed that our mind, as well as our perception of patterns, is much more than the sum of the individual components that constitute it. As an example, you can look at Figure 1. Viewers will perceive the presence of eight circles and a three-dimensional cube, although the image itself is only a composition of black shapes. This is because our mind creates the perception of this image, which is much more than the sensorial stimulus itself.

*"The whole is more than the sum of its parts."*

Just like our perception confers more information to certain visual stimuli, shaping the world around us, the interaction between substrate and microorganism creates products that are *so much more* than the sum of these two components. Think of any fermentable food substrate, of the thousands of micro-organisms that are currently used worldwide every day…The possibilities to develop novel fermented foods are virtually endless.

## 6.1 General discussion

In ancient times the fermentation of food products relied on the microorganisms present in the raw materials or in the fermentation vessel to conduct the desirable changes in the substrate. This so-called wild fermentation could be steered towards the formation of desirable end products by changing the fermentation conditions to give an advantage to the right microbial populations present in the substrate. For example, salt inhibits LAB and yeast less compared to Enterobacteriaceae or pseudomonads (collectively referred to as undesired microbes). The growth of yeasts and LAB effectively outcompete the mentioned undesired microbes. The tight compression of a substrate and limited contact with air and oxygen favours fermentative micro-organisms, and can prevent the outgrowth of undesirable moulds, which need oxygen to grow. Via backslopping a new batch of raw food material could be inoculated using aliquots of the previous ferments to make the process more robust. The beauty of wild spontaneous fermentation is the complexity of the final product obtained. This is because the different microorganisms produce a wider range of metabolites present, compared to fermentation processes that use a defined starter with less microbial variety. But beauty, although important, is not always enough…The use of a defined starter culture has many advantages over spontaneous fermentation. The characteristics of the product obtained will be more standardised, with fewer batch-to-batch variations, and the process will be overall more robust, with improved microbiological safety. Another important advantage of using a defined starter culture is that a selected strain will often have specific characteristics, such as the increased production of certain metabolites, desired for industrial applications. For instance, the production of vitamin $K_2$ and $B_1$ has been proven to be strain-dependent (**Chapter 5**, **Chapter 3**, Figure 2A), and certain strains of *B. subtilis* produce γ-PGA more effectively than others (**Chapter 5**).

The work we conducted aimed at exploring the potential of microbial vitamin $B_1$ production to deliver a meaty odour to plant-based substrates. Our framework demanded that the strains developed during this study had no genetic modification that would lead to the development of genetically modified organisms (GMO), as defined by the directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001.

This manuscript started with a review of the societal relevance and current knowledge of this research work (**Chapter 1**). In **Chapter 2** we described the newly developed thiamine extraction and quantification method based on mechanical cell disruption, derivatization of thiamine and phosphates to thiochrome and phosphates, and quantification via HPLC. In **Chapter 3** we applied this method to screen forty-eight

strains of *Saccharomyces cerevisiae* for their thiamine production capacity in a thiamine-free minimal medium. We found a large variation in thiamine production, and among the highest-producing strains, there was a variant isolated after adaptive laboratory evolution in a thiamine-free medium. From this screening work, we selected a high-thiamine producer and a low-thiamine producer strain, in **Chapter 4** we evolved them for 370 generations in the thiamine-free minimal medium. After evolution, we isolated variants from each WT and screened their phenotype in terms of maximum growth rate, thiamine production, and biomass formation. We also sequenced the genome of all the strains to look for the mutations that derived from the ALE. Afterwards, we selected a variant for each WT and analysed their proteome in exponential growth and stationary phase. We concluded this work by studying the impact of a unique combination of different strains of the thiamine-producing *Bacillus subtilis* and six different legumes, on the quality of the natto produced (**Chapter 5**).

Below we discuss the potential and challenges of this research work and reflect on the prospects.

### 6.1.1 Microbial thiamine production: potential and challenges

#### 6.1.1.1 Can we push *S. cerevisiae* to increase thiamine production?

The production of thiamine is an expensive metabolic process in *Saccharomyces cerevisiae*. We demonstrated it by showing that Thi11 and Thi4, responsible for the biosynthesis of the two moieties of thiamine, account for a large amount of the proteome fraction when *S. cerevisiae* is growing in thiamine-free minimal medium. This results in Thi11 and Thi4 being among the 10 most abundant proteins overall (**Chapter 4**). This unusual metabolic bottleneck of thiamine production has been attributed to the fact that certain thiamine precursors, such as pyridoxal phosphate (PLP), and $NAD^+$, are important cofactors in numerous metabolic reactions (Coquille et al., 2012; Iyanagi, 2019; Sellés Vidal et al., 2018; Wightman et al., 2003). Therefore, having a single-turnover enzyme could prevent their unlimited conversion to thiamine, avoiding their complete depletion.

While other organisms, such as *B. subtilis*, possess riboswitches for the regulation of thiamine production (Bettendorff et al., 2009; Blount et al., 2006; Gong et al., 2018; Miranda-Ríos, 2007), thiamine production in *S. cerevisiae* is controlled at the transcriptional level. In minimal medium and low thiamine levels, this process is under the regulation of the protein complex consisting of Pdc2, Thi2, and Thi3, which controls the transcription of Thi-genes. The binding of Thi3 to Thi2 and Pdc2 is necessary for the initiation of transcription of thiamine pyrophosphate (TPP) biosynthetic genes

(Hohmann et al., 1998). At high intracellular TPP levels, Thi3 binds to TPP, and when this happens, the complex formed by Thi3 with Pdc2 and Thi2 is disrupted, blocking the transcription of thiamine biosynthetic genes. In rich medium in which thiamine is not limiting, both the sirtuins Hst1 and Sir2 as well as high $NAD^+$ levels can repress the expression of thiamine biosynthetic proteins (Li et al., 2010). The sirtuins, however, bind to a DNA region upstream compared to where the Thi2, Pdc2, and Thi3 complex binds. Therefore, they can only repress thiamine production at the basal level, and cannot stop transcription of thiamine biosynthetic genes induced by the Thi2, Pdc2, and Thi3. The presence of thiamine in the growth medium thus induces thiamine transport and represses de novo biosynthesis. Since thiamine itself is an indispensable co-factor for plant metabolism (Goyer, 2010; Rapala-Kozik, 2011), it is found in all plant-based substrates. For this reason, in situ production of thiamine by a fermenting organism in a plant substrate is challenging.

In **Chapter 5** we showed that despite the inoculation with a thiamine-producing *B. subtilis* strain, the amount of thiamine during fermentation did not increase significantly in any of the fermented substrates. This is possible because thiamine was detected in all the unfermented legumes, which would inhibit *B. subtilis* de novo biosynthesis of thiamine. In *S. cerevisiae* the presence of thiamine in a selected substrate would probably lead to the same outcome. Indeed, we detected the thiamine transporter Thi10 (alias of Thi7) during proteomics analysis in all the tested samples analysed in **Chapter 4**, despite using thiamine-free synthetic medium as growth substrate. This indicates that the thiamine transporter, although at a minimal level, is expressed constitutively to sense the possible presence of thiamine, which will be taken up when present, and inhibit de novo synthesis. A solution to such a problem could be to use a specific yeast strain that cannot transport thiamine. This would lead to de novo biosynthesis and therefore an increase in the overall content of thiamine in the fermentation substrate.

