DOCTORAL THESIS

Oligopeptides as a Nitrogen Source for *Saccharomyces cerevisiae* in Industrial Fermentations.

A Study to Oligopeptide Uptake and Transporter Function in Practical Applications

Hidde Yaël Berg

TALLINNA TEHNIKAÜLIKOOL
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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for a doctoral or equivalent academic degree.

Hidde Yaël Berg

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TALLINNA TEHNIKAÜLIKOOL
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Oligopeptiidid *Saccharomyces cerevisiae* lämmastikuallikana tööstuslikes käärituspro tsessides.

Uuring oligopeptiidide omastamisest ja peptiidide transporterite funktsioonidest praktilistes rakendustes

HIDDE YAËL BERG

TAL TECH KIRJASTUS

Contents

List of Publications

The list of author's publications, based on which the thesis has been prepared:

I Arju, G., **Berg, H. Y.**, Lints, T., & Nisamedtinov, I. (2022). Methodology for Analysis of Peptide Consumption by Yeast during Fermentation of Enzymatic Protein Hydrolysate Supplemented Synthetic Medium Using UPLC-IMS-HRMS. *Fermentation*, *8*(4), 145.

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Author's Contribution to the Publications

Contribution to the papers in this thesis are:

I The author provided the biological framework and motivation for the study, and was involved in the study design, fermentation experiments, analysis, data processing, interpretation of results, and drafting the manuscript.

II The author was involved in the study design, conducted the experiments and analyses, interpreted the results, and wrote the manuscript.

III The author was involved in the study design, conducted the experiments and analyses, interpreted the results, and wrote the manuscript.

Preface

Achieving optimal efficiency in industrial fermentations requires adequate nitrogen levels in the fermentation substrate. *Saccharomyces cerevisiae*, the most commonly used organism in these processes, can obtain nitrogen from various sources, including ammonia, urea, free amino acids, and peptides. Fermentation substrates often lack sufficient nitrogen and may require external nitrogen supplementation. While most research carried out today has focused on the impact of adding simple nitrogen sources, the utilisation of oligopeptides by yeast and the effects of peptide supplementation during fermentation have not been sufficiently explored.

In 1994, the first oligopeptide transporter (Ptr2) in *Saccharomyces cerevisiae* was characterised as a di- and tripeptide transporter. Subsequently, two additional peptide transporters, Opt1 and Opt2, have been identified. Among these, Opt1 was reported to be the most active, transporting tetra- and pentapeptides, whereas Opt2 only transported tetrapeptides. In 2006, a comprehensive study by Wiles et al. was published detailing the nutrient regulation of oligopeptide transport. This research established a baseline understanding of peptide transport mechanisms in yeast, assuming that this ability is conserved within the species. However, in 2011, the conservation of the peptide uptake ability was already disproven by Damon et al., who identified a new class of di- and tripeptide transporters, called Fot, that were present only in certain wine yeasts. Although a wide range of *Saccharomyces cerevisiae* strains are available for numerous applications, there is a scarcity of research exploring the diversity in peptide uptake among different strains and its impact on fermentation kinetics. In addition, the upper limit of peptide uptake (which currently stands up to a chain length of five amino acids) has remained unchallenged for more than two decades.

There are good reasons why peptide utilisation has not been as thoroughly studied as simpler nitrogen sources such as ammonia and amino acids. The high diversity of physicochemical properties of peptides, due to the endless combinations of different amino acids that make up the peptide chain, makes their analytical determination and identification a challenge. Therefore, most previous studies have used growth experiments with single synthetic peptides as the sole nitrogen source. While studies on single peptide consumption have contributed to our general understanding of oligopeptide transporter specificities, they have not provided information on the kinetics of peptide utilisation during fermentation in complex media representing those utilised in industry.

This study aimed to provide a methodology to study peptide utilisation by yeast during fermentation in complex media containing peptides of varying lengths and amino acid composition. Using this methodology, we elucidated the variability between yeast strains in their peptide uptake capability and their respective response to nitrogen supplementation with peptides. In addition, the role and peptide length specificity of peptide transporters of the Fot and Opt family were studied. Lastly, in a practical use case, the role of oligopeptides as a nitrogen source in the industrial Scotch grain whisky fermentation process was demonstrated.

1 Introduction

1.1 Nitrogen limitation in the fermentation industry

Saccharomyces cerevisiae plays a key role in many industrial fermentation processes, most prominently in the production of alcoholic beverages and bioethanol. To ensure optimal fermentation kinetics and product quality, the substrate must satisfy the yeast's requirements for a number of nutrients, including nitrogen sources.

Being one of the most abundant elements in biomass ($CH_{1.613}O_{0.557}N_{0.158}$), nitrogen is crucial for successful fermentation serving various anabolic functions (2), for example, in the biosynthesis of structural and functional proteins and nucleic acids (2,3). In addition, catabolic reactions with amino acids result in the formation of higher alcohols and their associated esters, which contribute to the flavour properties of the final product (4,5). To supply its need for nitrogen, *S. cerevisiae* can consume ammonia, free amino acids (excluding proline), and low-molecular-weight oligopeptides (6,7). For the latter, di- to pentapeptides have been reported to be assimilable by yeast (8–16). The nitrogen derived from these three groups of compounds is in industrial practice often referred to as "Yeast Assimilable Nitrogen" (YAN, Eq. 1).

$$[YAN] = [N]_{NH_4{}^+} + [N]_{free\ amino\ acids} + [N]_{oligopeptides} \quad (1)$$

Producers of (potable) ethanol use various techniques to determine the nitrogen content of their substrates. Among these, free amino nitrogen (FAN) analysis is the most widely used. This method is based on a colorimetric assay that involves the use of ninhydrin, which reacts with ammonia and the free amino groups of amino acids and peptides to form Ruhemann purple (3). The resulting colour change can be measured using a spectrophotometer and FAN values (mg N/L) are usually calculated from a glycine-based standard curve (17). Owing to its low cost and simplicity compared to other methods, such as liquid chromatography and mass spectrometry, the yeast available nitrogen levels in different fermentation matrices are mostly reported based on this analysis. However, as the ninhydrin method reacts only with free amino groups, it does not measure the total available nitrogen from peptides, which consequently leads to an underestimation of the yeast available nitrogen in matrices with a more abundant content of potentially assimilable peptides (18). To avoid confusion with the term "YAN", this thesis references FAN values from the literature as "α-amino nitrogen".

Nitrogen deficiency is a typical issue in many fermentation matrices, including grape must and those used for bioethanol production (19). Suboptimal concentrations of YAN can cause various problems, such as prolonged fermentation times, undesired organoleptic properties of the final product, loss of product due to stuck or sluggish fermentation, and related microbial contamination issues (20–22).

Prolonged fermentation time caused by nitrogen limitation is usually related to insufficient biomass growth and low enzymatic activity necessary to finish fermentation within the desired process duration (23,24). For example, low nitrogen levels have been related to a high turnover rate of sugar transporters, leading to a reduced activity or loss of sugar uptake capacity by the cells (23,25,26). In industry, slow fermentation kinetics result in increased production costs due to longer production times and inefficient use of bioreactor volume, in addition to an increased risk of contamination by other microorganisms (27). For instance, lactic acid bacteria usually grow faster and often have

a lower demand for nitrogen than yeast (28,29). Moreover, changes in metabolic activity in yeast may have a negative impact on the sensory properties of potable fermentation products (7,30,31). For example, in nitrogen-deficient grape must, yeast may produce H_2S, resulting in a rotten egg odour in wine (7,32–34).

In the bioethanol industry, suboptimal nitrogen levels, which result in decreased ethanol yields, can cause substantial economic losses. In 2020, the international bioethanol industry production was worth approximately € 53.81 billion, and it is estimated to reach € 59.67 billion by 2028 (35). Thus, in an industry that aims to achieve the highest possible ethanol yield from the substrate used, even a small reduction in yield has a significant monetary impact (Table 1).

Table 1. Economic implications of decreased ethanol yields in the fuel ethanol industry. *These values are based on the yearly production output of one of the smallest (Dynamic Recycling LLC, Bristol, TN, 15 ML ethanol/year) and one of the largest (Marquis Energy LLC, Hennepin, IL, 1512 ML ethanol year) U.S. fuel ethanol plants (36) and an ethanol price of € 0.41/L.*

Ethanol loss (%v/v)	Ethanol loss (ML/year)	Revenue loss (€/year)
0.1%	0.02–1.51	6,208–620,808
0.3%	0.05–4.54	18,624–1,862,423
0.6%	0.09–9.08	37,248–3,724,845
0.9%	0.14–13.63	55,873–5,587,268
1.2%	0.18–18.17	74,497–7,449,690
1.5%	0.23–22.71	93,121–9,312,113

1.1.1 Adequate yeast assimilable nitrogen concentrations in fermentation

Preventing nitrogen limitation during fermentation is essential to avoid slow or stuck fermentation that can result in various secondary production problems, as described in the previous section. Limited studies exist that precisely describe the relationship between sugar content and the required amount of nitrogen for yeast. In addition, the required nitrogen content of a substrate is dependent on other factors, such as the specific yeast strain or the rate at which yeast cells are pitched at the start of fermentation (37–39). Therefore, most fields handle a rule-of-thumb value to determine whether a substrate contains sufficient nitrogen to support fermentation. In this context, for wine production, it has been suggested that a minimum of 140 mg/L of α-amino nitrogen is needed for optimal fermentation efficiency in grape must with moderate sugar concentrations (≈ 220 g/L) (7,40). However, higher levels of α-amino nitrogen are usually necessary for reliable fermentation of grape musts with higher sugar concentrations (34,41–44). As mentioned before, the nitrogen requirement during fermentation can also vary significantly depending on the yeast strain used (Table 2) (37,38).

***Table 2. Nitrogen demand classification for yeast in winemaking and corresponding nitrogen need per 1 g of fermentable sugars** (45).*

Nitrogen demand by yeast	**α-amino nitrogen** mg / g fermentable sugars
Low	0.75
Medium	0.90
High	1.25

Similar values have been reported in other fields, such as brewing. In normal gravity wort (100–120 g/L fermentable sugars), a minimum level of 100–140 mg/L α-amino nitrogen is required for satisfactory yeast growth and fermentation performance to achieve full attenuation (46). Similar to wine fermentation, the optimum α-amino nitrogen concentration in wort varies depending on the yeast strain and wort sugar concentration. In addition, it has been demonstrated that in a high-gravity wort (260 g/L fermentable sugars), the α-amino nitrogen requirement can be between 150 and 250 mg/L depending on the pitching rate and nutrient status of the wort (39).

It must be noted that the YAN concentration in industrial fermentation substrates is currently calculated based only on the available ammonia and free amino acid (α-amino nitrogen) content, while the contribution of peptides is underestimated, despite often providing a substantial amount of YAN in many substrates. The main reason for this is associated with challenges related to qualitative and quantitative analyses of peptides (47). For example, in grape must, the oligopeptide fraction is estimated to constitute 10–30% of the total nitrogen content (7,10,29,48), with up to 10% potentially assimilable by yeast (10,48). In an all-malt wort, polypeptides form about 30–40% of the total nitrogen (49,50), of which 30–50% is assumed to be available to yeast (6,51,52). Isotope labelling experiments with ^{15}N-labelled-NH_4Cl and yeast hydrolysate have shown that nitrogen assimilated from peptides constitutes up to 40% of the yeast protein fraction, compared to only 20% from ammonia (53). Furthermore, other studies have shown that the consumption of oligopeptides by yeasts during wine fermentation had a positive impact on cell viability, fermentation kinetics, and the production of volatile compounds (10,54,55). Thus, oligopeptides are important nitrogen sources for yeast, although their contribution to YAN is often underestimated.

1.1.2 General practices to overcome nitrogen limitation in industrial fermentations

The most common practice to increase the YAN content in fermentation is supplementation with urea or ammonium salts, usually mono- or diammonium phosphate (MAP, DAP). In winemaking, the European Union allows a maximum dose of 1 g DAP per litre of grape must (56), resulting in approximately 200 mg/L of YAN. Although cost-effective and convenient, this practice has limitations. The addition of DAP has been shown to result in slower fermentation rates and poorer synthesis of aroma compounds (e.g. higher alcohols and esters) than free amino acids delivering the same amount of nitrogen (33,37,40,57–59). Furthermore, excessive DAP usage can lead to the production of unwanted metabolites such as biogenic amines (60) and decreased microbiological stability in the final product (61). The application of urea for supplementation of YAN in the potable alcohol industry is prohibited because of its

reactivity with ethanol, resulting in the formation of carcinogenic ethyl-carbamate (62). Nonetheless, urea is still widely used in the production of bioethanol and has been demonstrated to result in higher ethanol yields compared to the use of ammonium salts (63,64).

An alternative solution to overcome nitrogen deficiency is the addition of organic supplements such as yeast extracts or plant peptones which contain amino acids and peptides (65). However, depending on the degree of protein hydrolysis and the allowed and/or economically feasible dosage rates, the impact of such organic nutrients on YAN concentration is often insufficient to make a significant contribution to YAN levels (66). Thus, strategies combining both organic and inorganic YAN-containing nutrients can also be applied, depending on the desired product quality.

Many raw materials used in industrial fermentation contain substantial amounts of protein which can be used as a source of YAN if hydrolysed into free amino acids and yeast-assimilable peptides. For example, in grape must 2–10% of the total nitrogen is derived from proteins (7,29), whereas in all malt worts used for brewing, this can reach up to 10% (w/v) (67). Thus, a third option to increase the YAN content during fermentation is liberation from the protein fraction using proteases. Since *S. cerevisiae* does not naturally excrete proteolytic enzymes (50,68,69), they must be present in the raw material (e.g. malt) or applied exogenously. For example, the bioethanol industry sometimes applies proteases to release additional YAN from feedstocks like wheat and corn, resulting in shorter fermentation times and increased ethanol yields (49,70,71). Exogenous proteases, used as process aids, have also been proven to increase the fermentability of high-gravity worts during brewing, increasing the formation of ethanol and volatile aroma compounds (49). In winemaking, the protease-assisted liberation of YAN is more difficult because several of the most abundant protein classes in grapes, particularly pathogenesis-related proteins (thaumatin-like proteins and chitinases), are resistant to proteolysis by conventional commercial proteases because of their compact globular structure, which prevents access to protease enzymes (72–75). Nevertheless, some non-*Saccharomyces* yeast species secrete proteases that can degrade grape proteins. For example, aspartic protease MpAPr1 from *Metschnikowia pulcherrima* was shown to be able to degrade grape proteins during fermentation, which resulted in a reduction of the protein haze in the final wine and significantly increased volatile compounds related to the metabolism of released amino acids by yeast, but not in an improvement of fermentation kinetics (76). Releasing YAN from the protein fraction in grape must to improve fermentation kinetics and thus remains a topic of research.

Lastly, the endogenous proteases present in the substrate could be used to increase YAN content. The best example is the use of malted raw materials. For example, grain mash for whisky fermentation is usually not heated to temperatures that inactivate all proteases (77–81), possibly resulting in retained proteolytic activity during fermentation and potentially releasing additional available YAN for yeast. The advantages and disadvantages of the different practices used to increase YAN content in industrial fermentation are given in Table 3.

Table 3. Advantages and disadvantages of different nitrogen supplementation strategies in industrial fermentation.

	Advantages	Disadvantages
Simple (in)organic supplements (MAP, DAP, urea)	• Relatively cheap • Easy to calculate the amount of added nitrogen	• Fermentation and growth rates have been shown to be lower than with organic N-sources (37,40,41,58,82,83). • High environmental impact due to being chemically synthesised with a high energy requirement of the production process (84)
Organic Supplements (yeast autolysates, peptones)	• In addition to nitrogen, also supplies other nutrients. • Positive influence on flavour development.	• Often insufficient at recommended/allowed dosages to make significant nitrogen adjustments (66). • Due to the complex composition, difficult to precisely calculate the added amount of YAN. • Expensive compared to inorganic nitrogen sources.
YAN liberation from endogenous proteins	• Cheaper due to making use of the endogenous nitrogen of the substrate • Can make use of endogenous proteases if present in the substrate. • Can potentially be accomplished with co-cultures containing protease-excreting strains	• More complex process • Not all substrates are compatible, e.g., grape must (72–75). • Due to substrate complexity and uncharacterised protease specificity/activity, the effect on YAN content is often difficult to estimate. • Excessive proteolysis may negatively affect the quality of some products, such as reduced foaming in beer and sparkling wines (85,86).

1.2 Nitrogen metabolism in *Saccharomyces cerevisiae*

1.2.1 Amino acid synthesis

As mentioned in the previous section, ammonia, urea, free amino acids, and certain oligopeptides can be utilised by yeast as nitrogen sources for growth. Once internalised, these nitrogenous compounds can be directly utilised in biosynthetic processes, stored in the vacuole, deaminated to generate ammonium, or serve as substrates for transaminases that transfer amino groups to α-ketoglutarate to form glutamate, which then acts as an amino group donor during the *de novo* synthesis of amino acids (Figure 1) (87).

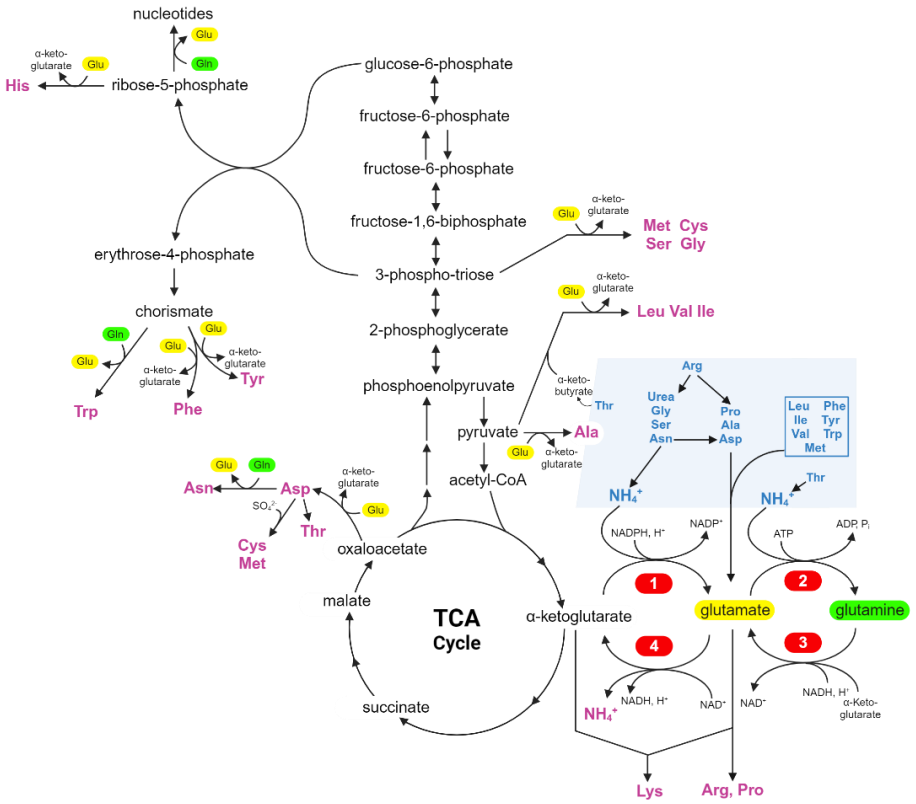

Figure 1. General Scheme for Amino Acid Biosynthesis. *The routes of amino acid entry into the central core reaction are shown in blue. Ammonia is incorporated during the formation of glutamate from α-ketoglutarate (Reaction 1) and glutamine from glutamate (Reaction 2). Additionally, glutamate can be synthesised from glutamine and α-ketoglutarate (Reaction 3) and ammonium can be released from glutamate (Reaction 4). Transamination reactions that transfer nitrogen from glutamate or glutamine are indicated in yellow and green, respectively. De novo synthesised amino acids and released ammonium are shown in purple. The scheme was adapted from Ljungdahl and Daignan-Fornier, 2012 (87) and created with BioRender.com.*

All amino acids can be synthesised *de novo* by yeast, with glutamate and glutamine playing central roles in this process (87–89). There are two anabolic processes (outlined in Figure 1) by which ammonium can be incorporated into one of these two amino acids: the first involves the synthesis of glutamate from ammonium and α-ketoglutarate (reaction 1), while the second involves the synthesis of glutamine from ammonium and glutamate (reaction 2) (87,90). Both processes require either free ammonium or amino acids, which can be deaminated to produce the required ammonium ions. When glutamine is the sole nitrogen source, glutamate can be synthesised from glutamine and α-ketoglutarate (reaction 3), and when glutamate is the sole source of nitrogen, ammonium can be released from glutamate (reaction 4) to be used in reaction 2 to generate glutamine. Thus, yeast cells have well-established pathways to produce glutamate and glutamine which play central roles in many transamination reactions required for the synthesis of other amino acids (Figure 1). Approximately 85% of the total *de novo* synthesised cellular amino acids are derived from the amino nitrogen of glutamate, and the remaining 15% are derived from the amide nitrogen of glutamine (89).

S. cerevisiae can utilise most L-amino acids, except for lysine, histidine, and cysteine, as the sole source of nitrogen (87). These amino acids are categorised into two distinct classes during biosynthesis. Class A amino acids (alanine, serine, aspartic acid, asparagine, glutamic acid, and glutamine) serve as substrates for transaminases or deaminases that incorporate nitrogen into glutamate. The resulting carbon skeletons are then converted into pyruvate (in the case of alanine and serine), TCA cycle intermediates oxaloacetate (in the case of aspartate and asparagine), or α-ketoglutarate (in the case of glutamate and glutamine).

Class B amino acids, including branched-chain amino acids (leucine, isoleucine, and valine), aromatic amino acids (phenylalanine, tyrosine, and tryptophan), and methionine, undergo transamination during which nitrogen is transferred to α-ketoglutarate to form glutamate. This process can influence the organoleptic properties of fermentation products, as the resulting carbon skeletons are converted via the Ehrlich pathway into higher alcohols (Figure 2) (4,87,91). The type of higher alcohol produced is determined by the amino acid, with common examples being n-propanol (from threonine), isobutanol (from valine), isoamyl alcohol (from leucine), active amyl alcohol (from isoleucine), and 2-phenylethyl alcohol (from phenylalanine) (91). The presence of these higher alcohols can impart a range of organoleptic attributes such as alcohol, fruity, pungent, solvent-like, and rose-like or floral flavours, depending on their concentration and type (91). Additionally, higher alcohols can be further converted into acetyl esters through a reaction with acetyl-CoA catalysed by acetyl transferases (92). Esters contribute fruity and floral qualities to the sensory attributes of fermented drinks (93–98).

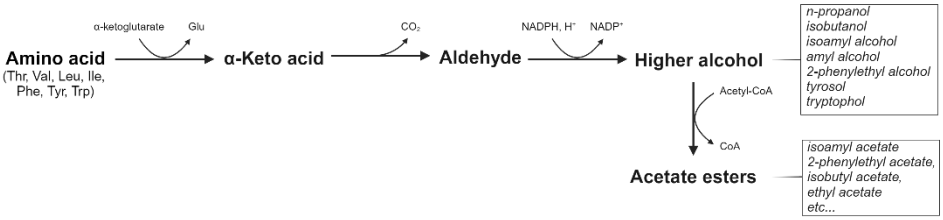

Figure 2. Schematic view of the formation of higher alcohols through the Ehrlich pathway and the formation of acetate esters. *The figure was created with BioRender.com.*

In addition to free amino acids*, S. cerevisiae* can also utilise oligopeptides as a source of nitrogen (87,99). This process involves internalisation of the peptides, followed by hydrolysis into individual amino acids by intracellular peptidases, which then enter the previously described catabolic and anabolic pathways. The cellular location of peptide hydrolysis remains undetermined, although most evidence suggests that it occurs in vacuoles (87,100–106). The vacuoles contain a diverse range of proteases and peptidases, and during vegetative growth, they account for 40% of cellular proteolysis (87,100,101). A good example of a peptidolytic process in vacuoles is the breakdown of the antioxidant tripeptide glutathione (GSH; L-γ-glutamyl-L-cysteinyl glycine) (102–106). There are a variety of ways for proteins to enter vacuoles, for example, through the vacuole protein sorting pathway, endocytosis, autophagy, and direct transport across the vacuole membrane (100,107–109). Unfortunately, peptide transport mechanisms in vacuoles have not been adequately researched and require further investigation. While *s*everal amino acid transporters are present in vacuoles (Figure 3) (110) the only known oligopeptide transporter located in the vacuole membrane is Opt2 (111,112).

This transporter has been related to vacuole membrane stability but has not yet been associated with oligopeptide translocation from the cytosol into the vacuole (111,112).

1.2.2 Yeast nitrogen source transporters and their regulation

Saccharomyces cerevisiae possesses a multitude of transporters that facilitate the transport of urea, ammonia, amino acids, and oligopeptides across the plasma membrane (Figure 3). For example, it encodes 24 amino acid permeases (113) and 7 oligopeptide transporters (99). Yeast is known to acquire nitrogen sources in an ordered manner, preferably by selecting the most favoured sources (NH_4^+, glutamic acid, glutamine, or arginine). This is achieved by controlling the expression and activity of the transport of nitrogen sources, which involves the regulation of transcription, transporter activity, and transporter turnover (87–89,99).

Yeast cells can detect the presence of amino acids through a specialised sensor complex situated in the plasma membrane (87,113,114). This complex comprises the proteins Ssy1, Ptr3, and Ssy5 (SPS) (115). Ssy1 is a permease-like protein that lacks transport activity and acts as an amino acid receptor (116,117), whereas Ssy5 is a protease responsible for the activation of transcription factors Stp1 and Stp2 (118). Thus, upon sensing external amino acids, Ssy1 activates the proteolytic activity of Ssy5 mediated by Ptr3, which in turn activates the transcription factors Stp1 and Stp2 (118). These transcription factors then activate the expression of a variety of amino acid-specific transporters, as well as transporters of oligopeptides (119–125).

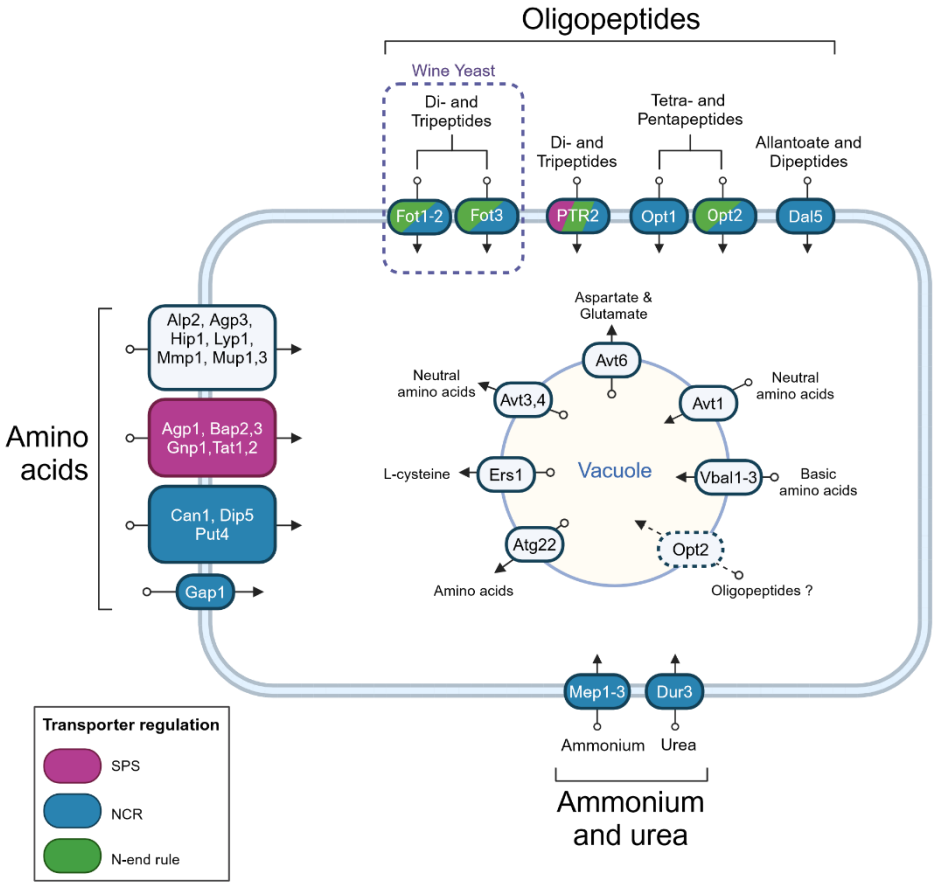

Figure 3. Membrane transporter systems for urea, ammonium, amino acids, and oligopeptides in S. cerevisiae. *The arrows depict the direction of catalysed transport. The figure was adapted from Ljungdahl and Daignan-Fornier, 2012 (87), modified with additional information on peptide transporters based on Becerra-Rodríguez et. al., 2020 (99) and created with BioRender.com.*

While some transporters are constitutively expressed, others are only expressed based on the nitrogen requirements of the cell and the quality of the nitrogen sources available in the environment (87,113). The best-known uptake regulation system in yeast is the Nitrogen Catabolite Repression (NCR) system, which involves several regulatory mechanisms for gene expression that suppress the transcription of genes encoding transporters for non-preferred nitrogen sources. A nitrogen source is preferred based on its capacity to support rapid cell growth (ammonium, glutamic acid, glutamine, or arginine), whereas non-preferred sources trigger de-repression of genes controlled by NCR (126). NCR-sensitive gene expression is regulated by key regulators such as the transcription activators Gln3 and Gat1/Nil1, as well as the negative regulator Ure2 (127–130). Under optimal nitrogen concentrations, Gln3 remains in the cytoplasm mediated by Ure2, whereas Gat1 is primarily distributed between the cytoplasm and nucleus, resulting in minimal NCR-sensitive gene expression (127). However, in the absence of preferred nitrogen sources, Gln3 and Gat1 are mainly located in the nucleus, where they bind to their target DNA sequences and promote the transcription of NCR-sensitive genes (130–132).

1.2.3 Oligopeptide transport and regulation in *S. cerevisiae*

To date, seven oligopeptide transporter systems have been identified in *Saccharomyces cerevisiae:* Ptr2, Dal5, Opt1, Opt2, Fot1, Fot2, and Fot3 (Figure 3). These transporters allow yeast to take up peptides with a chain length of two to five amino acids. The first oligopeptide transporter discovered in *S. cerevisiae* was the di- and tripeptide transporter Ptr2 (8). Additionally, Dal5, a transporter of diureidoacetate and ureidosuccinate, also exhibits the ability to transport dipeptides (9). Certain wine yeasts possess fungal oligopeptide transporters (Fot1-Fot2 and Fot3) that have been experimentally shown to transport di- and tripeptides (10–12). However, as the characterisation experiments for these transporters were limited to di- and tripeptides, it is possible that Fot may also be capable of transporting larger peptides (54,99). Oligopeptide transporters Opt1 and Opt2 in yeast account for the uptake of tetra- and pentapeptides (13–16).

NCR regulates the expression of several oligopeptide transporter genes, including *PTR2*, *DAL5*, *OPT1*, and *OPT2* (8,14,88,133–137). Due to Fot being a relatively recently discovered family of oligopeptide transporters, the regulation of their respective genes have been less extensively investigated. Nevertheless, it is hypothesised that *FOT* gene expression is also under NCR regulation (12,133,134). The expression of *OPT1* is specifically induced under conditions of sulphur deprivation, whereas the availability of sulphur-containing amino acids sustains suppression through NCR (138,139).

Oligopeptides, like amino acids, also play a role in regulating the expression of oligopeptide transporter genes. *PTR2* expression is upregulated through positive feedback when it transports dipeptides containing basic and bulky amino acids at the N-terminal position, known as “N-end rule dipeptides” (140). This process involves N-end rule dipeptides binding to Ubr1, which triggers the breakdown of Cup9, a transcriptional repressor of *PTR2* (141–144). Research has demonstrated that Cup9 also inhibits *OPT2* expression and identified potential Cup9 binding sites within the regulatory regions of *FOT* (12). However, it remains uncertain whether N-end rule peptides activate these transporters in a fashion similar to *PTR2*. Interestingly, Cup9 has an inducing effect on the expression on *DAL5*, while having no effect on *OPT1* (9,138). This varied yet complementary regulation of *PTR2*, *DAL5*, *OPT*, and *FOT* expression may enable *S. cerevisiae* to adapt to diverse environmental conditions and nitrogen availability. Figure 3 illustrates various mechanisms involved in peptide transporter regulation.

The ability of yeast to take up peptides has mostly been characterised by growth experiments using synthetic peptides as the sole nitrogen source. These experiments provided accurate information about the ability of yeast strains to utilise the selected peptide as a nitrogen source. Nevertheless, without proper analytical methods, they fail to deliver insights into the rate at which the peptides are utilised at varying stages of fermentation on intricate peptide-rich media, such as grape must and grain mash. Knowledge of the latter has mostly been indirectly derived from gene expression analyses (138). Moreover, the depth of analysis was limited by the length of the peptides provided. For example, there are no reports to date that have examined the uptake of peptides larger than pentapeptides by Opt1 and Opt2, even though Opt1 orthologous transporters in the yeast species *Candida albicans* have been reported to import peptides of at least up to eight amino acid residues in length (145,146). Similarly, Fot has only been characterised in media containing di- and tripeptides (10–12).

Therefore, it cannot be ruled out that these peptide transporters may also transport larger peptides in *S. cerevisiae* (54,99). To summarise, to better understand the role of peptides as a nitrogen source in yeast, there is a need for screening and (semi-) quantitative methods to measure the uptake of peptides from media with diverse peptide compositions.

1.3 Peptide analysis in fermentation matrices

1.3.1 Peptide identification and quantification with liquid chromatography mass spectrometry (LC-MS)

Mass spectrometry (MS) is one of the most promising techniques for peptide analysis (147–151). The fundamental concept of this method involves the separation of ionised molecules in a sample and measurement of their mass-to-charge ratio (*m/z*) to determine their molecular weight (152). The amino acid sequence of a peptide can be accurately determined in most cases by measuring the m/z before and after fragmentation.

The most common setup for peptide analysis by MS is separation of peptides by liquid chromatography using reversed-phase columns (i.e. C18), and a gradient of water and acetonitrile (153,154). The eluent (containing peptides and the mobile phase) is then transitioned into the gaseous phase and ionised using a high voltage during electrospray ionisation (ESI). This process may produce many different *m/z* signals for a single peptide due to different charge states, different carbon and nitrogen isotopes, various adducts present in the peptide, the neutral loss of H_2O or ammonia, and in-source fragmentation during ESI. Low- and high-resolution MS (LRMS/HRMS) can be used for peptide identification and quantification. Although LRMS excels in the quantification of a defined list of peptides, HRMS provides flexibility for both identification and quantification because of its higher resolving power. LRMS is typically represented by triple quadrupole (QQQ) and ion-trap (IT) systems, whereas HRMS is typically represented by time-of-flight (TOF) or Fourier-transform (FT)-based MS system hybrids with ion mobility and/or low-resolution mass analysers.

To confirm the identity of a peptide, the precursor ion is fragmented. The typically used method is collision-induced dissociation (CID), which breaks down the C-N amide bond and produces mostly b- and y-fragments, which are product ions containing the N-terminus and C-terminus, respectively (Figure 4). CID also results, to a lesser extent, in the formation of a- fragments (derived from b-ions after the loss of CO_2) and fragments with losses of ammonia and H_2O. The number of selective fragments produced during CID is directly related to the charge state of the precursor ions. Unlike longer peptides, such as pentapeptides and longer ones, which have a greater number of peptide bonds and therefore tend to ionise with a higher charge state (≥ 2+), shorter peptides, including di- to tetrapeptides, typically ionise as singly charged ions. This results in fewer selective fragments, leading to reduced identification accuracy (155).