The use of a toxic thiamine analogue potentially provides a straightforward way of isolating a thiamine transporter mutant of *S. cerevisiae*. A toxic analogue is a molecule with the almost identical molecular structure to the target molecule, that is toxic when taken up because it interferes with metabolic functions related to the target molecule (Burgess et al., 2006; Sudarsan et al., 2005; Tylicki et al., 2017). In our case as a thiamine toxic analogue, we chose oxythiamine, which is taken up in *S. cerevisiae* by Thi10 and can be phosphorylated by Thi80 to form oxythiamine pyrophosphate (oxy-PP) (Tylicki et al., 2017). When this happens oxy-PP interacts with the thiamine-dependent enzymes, leading to toxic effects. Only yeast cells that cannot uptake oxythiamine due to the lack of thiamine transport, or those that cannot phosphorylate oxythiamine to form the

pyrophosphate form, or those who can degrade this molecule to form the monophosphate form (Goyer et al., 2013), will survive and be resistant. This technique selects for the naturally occurring genetic variations in a microbial population and therefore allows to select for specific phenotypes of interest. We tested this hypothesis using oxythiamine as the selected thiamine-toxic analogue and isolated various oxythiamine-resistant variants. We did so by taking a fully grown *S. cerevisiae* cell suspension and spreading about $10^7$ cells in a petri dish containing thiamine-free medium and an oxythiamine solution. The only cells that formed colonies were the oxythiamine resistant ones. The resistant variants isolated had almost completely lost the thiamine uptake (Figure 2B), but had unaltered thiamine production phenotype in the thiamine-free medium compared to the WT. This proves that it is relatively simple to select a specific variant with no thiamine uptake, but the same production phenotype compared to the WT. Such strains potentially enrich a plant-based substrate with thiamine via fermentation, even in the presence of plant-derived thiamine. The advantage of using this technique over direct genetic modification for strain selection is that such strain is not considered GMO, and therefore can be directly used for food production without having to go through authorization by the European Food Safety Authority.

We also tested whether it is possible to stimulate production of thiamine in *S. cerevisiae*. We did this by supplementing the growth medium by adding certain thiamine precursors, hoping that this would lead to an increase in thiamine yield. The supplements we chose were glycine, histidine, and iron. Glycine is used as a substrate by Thi4 to produce the thiazole subunit of T (Chatterjee et al., 2011). Histidine is donated from the active site of Thi5 to form the aminopyrimidine moiety (Coquille et al., 2012; Wightman & Meacock, 2003). Iron is indispensable for the reactions catalysed by Thi4, as the sulphide transfer reaction is iron-dependent (Chatterjee et al., 2011). We chose *S. cerevisiae* 2888, a high producer selected from **Chapter 3**, and supplemented the growth medium with different concentrations and combinations of these precursors (Table 1). We found no significant change in thiamine productivity after 44 h of growth with any of the media supplementation (Figure 2 C, D), indicating that none of the tested compounds are limiting thiamine productivity in this chemically defined medium, nor could stimulate thiamine production. This approach was evaluated only on one single high-producing strain, the results of media supplementation may vary if other strains are used.

Table 1- List of supplements to the thiamine-free CDM (C) illustrated in Figure 2. H stands for histidine, and G for glycine

| Sample | Supplementation composition |
|---|---|
| C | thiamine-free CDM |
| F1 | 0.1mM $Fe^{2+}$ |
| F2 | 0.3mM $Fe^{2+}$ |
| FG2 | 0.3mM $Fe^{2+}$ 20mM G |
| FGH1 | 0.1mM $Fe^{2+}$ 20mM G + 1mM H |
| FGH2 | 0.3mM $Fe^{2+}$ 20mM G + 1mM H |
| G | 20mM G |
| H | 1mM H |

#### 6.1.1.2 To phosphorylate or not to phosphorylate?

Thiamine itself can be present in different phosphorylated forms in food substrates, but only the non-phosphorylated thiamine delivers meaty odours. This poses a challenge since in yeast as well as in bacterial biomass, TPP is the most prevalent vitamer found (**Chapter 3**,Figure 2), and TP and T concentrations are generally lower (**Chapter 2**,**3**,**4**). While heat treatment of yeast biomass or thiamine solutions leads to the partial conversion of TPP to TP, further conversion of TP to T does not occur in these conditions (**Chapter 2**). Therefore, it is important to take this into account for the potential applications. Different strategies could overcome this issue, starting with an appropriate choice of microorganism for thiamine production.

*S. cerevisiae* does not possess a one-step conversion of TP to the active form TPP (Kowalska et al., 2012), which is most common in bacteria such as *B. subtilis* (Begley et al., 1999). For this reason, in *S. cerevisiae,* TP is first converted to T, to be then converted to TPP (Nishimura et al., 1991). This is an advantage from an application perspective since *S. cerevisiae* already possesses the enzyme to carry out this desirable reaction for potential applications. On the contrary, in *B. subtilis* TP can be directly phosphorylated to TPP, via *thiL* (EC. 2.7.4.16). Although this route is preferred in *B. subtilis*, in a study by Schyns et al. (2005) the deletion of *thiL* did not completely stop the growth of *B. subtilis* in the thiamine-free medium as expected, but rather generated a slow-growing strain. This revealed the presence of an alternative route to produce TPP, like the yeast pathway, going from TP to T, to TPP. This last biosynthetic step is conducted by *thiN*. We conducted a screening of 6 *B. subtilis* strains and measured the intracellular thiamine production capacity, but we did not detect any other vitamer than TPP in the bacterial biomass (Figure 2 A). In the biomass of *S. cerevisiae* we found all the different vitamers, although their proportion is strain-dependent (**Chapter 3**). Certain strains showed a

high proportion of T over TP and TPP (**Chapter 3**). It could be possible to isolate a *S. cerevisiae* strain that produce elevated levels of unphosphorylated thiamine, which is promising for food applications.

Pho3 is a thiamine-phosphates phosphatase that hydrolyses TPP and TP in the periplasmic space (Nosaka, 1990; Nosaka et al., 2005), the free thiamine is then transported by Thi10 inside the cytosol. Studies showed that Pho3 is constitutively expressed, but the level of expression is dependent on the type of N-source provided (Savinov et al., 2012), rich N-sources like glutamine and ammonium sulphate (the same N-source used in **Chapter2**,**3,4**) favoured the expression of Pho3, while glutamate and urea decreased it. Proteomics analysis (**Chapter 4**) revealed the presence of Pho3 in all the tested samples, and an increase in the Pho3 proteomic fraction in stationary phase by the variants, compared to the relative WTs. Because of its phosphatase activity, and therefore its potential of aiding the conversion of T to meaty aromas, we tested growing *S. cerevisiae* in media with glutamine or ammonium sulphate as N-source. The aim was to see if this could lead to an altered proportion of the thiamine vitamers, increasing the unphosphorylated thiamine intracellularly or extracellularly, or perhaps increase thiamine production. We tested the effect of different N-sources (ammonium sulphate, urea, glutamine) on the growth, metabolite production, and thiamine production in *S. cerevisiae* 2888 (Figure 3) which produces elevated amounts of thiamine (**Chapter 3**). The highest intracellular thiamine specific productivity was obtained with ammonium sulphate as the main N-source, despite the pH of the culture being lowest in these conditions (Figure 3). We detected differences in the intracellular vitamer composition, for instance at time 16 h and 22 h with urea. This can, however, mainly be attributed to the slow growth of *S. cerevisiae*, as in exponential phase TP and T are more abundant compared to TPP (Kowalska et al., 2012). In stationary phase we could not find any difference in the vitamer composition of extracellular or intracellular thiamine as reported by the study of Savinov et al. (2012).



Since we did not find difference in vitamer distribution using N-sources, ammonium sulfate remains an optimal N-source to be used with selected strains (such as CBS 3012 **Chapter 3**,**4**, and 2888 **Chapter 3**) in minimal synthetic medium, as it is a cheap substrate that leads to elevated levels of thiamine compared to urea, and glutamine. Despite this, the elevated protein costs of thiamine production (**Chapter 4**) could limit thiamine productivity in minimal medium, even in the highest producing strains, compared to media that are rich in amino-acids and proteins. It is possible that a plant-based substrate, with elevated levels of proteins, such as legumes, would provide enough amino acids/protein to overcome the limitations dictated by the high protein

costs that occur in a chemical medium in which thiamine needs to be synthesized de novo. Therefore, isolating from a high thiamine-producing strain, such as CBS 3012 (**Chapter 3**,**4**), a variant lacking thiamine transport using a thiamine toxic analogue for instance, and then inoculating such strain in a protein rich substrate, could potentially lead to high thiamine levels produced in situ. This possibility needs to be further explored, possibly by conducting experiments with selected overproducing strains and screening different fermentation substrates such as legumes.

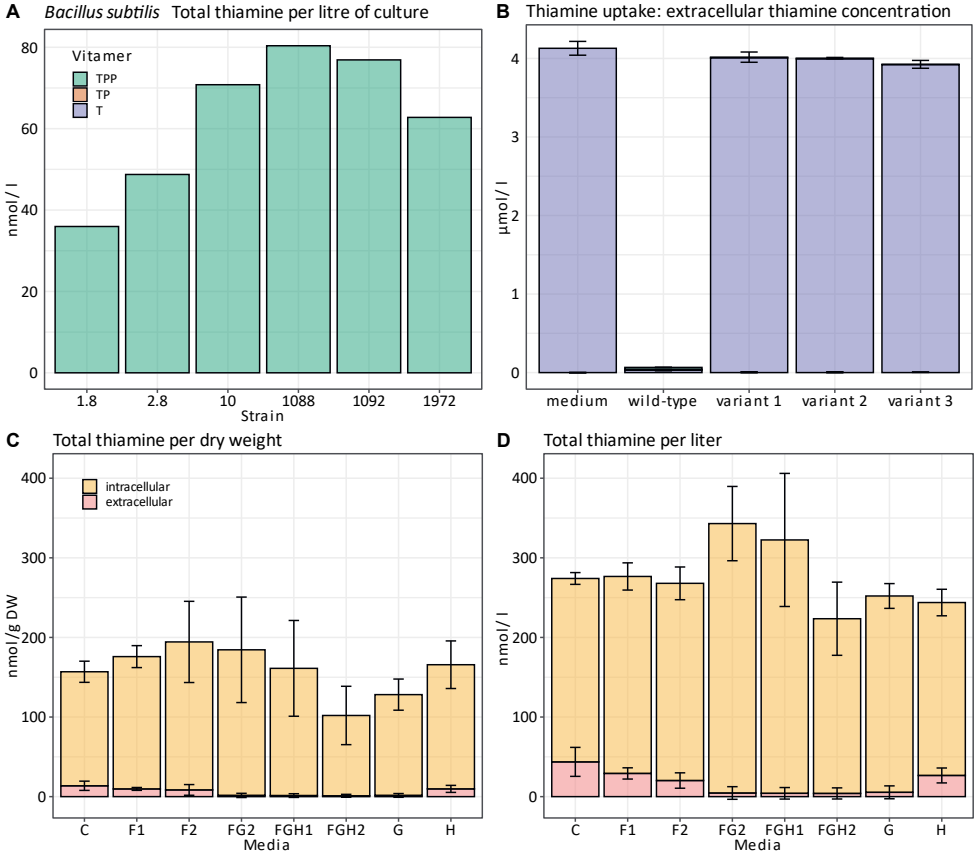


Figure 2- Intracellular thiamine yield (A) in 6 *Bacillus subtilis* strains (indicated by the facets) in C-minimal medium without thiamine after 36h of growth at 37°C and 250 rpm and with vitamin solution. Extracellular thiamine concentration (B) in the growth medium supplemented with thiamine (medium), inoculated with wild-type *S. cerevisiae* 2888 (wild-type) and oxythiamine-resistant variants (variant 1,2,3) and after 44h of growth at 30°C and 180 rpm. (C) Total thiamine specific productivity (C) and yield (D) in *S. cerevisiae* 2888 after 44h of growth in the thiamine-free medium at 30°C and 180 rpm supplemented with different combinations of iron, manganese, histidine, glycine (Table1). Error bars represent the standard deviation of three independent experiments.The X-axis indicates the strain name. Total extracellular thiamine is depicted in pink, while total intracellular thiamine is in yellow.

### 6.1.2 How much thiamine do we need?

Thiamine is present in all plant-based substrates. In **Chapter 5** we quantified the amount of thiamine in the boiled legumes before inoculation. Once cooked lupin had the highest thiamine content, 2.7 mg/ kg, and lupin had the lowest amount 1.3 mg/ kg. These quantities alone are not sufficient for delivering a meaty odour in a plant substrate. Pork meat is reported to have a concentration of thiamine of about 5.5-8.4 mg/kg on average (Dawson et al., 1988; Driskell et al., 1998; Leonhardt et al., 1997). Beef and veal have lower thiamine concentrations, compared to pork, with around 0.4 to 1.1 mg/kg (Leonhardt & Wenk, 1997). Chicken on the other hand is reported to have a large variation in thiamine concentration, ranging from 0.9 to 2.3 mg/kg (Graham et al., 1998; Leonhardt & Wenk, 1997). Studies on cooked ham found that the addition of about 100 mg/kg (Thomas et al., 2014, 2015) is sufficient for desired flavour formation of molecules such as 2-methyl-3-furanthiol, which is the main thiamine degradation product that has a meaty, roasted odour (Aliani et al., 2005; Alim et al., 2019; Cerny, 2007). A study by Thomas et al. (2014) found that its formation is directly proportional to the addition of thiamine, although the degradation of ribose can also yield this volatile (Aliani & Farmer, 2005). In this thesis, we found selected strains that have high thiamine productivity (**Chapter 3**,**4**), of around 40 mg/kg of biomass, which is much higher than the amount found in pork, chicken, or beef. Yet, if such strain would be used to inoculate a plant-based substrate, or yeast extract derived from it would be used to enhance the flavour of a meat-replacer, this would not deliver enough thiamine to be converted into a meaty odour. Perhaps then, delivering sufficient thiamine for flavour development via in situ fermentation leading to vitamin $B_1$ fortification might not be a viable option in the near future when using non-GMO *Saccharomyces cerevisiae* or *Bacillus subtilis*.