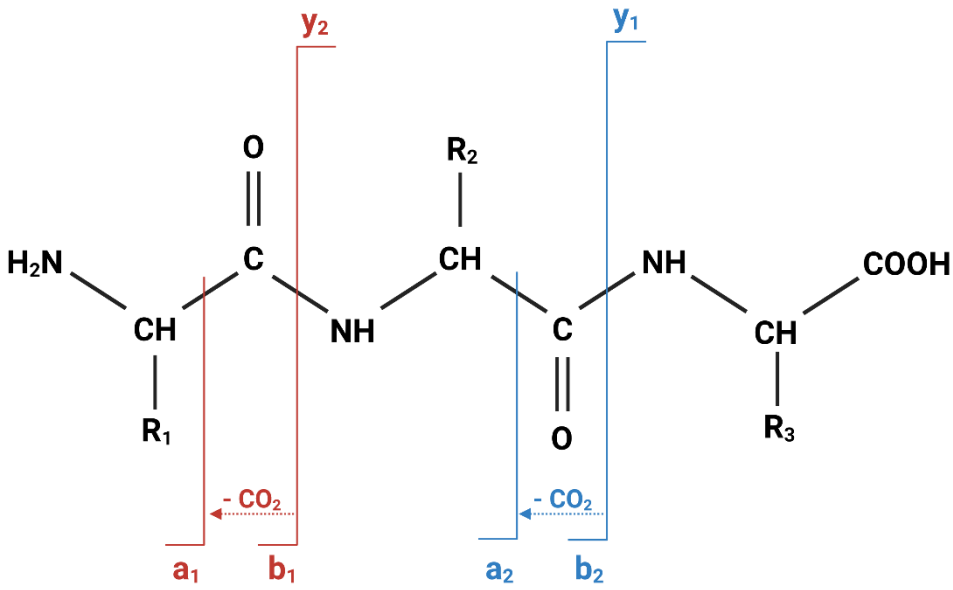

Figure 4. Tripeptide backbone fragmentation patterns using collision-induced dissociation (CID). *This type of fragmentation mostly yields y- and b-ions, and to a lesser extent, a-ions (derived from b-ions after the loss of CO_2) and fragments with losses of ammonium and H_2O. Figure was adapted from De Graaf et. al., 2022 (156) and created with BioRender.com.*

There are two major modes of data acquisition. With data-dependent acquisition (DDA), only the preselected precursor ions or ions with the most intense MS signals are isolated and fragmented. DDA is known to produce high-quality fragmentation spectra owing to the active isolation of precursor ions prior to fragmentation. This data-acquisition mode comes at the cost of a reduced acquisition rate, which often results in missing data and prolonged analytical gradients (157). The second data acquisition mode is data-independent acquisition (DIA), where all ions eluted at the same time are fragmented by altering the non-fragmenting and fragmenting collision energies, and the fragments are matched with the precursor ion based on their chromatographic MS peak. This approach minimises the likelihood of missing data but generates complex fragmentation spectra that are often caused by the simultaneous fragmentation of closely eluting precursor ions in samples containing an abundance of analytes, such as fermentation samples.

Conventional LC-MS can be enhanced by ion mobility (IM) separation (158). This technique generates an electric field and measures the effect of this electric field on ions moving through a gas phase. IM separations that use a “trap–release” mechanism can be used to measure the ions’ collisional cross section (CCS), which can be used as an additional identification qualifier and improve the spectral clarity.

1.3.2 MS data processing

The procedure for MS data processing can be divided into two approaches: targeted and non-targeted. A prerequisite for targeted data analysis is that the analyte of interest is known. Thus, the LC-MS data is searched for the theoretical mass of the peptide in question at the corresponding retention time to determine its presence. However, this approach is not applicable to peptide analysis of fermentation samples of natural

matrices, where the peptide composition is unknown. In such cases, a non-targeted approach is more suitable.

In the non-targeted approach, the precursor ion *m/z* is matched to that of the possible (theoretical) peptides. It is important to consider the mass accuracy of a mass spectrometer to avoid missing or false annotations. To determine which annotation is correct, the fragmentation spectra are compared with the theoretically possible fragments for each annotation. Although this analysis can be performed manually, fermentation samples generate complex fragmentation spectra, making the process time-consuming and labour-intensive. Alternatively, software can be used to return the fragments of each option.

Two software platforms were used in this thesis to annotate peptides in the fermentation samples. UNIFI (Waters Corporation, Milford, MA, USA) makes all possible matches between precursor ions and one or more target protein sequences, and reports the best match for each annotated peak. Criteria must be set to obtain a reliable and repeatable list of identified peptides. These criteria are mass error tolerance, minimum peak intensity for both the precursor ion and its fragments, and the minimum number or percentage of fragments that match the annotation. The other software used was Progenesis QI (Nonlinear Dynamics, Milford, MA, USA). This program allows for the identification of short peptides using a library search. The ChemSpider peptide database is an example of such a library, which contains an *in silico* database of di- to tetrapeptides. The search is performed using criteria similar to those described for UNIFI. The best match is then manually selected from a list of possible annotations, based on criteria on the fragmentation and overall score that Progenesis QI has assigned to the annotation, as well as possible foreknowledge of the identity of the peptide.

1.3.3 Quantification of peptides

Quantification is the final and the most challenging stage of peptide analysis. Mass spectrometry is not a quantitative technique. However, by using the ratio of the MS signal intensity between an internal and external standard, it can be calibrated to provide a precise concentration value for a specific analyte of interest. This approach for peptide quantification is complicated by the occurrence of many ions generated from single peptide ionisation. Furthermore, the signal intensity also depends on the ionisation efficiency of the peptide. Even when the analysis is performed under standardised conditions, the ion intensities can still be affected by ion suppression, matrix effects, and day-to-day variations (159–161). In peptide analysis of fermentation samples, it is not feasible to synthesise a standard for each possible peptide (to normalise intensities for every peptide of interest) because of the high cost and complexity of the sample.

Alternatively, label-free quantification approaches which do not require extensive sample preparation or standards have also been explored. One such method uses the UV absorbance of peptides by coupling a UV absorbance detector between the LC and MS. The UV peak areas can be converted to absolute concentrations based on the Lambert–Beer law, which requires the molar extinction coefficient of a peptide (162). UV-based peptide quantification has been successfully used in numerous studies, mainly to investigate simple protein hydrolysates (151,163–171). The main drawback of this method is that, in complex mixtures, the UV peaks are almost never baseline-separated. In this case, it has been suggested that one UV peak should be divided over multiple peptides using the ratio of the MS intensities of the co-eluting peptides, assuming that

co-eluting peptides have similar chemical properties and thus have similar ionisation efficiencies (151,162). While this might be true for longer peptides (over five amino acids in length), shorter peptides have been demonstrated to have vastly different ionisation efficiencies (up to 5000 times) (172). Attempts have been made to estimate the ionisation efficiencies of short peptides computationally. However, further research is required to make these models more accurate (172). Therefore, UV-based peptide quantification in complex fermentation samples is not applicable for studying peptide uptake by yeasts.

Considering the complexity associated with quantitative peptide analysis, relative quantification is the most viable option for studying peptide uptake by yeast. In this approach, a normalised sample load is combined with a spike consisting of one or more nonendogenous compounds to account for experimental variations. By normalising the response values against the spike standard, the relative abundance of the same analyte could be determined over different samples in the same run. While the quantitative accuracy of this method is highly limited, the use of relative abundances still allows for making observations of statistical importance for individual peptides between different samples.

2 The Aims of this Dissertation

The overall objective of this dissertation is to further deepen the understanding of the role of oligopeptides as a nitrogen source to yeast. To achieve this goal, the thesis is divided into four studies:

1. Development of an LC-MS-based screening methodology to investigate oligopeptides consumption in complex matrices by yeast during fermentation;
2. Elucidation on the intraspecific variability between wine strains in their response to nitrogen supplementation with peptides and in their peptide uptake capability;
3. Elucidation of the role and specificity of peptide transporters of the Fot and Opt family on complex, oligopeptide-rich synthetic media;
4. Elucidation of the role of oligopeptides in an industrial Scotch grain whisky fermentation process as a practical use case.

3 Materials and Methods

An LC-MS methodology was developed to analyse the peptide composition in a bovine serum albumin (BSA) proteolytic digest (peptide mapping, Study 1) and to monitor the relative concentration changes of these peptides during fermentation in a synthetic medium to characterise the peptide uptake capability by different yeast strains (Study 2) and elucidate the role of yeast oligopeptide transporters of Opt and Fot family (Study 3). The method was further adjusted for analysis of peptides and their consumption by yeast in real fermentation matrices containing several dominant proteins with known amino acid sequences (Study 4). The respective publications from the given studies are outlined in Table 4. The materials and methods refer to these studies by their respective study number (1 to 4).

Table 4. Studies and related publications included in this thesis. *The materials and methods refer to these studies by their respective study number (1 to 4).*

Study	Topic	Deliverables	Reference
1	LC-MS method development for peptide analysis	Method for peptide mapping	Publication I
2	Intraspecific variety in peptide uptake by yeast strains used for winemaking	Peptide consumption kinetics by different wine yeast strains	Supplementary study *(same procedures as Study 1)*
3	Characterisation of oligopeptide transporters in wine strain *Saccharomyces cerevisiae* 59A	Elucidation of the role and specificity of different peptide transporters	Publication II
4	Role of peptides as the nitrogen source for yeast in industrial Scotch grain whisky fermentation	Elucidation of the role of peptides in a complex, industrial fermentation matrix	Publication III

3.1 Preparation of the BSA hydrolysate

Bovine serum albumin (BSA) was enzymatically hydrolysed to produce the peptide source for yeast fermentation. For this purpose, the industrial protease COROLASE®7089 (AB Enzymes, Darmstadt, Germany) was selected based on its optimal endoproteolytic activity (153). To concentrate the yeast assimilable peptide fraction in the hydrolysate, it was filtered with a Vivaflow®200 Hydrosart crossflow cassette (Sartorius, Göttingen, Germany). In Studies 1 and 2, only a 10 kDa filtration step was applied. To produce the peptide source for studies 3 and 4, the 10 kDa fraction was subsequently filtered with a 2 kDa cut-off filter cassette. The filtered fractions were characterised on their content of free- and bound amino acids (as a quantitative estimate of the concentration of oligopeptides) as well as for the peptide composition (see below). The ≤1 kDa fraction of the hydrolysate used in Studies 1 and 2 was used to calculate the required amount of hydrolysate to add to the fermentations. In Studies 3 and 4, the whole hydrolysate (≤ 2 kDa) was considered in these calculations. The exact procedure for the preparation of the BSA hydrolysate for each study can be found in the corresponding publications (Table 4).

3.2 Synthetic media

Several synthetic media with varying nitrogen compositions were utilised in this thesis to characterise yeast strains on their peptide uptake capability. The composition and concentration of nitrogen sources can be found in Table 5. The synthetic grape musts (SGM) in Studies 1 to 3 were based on the MS300 medium reported by Salmon and Barre (173), but prepared with a reduced nitrogen content coming from amino acids and ammonia to which peptides were added as the additional nitrogen source. The synthetic whisky mash (SWM), used in Study 4, was designed based on the SGM. Citric and malic acids were excluded from the medium, and the sugar concentration, free amino acid ratio, and pH were adjusted to mimic grain whisky mash, based on measurements on an industrial mash sample. A more detailed description of the different media used for one study can be found in the introduction to each study in the Results and Discussion section.

Table 5. Synthetic media used in this thesis. *The letters in the medium code represent the available nitrogen sources: N = ammonium (NH_4Cl); D = ammonium (DAP); A: amino acids; P: peptides. The number in the medium code reflects the initial nitrogen concentration of the medium.*

Study	Medium code	Description	Purpose
Study 1, 2 *(synthetic grape must)*	**NA115**	Nitrogen-limited medium with only amino acids and ammonia (115 mg N/L)	Control
	NAP250	NA115 supplemented with peptides (+135 mg N/L)	Nitrogen supplementation with peptides
Study 3 *(synthetic grape must)*	**NA100**	Nitrogen-limited medium with only amino acids and ammonia (100 mg N/L)	Control
	NAP200	NA100 supplemented with peptides (+100 mg N/L)	Nitrogen supplementation with peptides
	P200	Same nitrogen level as NAP200, but supplied solely from peptides	Peptides as a single source of nitrogen
	P200-SO_4^{2-}	P200 with an 87% reduction of sulphur content	Stimulation of *OPT1* gene expression
Study 4 *(synthetic whisky mash)*	**AP230**	Medium with amino acids and peptides (230 mg N/L)	Control
	P230	Same nitrogen level as AP230, but supplied solely from peptides	Peptides as a single source of nitrogen, control for supplementation experiments
	PD300	P230 with supplementation of DAP (+70 mg N/L)	Nitrogen supplementation with ammonia
	PP300	P230 with supplementation of more peptides (+70 mg N/L)	Nitrogen supplementation with peptides

3.3 Yeast strains

All strains used in Studies 1, 2 and 4 were obtained from Lallemand Inc. (Montreal, QC, Canada). Due to commercial reasons, the strain names in Studies 2 and 4 have been anonymised.

The oligopeptide transporter knockout strains derived from *S. cerevisiae* strain 59A (a haploid variant of the commercial wine strain EC1118), used in Study 3, can be found in (Table 6). Because 59A does not natively express *FOT3*, a strain expressing the *FOT3* gene from another wine yeast strain was also included.

Table 6. Oligopeptide transporter knock-out/knock-in strains created from Saccharomyces cerevisiae strain 59A (a haploid derivative of wine strain EC1118) used in Study 3.

Strain name	Genotype	Purpose	Reference/ Source
59A (wt)	*MATa ; ho; AMN1::loxP*	Wild-type strain; control	Marsit et al., 2015 (10)
PepKO	*MATa ; ho; AMN1::loxP; FOT1-2::loxP-kanMx-loxP; dal5; opt1; opt2*	Control, complete KO for oligopeptide transport	Becerra-Rodríguez et al., 2021 (12)
Opt1	*MATa ; ho; AMN1::loxP; FOT1-2::loxP-kanMx-loxP; dal5 ;opt2*	Functionality of Opt1	Berg et al., 2023 (174)
Opt2	*MATa ; ho; AMN1::loxP; FOT1-2::loxP-kanMx-loxP; dal5 ;opt1*	Functionality of Opt2	Berg et al., 2023 (174)
opt1Δ	*MATa ; ho; AMN1::loxP; opt1*	opt1 KO	Berg et al., 2023 (174)
opt2Δ	*MATa ; ho; AMN1::loxP; opt2*	opt2 KO	Berg et al., 2023 (174)
Fot1	*MATa ; ho; AMN1::loxP; FOT1-2::FOT1 ; dal5; opt1; opt2*	Functionality of Fot1	Becerra-Rodríguez et al., 2021 (12)
Fot2	*MATa ; ho; AMN1::loxP; FOT1-2::FOT2 ; dal5; opt1; opt2*	Functionality of Fot2	Becerra-Rodríguez et al., 2021 (12)
Fot3	*MATa ; ho; AMN1::loxP; FOT1-2::FOT3 ; dal5; opt1; opt2*	Functionality of Fot3 (From *S. cerevisiae* strain K1)	Becerra-Rodríguez et al., 2021 (12)
Fot1Fot2	*MATa ; ho; AMN1::loxP; dal5 ; opt1 ; opt2*	Functionality Fot1 and Fot2 together	Becerra-Rodríguez et al., 2021 (12)
fot1fot2Δ	*MATa ; ho; AMN1::loxP; FOT1-2::loxP-kanMx-loxP*	fot KO / Functionality of Opt1 and Opt2 together	Becerra-Rodríguez et al., 2021 (12)

The procedure for the preparation of the inoculum for each study can be found in the corresponding publications (Table 4).

3.4 Fermentations

3.4.1 Fermentations on synthetic media

All fermentations on synthetic media (200 mL in Studies 1 and 2 or 100 mL in Studies 3 and 4) were performed at 24°C in 250 mL (Studies 1 and 2) or 100 mL (Studies 3 and 4) Pyrex™ bottles equipped with a GL45 open-top PBT screw cap and PYREX™ Media Bottle Septum (Corning Inc., Corning, NY, USA). A gas outlet was installed to prevent overpressure by piercing the septum with a Sterican® Ø 0.8 x 40 mm single-use hypodermic needle (B. Braun, Melsungen, Germany) attached to a Millex-FG 0.2 µm hydrophobic PTFE filter (Merck KGaA, Darmstadt, Germany).

The specific production rate of CO_2, monitored gravimetrically, was used as the main indicator of the fermentation progress. Samples (2 mL) were collected at varying time points, depending on the experimental setup and fermentation stage. The biomass density of the samples was assessed by measuring the optical density at 600 nm using an Ultrospec® 10 Cell Density Meter (Biochrom Ltd., Cambridge, UK). The samples were then centrifuged at 9600 × *g* for 10 min at 4°C in a MicroCL 21R microcentrifuge (Thermo Fisher Scientific, MA, USA) and the supernatant was filtered through a Minisart RC 0.2 µm syringe filter and subsequently stored at −20°C until further analysis. In Study 3, the biomass pellet was stored at –80°C for use in gene expression analysis.

3.4.2 Grain mash fermentations

Study 4 also included experiments on grain mash. The grain mash was obtained from a commercial Scotch grain whisky distillery and was produced from approximately 10% malted barley and 90% unmalted wheat. Fermentations (500 mL) were performed with three commercial whisky strains in 1 L Biobundle benchtop fermenters (Applikon, Delft, the Netherlands). Additionally, an uninoculated grain mash was incubated to study the background production of free amino acids and peptides due to residual proteolytic activity in the grain mash. The specific experimental conditions that were applied can be found in publication III.

3.5 Bound- and free amino acid analysis

The procedures for bound- and free amino acid analysis for each study can be found in the respective publications (Table 4).

3.5.1 Peptide-bound amino acid analysis in grain mash samples

The removal of reducing sugars from the grain mash samples was necessary to avoid amino acid losses during total amino acid (TAA) analysis. A combination of high temperature and acidic conditions is applied during TAA analysis, which facilitates the Maillard reaction between reducing sugars and amino acids. Reducing sugars were removed by fermentation with the peptide transporter knock-out (PepKO) strain which allowed to preserve the peptide composition (Table 6). The specific procedure can be found in publication III.

3.6 Peptide analysis

Peptides were analysed using ultra-high-pressure liquid chromatography ion mobility separation-enabled high-resolution mass spectrometry (UHPLC-IMS-HRMS). Prior to injection, any compound that would precipitate from the sample in organic solvents was removed by mixing the samples 1:1 with either MeCN (Studies 1 and 2) or MeOH (Studies 3 and 4) and subsequent centrifugation. In the case of grain mash, the samples were mixed 1:9 with MeOH. The specifics of the sample preparation, UPLC procedures and tuning of the Vion IMS-QTof Mass Spectrometer (Waters Corporation, Milford, MA, USA) for each study can be found in the respective publications (Table 4).

3.7 Gene expression analysis

In Study 3, the expression levels of the peptide transporter genes *DAL5*, *OPT1*, *OPT2*, *FOT1*, and *FOT2* were analysed in technical duplicates. RNA was extracted using the phenol-chloroform method. The resulting total RNA samples were treated with a gDNA Removal Kit (Jena Bioscience, Jena, Germany) and quantified using the Invitrogen Qubit RNA Broad Range (BR) Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was obtained from 1 µg of RNA using a FIREScript RT cDNA synthesis KIT (Solis BioDyne, Tartu, Estonia). For RT-PCR, 5× HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) was used. RT-PCR was conducted using a qTOWER G^3 system (Analytik Jena AG, Jena, Germany). A list of the primers used is shown in Table S2. Gene expression values were normalised to those of the housekeeping gene ACT1.

3.8 Data treatment and statistical analysis

Data was treated and analysed using R v4.3.2 (R Core Team 2023) and RStudio (RStudio Team 2023). All data in Studies 2, 3 and 4 were visualised using ggplot2 (175).

3.8.1 Fermentation kinetics

The growth rate and maximum cell population in the synthetic media were estimated by fitting the optical density curves with the summarizegrowth R-function from the GrowthCurver package (176). The significance of the analysis of variance between the mean values was assessed using Tukey's HSD method.

3.8.2 Peptide analysis

Peptide mapping for peptide analysis was conducted according to the method described in publication I (47). Peptide mapping in the grain mash samples in Study 4 was performed against five barley storage proteins and nine wheat storage proteins (Table S15). Data processing of amino acids and peptides in strain characterisation synthetic media in Studies 2 to 4 were performed as described in publication II (174).

3.8.2.1 Peptide analysis in a fully untargeted approach (Study I)

The ChemSpider data source "peptides" (PQI_CS_Peptides) was used for *de novo* identification of peptides. This database contains an *in-silico* database of short peptides (dipeptides to tetrapeptides). The precursor ion tolerance was set to 3 ppm and the fragment ion tolerance was set to 5 ppm. One or more fragment ions per peptide were required for ion matching.

A data matrix was compiled to compare the identifications obtained for di- and tetrapeptides from peptide mapping and untargeted analysis. The comparison was performed using in-house data analysis and visualisation scripts written in Python (Python Software Foundation, Wilmington, DE, USA). The identification accuracy and reproducibility assessment between peptide mapping and untargeted analysis was performed based on four categories: absolute amino acid sequence match (Absolute), amino acid sequence match while leucine (L) and isoleucine (I) were not differentiated and annotated as “J” (J Absolute), and amino acid composition match (J Composition) and peptide length match (Length).

4 Results and Discussion

4.1 Study 1: LC-MS Method Development for Peptide Assimilation Studies (Publication I)

4.1.1 Selection of protease for making the BSA hydrolysate

To date, studies on peptide consumption by *S. cerevisiae* have been focused on di- to pentapeptides, mainly in single synthetic peptide-based systems with small culture volumes (11–13,16,140). Unless a more diverse mixture of synthetic peptides is used (expensive), these experiments do not allow to study of consumption preference of peptides of varying chain length and amino acid composition in complex media. Therefore, an in-house-produced bovine serum albumin (BSA) hydrolysate as a cost-efficient model mixture of peptides for yeast studies was used in this study.

Several commercial proteases (COROLASE® 7089, 8000 and APC (AB Enzymes, Darmstadt, Germany)) were tested for their endoproteolytic activity that would allow for maximising the formation of small molecular weight (MW) peptides while minimising the formation of free amino acids (FAA) from BSA (47). The best effect was achieved with COROLASE® 7089 which produced hydrolysates with the highest small MW (smaller than 1 or 2 kDa) peptide and the lowest free amino acids content. The content of nitrogen in the form of small MW peptides in all three hydrolysates produced with COROLASE® 7089 is shown in Table 7. Based on this, the amount of BSA hydrolysate to be added to the experiments in the following peptide consumption studies was calculated.

Table 7. BSA hydrolysates used in this study. *Hydrolysates were prepared using the industrial endoprotease, COROLASE® 7089. The three hydrolysates were characterised to contain 123 di- to hexapeptides that could be followed by their consumption in publication I (Table S1), 72 di-to heptapeptides in publication II (Table S9) and 170 di- to nonapeptides publication 3 (Table S15).*

	FAA YAN mg/g	**Peptides <1kDa** mg N/g	**Peptides <2kDa** mg N/g	**Source**
Hydrolysate 1 (Studies 1 and 2)	8.1	81.4	-	Arju et al., 2022 (47)
Hydrolysate 2 (Study 3)	4	-	121.4	Berg et al., 2023 (174)
Hydrolysate 3 (Study 4)	15.6	-	114.9	Berg, Arju and Nisamedtinov, 2024 (177)

4.1.2 Optimisation of LC-MS method for peptide analysis

4.1.2.1 Reproducibility of the method

The Vion IMS-Qtof had to be tuned to be able to effectively follow peptide consumption in fermentation samples, as the default settings of the ion optics and quadrupole of the instrument initially resulted in low response of the small MW peptides, in particular di- and tripeptides. A combination of the labile ion mobility tune and a manual short peptide-specific quadrupole transmission profile resulted in an increased response of di- and tripeptides. The system performance was found to be highly reproducible as illustrated in Figure 5 (overlay of three technical replicates). The standard deviation of

the peptide retention times was below 0.1 min, and the Progenisis QI alignment score was over 95% for the 6 QC samples (pooled sample (100 µL) spiked with 20 µL of 1 pmol/µL of Hi3 E.coli STD) and over 92% for all 36 samples analysed for publication I (47).

Moreover, the starting points of all three fermentations (Section 4.1.2.2) displayed an average relative standard deviation of 5.3% for the signal intensities of the peptides of interest. The average peak width at 1/10 of peak height was 8 s. The suitable data acquisition rate resulted in a sufficient number of data points for reliable peak integration as well as for accurate tracking of the peak's lift-off, apex, and touch-down points allowing in combination with the IMS drift time for improved high-to-low energy spectra association.

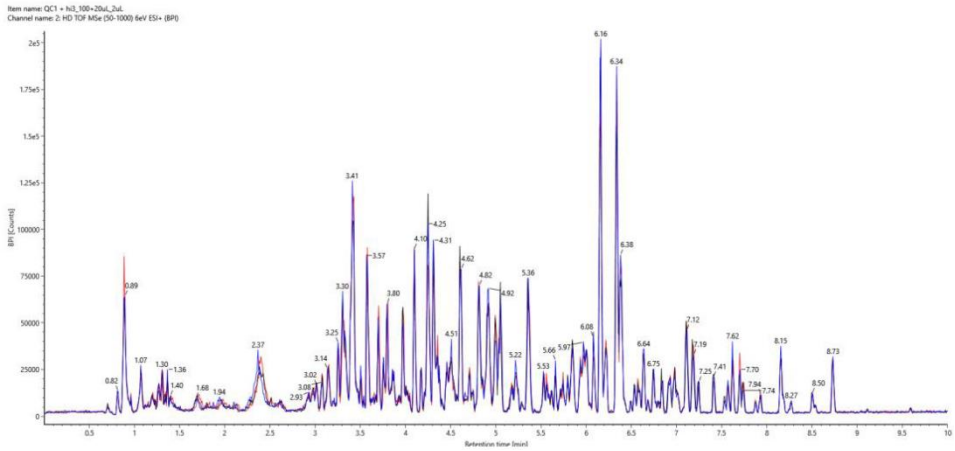

Figure 5. Overlay of the chromatogram of the triplicate injection of the QC sample.

4.1.2.2 Testing the applicability of the LC-MS-based peptide analysis methodology for yeast studies

Three wine yeast strains (Lalvin ICV Opale 2.0™, Lalvin Persy™ and Lalvin QA23™) were grown on synthetic grape must with added BSA hydrolysate (Table 8) to demonstrate the applicability of the optimised LC-MS method for analysis of peptide consumption by yeast. The results are part of a larger dataset that is further discussed in Study 2.

In total, 123 peptide candidates complying with the data analysis filtration criteria (19 dipeptides, 41 tripeptides, 31 tetrapeptides, 19 pentapeptides and 13 hexapeptides) were monitored throughout the fermentation experiments (Figure 6). The three strains demonstrated similar uptake trends of di-, tri- and tetrapeptides, however, with some strain variability. The uptake rate of di- and tripeptides was higher by Lalvin Persy™ compared to Lalvin ICV Opale 2.0™ and Lalvin QA23™, as several peptides were consumed by this strain already by 24 hours. Not all tetra- to hexapeptides were fully depleted (signal-to-noise ratio < 3:1), and their uptake ceased when the growth of cells entered the stationary phase (Lalvin ICV Opale 2.0™ and Lalvin QA23™) or slowed down remarkably (Lalvin Persy™) after 72 h (not illustrated). The differences in peptide uptake kinetics between strains were most notable for penta- and hexapeptides. Thus, while Lalvin ICV Opale™ consumed these larger peptides almost completely, their consumption by Lalvin QA23™ and Persy™ was only partial.

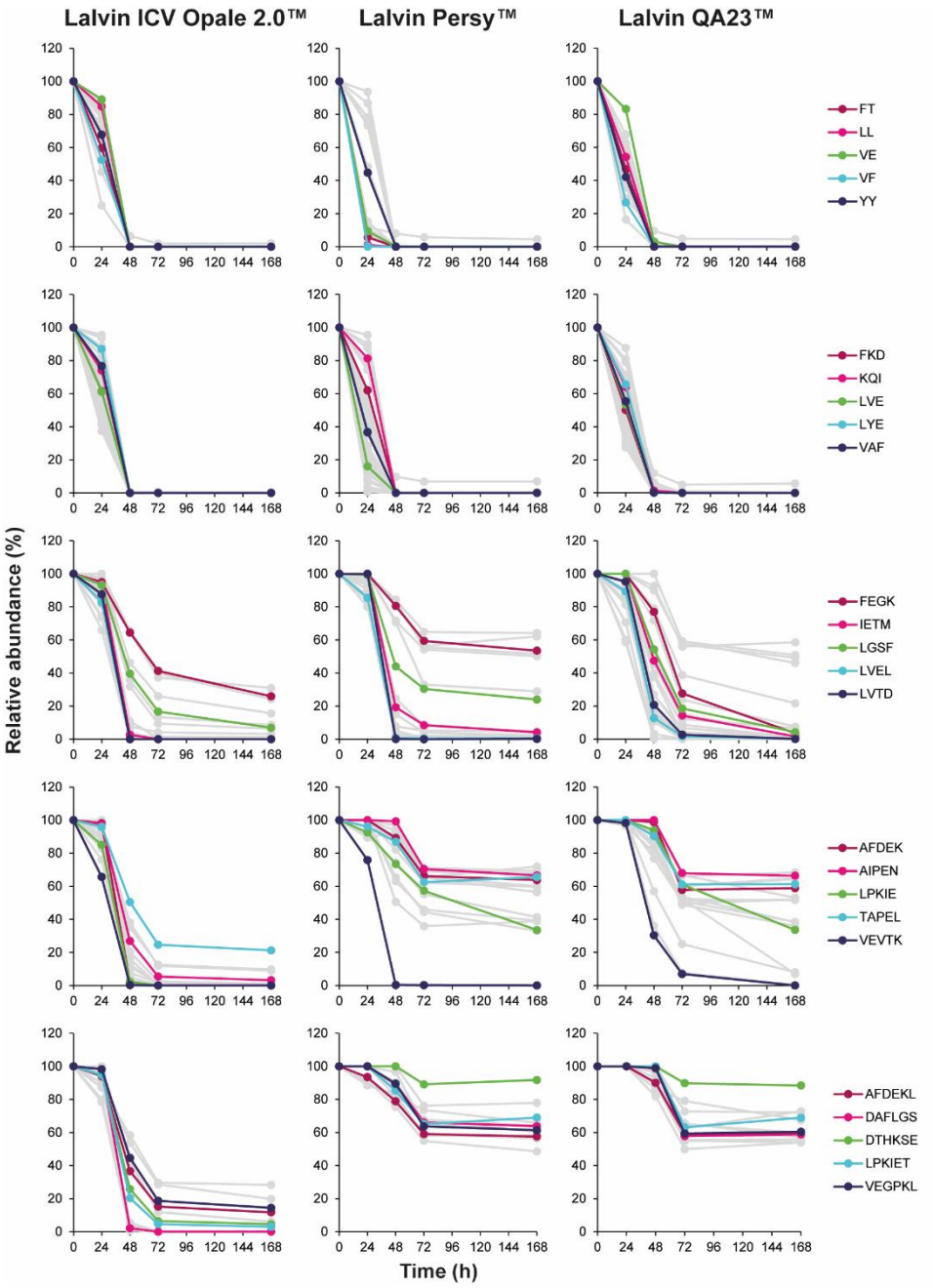

Figure 6. The relative consumption trends of 123 di- to hexapeptides in the fermentation with commercial wine yeast strains Lalvin ICV Opale 2.0™, Lalvin ICV Persy™ and Lalvin QA23™ on modified synthetic must with added BSA hydrolysate as the peptide source. *Five peptide candidates per peptide chain length group are displayed in colour to show the variation of peptide consumption within each group, the others are depicted in grey.*

The outcome of this study suggests that the optimised LC-MS method can be used to evaluate yeast strains' capability of consuming various peptides during fermentation on media that simulate a natural fermentation environment. Using high-resolution mass spectrometry (HRMS) for analysis of the fermentation samples revealed a significant number of di- to hexapeptides in BSA hydrolysate, which could be used for screening yeast strains for peptide uptake. However, due to differences in ionisation efficiency between small molecular weight peptides (172), consumption curves can only be used to track individual peptides semi-quantitatively across different samples, limiting the analysis of specific consumption rates of individual peptides by yeast.

4.1.2.3 Adjustment of the method for untargeted peptide analysis

Peptide composition analysis from a single protein digest with a known amino acid sequence allows for an additional degree of confidence in the identification accuracy. However, in many practical cases, protein sequences are not always available. In addition, peptide mixtures in industry are generally produced with crude proteases with unelucidated cleavage site specificities. Thus, an untargeted approach should be used for peptide composition analysis in natural fermentation feedstocks. To assess the identification accuracy of the untargeted workflow the results of the untargeted data analysis (using the PQI_CS_Peptides database) of the BSA hydrolysate were compared against the results obtained by peptide mapping.

Figure 7A highlights the ability of the untargeted peptide analysis approach to identify all di- to tetrapeptides in the BSA hydrolysate in comparison to peptide mapping. A trend emerged highlighting how the number of peptides assigned identically by both search engines depends on the identification specificity. Only 27.4% of the peptides were matched on the level of absolute amino acid sequence and 37.5% on the level of “J Absolute” where leucine (L) and isoleucine (I) were not differentiated and annotated as “J”. On the level of “J Composition”, where the order of the amino acids in the sequence was ignored and only the amino acid compositions of the peptide were considered, 71.4% of peptides were matched. Lastly, on the level of “Length”, where only the chain length of the peptide was considered, 90.5% of peptides were matched.

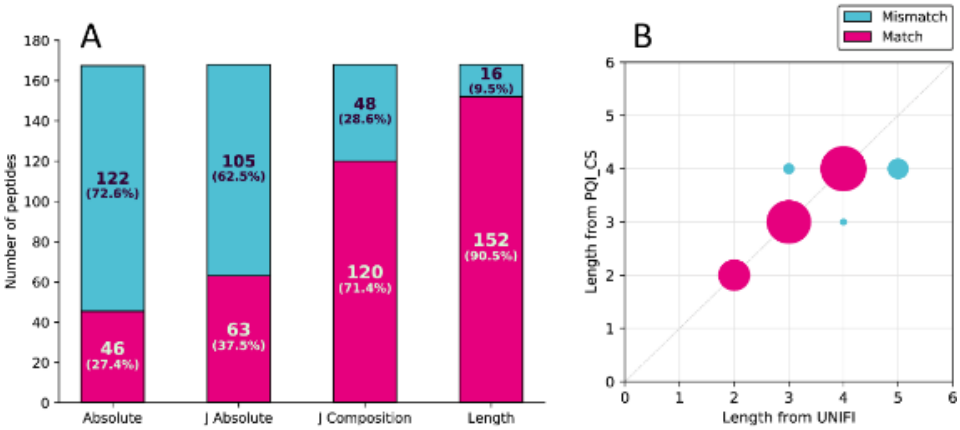

Figure 7. Comparison of the peptide analysis results in BSA hydrolysate obtained by untargeted data analysis (using the PQI_CS_Peptides database) and peptide mapping. *A) The dependence of the peptide matching specificity criteria on the peptide identification assignment discrepancy: absolute amino acid sequence match (Absolute), amino acid sequence match, where leucine (L) and isoleucine (I) were not differentiated and are annotated as "J" (J Absolute), amino acid composition match (J Composition) and peptide length match (Length). B) Peptide chain length assignment discrepancies by the untargeted method in relation to peptide mapping. Magenta circles represent peptide identification overlap between the two methods, whereas light blue circles represent mismatches between peptide identifications. Circle areas are scaled to represent the ratios between matched and mismatched peptides.*

Figure 7B displays the identification discrepancies between the two methods regarding the peptide length assignment for all di- to tetrapeptides in the BSA hydrolysate. For dipeptides, the match was 100%: all 29 dipeptides which were identified by peptide mapping were also assigned by the untargeted method. The tripeptide length assignment displayed minimal discrepancy: out of 62 tripeptides identified by the untargeted method, only one (1.6%) was assigned as a tetrapeptide by the peptide mapping. A larger discrepancy was observed for tetrapeptides: out of 77 tetrapeptides assigned by the untargeted method, three (3.9%) were assigned as tripeptides by peptide mapping, and 12 (15.6%) were assigned as pentapeptides by peptide mapping. The larger mismatch for tetrapeptides can be partly explained by the fact that the PQI_CS database contains sequences only up to tetrapeptides. While the peptide mapping approach resulted in a higher number of total identifications (not limited to di- to hexapeptides), a consistently low number (< 10%) of unique (picked only by PQI) peptides were detected by the untargeted approach.

These results suggest that the untargeted method does not allow for small peptide unambiguous absolute sequence identification, which might be crucial for studying peptide transporters' specificities. However, the method allows for peptide identification at the level of peptide length ("Length", 90.5%) and amino acid composition, when isoleucine or leucine are not differentiated ("J Composition", 71.4%), suggesting its potential for characterisation of the composition of unelucidated natural peptide-containing fermentation matrices, such as grain mash, wort, or grape must. However, due to the limitations of the used Chemspider library, the untargeted approach does not provide information on peptides larger than tetrapeptides, for which the most intraspecific variation was observed by the three wine yeast strains (Figure 6).

4.2 Study 2: Intraspecific Variety in Peptide Consumption by Yeast Strains used for Winemaking (Supplementary Study)

Eighteen *Saccharomyces cerevisiae* wine strains were characterised for their ability to take up peptides to identify the intraspecific variety for this ability using the developed peptide analysis workflow. Fermentations were conducted on synthetic grape must (SGM) containing 210 g/L of fermentable sugars consisting of glucose and fructose in equimolar concentrations and under two nitrogen supplementation conditions. The first medium (NA115) contained 115 mg N/L in the form of NH_4^+ and FAA (Table 8), while the second medium (NAP250) contained an additional 135 mg N/L from BSA-derived peptides with an MW ≤ 1 kDa (Table 8), supplied from hydrolysate 1 (Table 7).