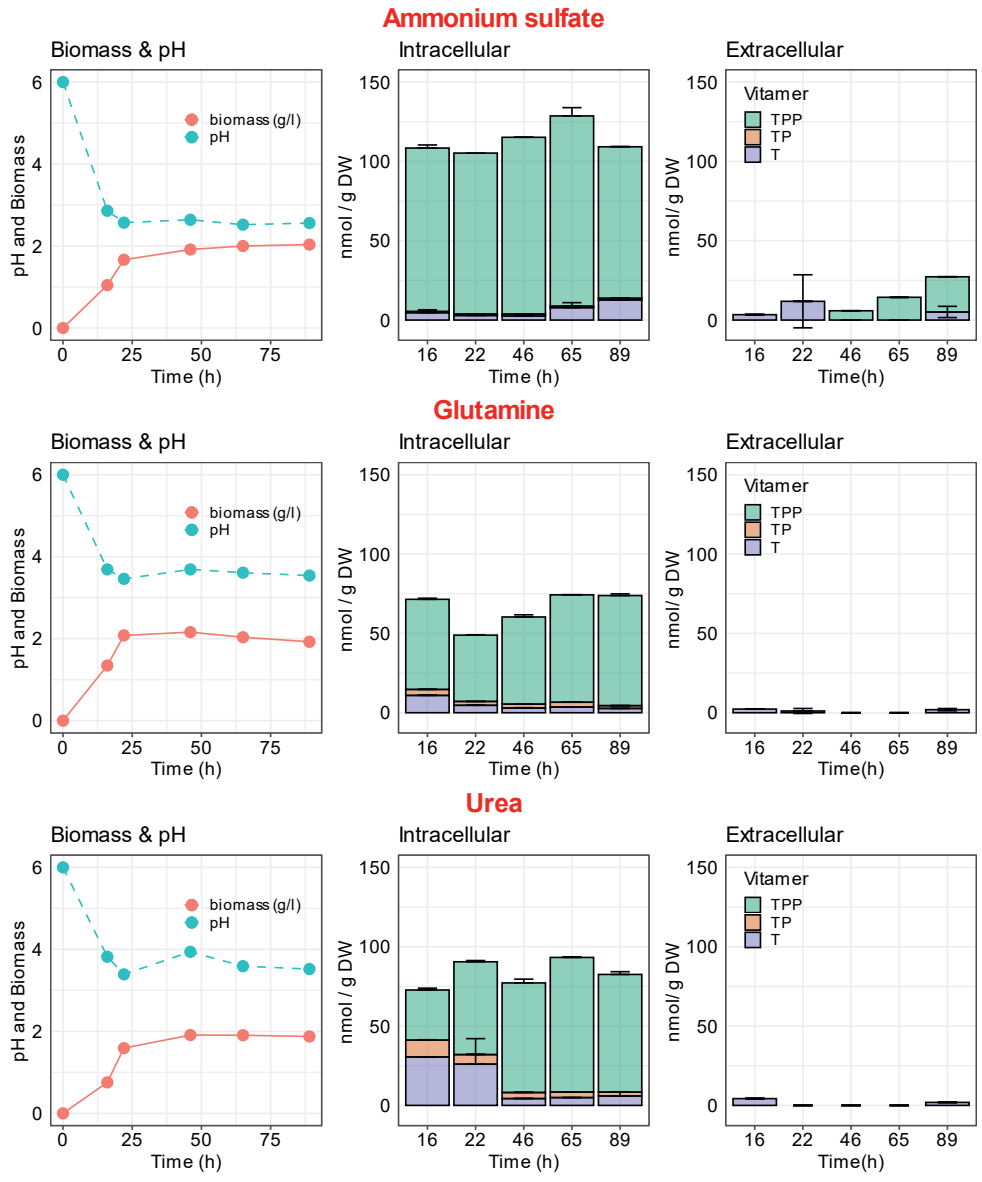


Figure 3-pH, biomass, intracellular and extracellular thiamine in 6 cultures of *Saccharomyces cerevisiae* 2888 (Chapter 3) over time. Cells were grown in thiamine-free CDM with the corresponding single N-source. All media had equimolar amount of nitrogen. Potassium sulfate was used to provide equimolar amount of sulphur compared to the reference ammonium sulfate. The pH of the media was adjusted to 6 to minimize the initial impact of a pH difference among the cultures. This study originated from a collaboration with Dr. Pranas Grigaitis from VU Amsterdam to study the effect of n-source on thiamine productivity and thiamine vitamer distribution.

### 6.1.3 What is left to discover?

Despite the findings described in this manuscript, there are still many unknowns to unveil regarding *S. cerevisiae* thiamine metabolism. An example of this is the role of Thi4 in conferring tolerance towards mitochondrial DNA damage (Machado et al., 1997), and towards oxidative stress (Kowalska et al., 2012). Not only Thi4, but thiamine itself was reported to confer resistance to different types of stress due to mechanisms independent of its co-factor activity (Wolak et al., 2014). Thiamine can be efficiently synthesised by *S. cerevisiae* in anoxic conditions, although the pathway that leads to the formation of HMP-P in an anaerobic environment is still unknown (Tanaka et al., 2000). Indeed the reaction catalysed by Thi5 is oxygen dependent, although oxygen is not directly incorporated in the reaction (Wightman & Meacock, 2003). In addition, TTP has been detected in yeast biomass (Makarchikov et al., 2003), while its biological role in certain bacteria has been elucidated already (Gigliobianco et al., 2010; Lakaye et al., 2004), its metabolic role in yeasts still remains to be discovered. Our research pointed out that in the thiamine overproducing strain CBS 3012, screened in **Chapter 3** and of which the proteome was analysed in **Chapter 4**, the total thiamine intracellular concentration increases in the stationary phase compared to in the exponential growth. This was not the case for all the other strains analysed, in which intracellular thiamine concentration decreased after the stationary phase, resulting in lower thiamine production. Unravelling the mechanism behind this increase in stationary phase, and on the unknown metabolic functions of thiamine and metabolism could be key to obtaining more thiamine-overproducing strains, which could find many potential applications to enrich plant-bases substrates.

Perhaps we could take a closer look to the current trends in gastronomy, fine dining, but also to cooks that experiment with fermentation, to gather inspiration to research novel fermented products. On my shelf of cookbooks I have two volumes that I am particularly fond of: the Noma guide to fermentation (Redzepi et al., 2018), and the book Of Cabbages and Kimchi (Read, 2023). Both books describe detailed recipes of novel types of soy-sauce, miso, and other fermented food products, which are innovated by switching the type of substrates used. I wanted to try with my own hands making and tasting different misos. I made three different types using green peas, red lentils, and a combination of the two. After waiting patiently for 6 months I was truly blown away by the floral smell of the miso made with peas, and the intense scent of caramel of my red lentil miso. But the biggest surprise was the miso made by combining these two substrates in a 1:1 ratio, which had an intense meaty, nutty, and super umami smell… *surely this miso does remind me of meat! An example of the whole being more than the sum of its parts.*

### 6.1.4 Outlook

The role of thiamine as a meat aroma precursor is well understood. Looking at the scientific literature available regarding the levels of thiamine in meat, it is obvious that these are quite comparable to those in plant-based substrates (**Chapter 5**), as they are in the same order of magnitude of a few mg/kg. Studies like the one carried out by Thomas et al. (2015) show that the amount of thiamine added to deliver a meaty odour (to ham…which is already meat!) is very high compared to the levels that fermentation using *S. cerevisiae* or *B. subtilis* could deliver. Even the high thiamine content of yeast biomass (**Chapter 3,4**) would still not match the required amount to have the desired impact on the aroma formation in plant-based substrates. Although this is discouraging, it is important to remember that thiamine itself is *only one* of the *many* compounds found in meat that deliver a meaty flavour. As already specified in **Chapter 1**, many factors add to the formation of flavour development in meat upon cooking, such as the degradation of lipids, amino acids, sugars, thiamine, heme and the maillard reaction (Ardö, 2006; Jayasena et al., 2013; Mottram, 1994). Focusing on delivering only *one* specific compound, compared to a wide array of metabolites, can be a reductive strategy that narrows down the horizon of delivering complex umami, meat-like flavours. Especially when there are big biological challenges to overcome to deliver enough quantities of that specific compound. **Chapter 5** showed that the fermentation of plant-based materials with *B. subtilis* can lead to the development of complex aroma profiles, characterised by the presence of numerous volatile compounds, like pyrazines, that have rich umami, roasted, and nutty flavour (Alim et al., 2019; Dajanta et al., 2011; Devaere et al., 2022; Fayek et al., 2021; Rizzi, 1987). By using six different substrates and strains to produce natto we obtained products that differ in their flavour profile, by having different proportions of pyrazines, ketones, and aldehydes. This shows that a vast "library of flavours" can be created by combining different strains with different substrates, to innovate traditional food products. Natto is only one of the many fermented products that have a complex volatile composition. Other examples are miso, koji, garum, soy sauce, fermented tofu (sufu), and doenjang. There is truly a vast horizon of possibilities in terms of flavour development, and the creation of umami and meaty aromas through fermentation in plant-based substrates.

As scientific progress increases, we will perhaps become more capable of grasping the complexity of fermentation processes and how to use them to our advantage. The journey towards the development of novel food products started a long time ago. While this craft took thousands of years to develop, the science to comprehend and harness the power of microbes in food production is still, beautifully, at its dawn.

## 6.2 References


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# APPENDIX



Rebecca Rocchi

## Summary

The thermal degradation of thiamine (vitamin $B_1$) generates a wide variety of volatile compounds with potent meaty and roasted odours. These compounds are essential in contributing to the aroma profile during the cooking of meat, giving it its characteristic flavour. Thiamine has already been used to produce flavourings for meat replacers due to its well-known importance as an aroma precursor. Currently, it is chemically synthesised, following an increase in demand for clean-label and fermented products, it is of interest to produce thiamine with food-grade organisms. The scope of this thesis was to explore the use of microbes to produce thiamine via fermentation, and to gain insights into various aspects of its production, metabolism in *Saccharomyces cerevisiae*, and possible applications.

**Chapter 1** of this thesis presents the societal relevance of microbial thiamine production, and describes the currently available scientific knowledge on this topic, while identifying the knowledge gaps, leading to this thesis's aim.

This research started by developing a new thiamine extraction and quantification method (**Chapter 2**). The extraction method relies on the use of beads for mechanical cell disruption, which, compared to the widely used hot-acid hydrolysis, has the advantage of preventing the conversion of thiamine pyrophosphate to thiamine phosphate. Afterwards, the extracts are derivatised using potassium hexacyanoferrate, to convert all the thiamine vitamers to their respective thiochrome derivatives, which emit a strong fluorescence. The derivatization targets only thiamine and thiamine vitamers, preventing the development of background fluorescence of other compounds that are present in the extract, which would lower the sensitivity and specificity of detection. The high sensitivity of the method, the specificity for thiamine and vitamers, and the selectivity of the fluorescence signal, allows for a broader spectrum of applications than the ones demonstrated here. This method is excellent at specifically quantifying all the thiamine vitamers, not only in fresh yeast biomass, but in extracts from different origins, with high impurity, and low thiamine concentrations, which are normally challenging to analyse.

We used this method to screen the differences in thiamine production in forty-eight strains of *S. cerevisiae* in a chemically defined thiamine-free minimal medium (**Chapter 3**). This is the first time that such a large screening of *S. cerevisiae* strains was carried out using a vitamer-specific detection method. We found an 8-fold difference in thiamine production capacity between the highest and lowest-producing strains. The results of this screening highlighted the critical role of choosing the right strain for

thiamine production. In addition to a variety of wild-type strains (WTs), the screening incorporated three variants isolated after adaptive laboratory evolution (ALE) in a thiamine-free medium. All the evolved isolates had increased maximum growth rate, and up to 60 % increased thiamine yield (intended as nmol of thiamine per litre of culture) compared to the corresponding WT. The large strain-to-strain variation in thiamine production, and the effectiveness of ALE in increasing thiamine yields, made us question the trade-offs between increased fitness and thiamine production.

Therefore, to shed light on this matter, we evolved a high and a low thiamine producing strain from the screening pool, in a thiamine-free chemically defined minimal medium (**Chapter 4**). After 370 generations, we isolated evolved variants for both the WTs. The low thiamine-producing strain evolved to have a higher maximum growth rate in a thiamine-free medium, and to significantly increase thiamine and biomass production. The high thiamine-producing strain halved its thiamine production after evolution, although the maximum growth rate didn't change significantly, and biomass increased by 10 %. We did not find any genetic mutation directly involving thiamine-dependent proteins or thiamine-biosynthetic ones. Proteomic analysis revealed that for the two WTs and two selected evolved variants, despite the differences in thiamine production, there was no significant difference in proteome allocation to thiamine biosynthesis. Thi4 and Thi11, which are the single-turnover enzymes producing the two thiamine moieties, were both among the top 10 most abundant proteins in the proteome. These findings highlight the extremely high metabolic costs of thiamine de novo biosynthesis and suggest the possible use of protein-rich substrates to produce thiamine to overcome these protein costs.

We concluded this work by using another thiamine-producing organism, *Bacillus subtilis*. We developed novel natto varieties based on unique combinations of six different legumes, and three different strains (**Chapter 5**). Natto is a food with a pungent smell, and umami flavour. It is of particular interest since it is enriched in vitamin $K_2$ via fermentation and rich in proteins. In total, we produced different types of natto using distinct combinations of strains and substrates and analysed the effect of these combinations on the attributes of the final product. We found large differences in aroma composition, amount of free amino acids present, and vitamin $K_2$ levels. Although we found no increase in thiamine after fermentation, there was a remarkably elevated level of vitamin $K_2$ produced by *B. subtilis*. One of the nattos made with red lentils as a substrate, reached over 2 mg/100 g of product, which is about three times higher than the commercially available natto based on fermented soybeans. We also found many volatiles that are associated with roasted, meaty, umami odours. This work

demonstrated that vitamin enrichment of a food product is possible, and that different strain and substrate combinations can lead to different characteristics of the final product. This offers opportunities for future applications.

In **Chapter 6** we presented a general discussion of the results, including the challenges and opportunities that emerge from this research work. We paid special attention to the prospects of using fermentation for the development of meaty flavours in plant-based food products.

## Layman summary for general audiences (English)

Everyone loves a good barbecue or a good roast, perhaps the smell of cooking meat is the really exciting part of it! During the process of cooking meat, many chemical reactions occur, which change the characteristics of the product. Thiamine, which you may know as vitamin $B_1$ is a potent precursor of many meaty aromas. This vitamin, when heated, degrades, and in doing so it forms different chemicals that smell delicious, meaty, roasted, and rich. This beautiful chemical compound, which is naturally produced by plants, some fungi, such as yeasts, and some bacteria, is therefore precious not only for keeping us healthy but also for giving a delicious smell to food. Because of its ability to deliver aromas, it is often used in the production of flavours, which are used as ingredients in plant-based meat replacers, and it is chemically synthesised. Nowadays consumers do not really love the idea of having many "chemicals" added to their food, and at the same time, started gaining interest in fermented foods as well. That's why, I researched the possibility of naturally producing thiamine (in the sense that I did not do any genetic modifications to the organisms that I used, at least according to the GMO definition), using a yeast, and a bacterium.

In this thesis, you will find a short review on the background of this study (**Chapter 1**). My research work started with the development of a method to measure thiamine produced by yeasts (**Chapter 2**), how can I work to produce this compound if I can't measure how much I make of it? My yeast of choice for the producing yeast was *Saccharomyces cerevisiae*, the same yeast you buy at the supermarket to make some bread at home. This method is relatively easy to implement and can accurately measure small amounts of this vitamin. First of all, I compared different extraction methods of thiamine, such as boiling the yeasts (I know… poor guys… boiled alive….) or crushing them using very tiny beads (I tried some that were very tiny like very fine sand, others bigger), or a combination of both. According to the result, the best way to extract the vitamin was to crush the yeasts with the finest beads. Afterwards, I measured the amount of vitamin in my extract, by converting thiamine to a fluorescence compound, via adding some weird-sounding chemicals, and using a special, very cool, machine called HPLC to quantify this, even when present in very small amounts. Once I could measure thiamine, I applied this newly developed method to screen as many samples of *Saccharomyces cerevisiae* as I could (48) to see which one of these guys could make the most thiamine (**Chapter 3**). There were large differences in the amount of thiamine that was produced by these yeasts, some had 8 times more than others! Its impressive! Afterwards I wanted to understand why there were such big differences in thiamine production among the strains. To do so I took one of the highest thiamine-producing

yeast, and a low producing one and grew them separately in a liquid yeast food that did not contain any thiamine. Every time the yeast was growing, I took a little part of it (**Chapter 4**) and propagated it again, I did this as many as 300 times. This means that my yeast over time got used to being in this thiamine-deprived environment, and since it cannot grow without it, it had to optimise its production in order to grow well. At this point I took the yeasts that evolved and looked at their DNA to see how they were different before and after this adaptation. For both strains I did not find any difference in the DNA parts that were encoding thiamine producing enzymes. But certainly, the low-thiamine producer increased thiamine productivity and grew faster than before the adaptation, while the high producing halves its thiamine production, but did not seem to grow faster. Afterwards, I tried a new approach of research (**Chapter 5**). I made some natto, this is a fermented food product that is sticky, pungent, and slimy, made from fermenting soybeans with a bacterium called *Bacillus subtilis*). This bacterium can make thiamine and can also produce other vitamins like vitamin $K_2$. I used different types of this bacterium and six different legumes. We found out that thiamine did not increase after fermentation, despite the bacterium can produce it, but we found that instead, a lot of vitamin $K_2$ is produced. Moreover, the combinations of different legumes and bacteria lead to nattos with different characteristics, showing that the choice of these different component can change the product dramatically. I concluded this thesis (**Chapter 6**) by discussing important aspects to consider to produce thiamine using microbes, gave an overview of what are still the things to investigate regarding this topic, explained the various limitations I encountered during my research, and concluded by discussing the prospects regarding microbial thiamine production.

## Riassunto in Italiano per il pubblico generale (Italiano)

Tutti amano una buona grigliata o un buon arrosto, forse il profumo della carne cucinata è la parte davvero speciale che ci fa amare queste ricette. Durante il processo di cottura della carne avvengono numerose reazioni chimiche che modificano le caratteristiche del prodotto stesso. La tiamina, che forse conoscete come vitamina $B_1$, è un potente precursore di molti aromi tipici della carne cotta. Questa vitamina, quando riscaldata, si degrada e così facendo forma diverse sostanze chimiche che hanno un profumo delizioso, carnoso, e ricco. Questo composto chimico, che viene prodotto naturalmente dalle piante, da alcuni funghi, come i lieviti, e da alcuni batteri, è quindi prezioso non solo per mantenerci in salute ma anche per conferire un delizioso profumo al cibo. A causa della sua capacità di generare odori, questo composto viene spesso utilizzato nella produzione di aromi, che vengono utilizzati come ingredienti nei sostituti della carne a base vegetale, ed è sintetizzato chimicamente. Al giorno d'oggi i consumatori non amano molto l'idea di aggiungere molte "sostanze chimiche" al loro cibo e, allo stesso tempo, hanno iniziato ad interessarsi anche ai cibi fermentati. Ecco perché, ho studiato la possibilità di produrre naturalmente tiamina (nel senso che non ho apportato alcuna modificazione genetica agli organismi che ho utilizzato, almeno secondo la definizione OGM), utilizzando un lievito, e un batterio.