Table 8. The composition and concentration of N sources in the synthetic grape must used in Studies 1 and 2. *The two media contained 210 g/L of glucose and fructose at equimolar concentrations. All values are displayed in milligrams of nitrogen per litre. Free proline was not considered, as it is poorly assimilated by yeasts during fermentation (2,178).*

	NA115 ($\underline{N}H_4^+$, $\underline{A}$mino acids)	**NAP250** ($\underline{N}H_4^+$, $\underline{A}$mino acids, $\underline{P}$eptides)
Amino acids	85	85
Peptides	-	135
Ammonia	30	30
Total	**115**	**250**

4.2.1 Different strains of wine yeast display distinct fermentation kinetics in response to peptide supplementation in a nitrogen-limited medium

The addition of peptides to the medium containing initially 115 mg N/L from NH_4^+ and free amino acids improved fermentation kinetics, indicating that all the strains were able to take up peptides from their environment and use peptides as a nitrogen source. However, there were substantial differences observed between the strains (Figure 8). Thus, the maximum CO_2 production rate (Vmax) increased 2–3 times for strains LV9-18, while it was less pronounced in the case of the other strains (Figure 8A). The mentioned strains also showed the highest maximum cell population, which on average doubled for these strains with the addition of peptides (Figure 8B).

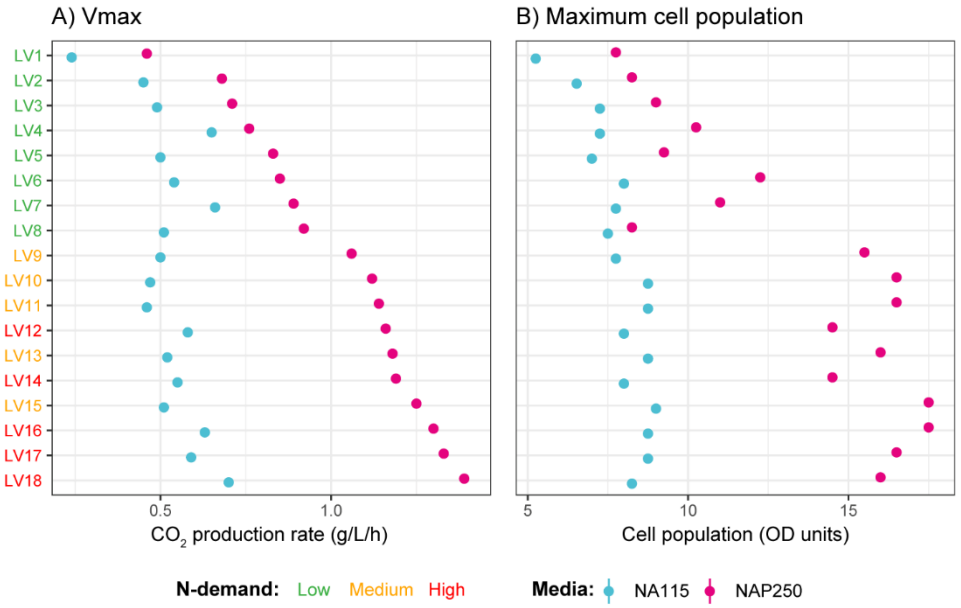

***Figure 8. Maximum CO_2 production rate (Vmax) and maximum cell population for the 18 tested wine yeast strains**. Fermentations were performed on a modified synthetic must with (NAP250) and without (NA115) added BSA-derived peptides.*

The obvious improvements in fermentation kinetics upon increasing the nitrogen content with peptides seem to follow the available data on the strains' nitrogen demand (Figure 8), with those strains having a higher N demand showing a more pronounced response in fermentation kinetics to the addition of peptides.

4.2.2 Peptide uptake capability varies between wine yeast strains

The consumption of peptides by the wine yeast strains in the medium supplemented with BSA-derived peptides was monitored during 72 hours of fermentation (Figure 9). In general, the level of peptide consumption by the strains was in accordance with their previous characterisation by the manufacturer based on their nitrogen demand. Thus, most of the strains that were characterised as high N demanding strains showing the biggest improvement in fermentation kinetics in response to the addition of peptides (LV9, LV10, LV11, LV14 LV15, LV16, LV17 and LV18) also had the highest consumption levels of di- and tripeptides, and in case of some strains (LV9, LV11, LV17 and LV18) also tetrapeptides. The exceptions were strains LV12 and LV13, which showed improved fermentation kinetics in response to the addition of peptides but had lower peptide consumption levels. The consumption of higher MW peptides (pentapeptides and larger) could also be observed by most strains, but not to the same extent as di- and tripeptides (Figure 9). In this case, the differences between the strains could not be aligned according to their nitrogen demand. Altogether, these results indicate an intraspecific variability between wine strains in their response to nitrogen supplementation with peptides and in their peptide uptake capability.

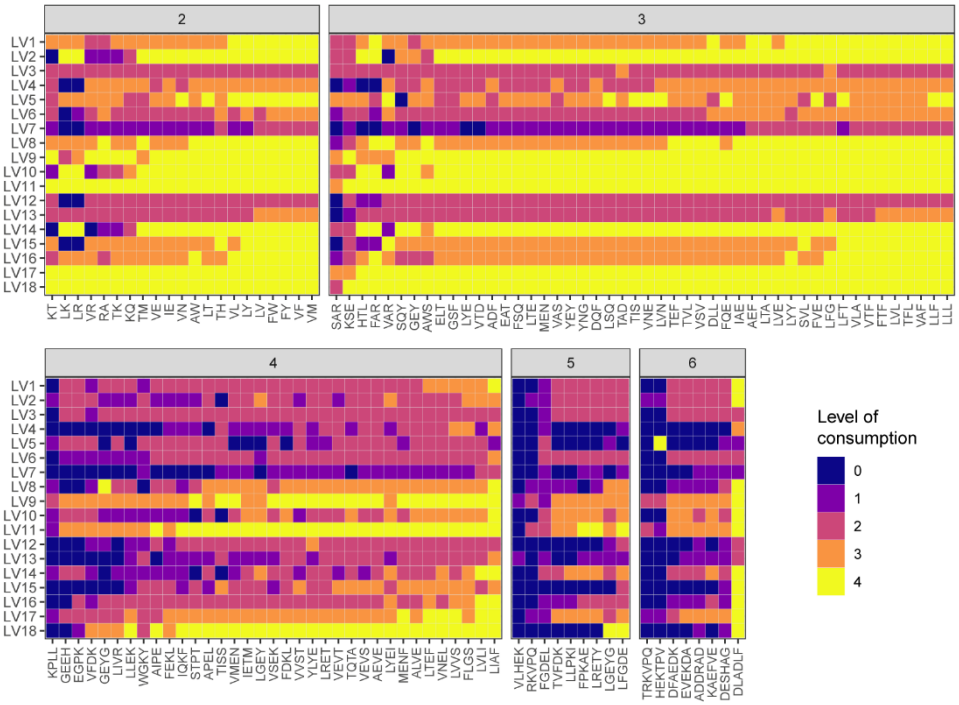

Figure 9. Relative consumption of peptides by 18 wine strains in media containing 135 mg N/L of BSA-derived peptides (NAP250). *Strains are listed in the same order as in Figure 8. Peptides are grouped according to their number of amino acid residues. The area under the curve (AUC) was calculated from the relative abundance curves of each peptide over 72 hours. The AUC values were compared to a virtual negative control (100% abundance over 72 hours). The consumption of a particular peptide is level 0 when its abundance AUC is equal to or higher than 80% of the negative control AUC; level 1, 60–80%; level 2, 40–60%; level 3, 20–40%, and level 4 when the peptide abundance AUC is lower than 20% of the control AUC.*

4.3 Study 3: Characterisation of Yeast Oligopeptide Transporters in Wine Strain *Saccharomyces cerevisiae* 59A (Publication II)

In this study, the role of yeast oligopeptide transporters of the Opt and Fot families in peptide assimilation during fermentation under different nitrogen conditions was studied. For this purpose, we worked with engineered strains derived from 59A, a haploid version of the commercial *S. cerevisiae* wine strain EC1118, which contains functional genes encoding Opt1, Opt2, Dal5, Fot1 and Fot2 oligopeptide transporters. The strains were engineered to express or lack the expression of single peptide transporter genes (Table 6) (12). Fermentation was conducted in four media containing 220 g/L of fermentable sugars consisting of glucose and fructose in equimolar concentrations, but with different conditions of nitrogen supply (Table 9). Medium NA100 contained ~ 100 mg/L YAN from NH_4^+ and FAA, medium NAP200 contained an additional ~ 100 mg N/L added from peptides (MW ≤ 2kDa), and medium P200 contained ~ 200 mg N/L delivered solely from peptides. In addition, sulphur-limited media conditions (P200-SO4) were applied in a separate experiment to promote the transcription of *OPT1* (138). Biomass density (OD_{600}) and CO_2 production were monitored during 12 days of fermentation. The consumption of peptides in NAP200 and P200 media was monitored

during the first 72 hours of fermentation, which covered the exponential and beginning of the stationary growth phases. In addition, changes in the FAA concentration in the NAP200 medium were measured during the same period.

Table 9. Composition and concentrations of nitrogen sources in synthetic grape musts used in Study 3. *The four media contained 220 g/L of glucose and fructose at equimolar concentrations. All values are displayed in milligrams of nitrogen per litre. Free proline was not considered a YAN source, as it is poorly assimilated by yeasts during fermentation (2,178).*

	NA100 (Ammonium, amino acids)	**NAP200** (Ammonium, amino acids, peptides)	**P200 and P200-SO4** (Peptides)
Amino acids	78	82	-
Peptides	-	100	8
Ammonia	30	30	200
Total	**108**	**212**	**208**

4.3.1 Nitrogen supplementation with peptides has a positive effect on the fermentation kinetics of wild-type strain 59A

In fermentations with NH_4^+ and FAA as the sole nitrogen sources (NA100), all strains displayed similar profiles of cell growth and fermentation kinetics (Figure 10). Doubling the YAN concentration by the addition of peptides to NA100 media or by providing nitrogen from peptides only (NAP200 and P200, respectively) increased the CO_2 production rate (Vmax) and the maximum cell population of most strains, resulting in at least 30% shorter fermentation time (Figure 10A, B, Figure 11A, B). This tendency was more pronounced in the strains containing at least one Fot transporter, all of which reached 40% faster fermentation times in the NAP200 and P200 media than in the NA100 medium (Figure 11B).

The exceptions to these observations were the full peptide transporter knockout strain (PepKO) and the *OPT1*-only expressing strain. These two strains showed no improvement in biomass density or fermentation time when peptides were added to the NA100 medium (Figure 11A, B). Additionally, these strains did not grow on the P200 medium, which contained only peptides. This suggests that the *OPT1*-only expressing strain, similar to the PepKO strain, was not able to utilise peptides as a nitrogen source. It should be noted that both strains showed an atypical increase in biomass density and CO_2 production rate in the later phases of the experiment in the P200 medium (Figure 10A, B) which was likely caused by the autolysis of part of the cell population and the consequential release of assimilable FAA for growth. Due to this deviant behaviour, the PepKO and Opt1 strains were excluded from the statistical analysis of growth and fermentation parameters under the P200 condition (Figure 11).

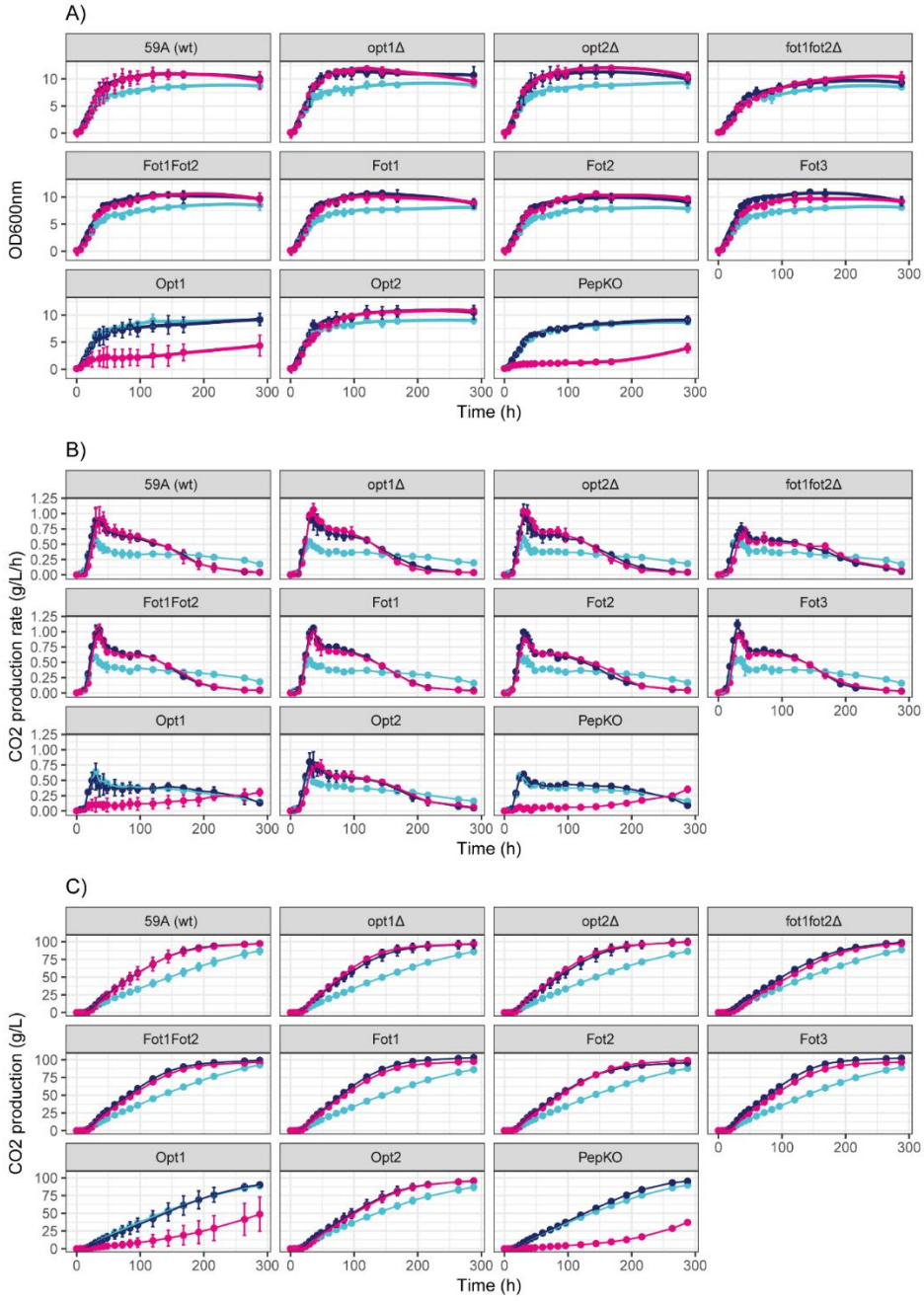

Figure 10. Growth and fermentation kinetics (represented by CO_2 production) of wild-type (59A) and its peptide transporter knockout variants under different nitrogen conditions. *A) Cell growth during fermentation. B) Rate of CO_2 production. C) Cumulative CO_2 production. The strain descriptions are listed in Table 6. The NA100 condition is represented in light blue, the NAP200 condition in dark blue, and the P200 condition in pink.*

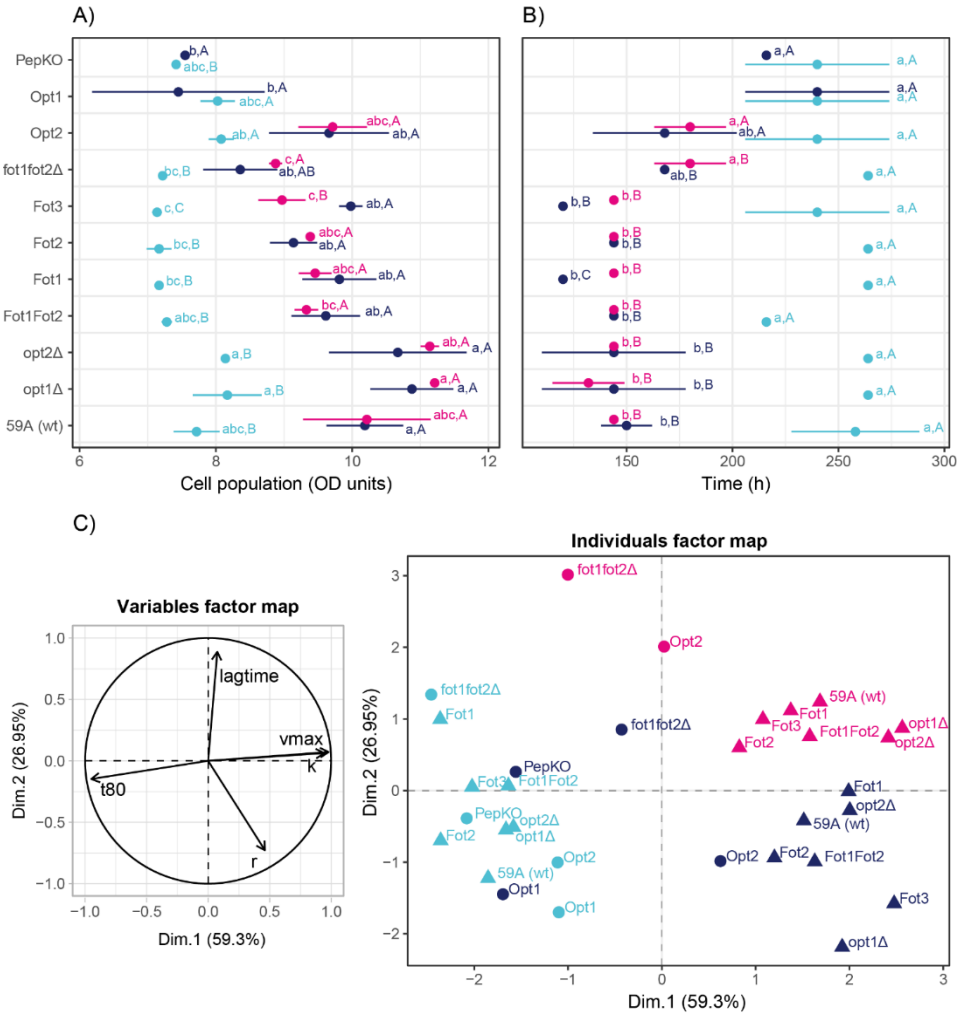

Figure 11. Growth and fermentation parameters of wild-type (59A) and its peptide transporter knockout variants under different nitrogen conditions. *The NA100 condition is represented in light blue, the NAP200 condition in dark blue, and the P200 condition in pink. Letters in maximum cell population* ***(A)*** *and time to reach 80% attenuation* ***(B)*** *denote the statistical groups from Tukey's tests ($p < 0.05$), with lower-case letters indicating different groups of strains per medium and upper-case letters indicating the groups of media for every strain.* ***C)*** *Principal component analysis of the growth and fermentation parameters of different strains in the three media. t80: time to reach 80% of attenuation; vmax: maximum CO_2 production rate; lagtime: time to produce 1 g of CO_2; k: maximum cell population; r: maximum growth rate. Triangles and circles in the individual factor map represent strains that have or do not have Fot.*

In the case of the fot1fot2Δ strain and *OPT2*-only expressing strain, an increase in the maximum cell population and a decrease in the fermentation time with the addition of peptides was observed in both the NAP200 and P200 media (Figure 11A, B); however, the obtained values were not significantly different from the values obtained in the NA100 medium. This suggests that Opt2 contributes to peptide uptake in the wild-type strain 59A but not to the same extent as Fot. These observations are further reinforced by the principal component analysis (Figure 11C), constructed from the maximum cell

population, the time to reach 80% attenuation, Vmax, the maximum growth rate, and the time to produce 1 g of CO_2 (Tables S3 through S7). Here, the *OPT2*-only expressing strain in NAP200 was the only strain not containing *FOT* that clustered together with the *FOT*-containing strains in the same medium, whereas the fot1fot2Δ strain in the NAP200 medium did not cluster with the rest of the NAP200 group. The *OPT2*-only expressing strain and fot1fot2Δ strain were more distinguishable in the P200 medium. These results again suggest that Opt2 was the only peptide transporter able to compensate for the lack of Fot in the 59A strain. However, as mentioned above, peptide import by Opt2 did not have the same impact on growth and fermentation kinetics as Fot.

In conclusion, fermentation kinetics analysis showed that wild-type strain 59A and most of its peptide transporter knockout variants were able to complete fermentation with peptides as the sole nitrogen source, demonstrating that peptides with suitable chain lengths can support yeast growth during fermentation without requiring NH_4^+ or FAA. These results reinforce the assumption that peptides can represent a powerful resource for coping with problems associated with nitrogen deficiency during fermentation.

4.3.2 Opt1 does not contribute to peptide uptake in wild type strain 59A

As mentioned above, not all 59A-derivative strains could complete fermentation with peptides as the sole nitrogen source (Figure 10 and Figure 11). These include the complete peptide transporter knockout strain (PepKO) and the strain with Opt1 as the sole peptide transporter. Opt1 was originally characterised as a tetra- and pentapeptide transporter in descendants of the reference strain *S. cerevisiae* S288C (13,16). In these strains, the transporter was overexpressed using a plasmid (13,16). Opt1 is also a glutathione (GSH) transporter, and it has been hypothesised that this function may be primary to peptide transport (15,138).

To exclude a potential incapacitating mutation of *OPT1* in 59A, the *OPT1* gene in the 59A and Opt1 strains was sequenced by Sanger sequencing, which showed that the *OPT1* gene sequence was identical in 59A and its derivative Opt1-only expressing strain (GenBank accession number: OR468328). Likewise, the *OPT1* gene sequence in the 59A and Opt1 strains was identical to the sequence in the EC1118 genome (NCBI entry: FN393075.2, positions 35292 to 37691). The *OPT1* gene in 59A/EC1118 shared 99.25% sequence identity with the reference strain S288C (NM_001181645.1) (Figure 12), which translates to 100% identity at the protein sequence level. Therefore, this result confirmed that the *OPT1* gene in 59A/EC1118 does not contain any mutations that could render the protein non-functional.

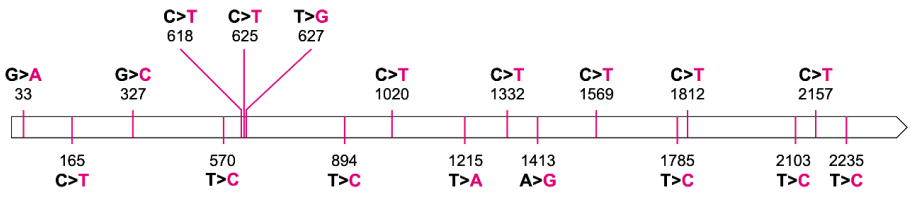

Figure 12. Variable sites in OPT1 between the S288c (black) and 59A (pink) strains.

Another option that could hinder the function of Opt1 in peptide uptake are alterations associated with gene expression. For this reason, fermentation was carried out with a reduced concentration of SO_4^{2-} salts (medium P200-SO4; Table 9). An abundance of sulphur compounds (SO_4^{2-} and sulphur-containing amino acids) in the medium has been shown to repress *OPT1* (138). Indeed, a more pronounced expression of *OPT1* was observed in all strains in which *OPT1* was present (Figure 13B). However, this did not lead to improved growth of the *OPT1*-only expressing strain in P200-SO4 medium, while other strains were still able to grow (Figure 13A). The fact that *OPT1* gene expression responded positively to sulphur limitation indicates that Opt1's inability to support growth as a peptide transporter is not due to alterations associated with gene expression.

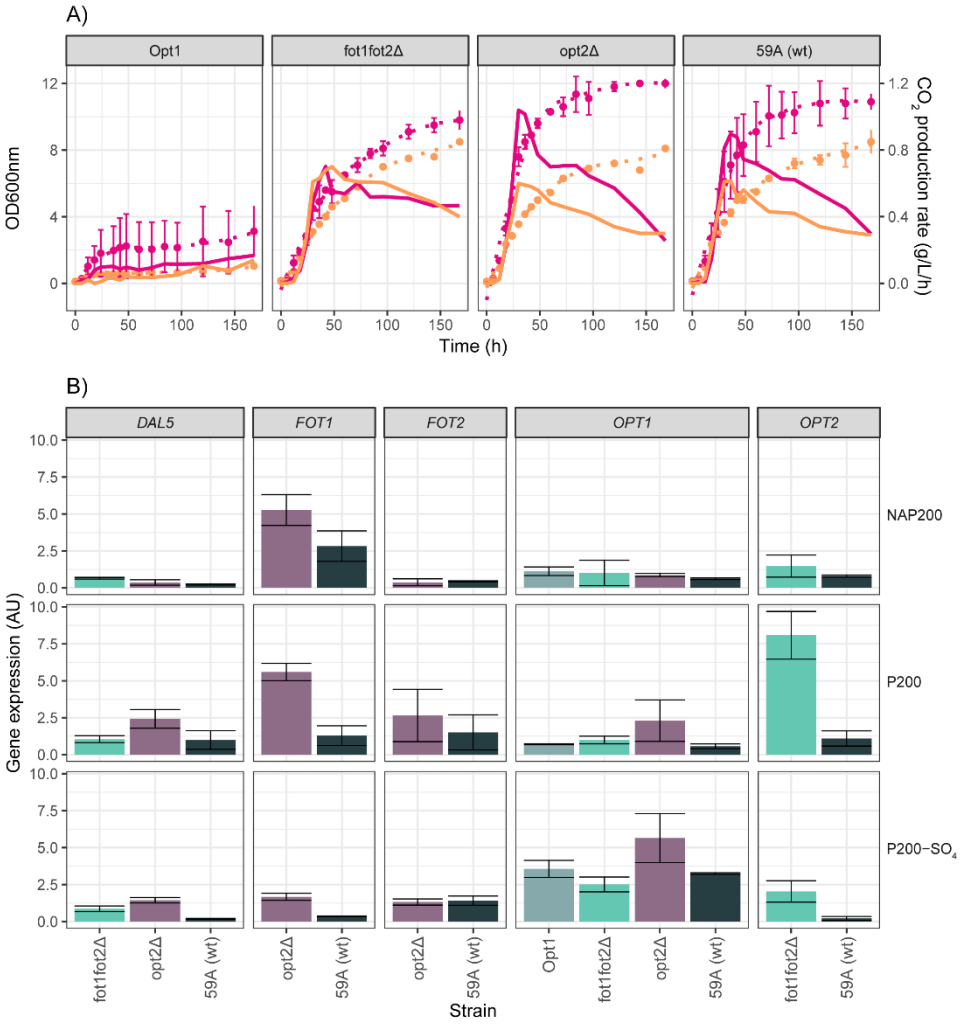

Figure 13. Effect of sulphur limitation on fermentation kinetics and expression of peptide transporter genes. *A) Comparison of biomass production (OD600, dashed line) and CO_2 production rate (straight line) between strains grown in P200 (pink) or P200-SO_4 (orange) SGM media. B) Expression of DAL5, FOT1, FOT2, OPT1, and OPT2 in OPT1-containing strains grown in NAP200, P200, and P200-SO4 media, measured at 48 hours of fermentation.*

As expected from the fermentation kinetics results, relative peptide concentration analysis showed that the *OPT1*-only expressing strain did not consume any peptides in the NAP200 and P200 media (Figure 14). Alternative to tetra- and pentapeptide uptake, it has been suggested that Opt1 is also a GSH transporter (15,138). Although conditions containing GSH were not investigated in this study, these conditions were tested on the wild-type strain 59A and the fot1fot2Δ strain in a previous study (10). In that study, the fot1fot2Δ strain showed a growth defect in fermentation with glutathione as the sole nitrogen source in comparison with the wild-type strain 59A, which does not support the role of Opt1 as a GSH transporter. Therefore, the role of Opt1 in 59A, and consequently EC1118, remains unclear.

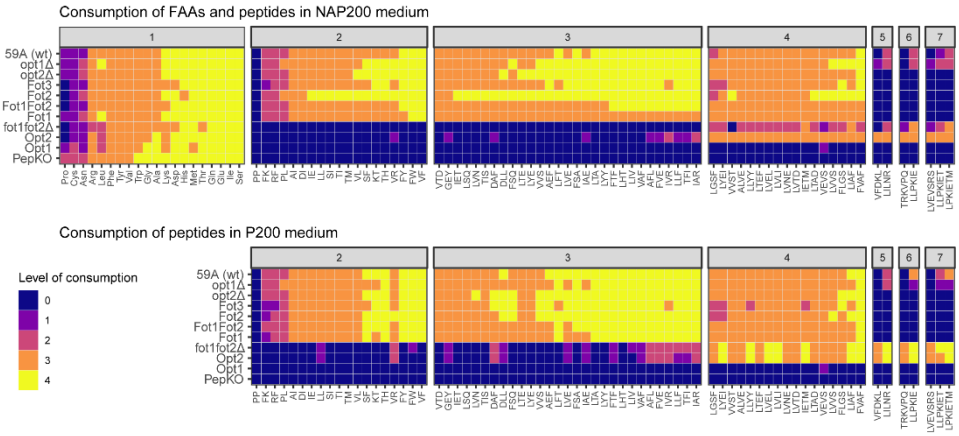

Figure 14. Consumption of FAA and peptides in NAP200 and P200 media. *The area under the curve (AUC) was calculated for the relative abundance curve of each peptide and FAA (Figure S1–Figure S4). The AUC values were compared to those of a virtual negative control (100% abundance over 72 hours). The consumption of a particular peptide or FAA was level 0 when its abundance AUC was equal to or higher than 80% of the negative control AUC; level 1, 60–80%; level 2, 40–60%; level 3, 20–40%, and level 4, when the peptide abundance AUC was lower than 20% of the control AUC.*

4.3.3 Fot1, 2 and 3 are di- to tetrapeptide transporters

The main factor contributing to the improvement in growth and fermentation kinetics was the increase in nitrogen concentration, regardless of the nitrogen source composition in the medium. Nevertheless, the most pronounced effect of peptide addition with the BSA hydrolysate was observed in strains containing *FOT* (Figure 10 and Figure 11). Fot transporters, therefore, seem to be the most important peptide transporters in strain 59A, which supports previous characterisation studies of these transporters in synthetic media, as well as in natural grape must (10,12). However, the exact concentration of yeast assimilable nitrogen delivered with different peptide chain length groups in the used BSA hydrolysate was unknown. Therefore, the effect of the peptide concentration should not be excluded.

Relative peptide concentration analysis at different points of fermentation showed that strain 59A could consume a wide variety of peptides, ranging from di- to heptapeptides (Figure 14). Fot1, Fot2, and Fot3, previously characterised as di- and tripeptide transporters (10–12), were also able to consume tetrapeptides, but did not transport penta-, hexa-, or heptapeptides (Figure 14). The strains expressing all Fot transporters (59A and Opt2Δ) showed active Fot-related peptide uptake during the early

stages of fermentation when preferable nitrogen sources (NH_4^+ and FAA) were also present (Figure S1–Figure S5). Indeed, the RT-PCR analysis showed that there was no difference in the gene expression of *FOT1* between the NAP200 and P200 media (Figure 13B). In contrast, the expression levels of *FOT2* in the 59A and opt2Δ strains were higher in the medium containing only peptides (P200) than in the medium containing peptides, FAA, and NH_4^+ (NAP200), suggesting that *FOT2* may be regulated by NCR.

Interestingly, by contrast to the regular P200 medium, the strains containing *FOT* (opt2Δ and 59A) displayed a much bigger gap in fermentation rates and biomass growth in the sulphur-limited P200-SO4 medium, compared to the fot1fot2Δ strain (Figure 13A). Based on these findings, it was proposed in Publication II that a metabolic imbalance caused by the activity of Fot may have been the reason why these strains did not perform the same way as in P200 (174). In hindsight, a more plausible explanation is the negative effect of sulphur limitation on *FOT* expression, particularly *FOT1* (Figure 13B), which may have led to reduced peptide uptake. Unfortunately, relative peptide concentration data is not available to confirm this hypothesis.

It must be noted that strain 59A also contained the genes encoding other peptide transporters with di- and tripeptide uptake capabilities, namely *PTR2* and *DAL5*. However, Ptr2, described as the main di- and tripeptide transporter in *S. cerevisiae*, is inactive in wild-type strain 59A due to a gene truncation (11). Comparing the peptide uptake of the fot1fot2Δ strain (containing *DAL5*, *OPT1* and *OPT2*) and the *OPT2*-only expressing strain, the dipeptide uptake activity of the allantoate and ureidosuccinate permease Dal5 could not be observed. As the focus of this study was to investigate the oligopeptide transporters of the Fot and Opt families, a separate strain containing only Dal5 was not included.

4.3.4 Opt2 is a transporter of tetra- to heptapeptides

The strains expressing *OPT2* while lacking *FOT* were also able to grow and carry out fermentation with peptides as a nitrogen source, although not to the same extent as the *FOT*-containing strains (Figure 10 and Figure 11). As stated before, the concentration of yeast assimilable nitrogen in peptides of different chain lengths were unknown, and only the relative decrease in the abundance of the identified peptides could be followed. Therefore, it was not possible to determine whether the better response to the addition of peptides by the *FOT*-containing strains was caused by a higher efficiency of Fot in internalising peptides or by a higher nitrogen content in the Fot-consumed peptide groups (di-to tetrapeptides).

Opt2 has been defined as a tetrapeptide transporter in *S. cerevisiae* (14). However, the strain with Opt2 as the sole peptide transporter preferred the consumption of tetra- to heptapeptides, while it only partially consumed a few di- and tripeptides (Figure 14). Thus, the function of Opt2 can be extended as a tetra- to heptapeptide transporter. Nevertheless, as in this work, we only analysed di- to heptapeptides it cannot be excluded that Opt2 can also take up peptides with a larger chain length.

The consumption of tetra- to heptapeptides by the *OPT2*-only expressing strain occurred within the first 36 hours of fermentation, regardless of the presence or absence of NH_4^+ and FAA (Figure S3 and Figure S4). In contrast, in the fot1fot2Δ strain (containing the *OPT1* and *OPT2* genes), in which the only functional peptide transporter was Opt2 (see in 4.3.2), the consumption of tetra- to heptapeptides was slower in the NAP200

medium than in the P200 medium. This was in correspondence with the lower expression level of *OPT2* in the fot1fot2Δ strain in the NAP200 medium than in the P200 medium and suggests, contrary to Fot1, that Opt2 oligopeptide transport activity may be negatively affected by the presence of NH_4^+ and FAA when other peptide transporters (e.g. *OPT1*) are also expressed.

4.3.5 Fot and Opt2 have complementary activities in peptide-rich media

Most FAA and di- to tetrapeptides were simultaneously and completely consumed during the first 18-36 hours of fermentation by all strains containing *FOT*. In contrast, Opt2-associated peptide transport in both the 59A and opt1Δ strains occurred only after most FAA and di-to-tetrapeptides were consumed, suggesting a preference for the consumption of di- to tetrapeptides by Fot over the uptake of longer peptides by Opt2. In addition, *OPT2* was more highly expressed in the absence of *FOT1* (fot1fot2Δ strain) and vice-versa (opt2Δ strain), suggesting a possible interplay between Fot and Opt2 activities.

Consequently, we hypothesise that there is a balance between Fot and Opt2 activity in wild-type strain 59A, where Fot-mediated di- to tetrapeptide consumption is the main system for oligopeptide import during the first stages of fermentation, of course, provided that these peptides are available in the growth environment. Due to the broad peptide length specificity of Fot, yeast can take up a wide range of nitrogen sources for fast growth, which is needed to become the dominant organism in a competitive environment, such as grape must. We suggest that Opt2 activity helps to obtain additional nitrogen from larger peptides when more readily available nitrogen sources are depleted.

In conclusion, this study highlights the importance of peptides as a nitrogen source for *S. cerevisiae* during fermentation. We demonstrated a broader peptide length specificity for Fot and Opt2 than previously reported. The results also showed that Opt2, and not Opt1, was the main tetra- to heptapeptide transporter in the *S. cerevisiae* wine strain 59A. Furthermore, the complementary peptide uptake specificities of Fot and Opt2 and their expression at different phases of fermentation, allowed yeast to consume preferred nitrogen sources in an orderly fashion. However, the fact that Opt1 as a single available peptide transporter could not support growth, together with the inactivity of Ptr2 in this strain due to a gene truncation (11), is indicative of the variability of oligopeptide uptake within yeast at the transporter level.

4.4 Study 4: Nitrogen Availability and Utilisation of Oligopeptides by Yeast in Industrial Scotch Grain Whisky Fermentation (Publication III)

Scotch grain whisky is usually produced from a mash containing approximately 90% (w/w) unmalted grains, most commonly wheat (80,179). The unmalted grains are mechanically milled to release starch from the grain's protein matrix and then mixed with process liquor, which is a combination of process water, backset, distillation condensate, and weak worts (79). The resulting grain slurry is subsequently cooked to gelatinise the starch. The cooking temperature varies based on the specific gelatinisation temperature of the starch. Nevertheless, most Scotch grain distilleries typically use much higher temperatures (95–145°C) for starch gelatinisation (79). The cooked grain slurry is then combined with milled malt slurry in a conversion tank and held at mashing

temperatures (63–65°C) for up to 30 min (79). The primary objective of this process, termed conversion, is to transform gelatinised starch into fermentable sugars (mostly maltose, glucose, and maltotriose) (80). Traditionally, wort has been separated from the grains to provide a clear fermentable liquid. However, the wort separation step has been largely eliminated in modern grain whisky distilleries and instead, the entire mash is transferred into the fermenter after cooling and pitching with yeast (79).