Innanzitutto, in questa tesi, troverete una breve rassegna sul contesto di questo studio (**Chapter 1**). Il mio lavoro di ricerca è iniziato con lo sviluppo di un metodo per misurare effettivamente la tiamina prodotta dai lieviti (**Chapter 2**), come posso lavorare per la produzione di questo composto se non posso misurare quanta ne produco? Il mio lievito preferito per la produzione del lievito era il *Saccharomyces cerevisiae*, lo stesso lievito che compri al supermercato per fare il pane in casa. Innanzitutto ho confrontato diversi metodi di estrazione della tiamina, come bollire i lieviti (lo so... poveri ragazzi... bolliti vivi...), oppure schiacciarli utilizzando delle perline piccolissime (ne ho provate alcune molto piccole come sabbia, altri più grandi), o una combinazione di entrambi. Secondo il risultato, il modo migliore per estrarre la vitamina era frantumare i lieviti con le perle più fini. Successivamente, ho misurato la quantità di vitamina nel mio estratto, convertendo la tiamina in un composto fluorescente, aggiungendo alcune sostanze chimiche dal nome strano e utilizzando una macchina speciale, molto interessante, chiamata HPLC per quantificarla, anche se presente in quantità molto piccole. Questo metodo è relativamente facile da implementare e può misurare con precisione piccole quantità di questa vitamina. Una volta che ho potuto misurare la tiamina, ho applicato questo metodo di nuova concezione per esaminare quanti più campioni possibile di *Saccharomyces cerevisiae* (48) per vedere quale di questi lieviti poteva produrre più

tiamina (**Chapter 3**). C'erano grandi differenze nella quantità di tiamina prodotta da questi lieviti, alcuni ne avevano 8 volte di più di altri! È impressionante! Successivamente volevo capire perché c'erano differenze così grandi nella produzione di tiamina tra i ceppi di lievito. Per fare ciò ho preso uno dei lieviti con la più alta produzione di tiamina e uno a bassa produzione e li ho coltivati separatamente in un alimento liquido per lievito che non conteneva tiamina. Ogni volta che il lievito cresceva, ne prendevo una piccola parte (**Chapter 4**) e l'ho propagato di nuovo, l'ho fatto fino a 300 volte. Ciò significa che il mio lievito col tempo si è abituato a stare in questo ambiente privo di tiamina e, poiché non può crescere senza di essa, ha dovuto ottimizzare la sua produzione per crescere bene. A questo punto ho preso i lieviti che si sono evoluti e ho osservato il loro DNA per vedere come fossero diversi prima e dopo questo adattamento. Per entrambi i ceppi non ho trovato alcuna differenza nelle parti del DNA che codificavano per gli enzimi che producono tiamina. Ma certamente i produttori a basso contenuto di tiamina hanno aumentato la produttività di tiamina e sono cresciuti più velocemente rispetto a prima dell'adattamento, mentre quelli ad alta produzione hanno dimezzato la produzione di tiamina, ma non sembravano crescere più velocemente. Successivamente, ho provato un nuovo approccio di ricerca (**Chapter 5**). Ho preparato del natto (un prodotto alimentare fermentato appiccicoso, pungente e viscido), ottenuto dalla fermentazione dei semi di soia con un batterio chiamato *Bacillus subtilis*. Questo batterio può produrre tiamina e può anche produrre altre vitamine come la vitamina $K_2$. Ho usato diversi tipi di questo batterio e sei legumi diversi. Abbiamo scoperto che la tiamina non aumenta dopo la fermentazione, nonostante il batterio riesca a produrla, e che durante la fermentazione viene prodotta moltissima vitamina $K_2$. Inoltre, le combinazioni di diversi legumi e batteri portano a natto con caratteristiche diverse, dimostrando che la scelta di questi diversi componenti può cambiare radicalmente il prodotto. Ho concluso questa tesi (**Chapter 6**) discutendo aspetti importanti da tenere in considerazione per la produzione di tiamina utilizzando i microbi, ho dato una panoramica di quali sono ancora le cose da indagare su questo argomento, ho spiegato le varie limitazioni che ho incontrato durante la mia ricerca, e discutendo le prospettive future riguardanti la produzione microbica di tiamina.

## Acknowledgements

The first sure thing that I want to acknowledge is that, although on the cover of this book, there is written only my name, this work is the result of the collective explicit and implicit effort of many people. This process taught me a lot of valuable lessons that I will cherish and need throughout my life. Therefore, I need to proceed with some well-deserved acknowledgements.

**Eddy**, thank you for all the support you gave me during my PhD, I always left our bi-weekly meetings feeling empowered by your words, excited, and hopeful. While doing a PhD I often had the feeling of being lost, overwhelmed, or doubtful, but your supervision turned these feelings around and gave me the motivation and confidence to believe in myself and finish this work. Thank you! **Tjakko**, thank you so much for your support, working with you was very inspiring. Your creativity is always very impressive, and it gave me very valuable points of view. I am really happy I had the chance to have you on board for this project!

**Judith**, my dear, all the hard work we did together in the lab, extracting tens of thousands of samples, derivatizing, and setting up the HPLC, which was not very cooperative. How much work you've put into helping me, and listening to me complaining about virtually everything, or having tantrums in front of the HPLC. Thank you also for your scientific guidance, and moral support. Thank you, a million times! **Kees**, how can I even start to thank you? Your knowledgeable help has been priceless (despite for the usual 25 euro bills). Thank you for always being helpful and always making a lot of effort to make my life in the lab easier. Thank you for cheering up my days with your loudness and humour. Thank you for always picking up the phone, and for always offering a hand to help me, even when you were on holiday! Thank you for being so kind. Collaborating with you gave me the possibility of doing my research without having to stress about the vitamin $B_1$ analysis, or at least not as much as I would if someone else was at your place. It has been a real pleasure! **Marcel T,** I had a lot of fun working together. You are very caring and supportive and I appreciate all the help you gave me during the past few years, thank you so much. **Dennis**, thank you for always finding time to help me, I appreciate it a lot. I had a lot of fun trying to figure out the MinIon set-up and wrap our heads around the data. It was really great to work together. **Ingrid**, despite your efforts to look strict, I know that deep down you are a sweetheart. Thanks for helping me out with my order emergencies, for the precious advice, for cutting me some slack when I needed it, and for always keeping an eye on me, and everyone. I appreciate that you are so attentive, and you always notice when

someone is having a rough time and go there to help them! **Anne**, you always have some joke ready up your sleeve, thank you for the company in the lab!

Dear **Joost** van den Heuvel, thank you a lot for jumping in on this project and helping me out just out of kindness, and doing it in a very timely manner. Thank you **Sijmen** Schoustra and **Ben** Auxier for helping me out and connecting me to Joost! It was truly impressive to see how you super quickly mobilised your network to come in help of a poor, ignorant, PhD that had very tight deadlines! Your willingness to help a colleague, and doing it so quickly, is unique.

To all the **TKI project partners**, it was fun to work all together on this big project, thank you for all the ideas shared, feedback and company! A special thanks to **Jacqueline** I feel so lucky that we worked together for the $B_{12}$Insight project, you have been so kind and patient with my last-minute changes, and my forgetfulness, and always ran to help me fix the little messes I made here and there. Besides, I also enjoyed our coffee together and chats. Thank you so much! **Chris Winkel**, your curiosity, and brightness inspired me a lot. Thank you for creating a nice and informal atmosphere, I enjoyed our collaborations a lot. Dear **Walter**, thank you for all the advice and guidance you gave me, it was truly helpful. **Gerben, Renata Dianne** and **WFBR**, thank you so much for all your support, I felt like our bond over the annoying HPLC brought us together. Thank you for your kindness, patience, and valuable help.

My beloved paranymphs **Claire** and **Anouk**. You two are the smartest, hardest-working, reliable, determined, strong and beautiful women I know. Your company has been a true inspiration and a strong support during my PhD route. Anouky, I can't believe so long has passed since the first time we went for our roller skates ride and soon after our daily appointment with the milky-shrimp lunch. I don't even know how I could have done all of this without your support and the motivation you gave me. You are a daily inspiration, I feel so lucky to have found a lifetime friend. Claire, I owe you an apology, for making fun of your lunches, during the last months of my PhD it was clear that there was a good reason for you to eat *that stuff* for lunch. You gave me so much help, not only content-wise, and always guided me with your experience. I feel very lucky to have you by my side, as you are always there for me when I need your help. I am glad we are friends, I feel lucky to have you in my life!