It is widely considered that a significant proportion of the yeast-assimilable nitrogen present in the final wort is derived from the malt. During the malting process, proteolytic enzymes are released into the endosperm to degrade grain storage proteins (i.e. hordeins, glutelins, albumins, and globulins) into oligopeptides and free amino acids (180). Many of these enzymes, including aminopeptidases and some endoproteinases, are sensitive to heat and are largely inactivated at temperatures above 55°C, used during the conversion step (78,181). However, studies have described the formation of soluble nitrogenous compounds during conversion as a result of heat-stable endoproteinases and carboxypeptidases (77,78). These proteases are inactivated only at temperatures over 70°C (78). Unlike in brewing, the mash in the Scotch grain whisky production process does not reach this temperature, nor does it contain a separation and boiling step (79–81). Consequently, in addition to the conversion step, the proteolytic activity can also be present during fermentation. In the context of Scotch grain whisky production where the malted substrate contains on average 10% of the mash, this may be crucial for supplying sufficient nitrogen for yeast to ensure good fermentation kinetics, especially as the addition of exogenous proteolytic enzymes or nitrogen supplements are prohibited in Scotland (80).

To study the uptake of peptides as a nitrogen source by three commercial whisky yeast strains, fermentation experiments were first performed on synthetic whisky mash (SWM) under different conditions of nitrogen supply (Table 10). In the first experiment, the fermentation medium (AP230) contained 130 mg N/L from free amino acids (FAA) and 100 mg N/L from peptides with a molecular weight (MW) ≤ 2 kDa. In the second medium (P230) the FAA were deficient (only 30 mg N/L), and peptides were the only substantial source of nitrogen (200 mg N/L). Finally, the effect of additional nitrogen supplementation (70 mg N/L) with either diammonium phosphate (DAP) or peptides was studied (media PD300 and PP300, respectively). The effect on the fermentation kinetics is shown in Figure 15.

Table 10. Nitrogen composition of the synthetic grain mash used in Study 4. *All media contained 150 g/L maltose and glucose (9:1, w/w). All values are displayed as milligrams of nitrogen per litre. Free proline was not considered a nitrogen source, as it is poorly assimilated by yeasts during fermentation (2,178).*

	AP230 (Amino acids + Peptides)	**P230** (Peptides)	**PP300** (Additional peptides)	**PD300** (Additional ammonia)
Amino acids	130	30	40	30
Peptides	100	200	260	200
Ammonia	-	-	-	70
Total	**230**	**230**	**300**	**300**

4.4.1 Oligopeptides can satisfy the nitrogen requirement for whisky yeast in synthetic media

Between the two synthetic media with a total N content of 230 mg N/L, containing either free amino acids (56.5% of N) and peptides (43.5% of N) (AP230) or mostly peptides (90% of N, P230), the three strains displayed only slight differences in the maximum cell population (Figure 15A), and no significant differences in maximum CO_2 production rate (Vmax, Figure 15B), and fermentation time (Figure 15C). This suggests that peptides with a suitable chain length as the sole nitrogen source can satisfy the nitrogen requirements of yeast, as was also demonstrated for wine yeast in Study 3 (Figure 11).

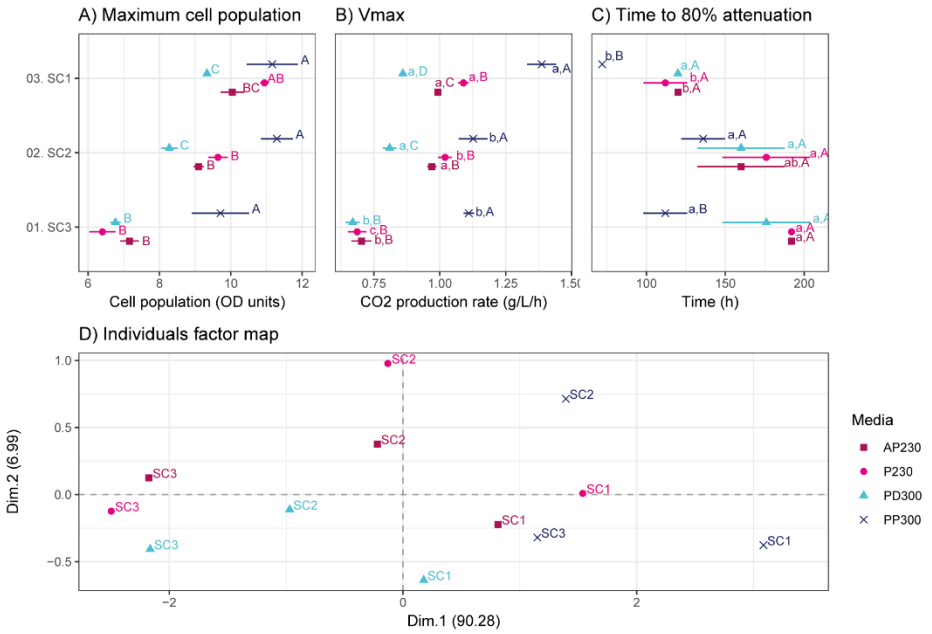

Figure 15. Cell growth and fermentation kinetics in synthetic whisky mash containing different nitrogen sources. *Letters denote the statistical groups from Tukey's tests ($P < 0.05$), with lower-case letters indicating different groups of strains per medium and upper-case letters indicating the groups of media for each strain. The "time to 80% attenuation" was chosen as not all fermentations reached 100% attenuation during the set experimental time.*

In Study 3 it was discovered that *Saccharomyces cerevisiae* wine yeast 59A, which is a haploid variant of the commercial wine strain EC1118 (10), had the ability to take up peptides with at least seven amino acids in length. This was the longest chain length that could be accurately measured for all strains in that study due to specific constraints applied in the LC-MS analysis (174). With further modifications applied to the MS conditions, peptides with higher chain lengths were included in the analysis in this study, demonstrating that the three whisky strains can take up peptides of up to at least nine amino acids in length (Figure 16).

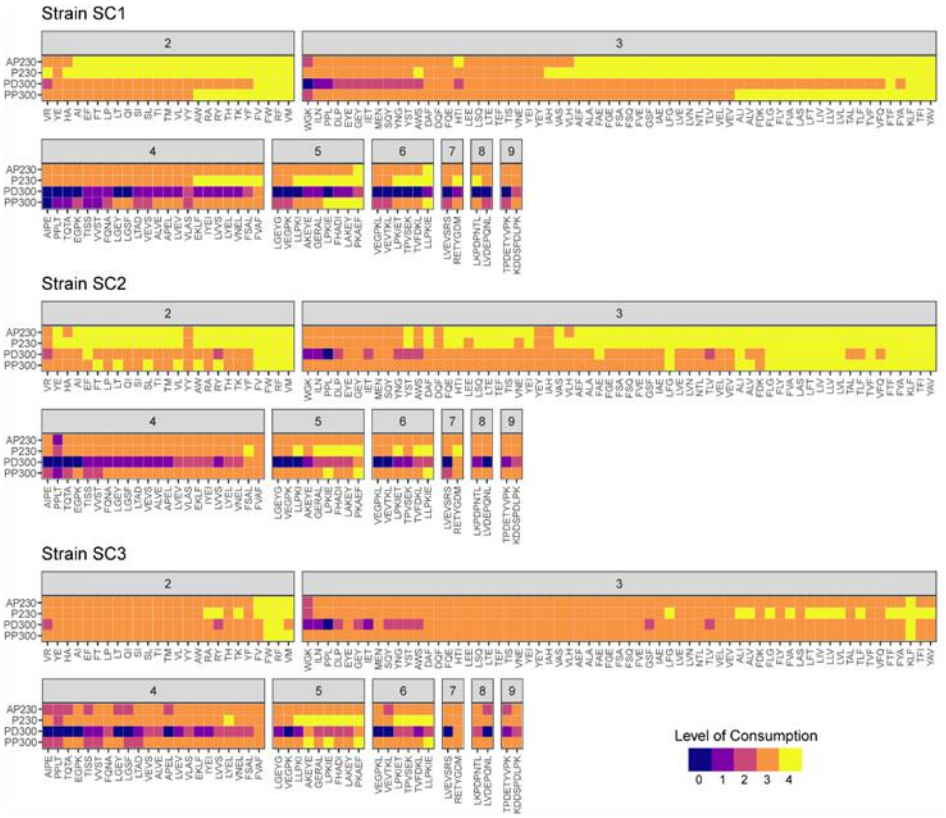

Figure 16. Consumption of peptides in synthetic whisky mash by the three whisky strains. *The area under the curve (AUC) was calculated from the relative abundance curve of each peptide. The AUC values were compared to those of a virtual negative control (100% abundance over 72 hours). The consumption of a particular peptide was level 0 when its abundance AUC was equal to or higher than 80% of the negative control AUC; level 1, 60–80%; level 2, 40–60%; level 3, 20–40%; and level 4, when the peptide abundance AUC was lower than 20% of the control AUC.*

Like the wine strain 59A in Study 3, these whisky strains took up smaller peptides first (Figure S11), with the only exception being that the whisky strains preferred di- and tripeptides, whereas in 59A tetrapeptides were also preferred (174). The simultaneous uptake of di-, tri- and tetrapeptides in strain 59A can be explained by the activity of the Fot family of peptide transporters, which have only been found in wine yeasts (10–12,174). Ptr2, previously characterised as a di- and tripeptide transporter in yeast (8), is presumably the active di- and tripeptide transporter in these whisky strains, explaining why tetrapeptides were taken up later with the other larger peptides.

Among the three whisky strains, strain SC3 exhibited the lowest fermentation performance (Vmax and fermentation time) at 230 mg N/L (AP230 and P230, Figure 15), which could be attributed to its higher nitrogen demand than that of the other strains. Therefore, the effects of additional nitrogen supplementation in the form of either peptides or by the often industrially applied ammonium salt DAP on the fermentation performance in synthetic whisky mash (SWM) were studied (Figure 15 and Figure 16).

Supplementation of the SWM base medium containing 230 mg N/L (medium P230) with peptides to bring the nitrogen level up to 300 mg N/L (medium PP300) resulted in higher biomass accumulation and CO_2 production rates in the case of all strains, and consequently, shorter fermentation times than with the medium with a lower nitrogen content (P230). This was in stark contrast to the effect of ammonium supplementation (PD300) on fermentation performance, which was neutral or even negative. These results seem counterintuitive considering the popularity of DAP in industry; however, they are in line with what has been observed in other studies. For example, Cruz et al. (2002) showed that nitrogen supplementation with a peptone resulted in higher biomass and ethanol production than ammonium sulphate (182). Another study revealed slower fermentation rates with ammonium compared to free amino acids, delivering equivalent amounts of nitrogen (82). As ammonium is considered an easily assimilable nitrogen source for yeast, questions about the biological rationale of such a phenomenon can be raised. Jiménez-Martí and del Olmo (2008) observed that gene expression of *de novo* amino acid biosynthetic pathways and those of protein synthesis were differentially reprogrammed based on the added nitrogen source (183). Thus, when ammonia was added, there was a higher expression of genes involved in amino acid biosynthesis, whereas the addition of amino acids resulted in a higher expression of genes related to protein biosynthesis. Thus, a possible explanation for the observed differences in the effect of nitrogen supplementation in our experiments is that (peptide-derived) amino acids can be directly used in protein biosynthetic processes, while ammonium must first be incorporated into amino acids through *de novo* biosynthesis, requiring cellular resources including carbon and energy (87). Under the conditions used in this study, ammonia delayed peptide uptake (Figure 16 and Figure S11), possibly through nitrogen catabolite repression (184). We hypothesise that the negative effect of ammonium supplementation on fermentation kinetics occurred due to the interplay between two factors: on the one hand, the cells needed to *de novo* synthesise all the required amino acids and on the other hand, the presence of ammonia also hinder the uptake of peptides which could otherwise supply readily usable amino acids for protein synthesis.

4.4.2 Oligopeptides play a significant role in the nitrogen supply to yeast during grain whisky fermentation

In addition to the synthetic media, experiments were also conducted on a grain mash from a Scotch grain whisky production process. The concentration of free amino acids and the ≤ 2kDa bound amino acid fraction (representing in part the yeast assimilable peptides) were analysed from grain mash fermentations carried out with the three beforementioned whisky stains and from an incubation experiment of uninoculated grain mash where the environmental conditions (decrease in pH and increase in temperature and ethanol concentration) were simulated to mimic the whisky mash fermentation (Figure 17).

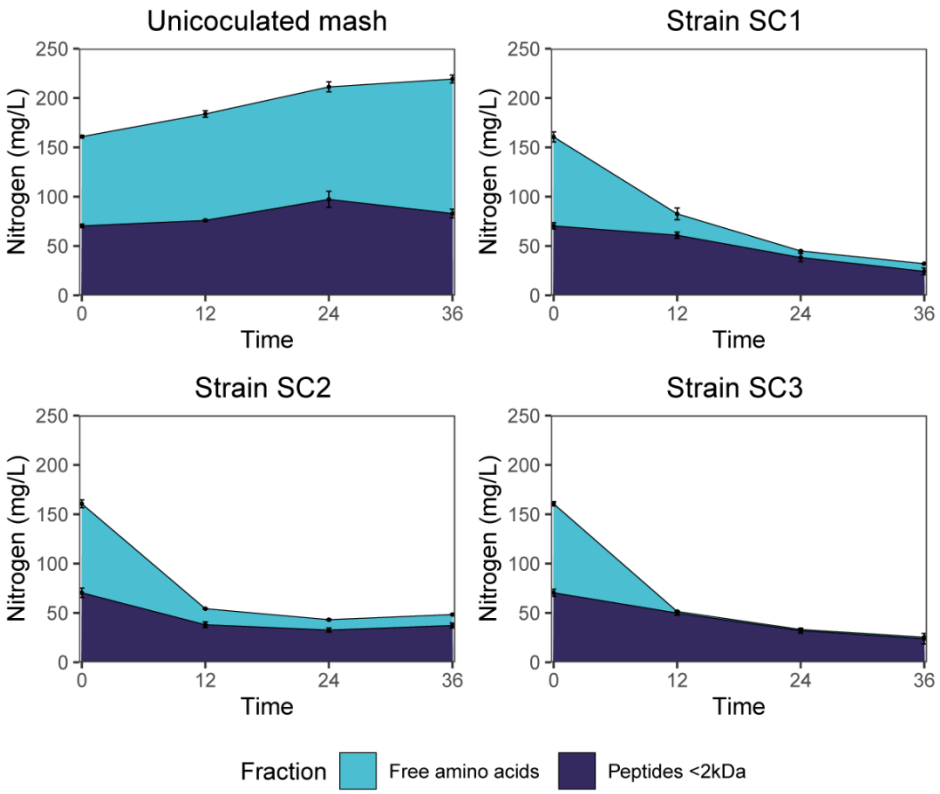

Figure 17. Changes in FAA and oligopeptide (≤ 2 kDa) content during fermentation of Scotch grain mash and uninoculated grain mash. *One experiment with uninoculated grain mash, simulating the fermentation process with changing pH and EtOH concentration was conducted to determine the background production of potentially available nitrogen sources for yeast (Table 10). Free proline was not taken into account in available N calculations as it is not or poorly assimilated by yeasts during fermentation (2,178).*

The results indicate that when a significant proportion of the mash bill is unmalted (approximately 90% in this case), the initial nitrogen content of ≈ 160 mg N/L in the MW ≤ 2 kDa fraction of the grain mash was relatively low, especially considering that not all peptides in the ≤ 2 kDa MW fraction might be fully assimilable by yeast. However, our study with the uninoculated mash incubation showed that the residual proteolytic activity that remained in the mash after conversion provided an additional ≈ 58 mg N/L (+36%) of potentially assimilable nitrogen to yeast during fermentation, of which the majority (80%) was in the form of free amino acids and 20% was contributed by peptides with MW ≤ 2 kDa. Therefore, a total of ≈ 220 mg N/L was available for fermentation, with 38% of it derived from peptides with a MW ≤ 2 kDa. This value was more in line with the required nitrogen range (150–250 mg/L) for satisfactory fermentation efficiency (3).

Indeed, all whisky yeast strains completed fermentation in the grain mash, albeit with distinct fermentation kinetics (Figure 18). Strain SC1 showed the fastest fermentation, with a rapidly decreasing CO_2 production rate by 24 hours and completing fermentation by approximately 48 hours. In contrast, with strains SC2 and SC3 the CO_2 production rate started to drop at 36 hours with fermentation completion also around 48 hours. The final

ethanol concentration was statistically in the same range for all strains (α = 0.05). The free and bound amino acid analysis in ≤ 2 kDa fraction suggested that all three strains took up on average ≈ 185 mg/L of nitrogen, of which ≈ 30% could be attributed to peptides.

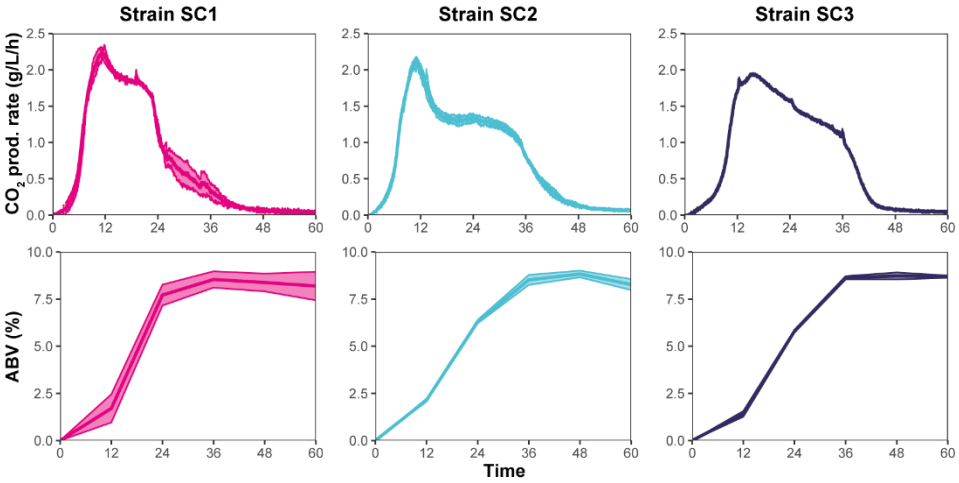

Figure 18. CO_2 production rate and ethanol concentration by volume (ABV) during laboratory-scale simulated Scotch grain whisky fermentation with three commercial whisky strains.

As it was mentioned before, very likely not all peptides in the ≤ 2 kDa fraction are utilisable by yeast; hence the question remains whether the yeasts had depleted all assimilable nitrogen sources and would have attained faster fermentation kinetics under conditions of even higher nitrogen concentration. In section 4.4.1, it was suggested that increasing the nitrogen supplementation with peptides might be more beneficial than "simple" ammonium salts like DAP. In the production of Scotch grain whisky, extra peptides could be supplied using malts with higher proteolytic strength or increasing the ratio of malted to unmalted grains. By contrast to the Scotch whisky industry, in many other jurisdictions and applications, it is also permissible to use exogenous proteases to liberate peptides from the protein fraction of the mash or to add external nitrogen sources to the fermentation process. Increasing the nitrogen content in grain fermentation by releasing more yeast assimilable peptides may thus open the door for the application of strains that otherwise are characterised as high nitrogen demanding.

In conclusion, these results indicate that the nitrogen content in the grain whisky mash was still sufficient to support complete fermentation, and peptides played a significant role in the nitrogen supply to yeast.

4.4.3 Peptide mapping in grain mash samples reveals the complex dynamics of peptide production and degradation due to malt proteases

The possibility of studying peptide consumption in unelucidated matrices using LC-HRMS with a fully untargeted approach was explored in Study 1. The outcome of that study suggests that peptide identification can be carried out in a fully untargeted approach on the level of peptide length and composition (without differentiating between the isobaric amino acids isoleucine and leucine). The primary limitation of this method is the limited size of the ChemSpider database, which only includes peptide entries up to tetrapeptides. Nevertheless, as all four studies included in this thesis have demonstrated, yeast can also take up peptides with a higher chain length than tetrapeptides. Therefore, the untargeted peptide identification approach explored in Study 1 would result in a significant amount of relevant data being overlooked. Hence, it was decided to apply the peptide mapping approach to analyse peptides in grain mash using the 15 most dominant protein sequences in barley and wheat (Table S11).

As mentioned in the introduction of this study, several heat-stable endoproteinases and carboxypeptidases originating from the malt may retain their activity in the grain mash during subsequent fermentation because the temperature of the conversion process does not exceed 70°C (77,78,185), which would cause inactivation of these enzymes (78). Indeed, the analysis of peptides in the uninoculated mash in which the temperature, pH, and ethanol concentration were changed to simulate the fermentation process, revealed the activity of both proteolytic enzyme classes of malt, resulting in the production of peptides and FAA (Figure 19A). However, this process was highly dynamic and depended on the fermentation stage. Thus, degradation of peptides occurred in the first 12 hours of incubation, as shown by the decreased relative concentration of most peptides initially present in the mash, as well as the increased concentration of free amino acids (Figure 17). The hydrolysis continued between 12 and 36 hours, first increasing relative concentrations of higher MW peptides (12 to 24 hours), which were then further degraded into smaller ones between 24 and 36 hours, suggesting an interplay between various proteases and peptidases. Because of this highly dynamic process, involving both production and degradation of peptides, limited variation in the total nitrogen concentration in the MW ≤ 2 kDa peptide fraction of the uninoculated mash was observed during the experiment (Figure 17).

The co-occurring kinetics of peptide formation and degradation due to proteolysis by malt-derived enzymes made it difficult to precisely interpret the extent of their consumption by yeast during the fermentation (Figure 19B-D). The changes in the relative concentration of smaller MW peptides (two to five AA in chain length) during fermentation in the first 12 hours were very similar to those observed in the uninoculated mash incubation experiment. In contrast, a higher number of peptides with a negative fold change in their relative concentration during later stages of fermentation when free amino acids were depleted suggests their consumption by yeast. In the case of all three strains, the net change of peptide abundancies between the start and end of fermentation suggested the consumption of di-to-pentapeptides (average fold change of 0.6 compared to 0.9 in the uninoculated mash).

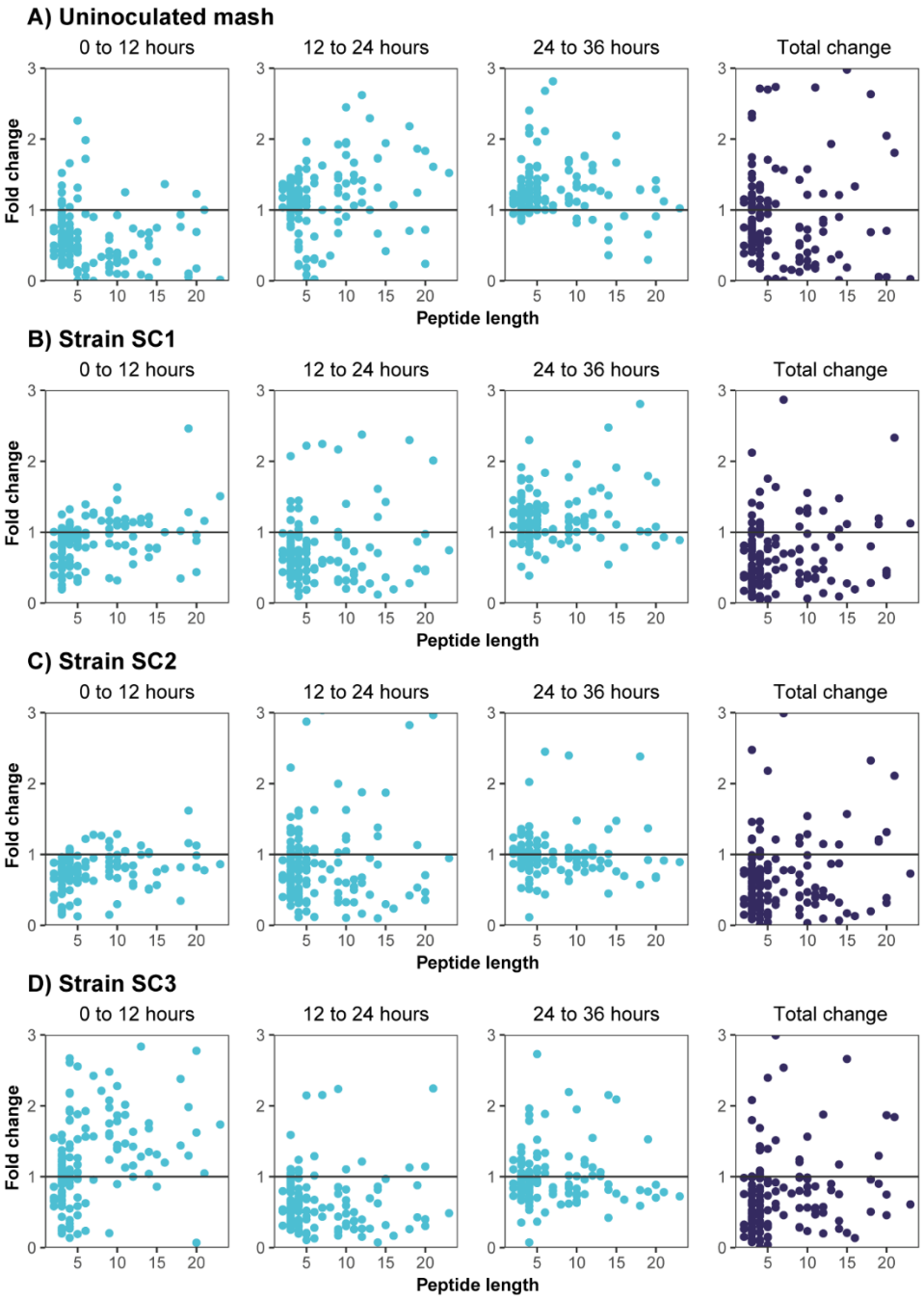

Figure 19. Fold changes in peptide abundance between samples of the uninoculated Scotch grain mash and the fermentations of the same mash with strains SC1 to 3. *The "total change" depicts the fold change between peptide candidates at the 0-hour sample and the 36-hour sample.*

The only major difference among the three yeast strains was observed in the experiment with strain SC3 (Figure 19D), in which the apparent increase in most of the identified peptides was much more pronounced during the first stage of fermentation (0 to 12 hours). The exact reasons for this are unknown but may be related to the longer lag phase observed for this strain compared to the other two strains during which the consumption of peptides did not take place yet. Bound amino acid analysis of the ≤ 2 kDa fraction in the experiment with strain SC3 did not show a substantial increase in the nitrogen content of the peptides fraction between 0 and 12 hours (Figure 17). Therefore, it was not possible to determine the significance of this observation without quantitative data on specific peptides at more frequent sampling points.

The applied peptide mapping methodology for studying the composition and relative concentration of peptides in unelucidated matrices also has its limitations. First, not all proteins in barley and wheat have been fully sequenced (186). Thus, in the present study, the obtained peptide masses were mapped only to the major (most abundant) storage proteins in barley (hordeins) and wheat (glutenins) for annotation (187,188). However, this approach was still subject to a degree of identification inaccuracy due to the high number of protein sequences (total of 15) that were included for peptide mapping, as well as potentially unknown proteins that were not included but were also degraded into detected peptides. This limits the annotation of peptides to their size (number of amino acid residues in the chain length) instead of the exact amino acid sequence. As the MS peak intensity of peptides can be highly dependent on the amino acid composition and sequence (172), only the relative quantification of peptides by fold-change analysis of individual detected peptides was performed in this study. In combination with fractionation and subsequent amino acid analysis of the obtained molecular fractions, it was possible to explain these trends more quantitatively. However, some inaccuracies may have occurred also in this case, for example, due to the retention of some smaller peptides on the cut-off filter. Ideally, the qualitative and quantitative analyses of peptides should be included in a single analysis. As discussed in the Introduction, efforts are being made to make the current qualitative MS methodologies quantitative using the UV absorbance of peptides to quantify peptide concentrations (162). However, more research is required to be able to accurately identify peptides and subsequently quantify them in complex mixtures.

To conclude, the results from study 4 demonstrate that peptides can act as a crucial nitrogen source for yeast during grain whisky fermentation. The three whisky yeast strains examined were capable of efficiently utilising a diverse range of peptides to complete the fermentation process. The production, degradation, and consumption of peptides during Scotch grain whisky fermentation is an intricate and dynamic process. Further advancement in both qualitative and quantitative peptide analysis techniques is necessary to achieve a more precise characterisation of their consumption by yeast.

5 Conclusions

This dissertation aimed to provide more insights into how *Saccharomyces cerevisiae* utilises oligopeptides as a nitrogen source. While previous research has primarily focused on the uptake of single peptides, our intention was to focus on conditions that are more representative of those in industry, with a highly diverse oligopeptide composition. The main conclusions that can be drawn from this thesis are as following:

An LC-HRMS-based peptide mapping methodology can be used for semi-qualitative characterisation (on amino acid composition and chain length level) of peptides and the relative-quantitative uptake of these peptides by yeast in complex substrate matrices.

The analysis of peptide uptake in BSA hydrolysate-containing media indicated variability between industrial wine yeast strains in peptide uptake and the resulting fermentation kinetics.

Oligopeptide transporters Fot 1–3 and Opt2 were shown to have broader peptide chain length specificities than previously characterised. The complementary peptide uptake specificities of Fot and Opt2, and their expression in wine strain 59A (a haploid derivative of the commercial wine yeast EC1118) at different phases of fermentation resulted in the consumption of peptides in an orderly fashion, primarily depending on their chain length.

Oligopeptides play a significant role in supplying nitrogen to yeast during grain whisky fermentation. Our findings indicate that the production and degradation due to intrinsic malt derived heat stable proteases, and consumption of peptides by yeast during Scotch grain whisky fermentation is a highly dynamic and complex process.

To conclude, this thesis has shown that peptides can play a crucial role in supporting yeast growth during industrial fermentations. The developed methodology presents an effective platform for future studies on peptide consumption by yeast in complex industrial fermentation matrices. Further methodology optimisation would be, nevertheless, required for absolute quantification of peptides.

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Abstract

Oligopeptides as a Nitrogen Source for *Saccharomyces cerevisiae* in Industrial Yeast Fermentations

The substrates used in industrial fermentations are often deficient in yeast-assimilable nitrogen content, which can lead to a slow or incomplete process unless external nitrogen sources, such as ammonium salts and peptones, are added. Nevertheless, many substrates contain proteins that can be broken down into peptides that yeast can utilise. However, the role of peptides as a nitrogen source for yeast has remained largely unexplored, primarily due to the challenges associated with analysis of peptide consumption by yeast in complex fermentation matrices. This doctoral dissertation aims to investigate the function of oligopeptides as a nitrogen source for yeast in conditions representative of industrial settings. The dissertation is based on three published studies.

The first study focused on developing an LC-HRMS-based screening methodology to analyse peptide uptake by yeast. The used approach employed an enzymatic digest of bovine serum albumin (BSA) as a pre-characterised peptide source with known composition in fermentations in synthetic media resembling grape must or whisky mash. The developed peptide mapping methodology served as a basis for tracking peptide assimilation by different yeast strains in the following studies.

The second study used the developed peptide mapping method for exploring the role of yeast peptide transporters in the peptide transporters knock-out mutants of *S. cerevisiae* wine strain 59A (haploid derivative of the commercial wine yeast EC1118). This study revealed that oligopeptide transporters Fot 1–3 and Opt2 have a broader peptide length specificity than previously characterised. The complementary peptide uptake specificities of Fot and Opt2, and their expression at different phases of fermentation allowed strain 59A to consume preferred nitrogen sources in an orderly fashion. Altogether, Fot and Opt2 activity assured completion of the fermentation process without necessarily requiring ammonia or free amino acids.

In the third study, the role of peptides as a nitrogen source to yeast in industrial grain whisky fermentation was researched as a practical use case. It was demonstrated that oligopeptides played a significant role in supplying nitrogen to yeast during grain whisky fermentation. Our findings indicate that the production and degradation of proteins and peptides by malt proteases, and the consumption of peptides by yeast during Scotch grain whisky fermentation is a highly dynamic and complex process.

All together, these studies contribute to a better understanding of peptide utilisation by *S. cerevisiae* and provide insights into the role of peptides as so far underestimated nitrogen source in industrial fermentations

Lühikokkuvõte

Oligopeptiidid *Saccharomyces cerevisiae* lämmastikuallikana tööstuslikes käärituprotsessides

Tööstuslikes fermentatsioonides kasutatavates substraatides on sageli puuduseks pärmile omastatava lämmastiku sisaldus, mille tulemuseks võib olla aeglane või mittetäielik (peatunud) käärituprotsess, juhul kui ei lisata väliseid lämmastikuallikaid, nagu näiteks ammooniumisoolad või peptoonid. Sellegipoolest sisaldavad paljud substraadid valke, mida saab lagundada peptiidideks, mida pärm saaks potentsiaalselt kasutada. Peptiidide roll pärmi lämmastikuallikana on aga jäänud suures osas uurimata, peamiselt pärmi peptiidide tarbimise analüüsiga seotud väljakutsete tõttu keerulistes fermentatsioonimaatriksites. Käesoleva doktoritöö eesmärk oli uurida oligopeptiidide funktsiooni pärmi lämmastikuallikana tööstuslikes tingimustes. Doktoritöö põhineb kolmel erialastes teadusajakirjades avaldatud uurimusel.

Esimene uuring keskendus LC-HRMS-põhise kvalitatiivse ja pool-kvantitatiivse metoodika väljatöötamisele, et analüüsida peptiidide omastamist pärmide poolt. Väljatöötatud meetodit kasutati veise seerumi albumiini (BSA) ensümaatilisel lõhustamisel saadud ning koostiseliselt karakteriseeritud peptiidiallikana uurimaks peptiidide tarbimist sünteetilises viinamarjavirdes. Väljatöötatud analüüsi metoodika oli järgmistes uuringutes aluseks erinevate *S. cerevisiae* tüvede peptiidide assimilatsioonivõime iseloomustamisele.

Teises uuringus kasutasime väljatöötatud peptiidide analüüsi meetodit uurimaks pärmi *S. cerevisiae* peptiiditransporterite rolli veinitüves 59A (kommertsialiseeritud veinipärmi EC1118 haploidne derivaat), kasutades vaigistatud geenidega mutante. See uuring näitas, et oligopeptiidi transporterid Fot 1–3 ja Opt2 transpordivad seniarvatust laiema peptiidahela pikkuse ulatusega oligopeptiide. Seejuures näitasime, et Fot ja Opt2 transporterite spetsiifilisus ning ekspressioonitasemed fermentatsiooni erinevates faasides on üksteist täiendavad, võimaldades pärmil tarbida eelistatud lämmastikuallikaid korrapäraselt. Kokkuvõttes tagasid Fot ja Opt2 aktiivsused käärimisprotsessi lõpuleviimise vabade aminohapete limiteerivais tingimustes isegi ilma täiendavate ammooniumsoolade lisamiseta.

Kolmandas töös uurisime praktilise näitena peptiidide rolli pärmide lämmastikuallikana tööstuslikul teraviljaviski kääritamisel. Näitasime, et oligopeptiidid mängivad teraviljaviski kääritamisel olulist rolli pärmi lämmastikuga varustamisel. Lisaks näitasid tulemused, et valkude ja peptiidide meskimisjärgne jätkuv hüdrolüüs käärituprotsessi ajal linnase proteaaside toimel ning tekkinud peptiidide tarbimine pärmide poolt on dünaamilineprotsess.