A special thanks to all the students I supervised, sometimes I have the feeling that I learnt as much from my students as they learnt from me! **Ferran**, I was so lucky to be your supervisor, your positive attitude, determination, precision, reliability, and passion were truly admirable. I enjoyed your ability to always remain cheerful and calm no

matter what! **Zhuotong** you are truly a kind, talented, sweet, smart, passionate, and hard-working person. During your thesis we supported each other, when I was feeling down you always cheered me up, and vice-versa, it was precious! **Maria**, what a kind, genuine, and warm person you are, you work hard and always manage to overcome the obstacles you are faced with. Besides, you can make, and cook, an amazing tempeh! Thanks for the little card you made for me, and for always coming to visit me. **Yuhan**, although our relationship has not always been easy, your stubbornness is something I look up to. You faced many challenges during your thesis and always worked incredibly hard to get on top of things. You are creative and fun. **Lukas**, you are one of the most calm and patient people I know, I need to learn that! Besides, you work hard! I am proud of the work you have done. **Merit** you are such a kind soul, you worked very hard and always showed a lot of interest in the research topic, I admire your gentleness and wish you the best of luck in your career! **Shantanu**, thank you for the nice chats, the coffees, and the lunches together! And for the delicious Indian food! You are fun, playful, and very honest. Best of luck with what's next! **Leonardo**, we clicked immediately! Maybe because we are both Italians? It was great to work together on the project, you always managed to push through the difficult research times with a smile and a good laugh! **Aditya**, I am glad I had the chance to work with you, even if for a very short while!

**Alberto G**. my partner in crime, my dear cow-painter friend and office mate for the first few months in the student's room! What a journey we started together, I am so glad we are friends. Thank you for teaching me how to use R, for always coming to help me out when I was facing troubles, for helping me with my research and for being a great friend. **Jeroen**, you always had my back. I can't even count the times I ran to you and asked for help and you never backed out! Even when it was a public holiday and I needed help in the lab at the very last minute! But not only this, you always gave me moral and psychological support, and precious advice, and were an excellent skinny-dipping buddy at the Rijn, what more to wish for in a colleague? You are such a fun nerd! And yes, you and I are so different, but it's been funny to see how two people who are almost opposite in everything can click so well.

I was lucky to be in the office x2125 during this PhD journey, and sorry guys from distracting you 6 hours a day, but I enjoyed it very much! What a great team we make! **Tamara**, our kitten pictures/memes/poems we exchanged brightened my day. Thank you for always helping me in the lab when things were going south, and for always supporting me, you are great! **Alberto B**., you were always by my side, and accommodated my weird requests, drove me around and lent me your car whenever I needed. Grazie Albi sei una forza, mi manchi! **Linda**, my oldest office mate, we have

been in this together from the beginning. If I still have some skin on my hands is thank to your generosity, in always sharing the lovely hand-creams (the panda, the ritual of ayurveda, the nivea… so many!). And of course, for always pulling a snack out of your drawer to share with us all! Guys you were the best office mates I could ever ask for, and I really appreciate that I could just be myself with you all, screaming included. **William**, we shared the office for a short time, but I enjoyed your company in the office and outside! Our dinners and drinks together were a lot of fun! **Domiziana**, thank you for the company, and taking over my spot in the office and keep the Italian share unchanged. Ti ringrazio anche per avermi aiutato con la casa, ho lasciato tutto in ottime mani.!

**George**, my dear food-loving colleague, and friend. Greeks and Italians just click on another level… μια φάτσα μια ράτσα. I had such a blast in your company, I felt truly understood and supported. I hope you will not stop sending me pictures of food and share with me your adventures. **Jasper B.** I really enjoyed sharing the lab with you. Thank you so much for the help you gave me, and the laughs we had in and out of the lab. And of course, for making the badass design of this thesis! Among my other lab mates, I can't forget **Maren**, and **Pjotr.** Maren you always a lot of fun and witty, and a great lab DJ. Pjotr, my dear fake frenemy, I actually really like your company, despite always saying the contrary, I wish we had more time together in the lab, I still have 1001 ideas on how I could piss you off, it is a pity not to have a ground to test them! **Xuchuan** thank you for always being so kind and helpful, and for the great hotpot and Chinese dinners. It is nice to finish this path together. **Johanna**, ho apprezzato molto la tua compagnia nel laboratorio, che ha reso i miei ultimi esperimenti molto meno noiosi, e la tua spontaneitá. **Xing**, I enjoyed your company and the good cups of tea we had, although for a short while! **Yingzhe** it was nice to know that there was someone else passionate about yeasts in our chairgroup!

**Jasper Z.** my dear! What an adventure with all that natto and the publication! I enjoyed our writing retreats very much. **Cristina** and **Soundarya**, thank you for always being sweet and caring, I had a lovely time together, I hope our paths will cross again. **Luuk**, I had a great time playing pool at the PhD weekend. **Angela**, we have known each other for a long time and our bond goes back to many years ago, during my MSc. Thank you so much for always being so kind and supportive. **Sylviani**, thank you for the nice chats and the fermentation brainstorms. **Thelma**, I enjoyed very much your calm and fun company during the PhD trip. Sweet **Clara** I enjoyed our chats and coffee breaks. **Yue**, thank you for your kindness and wisdom, your valuable experience in vitamin K analysis

helped me out a lot! **Oscar**, thank you for always finding time to brainstorm and help me out when I had technical difficulties, your support was very precious!

**Diego**, I loved our long chats in the fermentation lab when I was a MSc student, and you had just started with your PhD. I wish we had more of that during our PhDs. I miss you dearly, in a parallel universe we are lab buddies, making many more media vessels explode without control. You will always be in my mind and my heart. **Richard**, you are great fun! We had very good conversations and always felt like we clicked very well. I hope we stay in touch! **Gerda** thanks for explaining to me thousands of times the same bureaucratic things all over again, and for always doing it with a lot of patience and calm, everyone knows how much I need some calm around my messy self! **Jori**, we do not share the love for the same *type* of mayo (but one day you will discover that Zaanse wins above all), but sometimes I wonder whether, instead of focusing on these differences, we should have focused on our common love for this amazing sauce. Your company kept me cheerful and happy. I know it has been somehow hard to share a wall with my office, but please tell me that you miss my random screaming a little bit! And thanks for lending me your car, and always trying to help! **Frank**, thank you for your company, for always being so kind and sweet, and for coming with your appetite to help me finish that giant schnitzel during the PhD trip, I couldn't do that without your surprisingly stretchy stomach and eagerness to help, and eat! **Andy**, you are one fun man! Thanks for the advice, tips, and tricks you gave me. I enjoyed our hangouts and hope we can see each other again whenever you come to Europe! Thanks, you **Wilma** for the nice chats while sipping coffee. **Heidy** thank you for joining us during the PhD trip. **James** thank you for your company and your chill vibes. **Marcel Z.** thank you for always finding time to give precious feedback, and for being so down-to-earth and approachable.

**Pranas** I very much enjoyed our yeast and kittens talk, aren't these some of the best things in life after all? I am glad that besides an excellent scientific partner and collaborator, I also found a good friend!

To my friends, **Ivana, Lauren, Marne**, **Melissa, Jose**, **Francisco, Amanda**, **Rens**, and **Hanna**, I am so glad to have you all in my life. After all, a PhD is a hard job but forgetting about it during our time together, and finding people to share fun, food, and friendship with was the best fuel to recharge my mind! Thank you for that! Friendship is the most precious thing of this Dutch adventure!

**Daniela**, **Paolo** e **Federico**, grazie per il support che mi avete dato in questi anni, e per avermi sempre sostenuto e ascoltato.

To all the **Varotsis**' and **Maddie**, we took great holiday breaks together, thanks for all the company, the great food, and the beautiful adventures. I really enjoyed these relaxing moments together.