Kokkuvõttes aitavad antud doktoritöö raames läbiviidud uuringud paremini mõista peptiidide tarbimise võimekust pärmi *S. cerevisiae* poolt ning annavad ülevaate peptiidide kui seni üsna alahinnatud lämmastikuallika rollist tööstuslikes käärituprotsessides

Appendix 1

Supplemental Materials

Supplementary Tables

Supplementary tables to Study 1

Table S1. The proposed peptide sequences and observed relative consumption during fermentation with Lalvin ICV Opale 2.0™, Lalvin Persy™ and Lalvin QA23™ of the modified synthetic must. *Peptide sequences were acquired by peptide mapping to bovine serum albumin protein sequence (UniProtKB–P02769 (ALBU_BOVIN)). Additionally presented are the retention time, observed m/z, collision cross section values, as well as the averaged peptide intensities at the starting point and associated relative standard deviations.*

Peptide candidate	RT (min)	m/z	charge	CCS (Å²)	Average original intensity	%RSD	Opale (cons, %)	Persy (cons, %)	QA23 (cons, %)
AH	2.68	227.1134	1	150.41	807	4.86	99.97	100.00	99.98
AW	5.40	276.1338	1	160.10	1914	5.03	100.00	100.00	100.00
AY	4.56	253.1177	1	157.67	1470	6.44	100.00	100.00	100.00
FT	3.72	267.1335	1	163.99	5487	3.53	100.00	100.00	100.00
FW	7.03	352.1652	1	156.33	2617	5.18	100.00	100.00	100.00
FY	5.38	329.1487	1	179.32	698	3.61	97.94	95.49	95.50
JT	3.18	233.1492	1	156.95	2875	4.95	99.99	100.00	100.00
KQ	2.39	275.1709	1	160.14	1164	2.32	99.99	99.82	99.87
KT	1.40	248.1601	1	156.18	2201	8.75	99.97	99.98	100.00
LL	5.84	245.1856	1	166.83	20,475	3.04	100.00	100.00	100.00
LY	4.88	295.1648	1	171.64	4360	3.64	100.00	100.00	100.00
TF	4.78	267.1335	1	160.50	1507	3.99	100.00	99.87	100.00
TK	2.51	248.1600	1	156.18	2070	2.46	100.00	100.00	100.00
VE	2.14	247.1285	1	154.51	6009	6.03	99.96	99.98	99.95
VF	5.50	265.1542	1	164.08	5044	4.39	100.00	100.00	100.00
VN	1.37	232.1288	1	150.14	1012	2.78	99.98	100.00	100.00
VR	3.30	274.1866	1	167.20	1039	5.77	100.00	100.00	100.00
YE	3.72	311.1229	1	172.79	1091	4.24	99.67	100.00	100.00
YY	4.55	345.1441	1	184.22	5445	5.16	100.00	100.00	100.00
AEF	5.27	366.1656	1	181.69	5873	4.08	100.00	100.00	100.00
AWS	4.68	363.1658	1	181.79	2470	4.50	100.00	100.00	100.00
DAF	5.21	352.1496	1	176.71	1100	2.70	100.00	100.00	100.00
DLL	5.76	360.2124	1	189.23	2953	6.90	100.00	100.00	100.00
DQF	4.88	409.1712	1	195.14	728	3.28	100.00	100.00	100.00
ELT	4.01	362.1919	1	187.32	3543	2.99	99.98	99.62	99.92
FKD	3.53	205.1072	2	249.17	7981	3.00	100.00	100.00	100.00
FKG	3.33	176.1043	2	242.20	2685	2.41	100.00	100.00	100.00
FLG	5.65	336.1910	1	182.71	734	3.52	100.00	100.00	100.00
FQE	4.32	423.1866	1	198.49	658	3.50	100.00	100.00	100.00
FSA	4.73	324.1549	1	174.09	3223	2.23	100.00	100.00	100.00
FTF	7.23	414.2020	1	194.99	3313	6.62	100.00	100.00	100.00
FVE	4.88	394.1970	1	190.00	3242	3.60	100.00	100.00	100.00
GSF	4.64	310.1391	1	167.50	1014	6.12	100.00	100.00	100.00
IAE	3.72	332.1811	1	175.60	2444	4.33	100.00	100.00	100.00
IAF	6.63	350.2071	1	180.40	5291	6.60	100.00	100.00	100.00
IAH	2.66	340.1973	1	182.57	1628	7.50	100.00	100.00	100.00
IAR	3.08	359.2396	1	185.57	2132	6.89	100.00	100.00	100.00
ISL	6.42	332.2170	1	182.85	933	5.48	100.00	100.00	100.00
IVR	3.31	194.1387	2	253.10	4260	4.53	99.92	99.99	99.80
KIE	3.26	195.1228	2	243.74	2444	6.39	99.61	99.86	99.84
KQI	2.40	194.6309	2	246.86	8652	6.83	100.00	100.00	100.00
LAK	2.50	331.2332	1	181.06	482	3.99	100.00	100.00	100.00
LEE	3.90	390.1876	1	188.27	1557	5.17	100.00	93.10	94.29
LFT	5.92	380.2172	1	190.43	706	3.45	100.00	100.00	100.00
LLF	8.00	392.2539	1	197.53	1399	5.91	100.00	100.00	100.00
LSQ	3.30	347.1919	1	178.69	3179	5.20	100.00	100.00	100.00

Peptide candidate	RT (min)	m/z	charge	CCS (Å²)	Average original intensity	%RSD	Opale (cons, %)	Persy (cons, %)	QA23 (cons, %)
LVE	4.37	360.2126	1	183.71	18,437	5.06	100.00	100.00	100.00
LVN	3.77	345.2125	1	180.57	990	4.59	100.00	100.00	100.00
LYE	4.68	424.2076	1	196.58	9328	3.77	100.00	100.00	100.00
LYY	6.08	458.2284	1	207.03	3200	6.61	100.00	99.99	100.00
SQY	3.78	397.1714	1	186.22	3568	5.93	100.00	100.00	100.00
SVL	5.63	318.2013	1	176.11	265	7.45	100.00	100.00	99.94
TEF	5.23	396.1756	1	189.94	620	3.45	100.00	100.00	100.00
TLV	4.44	332.2174	1	181.03	1307	2.71	100.00	100.00	100.00
VAF	6.01	336.1915	1	175.46	10,184	2.70	100.00	99.96	100.00
VNE	3.12	361.1714	1	178.22	5809	4.62	100.00	100.00	100.00
VTF	5.96	366.2019	1	179.87	1740	6.69	100.00	100.00	100.00
VTK	1.40	347.2286	1	180.50	3469	6.77	100.00	100.00	100.00
YEY	5.06	474.1869	1	208.53	3085	5.50	99.98	100.00	99.99
YNG	3.07	353.1448	1	183.95	737	3.00	100.00	100.00	100.00
ALVE	5.13	431.2496	1	200.15	3448	7.14	100.00	98.07	99.34
FAKT	3.49	233.6360	2	266.06	2319	7.68	100.00	100.00	100.00
FEGK	5.44	269.1441	2	279.98	9697	5.25	74.05	46.42	96.43
FEKL	5.27	268.6570	2	276.78	1106	8.84	96.81	95.23	93.37
FHAD	3.66	245.1077	2	262.19	5008	5.24	100.00	100.00	99.99
FKDL	5.26	261.6491	2	277.18	3826	7.25	100.00	100.00	99.40
FLGS	5.62	423.2233	1	198.49	1214	1.92	100.00	100.00	100.00
FSQY	5.07	544.2400	1	222.40	5451	4.56	99.89	99.65	99.77
GERA	1.71	216.6131	2	254.59	646	5.90	84.37	71.04	78.31
IETM	5.11	493.2326	1	215.78	10,279	5.05	100.00	95.85	98.39
IKQN	1.91	251.6520	2	261.81	745	8.35	100.00	95.20	98.59
ISSK	1.99	217.6335	2	257.65	1951	10.49	100.00	99.71	99.07
KDAF	4.45	480.2451	1	204.55	4169	6.61	93.70	37.87	41.44
LEKS	2.52	238.6388	2	262.58	3371	5.37	100.00	100.00	99.97
LGSF	6.08	423.2236	1	196.61	7284	4.72	93.04	76.01	95.77
LILN	6.24	472.3125	1	218.28	2581	5.87	100.00	100.00	100.00
LIVR	4.82	250.6806	2	277.82	1165	3.78	75.70	49.87	50.76
LLEK	4.13	251.6648	2	271.33	4134	3.64	91.38	48.34	49.20
LRET	3.30	259.6495	2	267.68	915	7.21	100.00	100.00	100.00
LTAD	3.81	419.2132	1	194.85	2055	6.78	100.00	100.00	100.00
LTEF	6.28	509.2602	1	217.34	2520	6.27	100.00	100.00	100.00
LVEL	6.85	473.2970	1	214.35	7655	5.36	100.00	99.36	99.86
LVNE	4.15	474.2558	1	208.53	6118	6.06	99.95	99.71	99.98
LVTD	4.33	447.2446	1	201.61	8109	3.72	100.00	99.72	99.85
MENF	5.75	540.2122	1	222.49	2536	6.75	100.00	100.00	100.00
TQTA	2.58	420.2083	1	196.70	1160	6.33	100.00	100.00	100.00
VASL	2.93	195.1228	2	253.02	1457	5.55	99.77	99.68	99.85
VEVS	4.02	433.2284	1	198.21	2150	6.29	98.70	97.71	97.77
VFDK	4.02	254.6413	2	267.97	1976	5.98	69.07	35.82	53.99
VSEK	1.95	231.6310	2	259.87	2212	7.45	99.97	97.52	92.49
VVST	3.70	405.2340	1	191.53	3913	3.23	100.00	100.00	100.00
AFDEK	3.91	305.1472	2	274.91	15,458	6.56	99.93	36.21	41.18
AIPEN	4.24	543.2772	1	218.50	8777	4.98	96.76	33.49	33.68
ALVEL	7.32	544.3338	1	230.33	912	7.41	99.99	40.31	48.36
FDEKL	5.50	326.1703	2	300.05	5931	5.12	100.00	58.69	61.54
FLGSF	7.55	570.2920	1	231.74	934	6.74	99.96	33.44	31.64
FYAPE	5.74	626.2814	1	244.72	2299	4.18	100.00	66.49	66.40
GFQNA	4.50	536.2458	1	220.62	1214	7.16	78.76	34.47	38.77
KFWGK	5.00	333.1909	2	296.41	878	4.19	100.00	99.98	100.00
LAKEY	4.23	312.1727	2	277.79	4602	2.52	100.00	61.22	91.98
LFGDE	6.00	580.2609	1	227.53	825	7.83	99.79	28.10	35.07
LGEYG	4.95	538.2504	1	218.61	831	3.51	90.99	32.33	35.78
LILNR	5.33	314.7094	2	287.40	797	7.07	99.75	30.78	47.93

Peptide candidate	RT (min)	m/z	charge	CCS (Å²)	Average original intensity	%RSD	Opale (cons, %)	Persy (cons, %)	QA23 (cons, %)
LPKIE	5.42	300.1912	2	284.85	8843	6.91	100.00	60.78	99.87
LVELL	7.98	586.3803	1	241.49	707	5.49	90.22	33.34	64.02
LVEVS	5.21	546.3130	1	224.32	4239	0.63	99.26	39.13	54.87
TAPEL	6.26	592.2976	1	235.28	15,546	5.29	100.00	93.48	93.12
TVFDK	4.61	305.1650	2	284.60	2748	4.80	99.98	66.96	61.71
VEVTK	3.65	288.1730	2	275.74	7041	8.04	98.83	39.91	48.04
VVSTQ	3.53	533.2928	1	218.72	3638	5.90	98.97	43.60	46.92
AFDEKL	5.70	361.6892	2	288.60	36,673	6.08	88.2	42.5	40.9
DAFLGS	3.91	305.1472	2	274.91	15,458	6.56	99.9	36.2	41.2
DTHKSE *	1.19	358.6637	2	292.00	4537	2.90	95.4	8.3	11.6
KDAIPE	4.55	336.6810	2	286.40	952	7.69	71.7	35.6	27.2
KFGERA	3.66	354.1946	2	298.79	1133	3.60	93.9	22.1	27.9
KFPKAE	4.01	360.2070	2	308.56	2291	5.69	100.0	39.4	44.3
LFGDEL	7.43	693.3452	1	253.69	902	10.18	100.0	34.2	32.5
LLPKIE	6.10	356.7331	2	315.47	1720	7.65	100.0	41.7	46.1
LPKIET	5.60	350.7149	2	292.34	7905	6.27	96.9	31.0	31.1
NLPPLT	6.57	654.3815	1	248.25	1517	7.63	80.2	51.5	43.0
TVFDKL	6.40	361.7072	2	295.17	2718	5.65	100.0	35.9	40.2
VEGPKL	5.33	321.6941	2	280.58	9864	5.33	85.6	38.7	39.6
VSTPTL	6.14	617.3495	1	238.79	674	5.56	100.0	43.6	46.0

*** Elution within void volume.**

Supplementary tables to Study 3

Table S2. Real-time polymerase chain reaction (RT-PCR) primers used in Study 3.

Primer name	Sequence
ACT1-FW	CCACCATGTTCCCAGGTATT
ACT1-RV	CCAATCCAGACGGAGTACTT
DAL5-FW	ATCACCCAACGGTAAAATTG
DAL5-RV	ATCTTCTTCTGGTGTCACTT
OPT1-FW	CCACCAAGCACACCTTATAAC
OPT1-RV	ATTGCCACACCTGCTTCAACA
OPT2-FW	CCTGATGCTGTGACCTACTAT
OPT2-RV	ATGCATGCGCCTATCAACCAA
FOT1-FW	GCGGTTGGTTGTTGAACTTT
FOT1-RV	GGGCAGTGCTCAGAAGAATC
FOT2-FW	CGAGGGCTTATGACGAGGTA
FOT2-RV	GATCCCAGCGTAGTGGACAT

Tables S3-7. Mean values of growth and fermentation parameters within the different media. *An analysis of variance followed by a Tukey's test (p-value = 0.05) was performed to indicate the significant differences among the values, which are represented in the "groups" column. Significant differences among the strains within the same condition are indicated with low case letters, and significant differences between conditions for the same strain are indicated with upper case letters. 'sd': standard deviation.*

Table S3. Maximum rate of CO_2 production (Vmax).

	Vmax								
	NAP200			NA100			P200		
	Vmax (g/L/h)	sd	groups	Vmax (g/L/h)	sd	groups	Vmax (g/L/h)	sd	groups
59A (wt)	0.969	0.109	ab,A	0.570	0.040	a,B	1.024	0.039	a,A
Fot1	1.059	0.005	a,A	0.535	0.000	a,B	1.016	0.018	a,A
Fot1Fot2	1.065	0.004	a,A	0.586	0.041	a,B	1.053	0.042	a,A
fot1fot2Δ	0.762	0.080	abc,A	0.545	0.013	a,A	0.703	0.079	c,A
Fot2	0.997	0.038	ab,A	0.583	0.008	a,B	0.906	0.034	ab,A
Fot3	1.119	0.074	a,A	0.569	0.008	a,B	0.969	0.027	a,A
Opt1	0.561	0.217	c,A	0.642	0.017	a,A	-	-	-
opt1Δ	0.939	0.098	abc,A	0.542	0.039	a,B	1.071	0.090	a,A
Opt2	0.803	0.145	abc,A	0.583	0.032	a,A	0.781	0.027	bc,A
opt2Δ	0.987	0.167	ab,AB	0.580	0.057	a,B	1.060	0.010	a,A
PepKO	0.601	0.033	bc,A	0.589	0.027	a,A	-	-	-

Table S4. Time to reach 80% of attenuation.

	Time to reach 80% of attenuation								
	NAP200			NA100			P200		
	Time (h)	sd	groups	Time (h)	sd	groups	Time (h)	sd	groups
59A (wt)	150	12	b,B	258	30	a,A	144	0	b,B
Fot1	120	0	b,C	264	0	a,A	144	0	b,B
Fot1Fot2	144	0	b,B	216	0	a,A	144	0	b,B
fot1fot2Δ	168	0	ab,B	264	0	a,A	180	17	a,B
Fot2	144	0	b,B	264	0	a,A	144	0	b,B
Fot3	120	0	b,B	240	34	a,A	144	0	b,B
Opt1	240	34	a,A	240	34	a,A	-	-	-
opt1Δ	144	34	b,B	264	0	a,A	132	17	b,B
Opt2	168	34	ab,A	240	34	a,A	180	17	a,A
opt2Δ	144	34	b,B	264	0	a,A	144	0	b,B
PepKO	216	0	a,A	240	34	a,A	-	-	-

Table S5. Time to produce 1g of CO_2.

	Time to produce 1 g of CO2								
	NAP200			NA100			P200		
	Time (h)	sd	groups	Time (h)	sd	groups	Time (h)	sd	groups
59A (wt)	14	3	a,B	12	0	a,B	18	0	b,A
Fot1	15	4	a,A	18	0	a,A	18	0	b,A
Fot1Fot2	12	0	a,A	15	4	a,A	18	0	b,A
fot1fot2Δ	15	4	a,A	18	0	a,A	18	0	b,A
Fot2	12	0	a,A	12	0	a,A	15	4	b,A
Fot3	12	0	a,A	15	4	a,A	18	0	b,A
Opt1	12	0	a,A	12	0	a,A	24	8	ab,A
opt1Δ	9	4	a,A	15	4	a,A	18	0	b,A
Opt2	12	0	a,A	15	4	a,A	18	0	b,A
opt2Δ	15	4	a,A	15	4	a,A	18	0	b,A
PepKO	15	4	a,AB	12	0	a,B	36	8	a,A

Table S6. Maximum population (k).

	Maximum population (k)								
	NAP200			NA100			P200		
	k	sd	groups	k	sd	groups	k	sd	groups
59A (wt)	10.186	0.563	a,A	7.716	0.335	abc,B	10.215	0.936	abc,A
Fot1	9.812	0.543	ab,A	7.167	0.049	bc,B	9.457	0.241	abc,A
Fot1Fot2	9.613	0.502	ab,A	7.279	0.076	abc,B	9.328	0.172	bc,A
fot1fot2Δ	8.357	0.544	ab,AB	7.219	0.046	bc,B	8.876	0.097	c,A
Fot2	9.139	0.346	ab,A	7.167	0.182	bc,B	9.385	0.059	abc,A
Fot3	9.980	0.172	ab,A	7.137	0.070	c,C	8.971	0.346	c,B
Opt1	7.449	1.266	b,A	8.026	0.253	abc,A	-	-	-
opt1Δ	10.877	0.608	a,A	8.168	0.505	a,B	11.211	0.046	a,A
Opt2	9.660	0.881	ab,A	8.078	0.182	ab,A	9.714	0.503	abc,A
opt2Δ	10.669	1.008	a,A	8.140	0.058	a,B	11.139	0.135	ab,A
PepKO	7.549	0.020	b,A	7.417	0.021	abc,B	-	-	-

Table S7. Maximum growth rate (r).

	Maximum growth rate (r)								
	NAP200			NA100			P200		
	r	sd	groups	r	sd	groups	r	sd	groups
59A (wt)	0.110	0.025	a,A	0.107	0.023	a,A	0.103	0.038	a,A
Fot1	0.112	0.006	a,A	0.096	0.007	a,A	0.105	0.005	a,A
Fot1Fot2	0.113	0.012	a,A	0.100	0.017	a,A	0.113	0.005	a,A
fot1fot2Δ	0.087	0.008	a,A	0.088	0.014	a,A	0.057	0.006	a,A
Fot2	0.111	0.011	a,A	0.094	0.019	a,A	0.096	0.001	a,A
Fot3	0.128	0.016	a,A	0.099	0.013	a,A	0.107	0.010	a,A
Opt1	0.113	0.020	a,A	0.119	0.020	a,A	-	-	-
opt1Δ	0.121	0.015	a,A	0.112	0.009	a,A	0.113	0.005	a,A
Opt2	0.110	0.003	a,A	0.123	0.005	a,A	0.081	0.003	a,B
opt2Δ	0.117	0.020	a,A	0.111	0.019	a,A	0.115	0.009	a,A
PepKO	0.096	0.009	a,A	0.089	0.007	a,A	-	-	-

Table S8. Analysis of variance in gene expression. *ANOVA model: expression~Strain*Media*Time. Tukey's tests assign groups according to the significant differences found between the mean values by strain, media or timepoint.*

By strain										
	FOT1		***FOT2***		***OPT1***		***OPT2***		***Dal5***	
Strain	Mean Value	Groups	Mean Value	Groups	Mean Value	Groups	Mean Value	Groups	Mean Value	Groups
59A (wt)	0.951	b	0.705	a	1.078	b	0.540	b	0.354	b
Opt2Δ	2.350	a	0.918	a	1.845	a	-	-	0.899	a
Fot1Fot2Δ	-	-	-	-	1.094	b	2.364	a	0.811	a
Opt1	-	-	-	-	1.340	b	-	-	-	-

By media										
	FOT1		***FOT2***		***OPT1***		***OPT2***		***DAL5***	
Medium	Mean Value	Groups	Mean Value	Groups	Mean Value	Groups	Mean Value	Groups	Mean Value	Groups
NAP200	2.245	a	0.342	b	0.646	b	0.884	b	0.370	b
P200	1.966	a	1.206	a	0.827	b	2.581	a	0.960	a
P200-SO4	0.741	b	0.886	ab	2.513	a	0.891	b	0.752	a

By timepoint										
	FOT1		***FOT2***		***OPT1***		***OPT2***		***DAL5***	
Timepoint	Mean Value	Groups	Mean Value	Groups	Mean Value	Groups	Mean Value	Groups	Mean Value	Groups
18	0.466	b	0.353	b	0.722	b	0.620	b	0.458	b
48	2.835	a	1.286	a	1.984	a	2.284	a	0.935	a

Table S9. Mass spectrometric data of all di-heptapeptides followed during this study. *RT: retention time; m/z: mass to charge ratio; CCS: collisional cross section; RSD: relative standard deviation. Peptide candidates with a unique sequence (i.e., no other possible peptide sequences with the same AA composition found in BSA) are underscored.*

Peptide candidate	RT (min)	Observed m/z	Charge	CCS (Å²)	Average Original Intensity (NAP200)	RSD	Average Original Intensity (P200)	RSD
AI	2.03	203.1389	1	154.86	684	8%	1052	10%
DI	0.71	247.1288	1	155.86	1635	7%	2784	5%
FK	1.49	294.1811	1	167.80	1718	7%	3170	5%
FW	4.92	352.1656	1	181.90	3346	10%	6671	7%
FY	3.72	329.1495	1	182.71	1141	6%	1645	4%
IE	1.13	261.1445	1	158.67	981	10%	1431	7%
KT	1.25	248.1604	1	155.81	1005	9%	1628	7%
LL	3.74	245.1860	1	166.49	8075	11%	13052	9%
PL	1.00	233.1495	1	158.32	988	11%	1839	8%
PP	2.72	229.1546	1	155.06	5885	5%	9824	4%
RF	0.83	213.1233	1	147.39	1492	6%	2838	5%
SF	0.83	338.1823	1	176.91	839	9%	1500	6%
SI	2.40	253.1183	1	159.04	654	7%	1142	5%
TH	1.88	219.1338	1	155.62	2503	13%	4293	12%
TI	1.25	257.1244	1	158.85	811	6%	1346	5%
TM	0.64	248.1604	1	155.81	979	5%	1411	5%
VF	1.10	251.1060	1	155.67	3623	9%	6615	5%
VL	3.37	265.1547	1	163.74	4160	7%	6342	8%

Peptide candidate	RT (min)	Observed m/z	Charge	CCS (Å²)	Average Original Intensity (NAP200)	RSD	Average Original Intensity (P200)	RSD
VR	2.83	231.1703	1	161.93	1482	13%	2356	12%
AEF	3.14	366.1661	1	183.27	5625	6%	9727	5%
AFL	4.59	350.2076	1	180.13	6334	7%	11691	6%
DAF	3.07	352.1503	1	178.25	683	12%	1382	8%
DLL	3.75	360.2129	1	192.76	808	6%	1437	6%
FSA	2.77	324.1554	1	175.60	3728	7%	5820	6%
FSQ	1.84	381.1769	1	188.33	1310	9%	2372	6%
FTF	5.21	414.2025	1	196.70	4211	9%	8247	7%
FVE	2.96	394.1973	1	191.66	3353	9%	6158	8%
GEY	1.87	368.1453	1	181.37	1633	5%	2666	5%
IAE	1.77	332.1816	1	175.31	2526	12%	4690	8%
IAR	0.79	359.2401	1	187.19	2544	8%	4472	6%
IET	2.09	362.1922	1	187.09	3294	8%	5680	5%
IVR	1.21	387.2714	1	197.52	1033	6%	2004	8%
LFT	3.92	380.2180	1	192.09	1671	7%	2879	5%
LHT	1.26	370.2085	1	196.19	2748	6%	4894	6%
LIV	4.55	344.2543	1	187.71	786	9%	1620	8%
LLF	5.92	392.2544	1	197.36	3883	9%	8268	8%
LSQ	1.07	347.1926	1	180.24	4443	7%	7531	5%
LTA	2.24	304.1866	1	170.97	540	10%	1021	7%
LTE	1.90	362.1923	1	183.40	8691	6%	14156	5%
LVE	2.45	360.2132	1	185.31	13478	5%	21848	5%
LVN	1.84	345.2136	1	182.14	831	10%	1535	7%
LYE	2.78	424.2081	1	198.31	9323	5%	13865	4%
LYY	4.12	458.2289	1	206.93	7833	7%	13458	6%
TFI	5.04	380.2180	1	193.97	743	10%	1653	9%
TIS	1.19	320.1816	1	173.95	1201	5%	1867	11%
VAF	3.97	336.1919	1	175.17	11297	6%	18776	4%
VTD	0.81	334.1609	1	173.44	5024	4%	7147	4%
VVS	1.09	304.1865	1	170.97	1690	6%	2420	6%
ALVE	3.21	431.2501	1	201.91	2512	8%	4434	6%
FLGS	3.65	423.2238	1	200.23	588	12%	1201	8%
FVAF	5.53	483.2603	1	212.09	913	11%	1931	9%
IETM	3.18	493.2330	1	215.74	5788	7%	9550	6%
LGSF	4.08	423.2241	1	198.33	7620	10%	14327	6%
LIAF	5.74	463.2916	1	212.61	663	10%	1402	9%
LLYY	5.12	571.3122	1	233.82	775	10%	1724	9%
LTAD	1.89	419.2137	1	194.67	1765	8%	2861	6%
LTEF	4.27	509.2609	1	217.31	2100	8%	3800	6%
LVEL	4.87	473.2973	1	216.26	8030	7%	13870	6%
LVLI	6.17	457.3385	1	218.66	1830	10%	3780	9%
LVNE	2.32	474.2560	1	210.37	3492	6%	5374	5%
LVTD	2.48	447.2451	1	203.38	4508	6%	8247	6%
LVVS	3.31	417.2708	1	200.41	2257	10%	4266	7%
LYEI	4.95	537.2921	1	230.57	849	10%	1825	9%
VEVS	2.17	433.2293	1	199.95	637	10%	1170	8%
VVST	1.80	405.2345	1	193.20	4559	9%	8070	6%
LILNR	4.03	314.7101	1	149.70	541	6%	1020	8%
VFDKL	4.18	311.1835	2	296.71	619	13%	1251	12%
LLPKIE	4.47	356.7332	2	318.21	831	11%	1666	8%
TRKVPQ	1.55	364.7238	2	314.44	3214	19%	6547	9%
LLPKIET	4.57	407.2571	2	312.72	721	14%	1459	11%
LPKIETM	4.59	416.2353	2	312.38	1181	12%	2450	10%
LVEVSRS	2.88	395.2263	2	306.43	891	10%	1568	8%

Supplementary tables to Study 4

Table S10. Amino acid concentrations used in the synthetic whisky mash (SWM). *Total "yeast-assimilable nitrogen" was ~115 mg/L. Free proline was not taken into account as it is not or poorly assimilated by yeasts during fermentation (2,178)*

	Concentration (mg/L)	N (mg/L)	% total N		Concentration (mg/L)	N (mg/L)	% total N
Trp	45.47	6.24	4.76	**Ala**	50.48	7.94	6.06
Phe	54.12	4.59	3.50	**Thr**	25.15	2.96	2.26
Leu	72.13	7.70	5.88	**Glu**	50.28	4.79	3.65
Ile	27.16	2.90	2.21	**Asp**	47.15	5.00	3.82
Val	45.60	5.45	4.16	**Gly**	17.77	3.32	2.53
Met	15.36	1.44	1.10	**Arg**	59.53	19.15	14.62
Tyr	27.06	2.09	1.60	**Gln**	66.64	12.77	9.75
Lys	47.58	9.12	6.96	**Ser**	27.92	3.72	2.84
Cys	5.56	0.64	0.49	**Asn**	42.12	8.93	6.82
Pro	130.95	15.93	12.16	**His**	23.29	6.31	4.82

Table S11. Hordein and glutenin UniProt entries used in peptide mapping of the grain mash.

Hordeins - *Hordeum vulgare* (Barley)					
Protein name	**Entry name**	**UniProt Entry**	**Protein name**	**Entry name**	**UniProt Entry**
B1-hordein	HOR1_HORVU	P06470	Gamma-hordein 1	HOG1_HORVU	P17990
B3-hordein	HOR3_HORVU	P06471	Gamma-hordein 1	HOG3_HORVU	P80198
C-hordein	HOR7_HORVU	P06472			
Glutenins - *Triticum aestivum* (Wheat)					
Protein name	**Entry name**	**UniProt Entry**	**Protein name**	**Entry name**	**UniProt Entry**
Glutenin low MW subunit	GLTA_WHEAT	P10385	Glutenin low MW subunit PC237	GLT2_WHEAT	P02862
Glutenin low MW subunit 1D1	GLTB_WHEAT	P10386	Glutenin low MW subunit 12	GLT3_WHEAT	P08488
Glutenin low MW subunit PTDUCD1	GLTC_WHEAT	P16315	Glutenin low MW subunit PW212	GLT4 WHEAT	P08489
Glutenin low MW subunit DY10	GLT0_WHEAT	P10387	Glutenin low MW subunit DX5	GLT5_WHEAT	P10388
Glutenin low MW subunit PC256	GLT1_WHEAT	P02861			

Tables S13-15. Mean values of growth and fermentation parameters within the different media. *An analysis of variance followed by a Tukey's test (p-value = 0.05) was performed to indicate the significant differences among the values, which are represented in the "groups" column. Significant differences among the strains within the same condition are indicated with low case letters, and significant differences between conditions for the same strain are indicated with upper case letters. 'SD': standard deviation.*

Table S12. Maximum population (k). *The k is presented in OD units. Significant differences between media were not assessed as yeast strains might have different cell sizes.*

	DistilaMax® GW			DistilaMax® NT			DistilaMax® XP		
Medium	**k**	**SD**	**Groups**	**k**	**SD**	**Groups**	**k**	**SD**	**Groups**
AP230	10.04	0.33	BC	9.10	0.14	B	7.16	0.26	B
P230	10.94	0.12	AB	9.64	0.27	B	6.40	0.37	B
PD300	9.33	0.04	C	8.28	0.23	C	6.77	0.14	B
PP300	11.16	0.72	A	11.29	0.45	A	9.71	0.80	A

Table S13. Maximum rate of CO2 production (Vmax). *The Vmax is presented in g/L/h.*

	DistilaMax® GW			DistilaMax® NT			DistilaMax® XP		
Medium	**Vmax**	**SD**	**Groups**	**Vmax**	**SD**	**Groups**	**Vmax**	**SD**	**Groups**
AP230	0.99	0.01	a,C	0.97	0.02	a,B	0.70	0.04	b,B
P230	1.09	0.02	a,B	1.02	0.03	b,B	0.69	0.04	c,B
PD300	0.86	0.01	a,D	0.81	0.03	a,C	0.67	0.03	b.B
PP300	1.39	0.06	a,A	1.13	0.06	b,A	1.11	0.02	b,A

Table S14. Time to reach 80% of attenuation (t80). *The t80 is presented in hours.*

	DistilaMax® GW			DistilaMax® NT			DistilaMax® XP		
Medium	**t80**	**SD**	**Groups**	**t80**	**SD**	**Groups**	**t80**	**SD**	**Groups**
AP230	120	0	b,A	160	28	ab,A	192	0	a,A
P230	112	14	b,A	176	28	a,A	192	0	a,A
PD300	120	0	a,A	160	28	a,A	176	28	a,A
PP300	72	0	b,B	136	14	a,A	112	14	a,B

Table S15. Mass spectrometric data of all di-nonapeptide candidates followed in the synthetic whisky mash. *RT: retention time; m/z: mass to charge ratio; z: charge, CCS: collisional cross-section; RSD: relative standard deviation (calculated from each fermentation starting point).*

Peptide ID	RT (min)	m/z	z	CCS (Å^2)	RSD (%)
AI	3.33	203.1389	1	154.98	3.22
AW	5.13	276.1343	1	161.41	1.93
EF	4.39	295.1286	1	169.26	2.18
FT	2.92	267.1339	1	165.25	1.95
FV	5.02	265.1546	1	167.07	2.48
FW	7.99	352.1656	1	183.07	2.03
HA	1.43	227.1138	1	150.20	2.25
LP	4.42	229.1545	1	155.14	2.89
LT	2.03	233.1496	1	158.33	2.15
QI	3.29	260.1603	1	165.57	3.41
RA	1.89	246.1560	1	157.66	5.13
RF	2.89	322.1873	1	177.00	2.47
RY	1.54	338.1822	1	178.20	2.12
SI	1.02	219.1338	1	154.01	2.93
SL	3.10	219.1339	1	155.70	2.02
TH	2.58	257.1241	1	158.84	2.19
TI	3.44	233.1496	1	158.33	1.95
TK	2.57	248.1604	1	157.56	2.22
TM	2.07	251.1059	1	155.73	2.61
VL	4.57	231.1704	1	161.87	2.13
VM	3.32	249.1264	1	159.22	4.34

Peptide ID	RT (min)	m/z	z	CCS (Å^2)	RSD (%)
VR	2.60	274.1873	1	166.66	2.27
YE	2.16	311.1237	1	175.65	2.15
YF	6.03	329.1495	1	183.89	2.05
YY	4.17	345.1446	1	186.92	2.22
AEF	5.11	366.1660	1	184.41	2.17
ALA	3.59	274.1764	1	168.40	2.62
ALI	7.28	316.2230	1	180.79	2.47
ALV	5.64	302.2071	1	176.00	3.91
AWS	4.28	363.1662	1	184.51	2.67
DAF	5.01	352.1502	1	177.73	2.82
DLP	3.55	344.1811	1	181.56	3.35
DQF	4.61	409.1715	1	197.66	2.82
EYE	2.98	440.1661	1	200.48	4.07
FAE	4.11	366.1657	1	184.41	2.58
FDK	2.77	205.1076	2	252.55	1.85
FGE	4.03	352.1500	1	179.50	2.72
FLG	5.94	336.1915	1	185.43	3.61
FLY	7.94	442.2333	1	206.02	2.58
FQE	4.01	423.1872	1	200.95	3.03
FSA	4.55	324.1552	1	175.16	1.70
FSQ	3.20	381.1767	1	189.35	2.27
FTF	8.56	414.2023	1	197.51	2.43
FVA	5.27	336.1915	1	181.84	3.19
FVE	4.84	394.1973	1	194.42	2.49
FYA	8.45	400.1865	1	192.41	2.59
GEY	3.20	368.1452	1	180.77	2.53
GSF	4.06	310.1396	1	168.68	2.61
HTI	2.89	370.2081	1	191.52	2.98
IAE	3.11	332.1816	1	176.64	1.98
IAH	1.40	340.1977	1	183.49	2.34
IET	3.55	362.1921	1	188.15	2.23
ILN	4.19	359.2288	1	188.25	2.55
KLF	6.24	204.1360	2	252.62	2.44
LAS	3.70	290.1707	1	169.46	4.41
LEE	3.38	390.1871	1	190.89	2.32
LFG	7.53	336.1915	1	178.27	2.88
LFT	6.41	380.2178	1	193.03	2.02
LIV	7.42	344.2542	1	188.76	2.54
LLV	7.94	344.2545	1	188.76	2.28
LSQ	2.42	347.1924	1	181.45	2.15
LTE	3.27	362.1922	1	182.74	1.93
LVE	4.06	360.2130	1	184.60	2.00
LVL	7.69	344.2543	1	190.58	2.29
LVN	3.19	345.2136	1	181.52	2.42
MEN	1.42	393.1436	1	188.99	2.48
NTL	3.69	347.1923	1	188.66	3.44
PPL	5.33	326.2072	1	178.63	3.01
SQY	3.04	397.1717	1	188.87	1.73
TAL	4.37	304.1864	1	175.92	3.13
TEF	5.14	396.1763	1	192.53	2.27
TFI	8.25	380.2179	1	194.86	2.56
TIS	2.51	320.1815	1	175.31	2.76
TLF	7.74	380.2178	1	189.38	2.90
TLV	4.16	332.2178	1	181.98	2.84
TVF	6.38	366.2021	1	182.61	3.02
VAS	2.24	276.1558	1	164.85	3.44

Peptide ID	RT (min)	m/z	z	CCS (Å^2)	RSD (%)
VEL	6.11	360.2128	1	191.85	2.71
VEV	4.74	346.1967	1	186.88	5.02
VFQ	4.33	393.2129	1	192.62	2.50
WGK	5.84	390.2131	1	189.07	3.28
VLH	2.72	184.6178	2	248.00	3.64
VNE	1.91	361.1716	1	180.99	1.96
YAV	6.34	352.1866	1	179.50	2.44
YEI	6.61	424.2076	1	210.29	3.33
YEY	5.14	474.1870	1	210.82	2.43
YNG	1.70	353.1453	1	184.84	2.40
YST	3.01	370.1606	1	187.89	3.22
AIPE	4.70	429.2340	1	198.93	3.26
ALVE	5.25	431.2499	1	202.58	2.42
APEL	5.84	429.2340	1	202.64	2.73
EGPK	2.74	430.2294	1	195.22	3.49
EKLF	8.79	536.3076	1	226.55	3.46
FQNA	3.61	479.2247	1	214.48	3.17
FSAL	7.33	437.2391	1	206.15	3.23
FVAF	9.07	483.2601	1	212.48	3.25
IYEI	8.14	537.2917	1	230.43	2.93
LGEY	5.31	481.2292	1	206.87	2.21
LGSF	6.70	423.2239	1	199.10	2.69
LTAD	3.28	419.2134	1	195.53	3.34
LVEV	6.67	459.2809	1	211.20	3.53
LVVS	5.42	417.2705	1	202.98	2.62
LYEL	6.92	537.2916	1	228.47	3.81
PPLT	4.85	427.2547	1	202.70	3.09
TISS	2.19	407.2129	1	195.87	2.12
TQTA	1.37	420.2086	1	199.18	3.50
VEVS	3.65	433.2292	1	200.67	2.31
VLAS	4.41	389.2391	1	192.74	3.79
VNEL	5.23	474.2556	1	214.61	3.58
VVST	3.16	405.2344	1	194.09	2.12
AKEYE	2.78	320.1521	2	280.41	3.47
FHADI	5.11	301.6495	2	281.28	3.62
GERAL	3.77	273.1556	2	276.39	2.47
LAKEY	4.12	312.1724	2	280.78	3.06
LGEYG	5.04	538.2506	1	218.79	3.31
LLPKI	7.14	292.2121	2	291.39	2.58
LPKIE	6.13	300.1913	2	287.76	2.58
PKAEF	4.47	296.1597	2	287.96	2.31
VEGPK	2.74	265.2988	2	273.65	3.23
LLPKIE	7.36	356.7333	2	317.94	2.58
LPKIET	6.48	350.7151	2	295.13	2.48
TPVSEK	2.93	330.6806	2	292.77	3.46
TVFDKL	7.79	361.7073	2	297.92	2.51
VEGPKL	5.87	321.6944	2	283.53	2.32
VEVTKL	6.22	344.7151	2	298.65	2.66
LPKIETM	7.53	416.2352	2	312.22	3.01
LVEVSRS	4.74	395.2264	2	306.41	3.13
RETYGDM	4.93	436.1833	2	311.50	2.42
LKPDPNTL	6.15	449.2549	2	317.69	3.41
LVDEPQNL	7.14	464.2414	2	320.52	3.57
KDDSPDLPK	4.71	507.7574	2	332.59	3.50
TPDETYVPK	5.58	525.2604	2	356.14	5.18

Supplementary Figures

Supplementary figures to Study 3

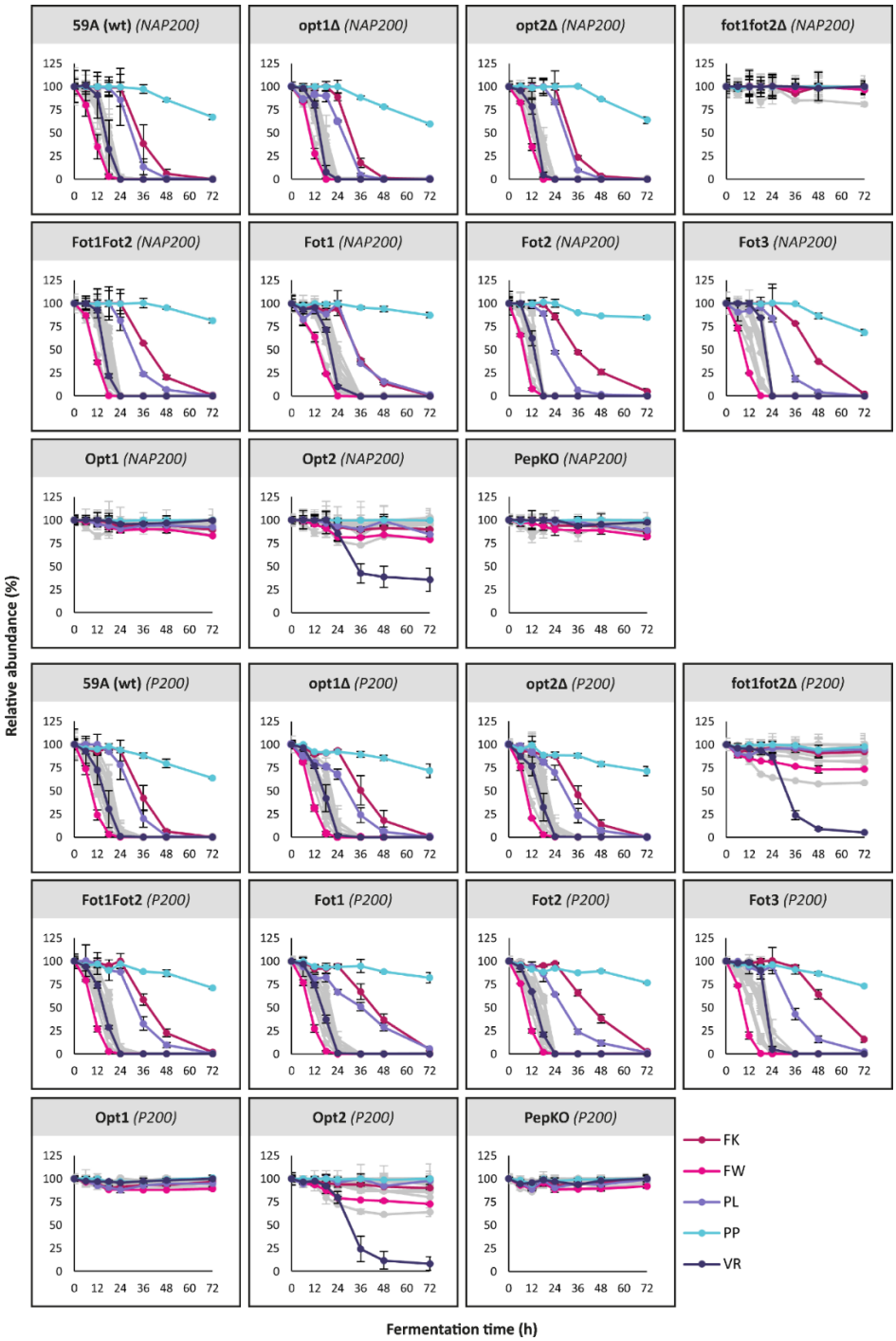

Figure S1. Dipeptide consumption curves of the strains tested on NAP200 and P200. *Five dipeptides are depicted in colour to represent their peptide length group. All other dipeptide consumption curves are depicted as 'shadow' in the background.*

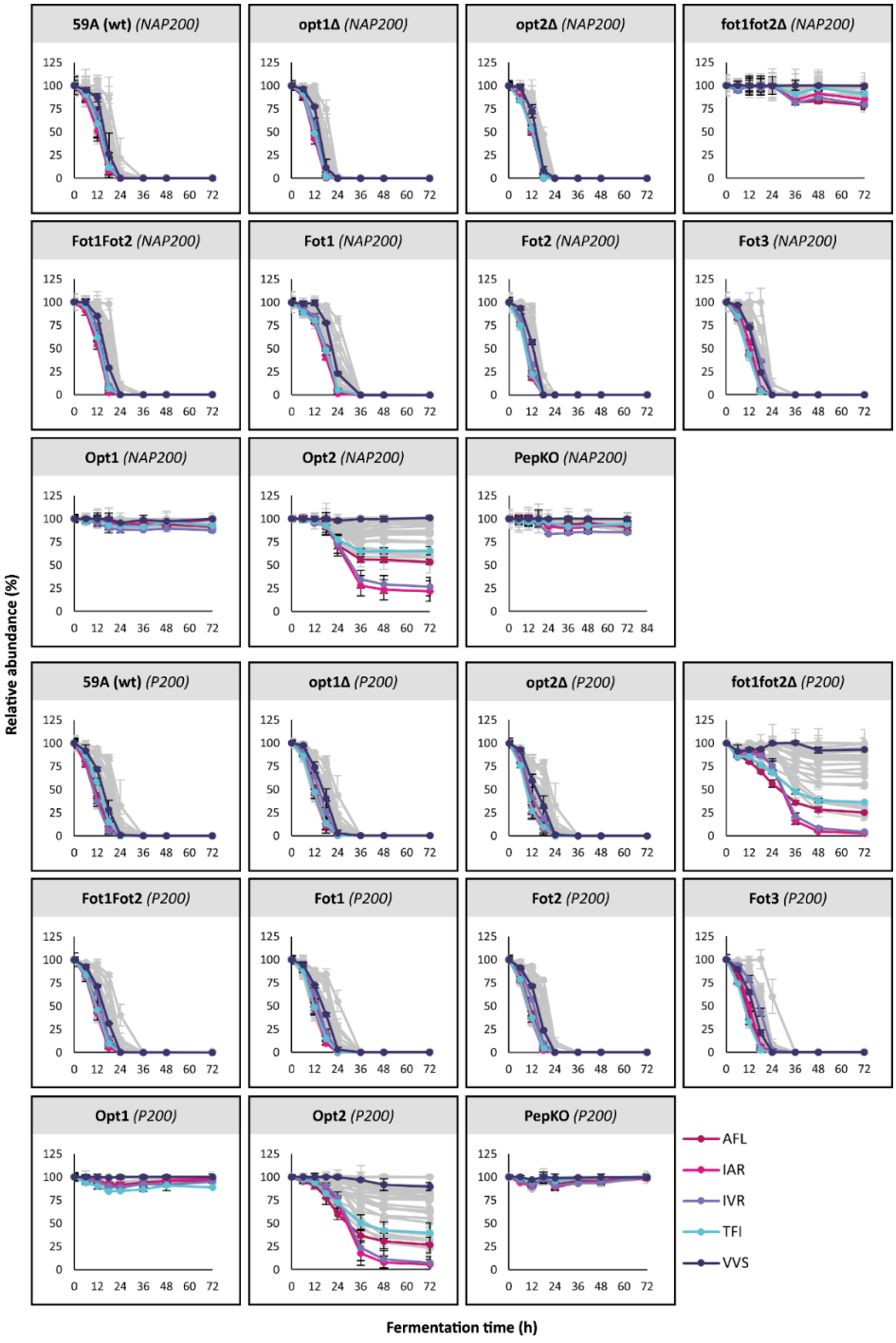

Figure S2. Tripeptide consumption curves of the strains tested on NAP200 and P200. *Five tripeptides are depicted in colour to represent their peptide length group. All other tripeptide consumption curves are depicted as 'shadow' in the background.*

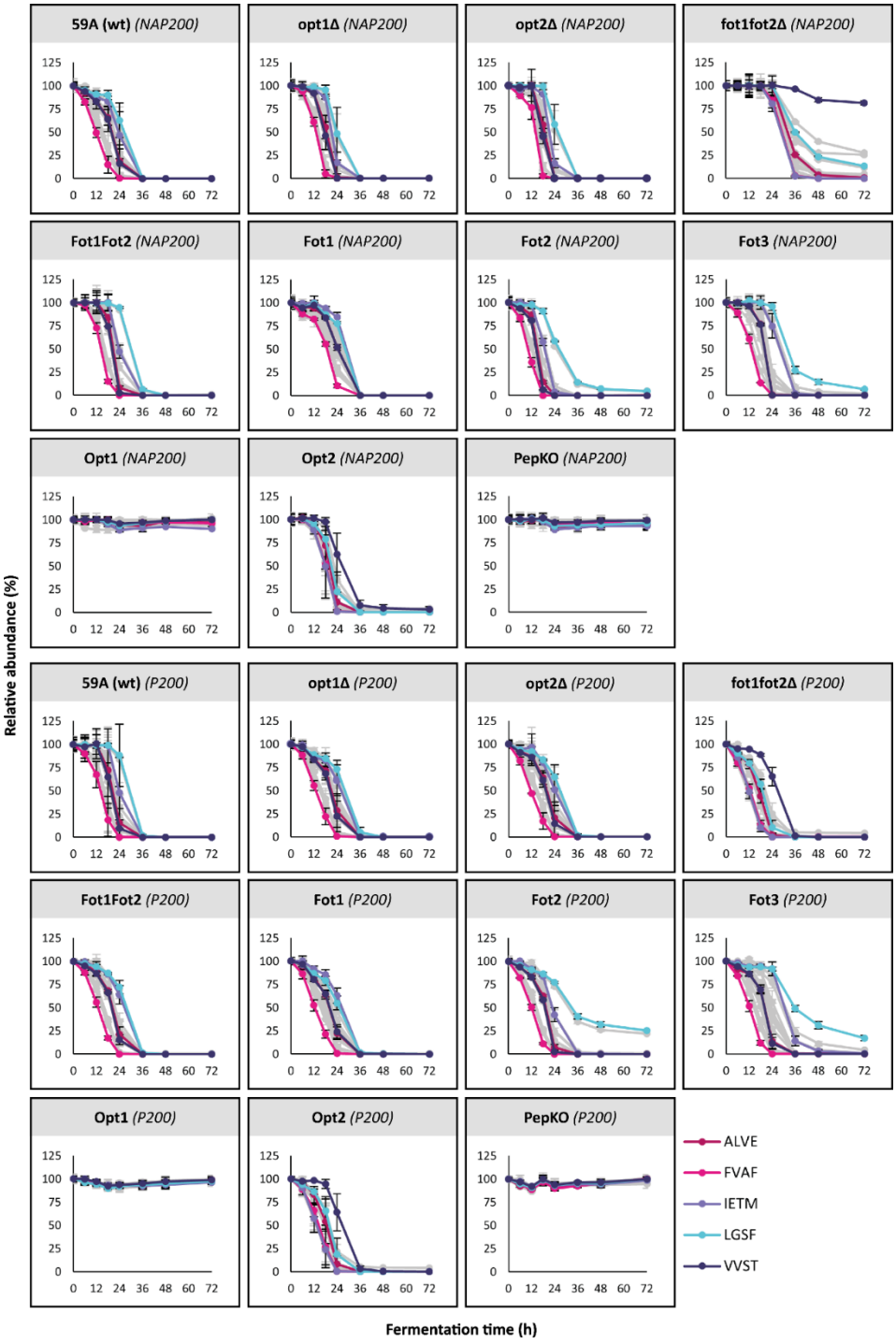

Figure S3. Tetrapeptide consumption curves of the strains tested on NAP200 and P200. *Five tetrapeptides are depicted in colour to represent their peptide length group. All other tetrapeptide consumption curves are depicted as 'shadow' in the background.*

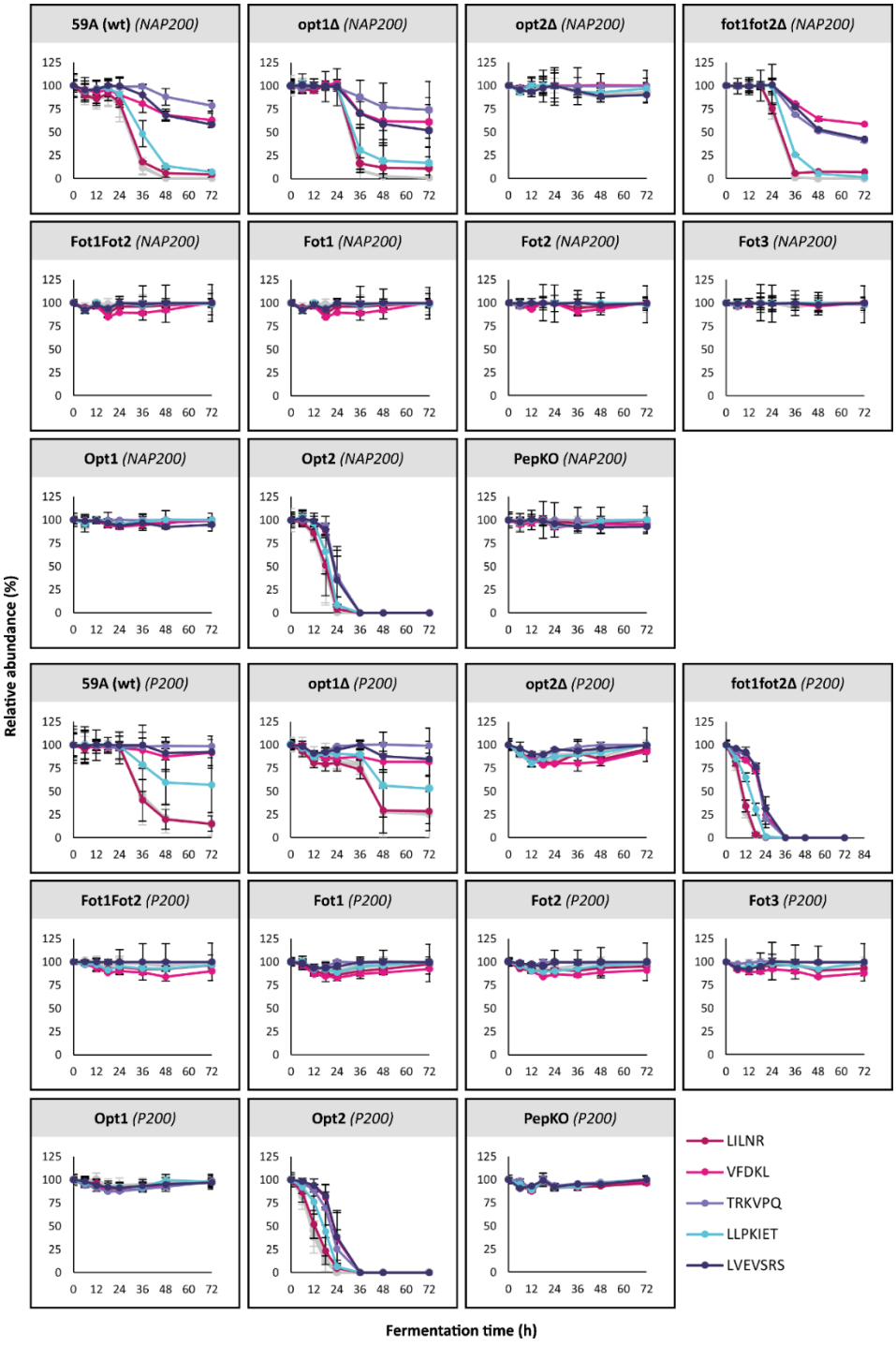

Figure S4. Penta-heptapeptide consumption curves of the strains tested on NAP200 and P200. *Five peptides are depicted in colour to represent this peptide length group. All other penta- to heptapeptide consumption curves are depicted as 'shadow' in the background.*

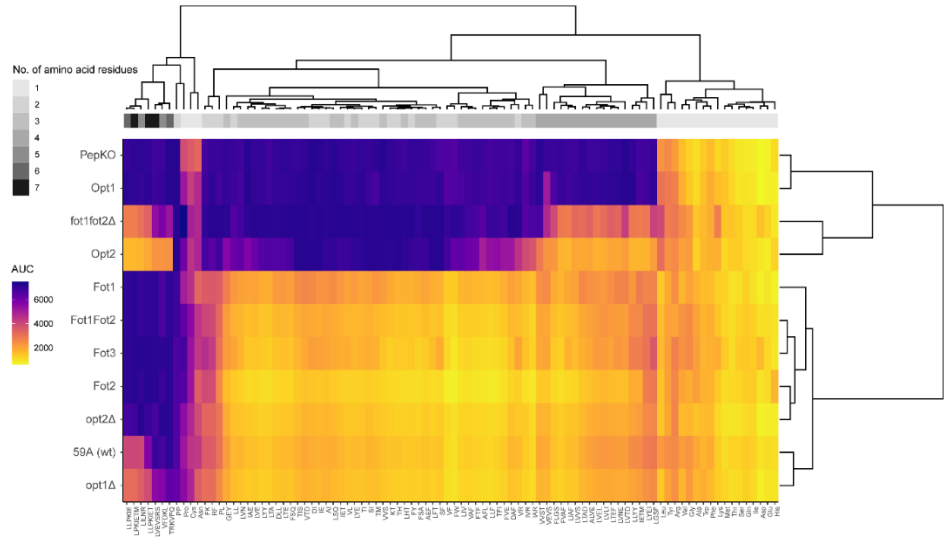

Figure S5. Consumption of FAA and peptides in NAP200. The heatmap represents the consumption of FAA and di- to heptapeptides (columns) by the researched strains (rows). Strains are sorted by consumption preferences similarity, represented by the cladogram to the right. The cladogram on top of the heatmap ranks the FAA and di- to heptapeptides according to their preferential consumption.

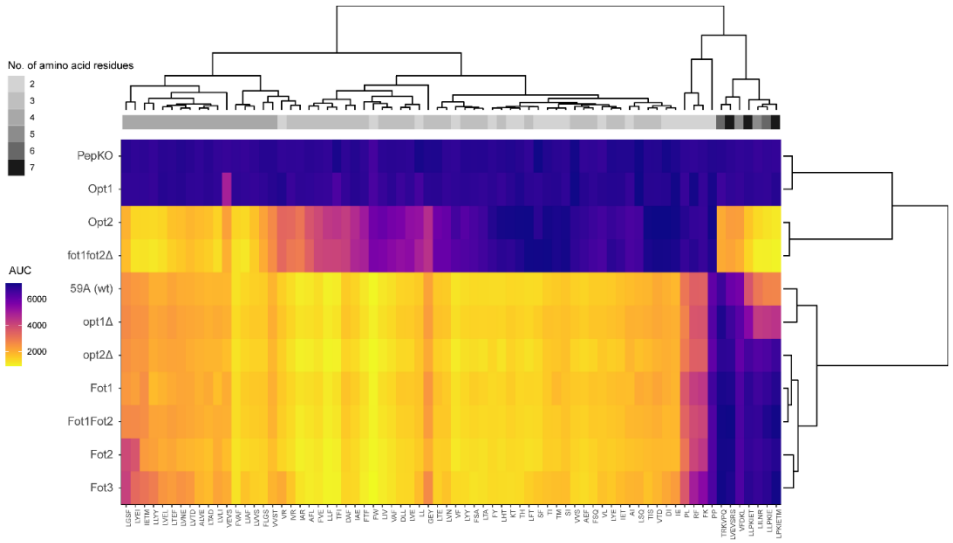

Figure S6. Consumption of peptides in P200. The heatmap represents the consumption of di- to heptapeptides (columns) by the researched strains (rows). *Strains are sorted by consumption preferences similarity, represented by the cladogram to the right. The cladogram on top of the heatmap ranks the di- to heptapeptides according to their preferential consumption.*

Supplementary figures to Study 4

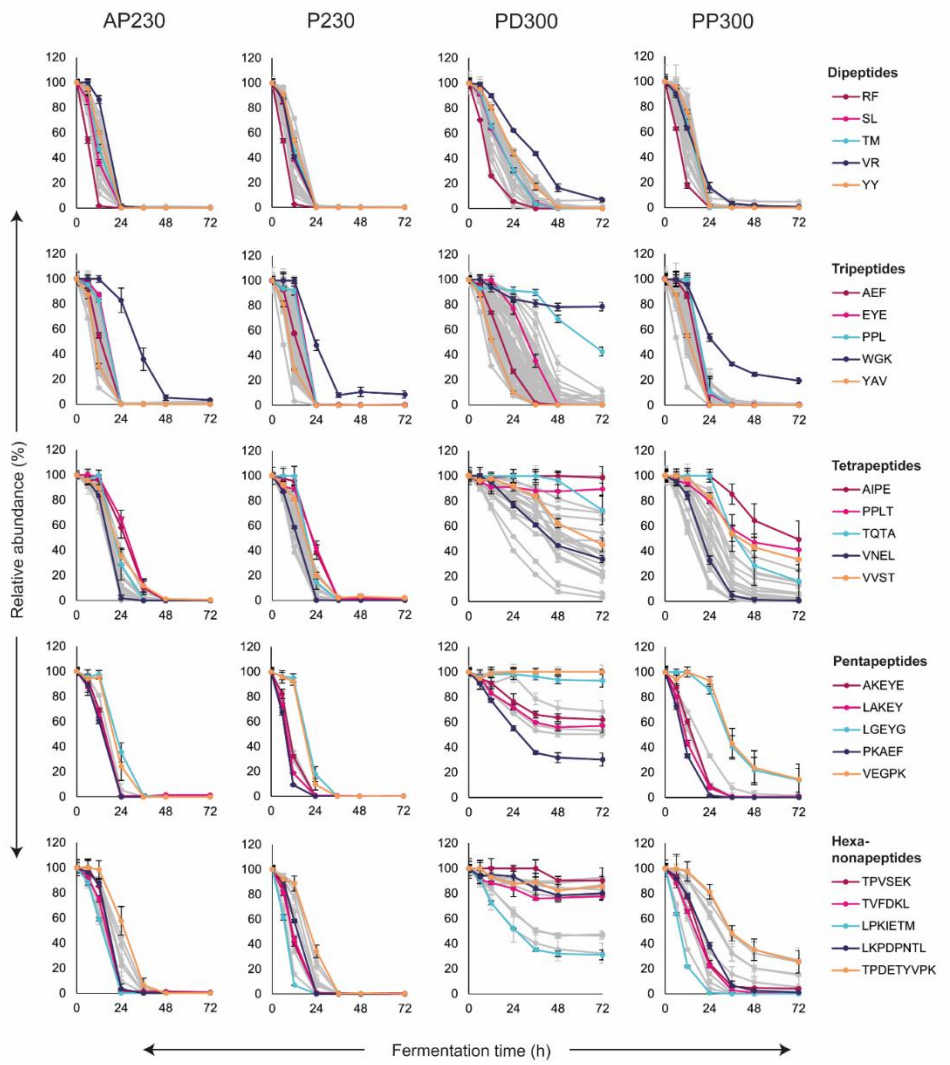

Figure S7. Oligopeptide consumption curves of Strain SC1 in synthetic whisky mash with different nitrogen conditions. *Five oligopeptides are depicted in colour to represent their peptide length group. All other oligopeptide consumption curves are depicted as 'shadow' in the background.*

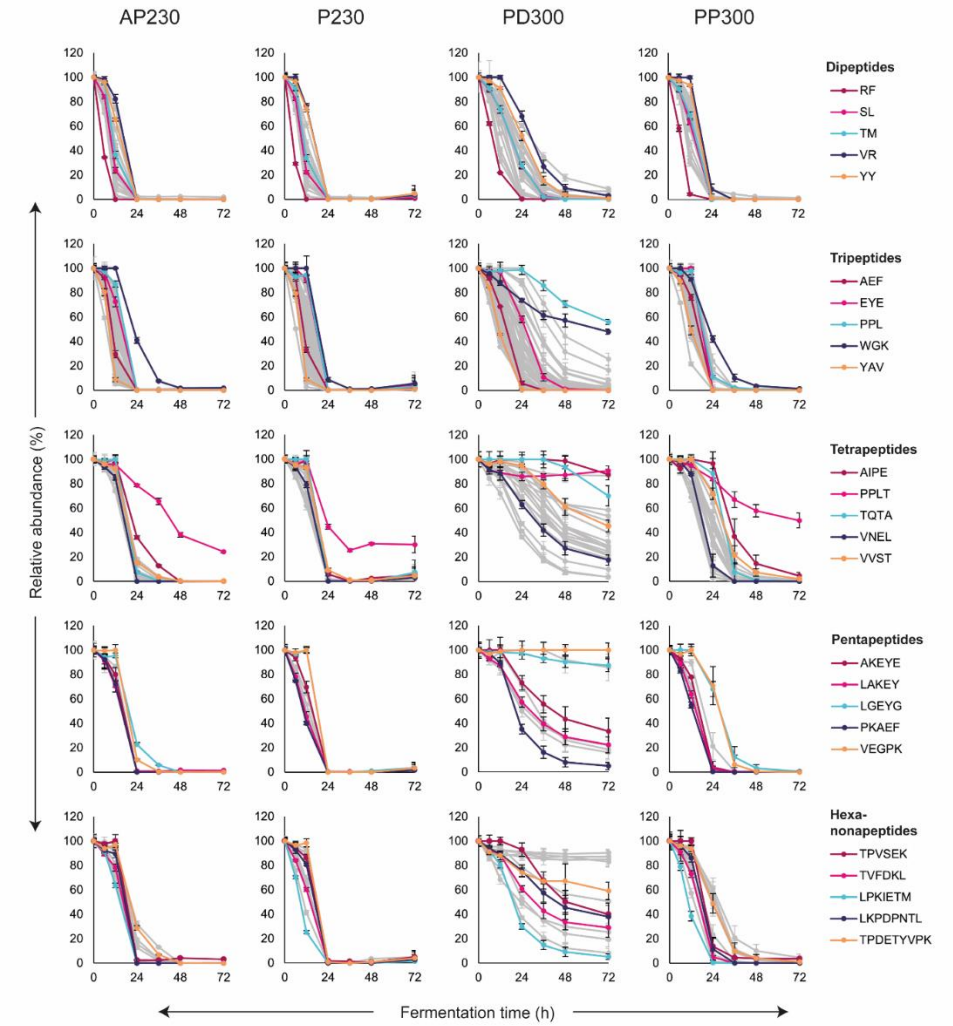

Figure S8. Oligopeptide consumption curves of Strain SC2 in synthetic whisky mash with different nitrogen conditions. *Five oligopeptides are depicted in colour to represent their peptide length group. All other oligopeptide consumption curves are depicted as 'shadow' in the background.*

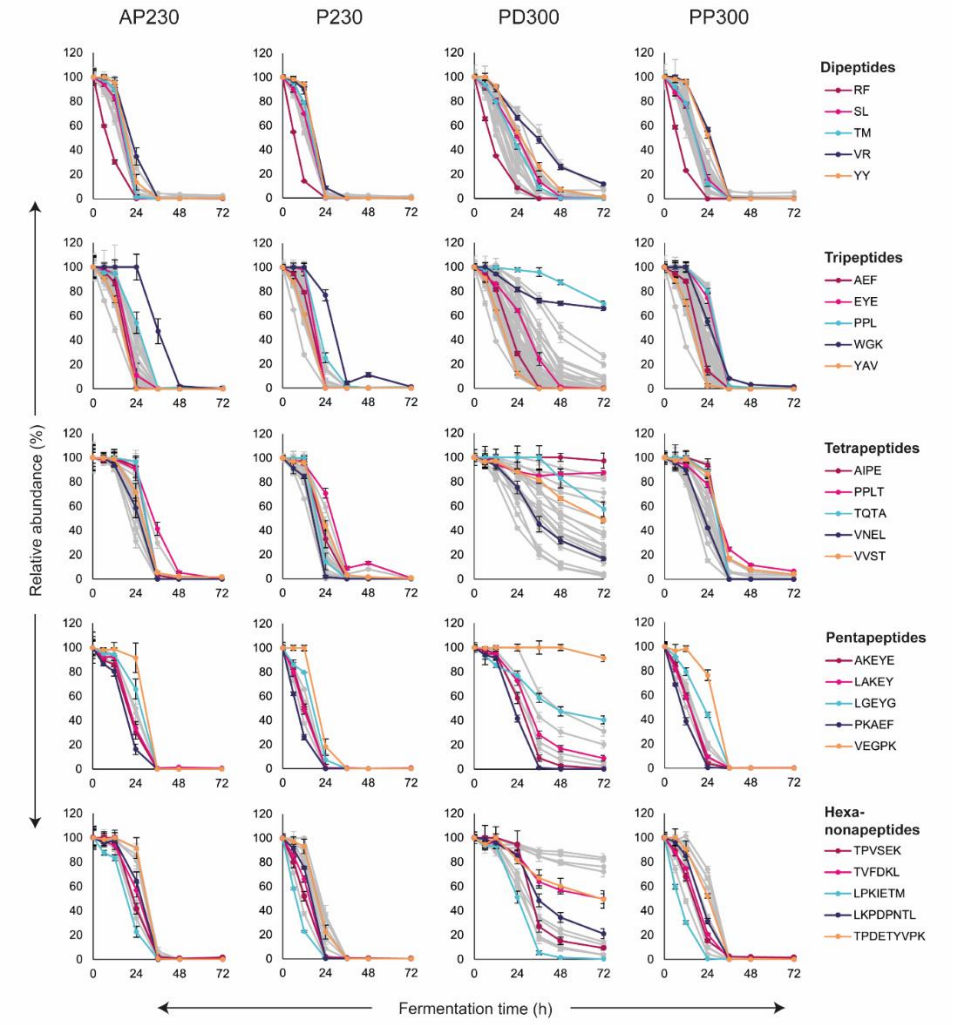

Figure S9. Oligopeptide consumption curves of Strain SC3 in synthetic whisky mash with different nitrogen conditions. *Five oligopeptides are depicted in colour to represent their peptide length group. All other oligopeptide consumption curves are depicted as 'shadow' in the background.*

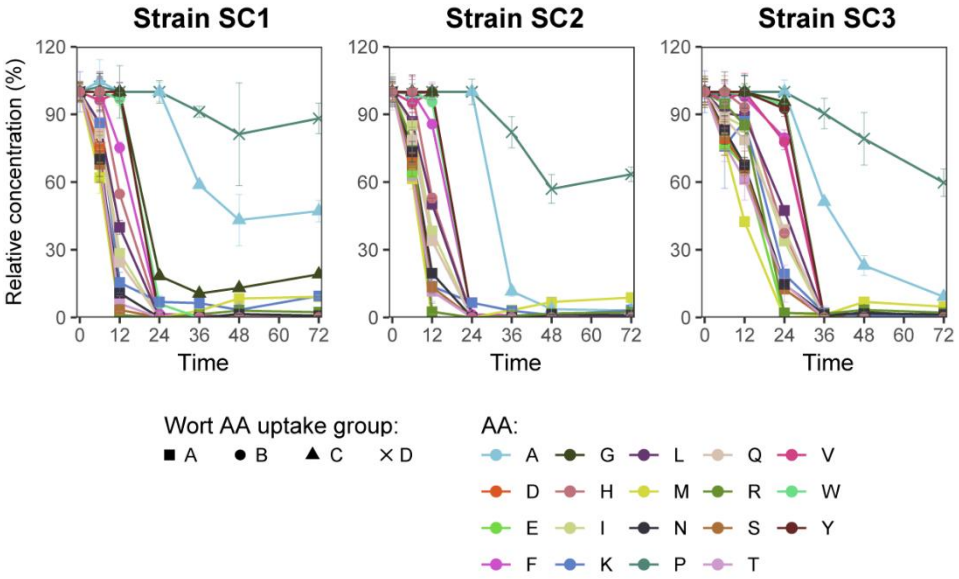

Figure S10. Relative amino acid consumption by strains SC1-3 in synthetic whisky mash (AP230). *Individual amino acids have been marked according to their AA uptake order group in wort defined by Jones and Pierce (1964) and revised by Lekkas et al. (2007).*

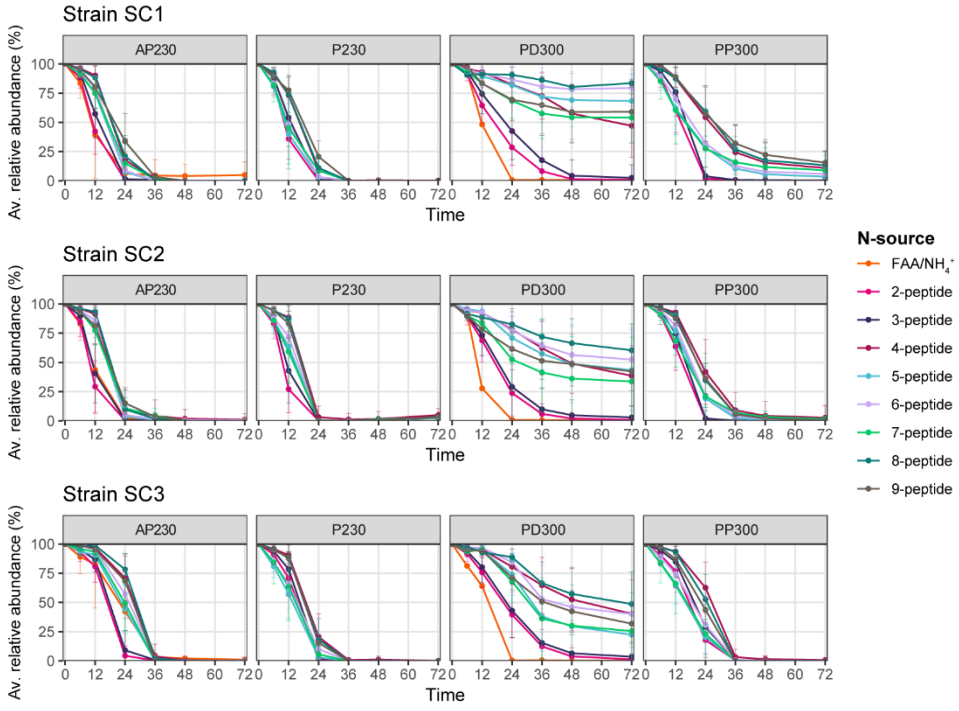

Figure S11. Average relative consumption curves sorted by the peptide length group in fermentation with three whisky strains in synthetic whisky mash with different nitrogen conditions. *The orange line in the AP230 medium represents FAA (individual consumption of AAs can be found in Figure S10), while in PD300 it represents ammonium (NH_4^+).*

Appendix 2

Publication I

Arju, G., Berg, H.Y., Lints, T., Nisamedtinov, I. (2022) Methodology for Analysis of Peptide Consumption by Yeast during Fermentation of Enzymatic Protein Hydrolysate Supplemented Synthetic Medium Using UPLC-IMS-HRMS. *Fermentation 8*, 145. https://doi.org/10.3390/fermentation8040145

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Article

Methodology for Analysis of Peptide Consumption by Yeast during Fermentation of Enzymatic Protein Hydrolysate Supplemented Synthetic Medium Using UPLC-IMS-HRMS

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Abstract: Several studies have shown the ability of yeast to consume peptides as a nitrogen source in single-peptide containing media. However, a suitable and cost-effective methodology to study the utilization of peptides by yeast and other microorganisms in a complex peptide mixture has yet to be put forward. This article addresses this issue by presenting a screening methodology for tracking the consumption of peptides by yeast during alcoholic fermentation. As a peptide source, the methodology makes use of an in-house prepared peptide-mapped bovine serum albumin (BSA) proteolytic digest, which was applied to a synthetic grape must. The peptide uptake was analyzed using high-throughput ultra-high-pressure liquid chromatography coupled to data-independent acquisition-based ion mobility separation-enabled high-resolution mass spectrometry (UPLC-DIA-IMS-HRMS) analysis. The relative changes of abundance of 123 di- to hexapeptides were monitored and reported during fermentations with three commercial wine strains, demonstrating different uptake kinetics for individual peptides. Using the same peptide-mapped BSA hydrolysate, the applicability of an untargeted workflow was additionally assessed for peptide profiling in unelucidated matrixes. The comparison of the results from peptide mapping and untargeted analysis experiments highlighted the ability of untargeted analysis to consistently identify small molecular weight peptides on the length and amino acid composition. The proposed method, in combination with other analytical techniques, such as gene or protein expression analysis, can be a useful tool for different metabolic studies related to the consumption of complex nitrogen sources by yeast or other microorganisms.