Grazie alla mia rumorosa **famiglia Rocchi**, quando torno a casa e ritrovo questo familiare casino mi sento meno aliena in questo vasto mondo dominato da anime noiose e calme. Grazie anche alla mia meta' di famiglia **Brughini**. **Zia Stefi** grazie per trattarmi sempre come una principessa, **Eli** io e te siamo due poli opposti, ma nessuno mi fa sentire compresa come te. Grazie **Leo**, sei un testone pazzesco, grazie per essermi venuto a trovare, e per sorprendermi ogni volta.

**Mamma** e **Papà**, grazie per avermi sempre supportata senza mai avermi fatto mancare nulla, rimuovendo tutte le preoccupazioni possibili così da farmi stare sempre serena. Sono molto fortunata ad avere voi come genitori, non è roba da poco! Vi voglio bene.

**Dimitri**, your support was crucial in all stages of my PhD, as it is in every other aspect of my life. I feel lucky every day to have you by my side. You are a constant source of inspiration and motivation, and looking ahead to our future together is the fuel for my mind and my soul. May we continue to grow, have fun, make mistakes, laugh, and learn together, I love you. ps: I left this last paragraph unjustified just for you, little freak.

## About the author

Rebecca was born in Perugia on January 2, 1994. She always loved science and fondly remembers her first science lectures in primary school. For instance, one day in class, she extracted DNA from a banana during a brief lab work activity. She also enjoyed multiple mesmerizing visits to the POST museum (Perugia Officina per la Scienza e la Tecnologia) in Perugia. During one of these visits, she learned the concept of atmospheric pressure with a small experiment. The experiment involved drilling holes in the bottom of a plastic bottle, filling it up with water, and seeing the waterfall only if the cap was not on. Rebecca's mother, Antonella, was not amused when she returned from work and opened the bottle to drink from, spilling water on herself. According to Pina, Rebecca's beloved nanny, Rebecca has always been passionate about food. As a toddler, she had to be fed using two spoons because she ate so quickly and greedily. When choosing a BSc program, Food Technology, which merged her two big passions, science, and food, was the obvious choice. Despite being terrified of the general microbiology professor, Rebecca studied hard for the exam and proceeded with interest to study Food Microbiology. With hard work, she turned her worst nightmare into a passion and graduated in 2016 from Universita' degli studi di Perugia with a BSc in Scienze e Tecnologie Agroalimentari.

In September 2016, Rebecca started an MSc in Food Technology at Wageningen University, specializing in Food Biotechnology and Biorefining. She was struck by the cultural differences between her birthplace and her new home. On the first day of lectures, Professor Dr. Eddy J. Smid looked at the digital clock, and when it indicated 9:00:00, he pronounced "Good morning" and started talking about fermentation, such punctuality was unheard of in her world. One year and a half later, after a lot of hard work, stinking up the fermentation lab, and driving across the Netherlands to gather samples, Rebecca completed her MSc thesis about seaweed fermentation under the supervision of Professor Dr. Eddy J. Smid and Judith Wolkers-Rooijackers, she started a fantastic 7-month internship at The KraftHeinz Company, which truly changed her life! Not only she met her beloved boyfriend and future husband Dimitri, after a very awkward encounter while sitting across each other on the first day, but she made many “cool friends” with a “real job” (not like a PhD…).

Rebecca graduated in February 2019 and accepted a position as a product development technologist at the same company. Finally, she had a real job, but the excitement for this new chapter of her life as a young professional did not last that long. Indeed, on the first hour of the first day of work, she received a call from Professor Dr. Eddy J. Smid, who offered her a great opportunity to apply for a PhD under his supervision at the Food Microbiology department at Wageningen University. Although the choice was difficult, Rebecca followed her heart.

Four and a half years later, Rebecca is about to complete her PhD in Food Microbiology and writing this biography in 3rd person.

You can scan this QR code to connect with me on LinkedIn:

www.linkedin.com/in/rebecca-rocchi

Or send an email at:

rocchi.rebecca@gmail.com

## List of publications

1. **Rebecca Rocchi**, Kees van Kekem, Walter H. Heijnis, Eddy J. Smid. A simple, sensitive, and specific method for the extraction and determination of thiamine and thiamine phosphate esters in fresh yeast biomass, Journal of Microbiological Methods, 2022, ISSN 0167-7012
https://doi.org/10.1016/j.mimet.2022.106561

2. **Rebecca Rocchi**, Judith C. Wolkers-Rooijackers, Zhuotong Liao, Marcel H. Tempelaars, Eddy J. Smid. Strain diversity in *Saccharomyces cerevisiae* thiamine production capacity, Yeast, 2023, ISSN 0749-503
https://doi-org.ezproxy.library.wur.nl/10.1002/yea.3906

3. **Rebecca Rocchi**, Jasper Zwinkels, Merit Kooijman, Alberto Garre Perez, Eddy J. Smid. Development of novel natto using legumes produced in Europe. Heliyon, 2024, ISSN 2405-8440
https://doi.org/10.1016/j.heliyon.2024.e26849

## Overview of completed

### Category A: Discipline specific activities

| Name of the course/meeting | Organizing institute | Country | Year |
|---|---|---|---|
| Emerging applications of microbes | VIB | Belgium | 2019 |
| MB 6.0 | TU Delft | Netherlands | 2019 |
| Microbial Physiology and Fermentation Technology | TU Delft | Netherlands | 2020 |
| World microbe forum | FEMS | Online | 2021 |
| Microbial Food and Feed ingredients | CAP Partner | Denmark | 2021 |
| Ecophysiology of food associated microorganisms | VLAG | Netherlands | 2021 |
| A to W Basic Course in Electron Microscopy | EPS | Netherlands | 2022 |
| MB 8.0 | VU Amsterdam | Netherlands | 2022 |

### Category B: General courses

| Name of the course | Organizing institute | Country | Year |
|---|---|---|---|
| VLAG PhD week | VLAG | Netherlands | 2019 |
| PhD workshop carousel | WGS | Netherlands | 2019 |
| PhD Competence assessment | WGS | Netherlands | 2019 |
| Introduction to R | VLAG | Netherlands | 2020 |
| Applied statistics | VLAG | Netherlands | 2020 |
| Career perspectives | WGS | Netherlands | 2022 |
| Critical thinking and argumentation | WGS | Netherlands | 2021 |
| Project and time management | WGS | Netherlands | 2020 |
| Scientific Artwork: Data Visualisation and Infographics with Adobe Illustrator | WGS | Netherlands | 2021 |
| Research data management | WGS | Netherlands | 2021 |
| UnBox your PhD | WGS | Netherlands | 2021 |

**Category C: Assisting in teaching and supervision activities**

| Name of the assisting activity | Organizing institute | Country |
|---|---|---|
| MSc students thesis supervision | FHM | Netherlands |
| MSC courses course supervision | FHM | Netherlands |

**Category D: Other activities**

| Name of the course | Organizing institute | Year | Country |
|---|---|---|---|
| Preparation of research proposal | FHM | 2019 | Netherlands |
| PhD study trip | FHM | 2022 | Germany and Switzerland |
| Weekly group meetings | FHM | 2019-2023 | Netherlands |
| Discussion group at FHM | FHM | 2019-2023 | Netherlands |
| Alternative protein project podcast | N/A | 2020 | Netherlands |

**Student supervision activities**

| Name of the student | PhD year |
|---|---|
| Leonardo Gaiani | 1 |
| Yuhan Wang | 1 |
| Ferran Porte' Vall | 2 |
| Maria Puspitasari Schonherr | 2 |
| Zhuotong Liao | 3 |
| Merit Kooijman | 3 |
| Shantanu Tiwari | 3 |
| Aditya Balakrishnan | 3 |
| Lukas Schneider | 4 |

**Academic courses supervision**

| Code and name of the course | PhD Year |
|---|---|
| Advanced Fermentation Science | 2-3 |
| Food Fermentation | 2 |

The research described in this thesis was performed at the Laboratory of Food Microbiology, Wageningen University & Research. We gratefully acknowledge the financial support from Wageningen University for printing this thesis. This research is part of the project B-Twelve Insight, which is co- financed by Top Consortium for Knowledge and Innovation Agri & Food by the Dutch Ministry of Economic Affairs. The project is registered under contract number TKI AF18081.

Cover design by Elisa Reali.

The layout of this thesis was designed by Jasper Bannenberg, taking inspiration from the thesis layout designed by Kah Yen Claire Yeak, and with the artistic direction of Rebecca Rocchi.

Printed by Proefschrifmaken.