Keywords: liquid chromatography mass spectrometry; ion mobility separation; yeast assimilable peptides; alcoholic fermentation; protein hydrolysate

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1. Introduction

Sufficient assimilable nitrogen content is a prerequisite for successful alcoholic fermentation. *Saccharomyces cerevisiae*, the main microorganism used in the production of alcoholic beverages and bioethanol, is known to utilize ammonia, free amino acids and short peptides as nitrogen sources [1]. While the consumption preferences of *S. cerevisiae* are well studied for ammonia and free amino acids, the role of peptides as a yeast nitrogen source requires further elucidation.

Many feedstocks used in alcoholic fermentation contain a significant proportion of peptides that could be considered as an important nitrogen source for yeast. Peptides thus have a significant technological value. For example, in grape must, about 17% of the total nitrogen can be attributed to oligopeptides, while for beer wort, this number is estimated at around 40% [2,3]. The nitrogen levels in grape must are dependent on the grape variety

and grape cultivation conditions used. Due to these factors, the nitrogen levels in some cases can be deficient [4]. Similarly, high gravity fermentation with substantial content of adjuncts in beer and whisky production is a process that may suffer from nitrogen limitation [2,5].

In any fermentation, nitrogen deficiency can result in premature cessation or even complete arrest of the fermentation process, as well as in poor flavor properties of the product [6–8]. Fermentation nutrients, such as yeast autolysates, rich in free amino acids and peptides, are often used in industry to overcome this problem. Regardless of whether the peptides are present in the fermentation feedstock naturally or are supplemented with nutrients, a suitable screening methodology is required to characterize yeast strains for their ability to take up different peptides. The information obtained from such a screening method would be useful to better anticipate nitrogen deficiency and to avoid related fermentation problems through either specific nitrogen supplementation or the selection of yeast strains with broader peptide consumption capabilities.

S. cerevisiae can internalize peptides of different sizes through multiple membrane peptide transporters [9]. Di- and tripeptide transport is facilitated by Ptr2p, Dal5p and fungal oligopeptide transporters (Fot1-3p) found in oenological yeast [10–14]. However, it is not excluded that FOT transporters can also transport longer peptides [9]. Oligopeptide transporters (Opt1p and Opt2p) have been shown to be involved in tetra- and pentapeptide transport [15–17].

The current knowledge on functionality of different yeast peptide transporters has been mainly obtained using experiments with single synthetic peptides as the sole nitrogen source [10,14–18]. This type of experiment provides accurate information about the ability of yeast strains to utilize the selected peptide as a nitrogen source. However, they do not provide information on the kinetics of peptide utilization throughout different time points during fermentation on complex peptide-rich media, such as wort, grain mash and grape must. Moreover, the characterization of peptide transporters in more complex matrices and peptide utilization capability by different yeasts is challenging due to the lack of suitable cost-efficient screening media with defined and diverse peptide composition. Another challenge is the lack of accurate semi-quantitative analyses for monitoring the uptake of these peptides during fermentation.

Liquid chromatography coupled to mass spectrometry (LC-MS) is currently one of the most promising techniques for short peptide analysis [19–22]. The two biggest analytical challenges are insufficient short peptide identification accuracy for unambiguous identity assignment and throughput limitation caused by data-dependent acquisition (DDA). Unlike longer peptides (pentapeptides and longer) that, due to a higher number of peptide bonds, tend to ionize with a higher charge state ($\geq$2+), shorter peptides (di- to tetrapeptides) most commonly ionize as singly charged ions. The singly charged peptides produce fewer selective fragments, which leads to reduced identification accuracy [23].

Even though DDA is known to produce higher quality fragmentation spectra due to active quadrupole-based selection of precursor ions prior to fragmentation, it comes at he cost of a reduced acquisition rate that often results in missing data and prolonged analytical gradients [24]. In contrast, data-independent acquisition (DIA) minimizes the likelihood of missing data by rapidly alternating between low and high collision energies and assigning fragments based on the chromatographic elution profile [25]. For fermentation matrices with highly diverse peptide composition, the main drawback of such data acquisition is the generation of complex fragmentation spectra that are often caused by simultaneous fragmentation of closely eluting precursor ions.

A rapid gas-phase separation of ions, also commonly referred to as ion mobility (IM) separation, is often used as an orthogonal method of separation that complements chromatography and mass spectrometry [26]. Hyphenation of travelling wave ion mobility separation (TWIMS) with DIA results in similar drift times for both fragment and precursor ions, allowing alignment by both the chromatographic and ion mobility elution profiles and thus improving the spectral clarity. Moreover, the "trap–release" mode of operation

of the TWIMS results in not only increased peak capacity but also in increased duty cycle of the mass spectrometer. This allows for shorter analytical gradients and the use of the collisional cross section (CCS) as an additional identification qualifier.

This study reports a screening methodology for tracking the consumption of a wide range of peptides by yeast during alcoholic fermentation. The methodology makes use of an in-house prepared peptide-mapped (against a known protein sequence) proteolytic digest of bovine serum albumin (BSA) as a peptide source that was applied to a synthetic grape must. The peptide composition in the hydrolysate as well as the consumption of peptides by yeast in the synthetic grape must were analyzed using high-throughput ultra-high-pressure liquid chromatography coupled with data-independent acquisition-based ion mobility separation-enabled high-resolution mass spectrometry (UPLC-DIA-IMS-HRMS).

To simulate the analysis of an unelucidated matrix, untargeted data analysis (that does not rely on a protein sequence) of the BSA hydrolysate was additionally performed and compared to the results of peptide mapping to assess the identification accuracy of the untargeted analysis.

2. Materials and Methods

An LC-MS methodology was developed for analyzing peptide composition in BSA proteolytic digest (peptide mapping), as well as for monitoring the relative concentration changes of these peptides during fermentation with three commercial wine yeast strains (screening). A modified synthetic grape must with a reduced concentration of free amino acids and ammonia was used as a base fermentation medium and was supplemented with additional nitrogen in the form of peptides from proteolytic digest of BSA. The relative changes of peptide abundances during fermentation were monitored and reported.

Peptide mapping requires a protein sequence to identify peptides. However, when analysing fermentation feedstocks with unknown protein/peptide content, such information is not always available. Therefore, the peptide identification accuracy of an untargeted workflow should be investigated. For this purpose, the peptide identification results obtained by untargeted data analysis against a short peptide database were compared to the results obtained by peptide mapping.

2.1. Chemicals

Hi3 *E.coli* STD (p/n: 186006012) and Leucine-Enkephalin (p/n: WT186006013) were purchased from Waters Corporation (Milford, MA, USA). Ultrapure water (18.2 MΩ×cm) was prepared with MilliQ® IQ 7000 equipped with LC-Pak (Merck KGaA, Darmstadt, Germany). Acetonitrile (MeCN; LiChrosolv, hypergrade for LC-MS), formic acid (FA; LC-MS grade), bovine serum albumin (lyophilized powder, ≥96%), fructose, cobalt chloride, boric acid, ammonium molybdate tetrahydrate, ergosterol, oleic acid, all vitamins and cysteine were acquired from Sigma-Aldrich (Darmstadt, Germany).

Histidine, methionine, glutamic acid and arginine were acquired from SERVA Electrophoresis GmbH (Heidelberg, Germany). Alanine, aspartic acid, cysteine, glutamine, glycine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine were acquired from Thermo Fisher Scientific (Waltham, MA, USA). Hydrogen chloride (HCl, 36%) was acquired from Labbox Labware (Barcelona, Spain). Liquified phenol was acquired from Avantor® (Wayne, PA, USA).

2.2. Fermentation Media

2.2.1. BSA Hydrolysate

Hydrolysis of BSA (40 g/L dH_2O) was conducted in a 1 L benchtop fermenter (Applikon, Delft, The Netherlands) using the industrial protease COROLASE®7089 (AB Enzymes, Darmstadt, Germany), which was selected based on its endoproteolytic activity. This facilitated the greater production of low molecular weight peptides, which were potentially assimilable by yeast. The protease dose rate was 0.5% *w/w* of BSA, and hydrolysis was performed at pH 7 (maintained by titration using 2 M NaOH) for 20 h at 50 °C. Af-

ter completion of the BSA hydrolysis, the hydrolysate was filtered with a Vivaflow®200 10 kDa cut-off Hydrosart crossflow cassette (Sartorius, Göttingen, Germany) to remove the protease as well as larger peptides. It was then freeze-dried and stored at −20 °C until further use.

As the scope of this research was to develop a methodology for following small molecular weight peptide consumption by yeast during alcoholic fermentation, the nitrogen content of peptides smaller than 1 kDa in the fraction was determined from the bound amino acid content and used to calculate the amount of total hydrolysate to be added into the nitrogen deficient synthetic grape must. The sub-1-kDa fraction for amino acid analysis was prepared by rehydrating the BSA hydrolysate (1 g/L dH_2O) and filtering through a Pall® Microsep Advance 1K Omega™ centrifugal filter device (Pall® Corporation, Port Washington, NY, USA).

The amounts of free and total amino acids in this fraction were analyzed on a Waters ACQUITY UPLC® system (Waters Corporation, Milford, MA, USA) that was coupled to a TUV detector after derivatization using Waters AccQ-Tag chemistry as described by Fiechter and Mayer [27]. For the total amino acids, the samples were first hydrolyzed with 6M HCl + 1% (*v/v*) phenol for 22 h in a vacuum using an Eldex H/D workstation (Eldex Laboratories Inc., Napa, CA, USA). The amount of bound amino acids was calculated by subtracting the free amino acids from the total amino acid concentration.

2.2.2. Synthetic Grape Must

A synthetic grape must (MS300) as described by Salmon and Barre [28] was prepared with a reduced initial nitrogen content. The free amino acid and NH_3 concentrations were both reduced by 75% to provide 116 mg/L of nitrogen, which was considered insufficient to ferment 210 g/L of fermentable sugars in the synthetic grape must [29,30]. The amino acid and NH_3 concentrations can be found in Table A2. An additional 136 mg/L of nitrogen was then supplemented in the form of peptides from the BSA hydrolysate, resulting in 252 mg/L nitrogen in the synthetic grape must.

2.3. Fermentation

Three commercial *S. cerevisiae* wine yeast strains (Lalvin™ ICV Opale 2.0, Lalvin™ Persy and Lalvin™ QA23), provided by Lallemand Inc. (Montreal, QC, Canada), were used in this study. All strains were obtained as active dry yeast cultures. Inoculum was prepared by rehydrating 1 g of dry culture in 10 mL of sterile 0.9 % NaCl for 15 min at room temperature. The synthetic grape must was then pitched to give an initial yeast concentration of about $5 \cdot 10^6$ cells/mL.

All fermentations were performed at 24 °C in 250 mL Pyrex™ bottles equipped with a GL45 open top PBT screw cap and PYREX™ Media Bottle Septum (Corning Inc., Corning, NY, USA). A sampling port was assembled with size 13 Masterflex™ Norprene™ Food L/S™ Precision Pump Tubing (Masterflex SE, Gelsenkirchen, Germany), connected with a metal tubing piercing through the septum. A gas outlet was installed to prevent overpressure by piercing the septum with a Sterican® Ø 0.8 × 40 mm single-use hypodermic needle (B. Braun, Melsungen, Germany) attached to a Millex-FG 0.2 µm hydrophobic PTFE filter (Merck KGaA, Darmstadt, Germany).

The initial volume of synthetic grape must was 200 mL. Samples (3 mL) were collected at 0, 24, 48, 72 and 168 h. For each strain, a control fermentation was performed in parallel without the addition of BSA hydrolysate.

2.4. Sample Preparation

Prior to peptide analysis, samples (200 µL) were mixed with MeCN (200 µL) in an Eppendorf® tube (Eppendorf AG, Hamburg, Germany) to precipitate proteins, vortexed for 30 s and centrifuged at 11,200 × *g* for 15 min. The supernatant (100 µL) and MilliQ® water (900 µL) were then transferred into a new Eppendorf® tube and vortexed for 30 s.

The obtained mixture (100 µL) was transferred into a low-volume insert and spiked with 20 µL of 1 pmol/µL of Hi3 *E.coli* STD.

Before and after injection of the batch of samples, a blank (MilliQ® water) and a blank (100 µl MilliQ® water) spiked with 20 µL of 1 pmol/µL of Hi3 *E.coli* STD were injected to determine the baseline and system variability. A pooled sample (100 µL) spiked with 20 µL of 1 pmol/µL of Hi3 *E.coli* STD was also injected three times at the beginning of the experiment and one time after each strain to determine the intra-batch variability.

2.5. Liquid Chromatography Mass Spectrometry

Samples were analyzed using Waters I-Class Plus (SM-FL) UPLC® system (Waters Corporation, Milford, MA, USA) coupled with a Waters Vion IMS-QTof Mass Spectrometer equipped with LockSpray II Exact Mass source enclosure and ESI Tool-free Mk3 UPC^2 probe (50 µm × 750 mm, p/n: 700011376) directly connected to the column outlet. Nitrogen was used as collision gas. The instrument was controlled by Waters UNIFI 1.9.4 (3.1.0, Waters Corporation, Milford, MA, USA).

Mobile phases (MP) were as follows: (A) MilliQ® + 0.1% formic acid and (B) MeCN + 0.1% formic acid. The weak needle wash was 90:10 (MilliQ®:MeCN), the strong needle wash was 10:90 (MilliQ®:MeCN), and the seal wash was 50:50 (MilliQ®:MeCN). Injection volume was 2 µL. Samples were analyzed using Acquity UPLC® HSS T3 Column (1.8 µm, 1 × 150 mm, Waters Corporation, Milford, MA, USA) kept at 40 °C. The initial flow rate was 0.1 mL/min. The gradient was as follows: a 0–0.5 min hold at 1% B, 0.5–10.5 min linear gradient 1–50% B, 10.5–12.5 min linear gradient, 50–99% B accompanied by a linear flow rate increase of 0.1–0.2 mL/min, 12.5–14.5 min hold at 99% B, 14.5–15 min linear gradient 99–1% B accompanied by a linear flow rate decrease of 0.2–0.1 mL/min, and 15–18.5 min hold at 1% B.

The instrument was operated in positive polarity, sensitivity mode (33,000 FWHM at 556.2766 *m/z*) and labile ion mobility tune. The analysis type was set as Peptide Map (IMS) and the experiment type was set to MSe. Data was acquired in HDMSe mode with a scan time of 0.2 s. The following manual quadrupole profile was used: mass 150/300/600 (*m/z*), dwell time 50/30 (%scan time), ramp time 10/10 (%scan time). The recorded mass range was from 50 to 1000 *m/z* for both low and high energy spectra. The collision energy was ramped from 10 to 63 V.

The cone voltage was set to 30 V, capillary voltage was set to 0.5 kV and source offset was set to 50 V. Source temperature was set to 120 °C and desolvation temperature set to 500 °C. Cone gas flow rate was set to 50 L/h and desolvation gas flow rate was set to 700 L/h. Leucine-enkephalin (50 pg/µL at 15 µL/min) was used as LockMass for mass axis correction and was acquired before and after each acquisition as well as every 5 min during the acquisition.

2.6. Data Processing

For peptide identification in the BSA hydrolysate, peptide mapping was conducted using UNIFI Large Molecule Package (LMP) by searching the peptides against the sequence of bovine serum albumin (ALBU_BOVIN; P02769). Low energy intensity threshold of 2000 counts and high energy intensity threshold of 200 counts were used. The retention time range was set between 1 and 12 min. Peptide search was set to non-specific digest with no missed cleavages allowed, and the minimum sequence length was set to 2.

The following filters were applied: mass error between −3 and 3 ppm, matched first gen primary ions greater than or equal to 1, fragment label not containing in-source fragments, loss of H_2O and loss of NH_3. For relative quantification (screening of peptide uptake by yeast) and untargeted analysis of short peptides the data was exported using UNIFI Export Package (UEP) to Progenesis QI (PQI) software (Nonlinear Dynamics, Newcastle, UK). During the import, masses were lock mass corrected with 556.2766 *m/z*, corresponding to singly charged Leucine-enkephalin. Data was subjected to normalization of compound

abundances based on a set of reference housekeeping peptides (spiked Hi3 *E.coli* STD). Automatic sensitivity at level 2 was used.

Automatic retention time alignment was conducted, and the retention time limits were set between 1 and 12 min. Fragment sensitivity of 1% of the base peak was used. The following adducts were used for peptide mapping: M+H, M+2H and M+3H. ChemSpider data source: peptides (PQI_CS_Peptides), containing in-silico database of short peptides (2-4 AA), were used for de novo identification. The precursor ion tolerance was set to 3 ppm and fragment ion tolerance was set to 5 ppm. One or more fragment ions per peptide were required for ion matching.

2.7. Data Analysis

Peptide peaks identified by peptide mapping and untargeted analysis of short peptides were aligned using observed mass to charge ratio, charge, observed retention time (min) as well as collision cross section ($Å^2$). Peptides without a distinct consumption trend were filtered out using PQI software. Peptides with up to six amino acids in length and an initial abundance higher than 0.1% of the summed abundance of all peptides were considered and reported for each fermentation experiment.

The aligned data was further used to construct a data matrix for the aforementioned data processing methods. The comparison between two identification methods was performed with the help of in-house data analysis and visualization scripts written in the Python™ programming language (Python Software Foundation, Wilmington, DE, USA). The identification accuracy and reproducibility assessment between peptide mapping and untargeted analysis was performed based on four categories: absolute amino acid sequence match (Absolute), amino acid sequence match while leucine (L) and isoleucine (I) were not differentiated and annotated as "J" (J Absolute), and amino acid composition match (J Composition) and peptide length match (Length).

3. Results

3.1. BSA Hydrolysate Composition

The total nitrogen in the BSA hydrolysate was determined in three fractions: free amino acids, peptides with molecular weight (MW) smaller than 1 kDa and peptides with MW higher than 1 kDa. Most of the nitrogen (69%; 81.4 mg/g) came from peptides with MW below 1 kDa. Endoproteolytic activity of COROLASE® 7089 was apparent as only 7% of total nitrogen could be assigned to free amino acids (8.1 mg/g). The remaining 24% of nitrogen (29 mg/g) was present in the higher than 1 kDa fraction. The degree of hydrolysis was calculated, as described by Adler-Nissen [31] and was 40%.

3.2. LC-MS Method Reproducibility

We observed that the default settings of the ion optics and quadrupole of Vion IMS-Qtof resulted in a lower response of the lower molecular weight peptides. Combination of the labile ion mobility tune and a manual short peptide specific quadrupole transmission profile resulted in an increased response of di- and tripeptides. The system performance was found to be highly reproducible as illustrated by Figure 1 (overlay of three technical replicates). The standard deviation of the peptide retention times was below 0.1 min, and the PQI alignment score was over 95% for the six QC samples and over 92% for all 36 samples.

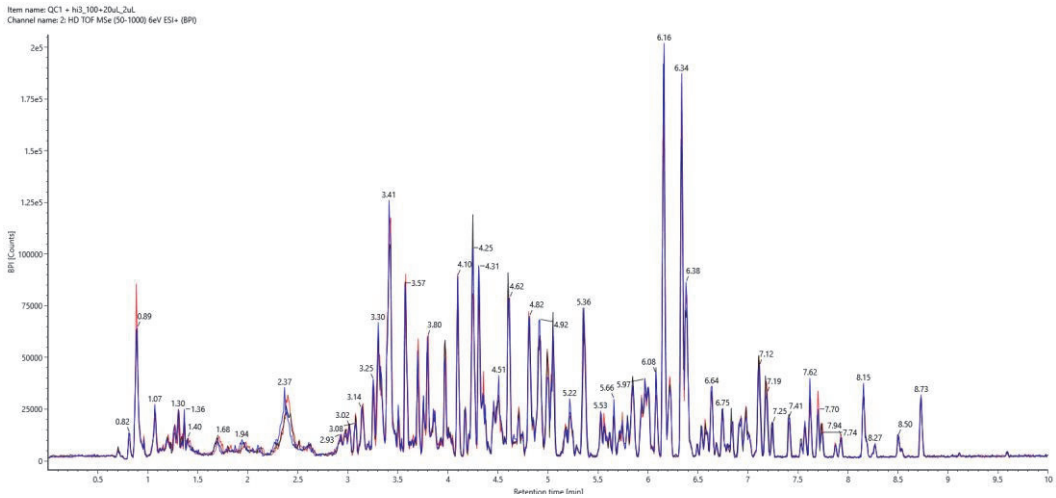

Figure 1. Overlay of triplicate injection of Hi3 *E.coli* STD spiked pooled-sample base peak intensity (BPI) chromatograms.

Moreover, the starting points of all three fermentations display an average relative standard deviation of 5.27% for the intensities of the peptides of interest. The average peak width at 10% of peak height was 8 s, thus, resulting in not only enough data points for reliable qualitative and quantitative integration but also in a sufficient number of data points for accurate tracking of the peak's lift-off, apex and touch-down points for improved high-to-low energy spectra association.

3.3. Fermentation and Peptide Uptake Kinetics

The maximum CO_2 production rate was reached at about 48 h in case of all strains. However, the fermentations supplemented with the BSA hydrolysate showed increased sugar consumption, higher CO_2 production and faster biomass accumulation compared to the corresponding controls without the hydrolysate (Figure 2). At the final experimental point (168 h), up to 95% of sugars were consumed in fermentations with added BSA hydrolysate, whereas up to 83% of sugars were consumed without hydrolysate addition (Table A1).

In total, 123 peptide candidates complying with data analysis filtration criteria (19 dipeptides, 41 tripeptides, 31 tetrapeptides, 19 pentapeptides and 13 hexapeptides) were monitored throughout the fermentation experiments. The relative changes of individual peptide candidate intensities during the fermentation of the synthetic grape must are shown in Figures 3–7 and Tables A4–A8.

All three strains demonstrated similar uptake trends of di-, tri- and tetrapeptides. For all strains, di- and tripeptides were consumed during the first 48 h of fermentation, simultaneously with the free amino acids (Figure 8). The uptake rate of di- and tripeptides was higher by Lalvin Persy™ compared to Lalvin ICV Opale 2.0™ and Lalvin QA23™, as several peptides were consumed by this strain already at 24 h.

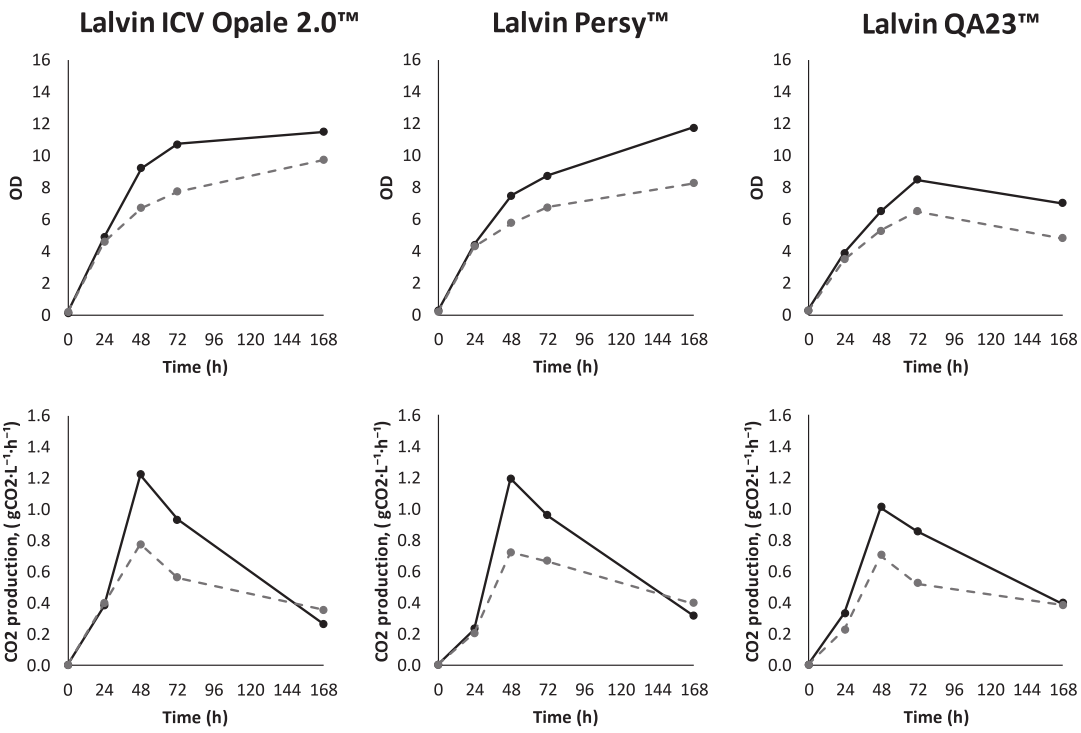

Figure 2. The optical density (OD) and CO_2 production rate in fermentations with Lalvin ICV Opale 2.0™, Lalvin Persy™ and Lalvin QA23™ of the modified synthetic must with (solid line) and without (dashed line) added BSA hydrolysate as the peptide source.

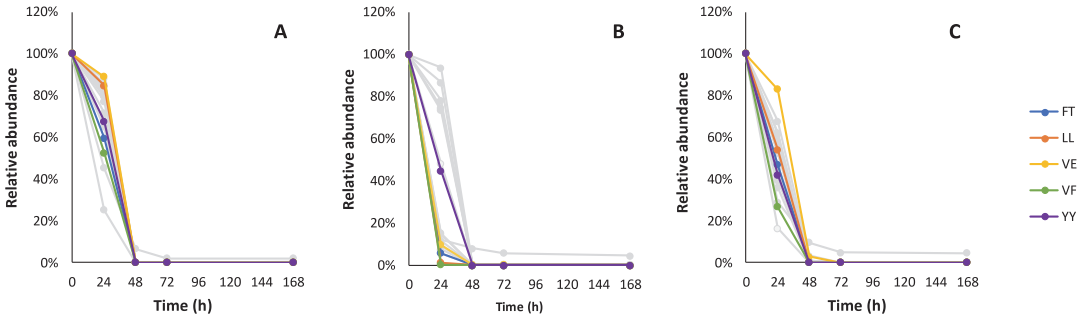

Figure 3. The relative consumption trends of 19 dipeptides in fermentation with Lalvin ICV Opale 2.0™ (**A**), Lalvin Persy™ (**B**) and Lalvin QA23™ (**C**) of the modified synthetic must with added BSA hydrolysate as the peptide source. The five dipeptide candidates with the highest abundance are shown in colour. Other dipeptide candidate sequences and their observed relative consumption trends are shown in Table A4.

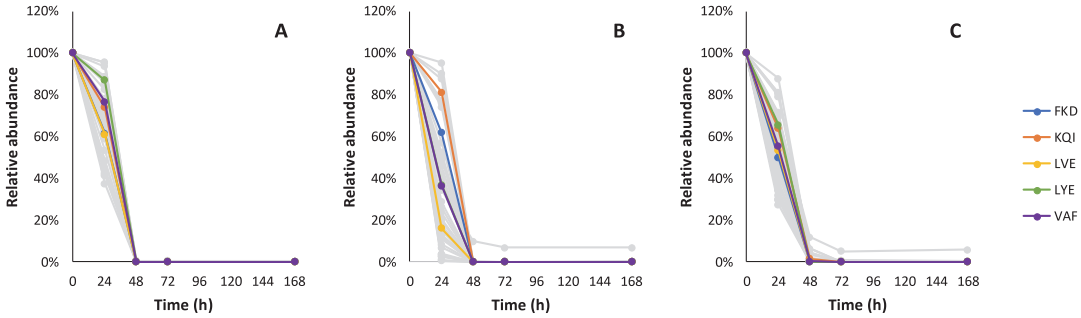

Figure 4. The relative consumption trends of 41 tripeptides in fermentation with Lalvin ICV Opale 2.0™ (**A**), Lalvin Persy™ (**B**) and Lalvin QA23™ (**C**) of the modified synthetic must with added BSA hydrolysate as the peptide source. The five tripeptide candidates with the highest abundance are shown in colour. Other tripeptide candidate sequences and their observed relative consumption trends are shown in Table A5.

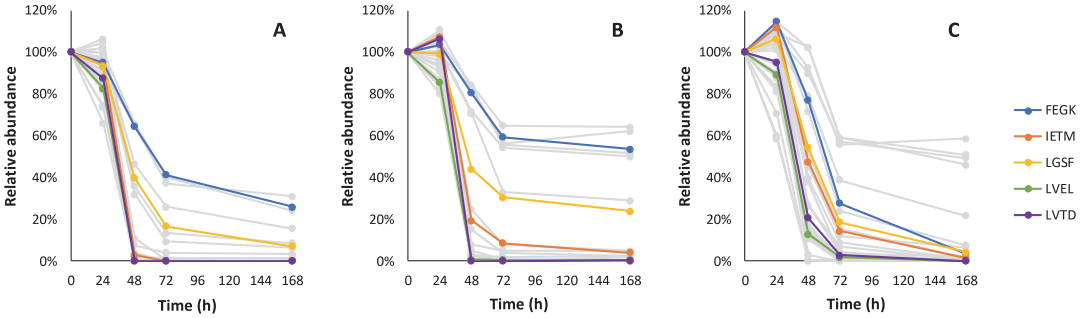

Figure 5. The relative consumption trends of 31 tetrapeptides in fermentation with Lalvin ICV Opale 2.0™ (**A**), Lalvin Persy™ (**B**) and Lalvin QA23™ (**C**) of the modified synthetic must with added BSA hydrolysate as the peptide source. The five tetrapeptide candidates with the highest abundance are shown in colour. Other tetrapeptide candidate sequences and their observed relative consumption trends are shown in Table A6.

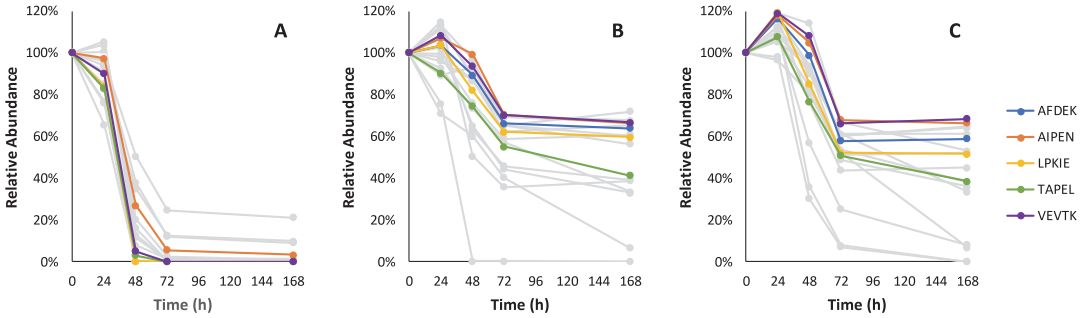

Figure 6. The relative consumption trends of 19 pentapeptides in fermentation with Lalvin ICV Opale 2.0™ (**A**), Lalvin Persy™ (**B**) and Lalvin QA23™ (**C**) of the modified synthetic must with added BSA hydrolysate as the peptide source. The five pentapeptide candidates with the highest abundance are shown in colour. Other pentapeptide candidate sequences and their observed relative consumption trends are shown in Table A7.

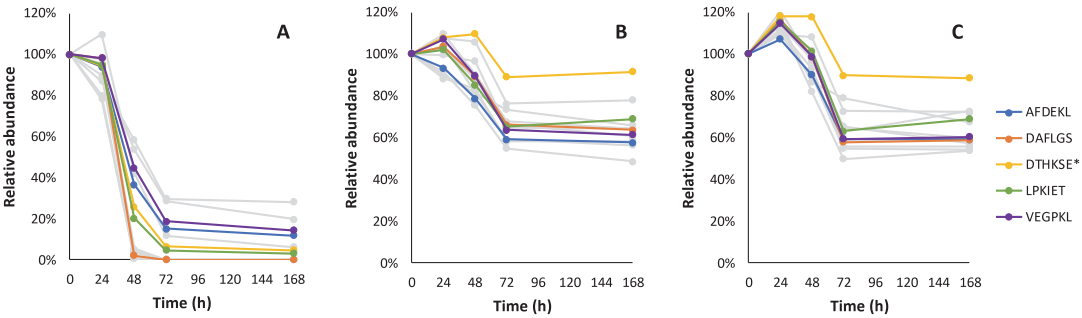

Figure 7. The relative consumption trends of 13 hexapeptides in fermentation with Lalvin ICV Opale 2.0™ (**A**), Lalvin Persy™ (**B**) and Lalvin QA23™ (**C**) of the modified synthetic must with added BSA hydrolysate as the peptide source. The five hexapeptide candidates with the highest abundance are shown in colour. Other hexapeptide candidate sequences and their observed relative consumption trends are shown in Table A8. * DTHKSE elutes within the void volume.

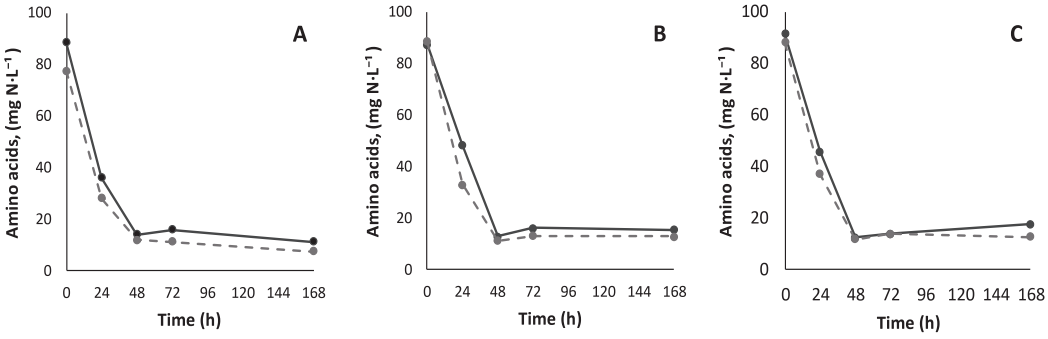

Figure 8. Free amino acid consumption trends in fermentations with Lalvin ICV Opale 2.0™ (**A**), Lalvin Persy™ (**B**) and Lalvin QA23™ (**C**) of the modified synthetic must with (solid line) and without (dashed line) added BSA hydrolysate as the peptide source.

Unlike di- and tripeptides, not all tetra- to hexapeptides were fully depleted (signal to noise ratio < 3:1), and their uptake ceased when the growth of cells entered the stationary phase (Lalvin ICV Opale 2.0™ and Lalvin QA23™) or slowed down remarkably (Lalvin Persy™) after 72 h. The differences in peptide uptake between strains were most notable for penta- and hexapeptides. Thus, the relative abundances of pentapeptides at 168 h decreased on average by 49% for Lalvin Persy™ and 58% for Lalvin QA23™, and hexapeptides decreased by 35% for both strains. The respective values for Lalvin ICV Opale 2.0™ were 98% for pentapeptides and 93% for hexapeptides.

3.4. Untargeted Peptide Analysis

The results of the untargeted data analysis (using the PQI_CS_Peptides database) were compared against the results obtained by peptide mapping to assess the identification accuracy of the untargeted workflow.

Figure 9 highlights the ability of the untargeted approach to identify all di- to tetrapeptides in the BSA hydrolysate in comparison to the peptide mapping. A trend emerged highlighting how the number of peptides assigned identically by both search engines depends on the identification specificity. Only 27.4% of the peptides were matched on the level of absolute amino acid sequence and 37.5% on the level of "J Absolute" where leucine (L) and isoleucine (I) were not differentiated and annotated as "J". On the level of "J

Composition", where the order of the amino acids in the sequence was ignored and only the amino acid compositions of the peptide was considered, 71.4% of peptides were matched. Lastly, on the level of "Length", where only the length of the peptide was considered, 90.5% of peptides were matched.

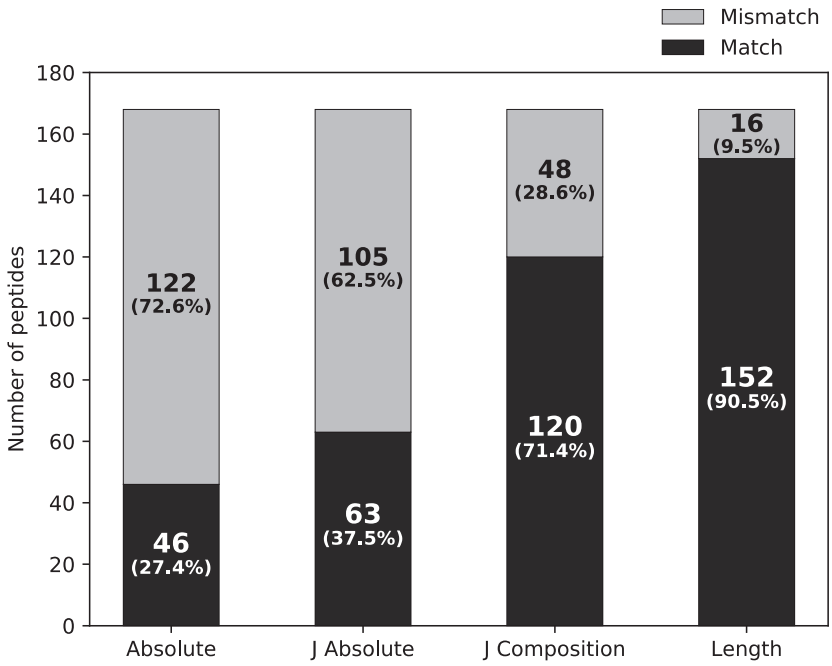

Figure 9. The dependence of the peptide matching specificity criteria on the peptide identification assignment discrepancy: absolute amino acid sequence match (Absolute), amino acid sequence match, where leucine (L) and isoleucine (I) were not differentiated and are annotated as "J" (J Absolute), amino acid composition match (J Composition) and peptide length match (Length).

Figure 10 displays the identification discrepancies between the two methods regarding the peptide length assignment for all di- to tetrapeptides. For dipeptides, the match was 100%: all 29 dipeptides were assigned by the untargeted method and the peptide mapping. The tripeptide length assignment displayed minimal discrepancy: out of 62 tripeptides identified by the untargeted method, only one (1.6%) was assigned as a tetrapeptide by the peptide mapping.

A larger discrepancy was observed for tetrapeptides: out of 77 tetrapeptides assigned by the untargeted method, three (3.9%) were assigned as tripeptides by peptide mapping, and 12 (15.6%) were assigned as pentapeptides by peptide mapping. The larger mismatch for tetrapeptides can be partly explained by the fact that the PQI_CS database contains sequences only up to tetrapeptides. While the peptide mapping approach resulted in a higher number of total identifications (not limited to di- to hexapeptides), a consistently low number (<10%) of unique (picked only by PQI) peptides were detected by the untargeted approach.

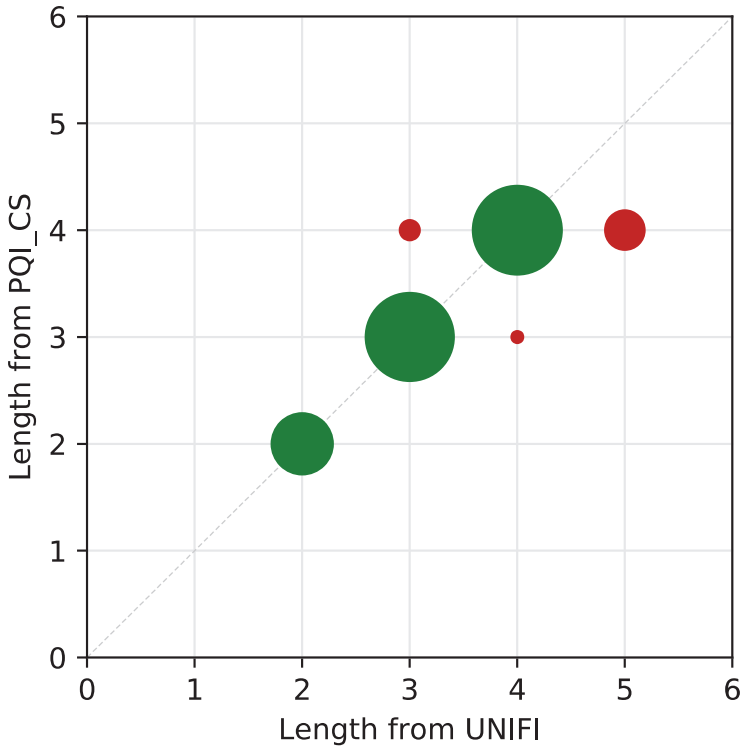

Figure 10. Length assignment discrepancies by the untargeted method in relation to peptide mapping. Green circles represent peptide identification overlap between the two identification methods, whereas red circles represent mismatches between peptide identifications. Circle areas are scaled to represent the ratios between matched and mismatched peptides.

4. Discussion

In this study, we explored the application of an in-house produced BSA protein hydrolysate as a cost-efficient model mixture of peptides for yeast studies. The results indicate the potential of this method for screening yeast strains for their ability to consume different peptides during alcoholic fermentation, for studying peptide transporters, as well as for evaluating the effect of peptides as a nitrogen source on fermentation kinetics. The hydrolysis of BSA with COROLASE® 7089 resulted in 123 di- to hexapeptides, followed by their consumption by different wine yeast strains during fermentation experiments.

The results highlight differences in penta- and hexapeptide consumption trends, indicating the need for further research. Until now, the studies of peptide consumption by *S. cerevisiae* have focused on di- to pentapeptides, mainly in single peptide-based systems and small volumes, mostly due to the higher cost of synthetic peptides. An example of such a system is the Biolog (Hayward, CA, USA) Phenotype MicroArrays (PM) used by Becerra-Rodríguez et al. [10] to characterize FOT transporter knock-out strains for their ability to utilize 270 dipeptides and 14 tripeptides as nitrogen sources using a 96-well plate system. The ability of yeast to consume an individual peptide was assessed based on the growth in the medium containing a single peptide as the nitrogen source.

Despite being a high-throughput method for screening, this approach, due to its set-up (one single peptide per experiment) and very small volume, imposes several limitations for more in-depth studies of peptide consumption. These limitations exclude the possibility to determine fermentation and peptide uptake kinetics as well as peptide transporter

expression analysis in the continuously changing environment of fermentation processes. The application of in-house-prepared protein hydrolysates allows for a more versatile and cost-effective approach to studying yeasts and other microorganisms for their ability to utilize various peptides in environments with more complex peptide compositions, which resemble more natural fermentation environments.

For this study, COROLASE® 7089 was chosen due to its high and broad endoproteolytic activity, which resulted in a higher number of di- to hexapeptides, which is important when peptide consumption by yeast is studied. However, the endoprotease and the protein source could be easily substituted with alternatives producing hydrolysates with different peptide compositions, allowing the tailor-made production of peptide mixtures based on various (micro-)organism-specific requirements [32–35].

For peptide composition analysis, it is typically recommended to hydrolyze a single protein with a known amino acid sequence. The identification of peptides against a known protein sequence allows for an additional degree of confidence in the identification accuracy. However, a protein sequence is not always available, and thus de novo identification should be used. The challenge of characterizing peptide mixtures using industrial proteases is often accompanied by unelucidated cleavage specificity of the proteases. This is also evident when peptide composition in natural fermentation feedstocks is to be analyzed, emphasizing the importance of the identification accuracy.

The results generated in this work by the untargeted analysis suggest that the method does not allow for small peptide unambiguous absolute sequence identification, which might be crucial for studying peptide transporters specificities. However, the method allows for peptide identification at the level of peptide length ("Length", 90.5%) and amino acid composition, where isoleucine or leucine are not differentiated ("J Composition", 71.4%), suggesting its potential for characterization of the composition of unelucidated natural peptide-containing fermentation matrices, such as grain mash, wort or grape must.

Even though the TWIMS used in this study increased the peak capacity and allowed for a shorter gradient time, the IMS resolution of the system was insufficient to reliably distinguish between isobaric peptides of the same length and different sequences. However, the collision cross section values were found to provide an extra qualifier when aligning features between two peak picking methods.

Quantitative representation of the content and consumption of all peptides remains a topic of further research. Due to the variation in the ionization efficiency of different peptides [36], consumption trends could only be used for tracking individual peptides across different samples, setting a limitation regarding the analysis of specific consumption rates of individual peptides by yeast in complex environments. The absolute quantitation of peptides remains reliant on labelled isotope dilution or tandem-mass-tag labelling [37–39].

5. Conclusions

We developed a methodology for characterization of the peptide composition in enzymatically produced hydrolysates of bovine serum albumin and implemented high-throughput monitoring of the peptide consumption trends of yeast during the alcoholic fermentation of a BSA-hydrolysate-supplemented synthetic grape must.

A correlation between the identification results of peptide mapping and untargeted analysis of di- to tetrapeptides (limited by the PQI_CS_Peptides database) suggests the potential of the untargeted analysis for following the peptide composition in unelucidated matrices, such as grain mash, wort or grape must. The method can additionally be used for profiling various enzymatically produced protein hydrolysates. However, consumption trends can only be used for tracking individual peptides across different samples. For the absolute quantitation of short peptides, the applications of tandem-mass-tag labelling must be further studied. The proposed methods, in combination with other analytical techniques, such as gene or protein expression analysis, can be a useful tool for different metabolic studies related to the consumption of complex nitrogen sources by yeast or other microorganisms.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Sugars and ethanol were measured using the Waters Alliance 2695 system (Waters Corporation, Milford, MA, USA). The flow rate used was 0.6 mL/min. The mobile phase was isocratic for 25 min on 100% *v/v* 0.5 mmol H_2SO_4 in MilliQ water. The column used for analyses was Bio-Rad HPX-87H column (Bio-Rad Laboratories Inc, Hercules, CA, USA) with the dimensions 7.8 × 300 mm and 9 µm particle size, coupled to a Micro-Guard Cation H Cartridge pre-column (Bio-Rad Laboratories Inc, Hercules, CA, USA). The analytes were detected by the refractive index detector.

Table A1. Glucose, fructose and ethanol measurements at the start and end of fermentation with Lalvin ICV Opale 2.0™, Lalvin Persy™ and Lalvin QA23™ of the modified synthetic must. 'Control' is without added peptides. 'BSA' is with added peptides.

Strain		Glucose Consumed ($g \cdot L^{-1}$)	Fructose Consumed ($g \cdot L^{-1}$)	Ethanol Produced ($g \cdot L^{-1}$)
Lalvin ICV Opale 2.0™	Control	97.3	72.4	77.5
	BSA	102.8	97.3	95.4
Lalvin Persy™	Control	97.4	76.8	81.4
	BSA	104.4	101.2	95.7
Lalvin QA23™	Control	90.1	66.9	75.5
	BSA	100.0	92.1	96.0

Appendix B

Free amino acids and ammonium chloride in a modified synthetic must

Table A2. Free amino acid and ammonium chloride concentrations in a modified synthetic must.

Compound	Amount ($mg \cdot L^{-1}$)	Compound	Amount ($mg \cdot L^{-1}$)
Tryptophan	38.7	Alanine	31.4
Phenylalanine	8.2	Threonine	16.4
Isoleucine	7.1	Glutamic acid	26.0

Table A2. *Cont.*

Compound	Amount ($mg \cdot L^{-1}$)	Compound	Amount ($mg \cdot L^{-1}$)
Leucine	10.5	Aspartic acid	9.6
Valine	9.6	Glycine	4.0
Methionine	6.8	Arginine	80.8
Tyrosine	4.0	Glutamine	109.1
Lysine	3.7	Serine	17.0
Cysteine	2.8	Asparagine	0.0
Proline	132.2	Histidine	7.1
NH_4CL	102.5		

Appendix C

Observed nitrogen consumption

Table A3. The observed nitrogen consumption of free amino acids (FAA), bound amino acids (BAA) and ammonia during fermentation with Lalvin ICV Opale 2.0™, Lalvin Persy™ and Lalvin QA23™ of the modified synthetic must. 'Control' is without added peptides, 'BSA' is with added peptides. Standard deviations represent measurements in three technical replicates.

Strain		N from FAA ($mg \cdot L^{-1}$)	N from BAA ($mg \cdot L^{-1}$)	N from NH_4 ($mg \cdot L^{-1}$)	N Total ($mg \cdot L^{-1}$)
Lalvin ICV Opale 2.0™	Control	70.20 ± 0.07	-	18.08 ± 6.87	88.2 ± 6.87
	BSA	77.44 ± 0.22	74.4 ± 2.12	22.49 ± 2.23	174.3 ± 3.08
Lalvin Persy™	Control	75.8 ± 0.17	-	26.96 ± 6.35	102.8 ± 6.65
	BSA	72.0 ± 0.18	57.3 ± 0.53	25.41 ± 5.07	154.7 ± 5.10
Lalvin QA23™	Control	75.50 ± 0.20	-	25.13 ± 6.19	100.6 ± 6.49
	BSA	73.79 ± 0.16	51.7 ± 5.87	25.41 ± 5.07	150.9 ± 7.76

Appendix D

The proposed peptide candidates and their relative consumption by yeast

Table A4. The proposed dipeptide sequences and observed relative consumption during fermentation with Lalvin ICV Opale 2.0™, Lalvin Persy™ and Lalvin QA23™ of the modified synthetic must. Peptide sequences were acquired by peptide mapping to bovine serum albumin protein sequence (UniProtKB–P02769 (ALBU_BOVIN)). Additionally presented are the retention time, observed *m/z*, collision cross section values, as well as the averaged peptide intensities at the starting point and associated relative standard deviations.

Peptide Candidate	Retention Time (min)	Observed *m/z*	Charge	CCS (Å2)	Average Original Intensity	RSD	Lalvin ICV Opale 2.0™ (Consumption, %)	Lalvin Persy™ (Consumption, %)	Lalvin QA23™ (Consumption, %)
AH	2.68	227.1134	1	150.41	807	4.86	99.97	100.00	99.98
AW	5.40	276.1338	1	160.10	1914	5.03	100.00	100.00	100.00
AY	4.56	253.1177	1	157.67	1470	6.44	100.00	100.00	100.00
FT	3.72	267.1335	1	163.99	5487	3.53	100.00	100.00	100.00
FW	7.03	352.1652	1	156.33	2617	5.18	100.00	100.00	100.00
FY	5.38	329.1487	1	179.32	698	3.61	97.94	95.49	95.50
JT	3.18	233.1492	1	156.95	2875	4.95	99.99	100.00	100.00
KQ	2.39	275.1709	1	160.14	1164	2.32	99.99	99.82	99.87
KT	1.40	248.1601	1	156.18	2201	8.75	99.97	99.98	100.00

Table A4. *Cont.*

Peptide Candidate	Retention Time (min)	Observed *m/z*	Charge	CCS (Å2)	Average Original Intensity	RSD	Lalvin ICV Opale 2.0™ (Consumption, %)	Lalvin Persy™ (Consumption, %)	Lalvin QA23™ (Consumption, %)
LL	5.84	245.1856	1	166.83	20,475	3.04	100.00	100.00	100.00
LY	4.88	295.1648	1	171.64	4360	3.64	100.00	100.00	100.00
TF	4.78	267.1335	1	160.50	1507	3.99	100.00	99.87	100.00
TK	2.51	248.1600	1	156.18	2070	2.46	100.00	100.00	100.00
VE	2.14	247.1285	1	154.51	6009	6.03	99.96	99.98	99.95
VF	5.50	265.1542	1	164.08	5044	4.39	100.00	100.00	100.00
VN	1.37	232.1288	1	150.14	1012	2.78	99.98	100.00	100.00
VR	3.30	274.1866	1	167.20	1039	5.77	100.00	100.00	100.00
YE	3.72	311.1229	1	172.79	1091	4.24	99.67	100.00	100.00
YY	4.55	345.1441	1	184.22	5445	5.16	100.00	100.00	100.00

Table A5. The proposed tripeptide sequences and observed relative consumption during fermentation with Lalvin ICV Opale 2.0™, Lalvin Persy™ and Lalvin QA23™ of the modified synthetic must. Peptide sequences were acquired by peptide mapping to bovine serum albumin protein sequence (UniProtKB–P02769 (ALBU_BOVIN)). Additionally presented are the retention time, observed *m/z*, collision cross section values, as well as the averaged peptide intensities at the starting point and associated relative standard deviations.

Peptide Candidate	Retention Time (min)	Observed *m/z*	Charge	CCS (Å^2)	Average Original Intensity	%RSD	Lalvin ICV Opale 2.0™ (Consumption, %)	Lalvin Persy™ (Consumption, %)	Lalvin QA23™ (Consumption, %)
AEF	5.27	366.1656	1	181.69	5873	4.08	100.00	100.00	100.00
AWS	4.68	363.1658	1	181.79	2470	4.50	100.00	100.00	100.00
DAF	5.21	352.1496	1	176.71	1100	2.70	100.00	100.00	100.00
DLL	5.76	360.2124	1	189.23	2953	6.90	100.00	100.00	100.00
DQF	4.88	409.1712	1	195.14	728	3.28	100.00	100.00	100.00
ELT	4.01	362.1919	1	187.32	3543	2.99	99.98	99.62	99.92
FKD	3.53	205.1072	2	249.17	7981	3.00	100.00	100.00	100.00
FKG	3.33	176.1043	2	242.20	2685	2.41	100.00	100.00	100.00
FLG	5.65	336.1910	1	182.71	734	3.52	100.00	100.00	100.00
FQE	4.32	423.1866	1	198.49	658	3.50	100.00	100.00	100.00
FSA	4.73	324.1549	1	174.09	3223	2.23	100.00	100.00	100.00
FTF	7.23	414.2020	1	194.99	3313	6.62	100.00	100.00	100.00
FVE	4.88	394.1970	1	190.00	3242	3.60	100.00	100.00	100.00
GSF	4.64	310.1391	1	167.50	1014	6.12	100.00	100.00	100.00
IAE	3.72	332.1811	1	175.60	2444	4.33	100.00	100.00	100.00
IAF	6.63	350.2071	1	180.40	5291	6.60	100.00	100.00	100.00
IAH	2.66	340.1973	1	182.57	1628	7.50	100.00	100.00	100.00
IAR	3.08	359.2396	1	185.57	2132	6.89	100.00	100.00	100.00
ISL	6.42	332.2170	1	182.85	933	5.48	100.00	100.00	100.00
IVR	3.31	194.1387	2	253.10	4260	4.53	99.92	99.99	99.80
KIE	3.26	195.1228	2	243.74	2444	6.39	99.61	99.86	99.84
KQI	2.40	194.6309	2	246.86	8652	6.83	100.00	100.00	100.00
LAK	2.50	331.2332	1	181.06	482	3.99	100.00	100.00	100.00
LEE	3.90	390.1876	1	188.27	1557	5.17	100.00	93.10	94.29
LFT	5.92	380.2172	1	190.43	706	3.45	100.00	100.00	100.00
LLF	8.00	392.2539	1	197.53	1399	5.91	100.00	100.00	100.00
LSQ	3.30	347.1919	1	178.69	3179	5.20	100.00	100.00	100.00
LVE	4.37	360.2126	1	183.71	18,437	5.06	100.00	100.00	100.00
LVN	3.77	345.2125	1	180.57	990	4.59	100.00	100.00	100.00
LYE	4.68	424.2076	1	196.58	9328	3.77	100.00	100.00	100.00
LYY	6.08	458.2284	1	207.03	3200	6.61	100.00	99.99	100.00
SQY	3.78	397.1714	1	186.22	3568	5.93	100.00	100.00	100.00
SVL	5.63	318.2013	1	176.11	265	7.45	100.00	100.00	99.94
TEF	5.23	396.1756	1	189.94	620	3.45	100.00	100.00	100.00
TLV	4.44	332.2174	1	181.03	1307	2.71	100.00	100.00	100.00
VAF	6.01	336.1915	1	175.46	10,184	2.70	100.00	99.96	100.00
VNE	3.12	361.1714	1	178.22	5809	4.62	100.00	100.00	100.00

Table A5. *Cont.*

Peptide Candidate	Retention Time (min)	Observed *m/z*	Charge	CCS (Å^2)	Average Original Intensity	%RSD	Lalvin ICV Opale 2.0™ (Consumption, %)	Lalvin Persy™ (Consumption, %)	Lalvin QA23™ (Consumption, %)
VTF	5.96	366.2019	1	179.87	1740	6.69	100.00	100.00	100.00
VTK	1.40	347.2286	1	180.50	3469	6.77	100.00	100.00	100.00
YEY	5.06	474.1869	1	208.53	3085	5.50	99.98	100.00	99.99
YNG	3.07	353.1448	1	183.95	737	3.00	100.00	100.00	100.00

Table A6. The proposed tetrapeptide sequences and observed relative consumption during fermentation with Lalvin ICV Opale 2.0™, Lalvin Persy™ and Lalvin QA23™ of the modified synthetic must. Peptide sequences were acquired by peptide mapping to bovine serum albumin protein sequence (UniProtKB–P02769 (ALBU_BOVIN)). Additionally presented are the retention time, observed *m/z*, collision cross section values, as well as the averaged peptide intensities at the starting point and associated relative standard deviations.

Peptide Candidate	Retention Time (min)	Observed *m/z*	Charge	CCS (Å^2)	Average Original Intensity	%RSD	Lalvin ICV Opale 2.0™ (Consumption, %)	Lalvin Persy™ (Consumption, %)	Lalvin QA23™ (Consumption, %)
ALVE	5.13	431.2496	1	200.15	3448	7.14	100.00	98.07	99.34
FAKT	3.49	233.6360	2	266.06	2319	7.68	100.00	100.00	100.00
FEGK	5.44	269.1441	2	279.98	9697	5.25	74.05	46.42	96.43
FEKL	5.27	268.6570	2	276.78	1106	8.84	96.81	95.23	93.37
FHAD	3.66	245.1077	2	262.19	5008	5.24	100.00	100.00	99.99
FKDL	5.26	261.6491	2	277.18	3826	7.25	100.00	100.00	99.40
FLGS	5.62	423.2233	1	198.49	1214	1.92	100.00	100.00	100.00
FSQY	5.07	544.2400	1	222.40	5451	4.56	99.89	99.65	99.77
GERA	1.71	216.6131	2	254.59	646	5.90	84.37	71.04	78.31
IETM	5.11	493.2326	1	215.78	10,279	5.05	100.00	95.85	98.39
IKQN	1.91	251.6520	2	261.81	745	8.35	100.00	95.20	98.59
ISSK	1.99	217.6335	2	257.65	1951	10.49	100.00	99.71	99.07
KDAF	4.45	480.2451	1	204.55	4169	6.61	93.70	37.87	41.44
LEKS	2.52	238.6388	2	262.58	3371	5.37	100.00	100.00	99.97
LGSF	6.08	423.2236	1	196.61	7284	4.72	93.04	76.01	95.77
LILN	6.24	472.3125	1	218.28	2581	5.87	100.00	100.00	100.00
LIVR	4.82	250.6806	2	277.82	1165	3.78	75.70	49.87	50.76
LLEK	4.13	251.6648	2	271.33	4134	3.64	91.38	48.34	49.20
LRET	3.30	259.6495	2	267.68	915	7.21	100.00	100.00	100.00
LTAD	3.81	419.2132	1	194.85	2055	6.78	100.00	100.00	100.00
LTEF	6.28	509.2602	1	217.34	2520	6.27	100.00	100.00	100.00
LVEL	6.85	473.2970	1	214.35	7655	5.36	100.00	99.36	99.86
LVNE	4.15	474.2558	1	208.53	6118	6.06	99.95	99.71	99.98
LVTD	4.33	447.2446	1	201.61	8109	3.72	100.00	99.72	99.85
MENF	5.75	540.2122	1	222.49	2536	6.75	100.00	100.00	100.00
TQTA	2.58	420.2083	1	196.70	1160	6.33	100.00	100.00	100.00
VASL	2.93	195.1228	2	253.02	1457	5.55	99.77	99.68	99.85
VEVS	4.02	433.2284	1	198.21	2150	6.29	98.70	97.71	97.77
VFDK	4.02	254.6413	2	267.97	1976	5.98	69.07	35.82	53.99
VSEK	1.95	231.6310	2	259.87	2212	7.45	99.97	97.52	92.49
VVST	3.70	405.2340	1	191.53	3913	3.23	100.00	100.00	100.00

Table A7. The proposed pentapeptide sequences and observed relative consumption during fermentation with Lalvin ICV Opale 2.0™, Lalvin Persy™ and Lalvin QA23™ of the modified synthetic must. Peptide sequences were acquired by peptide mapping to bovine serum albumin protein sequence (UniProtKB–P02769 (ALBU_BOVIN)). Additionally presented are the retention time, observed *m/z*, collision cross section values, as well as the averaged peptide intensities at the starting point and associated relative standard deviations.

Peptide Candidate	Retention Time (min)	Observed *m/z*	Charge	CCS (Å^2)	Average Original Intensity	RSD	Lalvin ICV Opale 2.0™ (Consumption, %)	Lalvin Persy™ (Consumption, %)	Lalvin QA23™ (Consumption, %)
AFDEK	3.91	305.1472	2	274.91	15,458	6.56	99.93	36.21	41.18
AIPEN	4.24	543.2772	1	218.50	8777	4.98	96.76	33.49	33.68
ALVEL	7.32	544.3338	1	230.33	912	7.41	99.99	40.31	48.36
FDEKL	5.50	326.1703	2	300.05	5931	5.12	100.00	58.69	61.54
FLGSF	7.55	570.2920	1	231.74	934	6.74	99.96	33.44	31.64
FYAPE	5.74	626.2814	1	244.72	2299	4.18	100.00	66.49	66.40
GFQNA	4.50	536.2458	1	220.62	1214	7.16	78.76	34.47	38.77
KFWGK	5.00	333.1909	2	296.41	878	4.19	100.00	99.98	100.00
LAKEY	4.23	312.1727	2	277.79	4602	2.52	100.00	61.22	91.98
LFGDE	6.00	580.2609	1	227.53	825	7.83	99.79	28.10	35.07
LGEYG	4.95	538.2504	1	218.61	831	3.51	90.99	32.33	35.78
LILNR	5.33	314.7094	2	287.40	797	7.07	99.75	30.78	47.93
LPKIE	5.42	300.1912	2	284.85	8843	6.91	100.00	60.78	99.87
LVELL	7.98	586.3803	1	241.49	707	5.49	90.22	33.34	64.02
LVEVS	5.21	546.3130	1	224.32	4239	0.63	99.26	39.13	54.87
TAPEL	6.26	592.2976	1	235.28	15,546	5.29	100.00	93.48	93.12
TVFDK	4.61	305.1650	2	284.60	2748	4.80	99.98	66.96	61.71
VEVTK	3.65	288.1730	2	275.74	7041	8.04	98.83	39.91	48.04
VVSTQ	3.53	533.2928	1	218.72	3638	5.90	98.97	43.60	46.92

Table A8. The proposed hexapeptide sequences and observed relative consumption during fermentation with Lalvin ICV Opale 2.0™, Lalvin Persy™ and Lalvin QA23™ of the modified synthetic must. Peptide sequences were acquired by peptide mapping to bovine serum albumin protein sequence (UniProtKB–P02769 (ALBU_BOVIN)). * Elution within void volume. Additionally presented are the retention time, observed *m/z*, collision cross section values, as well as the averaged peptide intensities at the starting point and associated relative standard deviations.

Peptide Candidate	Retention Time (min)	Observed *m/z*	Charge	CCS (Å^2)	Average Original Intensity	%RSD	Lalvin ICV Opale 2.0™ (Consumption, %)	Lalvin Persy™ (Consumption, %)	Lalvin QA23™ (Consumption, %)
AFDEKL	5.70	361.6892	2	288.60	36,673	6.08	88.2	42.5	40.9
DAFLGS	3.91	305.1472	2	274.91	15,458	6.56	99.9	36.2	41.2
DTHKSE *	1.19	358.6637	2	292.00	4537	2.90	95.4	8.3	11.6
KDAIPE	4.55	336.6810	2	286.40	952	7.69	71.7	35.6	27.2
KFGERA	3.66	354.1946	2	298.79	1133	3.60	93.9	22.1	27.9
KFPKAE	4.01	360.2070	2	308.56	2291	5.69	100.0	39.4	44.3
LFGDEL	7.43	693.3452	1	253.69	902	10.18	100.0	34.2	32.5
LLPKIE	6.10	356.7331	2	315.47	1720	7.65	100.0	41.7	46.1
LPKIET	5.60	350.7149	2	292.34	7905	6.27	96.9	31.0	31.1
NLPPLT	6.57	654.3815	1	248.25	1517	7.63	80.2	51.5	43.0
TVFDKL	6.40	361.7072	2	295.17	2718	5.65	100.0	35.9	40.2
VEGPKL	5.33	321.6941	2	280.58	9864	5.33	85.6	38.7	39.6
VSTPTL	6.14	617.3495	1	238.79	674	5.56	100.0	43.6	46.0

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Appendix 3

Publication II

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Unlocking the secrets of peptide transport in wine yeast: insights into oligopeptide transporter functions and nitrogen source preferences

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ABSTRACT Oligopeptides from grape must represent a secondary source of nitrogen for yeasts to grow and carry out fermentation. *Saccharomyces cerevisiae* takes up oligopeptides from the environment through multiple oligopeptide transporters with different peptide length specificities. However, due to difficulties associated with the qualitative and quantitative measurement of peptides in natural matrices, peptide transporter specificities have been mostly researched in single peptide environments. Using a peptide mapping method, we monitored the relative consumption of peptides derived from a protein hydrolysate by a set of CRISPR-Cas9-engineered *S. cerevisiae* wine strains to study oligopeptide transporters from the Opt and Fot families. Results show that Opt2 can import peptides containing three to at least seven amino acid residues, which is a broader peptide length specificity than previously reported, while Opt1 activity was not sufficient to support growth on peptides as nitrogen source. Fot1, Fot2, and Fot3, previously referred to as di-tripeptide transporters in *S. cerevisiae* wine strains, could also import tetrapeptides. The consumption order of peptides was determined by the peptide length as higher chain length peptides were taken up by Opt2 only after most di-tetrapeptides were depleted from the media. Altogether, Fot and Opt2 activity assured completion of the fermentation process without necessarily requiring ammonia or free amino acids. Analysis of peptide transporter gene expression during fermentation showed an effect of SO_4^{2-} not only on *OPT1* but also on *FOT* and supported the assumption of a possible interplay between Fot and Opt2 activities.

IMPORTANCE Limited nitrogen supply can prevent the completion of alcoholic fermentation. Supplementation through peptides as an alternative, natural source of nitrogen for yeast offers an interesting solution for this issue. In this work, the *S. cerevisiae* peptide transporters of the Opt and Fot families were studied. We demonstrated that Fot and Opt2 have a broader peptide length preference than previously reported, enabling yeasts to acquire sufficient nitrogen from peptides without requiring additional ammonia or amino acids to complete fermentation. On the contrary, Opt1 was unable to consume any peptide in the given conditions, whereas it has been described elsewhere as the main peptide transporter for peptides longer than three amino acid residues in experiments in laboratory conditions. This controversy signifies the need in applied sciences for approaching experimental conditions to those prevalent in the industry for its more accurate characterization. Altogether, this work provides further evidence of the importance of peptides as a nitrogen source for yeast and their consequent positive impact on fermentation kinetics.

KEYWORDS oligopeptides, *Saccharomyces cerevisiae*, yeast physiology, oligopeptide transporters (opt), fungal oligopeptide transporters (fot), alcoholic fermentation

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Nitrogen is an essential element for yeast growth and activity during alcoholic fermentation. A deficiency of nitrogen in the environment inherently leads to a sluggish or incomplete fermentation (1). Additionally, the synthesis of a variety of volatile compounds, which shape the aroma profile of fermented beverages, is also affected by the availability and source of nitrogen (2, 3). In the fermentation industry and related applied research, the term yeast assimilable nitrogen (YAN) is typically used to refer to NH_4^+ and free amino acids (FAAs) which are metabolized by yeast, the content of which can vary to a great extent within different fermentation matrices (1). For example, the YAN values in natural grape musts remain in the range of 60 to 500 mg/L (4), depending on different factors such as grape variety, climate, or vinification conditions and practices. In addition to NH_4^+ and FAA, other sources of nitrogen, such as oligopeptides, can also be assimilated by yeast (5). The monitoring of nitrogen assimilation during fermentation using ^{15}N-labeled NH_4Cl and yeast hydrolysate showed that nitrogen derived from peptides constituted 40% of the yeast protein fraction (6). Furthermore, higher consumption of oligopeptides by yeasts during wine fermentation has demonstrated to positively impact cell viability, fermentation kinetics, and the production of volatile compounds (7–9). Thus, oligopeptides have an important anabolic role during fermentation. However, despite obvious advantages on fermentation kinetics, oligopeptide-derived YAN is rarely considered due to difficulties associated with their qualitative and quantitative analysis (10).

Oligopeptide import systems in yeast have been best characterized in *Saccharomyces cerevisiae*, the most widely used yeast species in industrial fermentations. These include seven individual transporters (Ptr2, Dal5, Opt1–2, and Fot1–3) which all mediate the uptake of oligopeptides across the plasma membrane in a proton-coupled mechanism. The first oligopeptide transporter identified in *S. cerevisiae* was the proton-dependent oligopeptide transporter Ptr2 [Pot/Ptr, transporter classification (TC) number: 2.A.17], which imports di- and tripeptides (11). The allantoate and ureidosuccinate permease Dal5 (TC 2.A.1.14.4) has also displayed dipeptide uptake activity (12). Fungal oligopeptide transporters (Fot1-Fot2 and Fot3) are found in several *S. cerevisiae* wine strains and other fungi, being experimentally characterized as di- and tripeptide importers as well (7, 13, 14); nonetheless, it has been speculated, albeit without solid evidence, that Fot may also transport longer-chain peptides (8, 15). To date, the uptake of longer-chain peptides has been shown to be mediated by the oligopeptide transporters Opt1 and Opt2 (TC 2.A.67). Although Opt1 orthologous transporters in the yeast species *Candida albicans* have been reported to import peptides of at least up to eight amino acid residues in length (16, 17), Opt1 and Opt2 in *S. cerevisiae* have been characterized as tetra- and pentapeptide transporters (18–21).

Regulation of oligopeptide transporters' gene expression depends on the availability of assimilable nitrogen compounds in the medium. In yeast, the nitrogen catabolite repression (NCR) system involves several regulatory mechanisms for gene expression that repress the transcription of genes encoding for transporters of non-preferred nitrogen sources. A nitrogen source is considered to be preferred based on their capacity to support fast cell growth (e.g., NH_4^+, Glu, Gln, or Asn), while non-preferred sources trigger the de-repression of genes under control of the NCR (22–28). In *S. cerevisiae*, peptide transporter genes *PTR2*, *DAL5*, *OPT1*, and *OPT2* are all under the regulation of the NCR (11, 19, 26, 29–33). Interestingly, *OPT1* gene expression is specifically induced in sulfur starvation conditions, while the presence of sulfur-containing amino acids, Met and Cys, maintains the repression by NCR (24, 34). Regulation of *FOT* gene expression has not yet been thoroughly studied. Transcriptomic analyses on the *S. cerevisiae* wine strain EC1118, which contains *FOT1* and *FOT2*, revealed that these genes are upregulated during wine fermentation in nitrogen-limiting conditions (60–70 mg/L YAN), while being downregulated in nitrogen-rich synthetic musts (29, 30). A recent study on the expression levels of *FOT1*, *FOT2*, and *FOT3* in different *S. cerevisiae* wine strains during fermentation on natural and synthetic musts has shown that their expression levels are low overall, although they increase during the stationary phase in comparison to the

mid-log phase (14). Furthermore, several binding motifs for the NCR regulators Gln3 or Cup9 were predicted in the promoter regions of *FOT* genes, corroborating the assumption that *FOT* gene expression is also regulated by the NCR (14).

Apart from amino acids, oligopeptides also exert a regulatory activity on the expression of peptide transporter genes. The uptake of dipeptides with basic and bulky amino acids at the N-terminal position (the "N-end rule dipeptides") by Ptr2 upregulates its own expression by positive feedback (35). In this case, the N-end rule dipeptides bind to Ubr1, the mediator in ubiquitination processes, activating the degradation of Cup9, which otherwise acts as a transcriptional repressor of *PTR2* (36–39). Although Cup9 also represses the expression of *OPT2*, it is unknown whether the N-end rule peptides have the same inducing effect as in the case of *PTR2*. Contrary to the effect on *PTR2* and *OPT2*, Cup9 upregulates the expression of *DAL5*, while *OPT1* is not affected (12, 34). It is suggested that such divergent but complementary regulation of *PTR2*, *DAL5*, *OPT*, and *FOT* expression can allow yeasts to adapt to various environmental conditions and nutrient availabilities.

Substrate-dependent specificity of the different oligopeptide transporters has mostly been characterized by growth experiments with synthetic peptides as the sole nitrogen source. For example, tetra- and pentapeptide transport by Opt1 and Opt2 has been studied using peptide transporter gene(s) knockout (KO) strains cultured on different media containing single peptides, some of which exhibited a toxic/antimicrobial activity, such as KLAE (18, 21). In the case of toxic peptides, growth inhibition indicated the ability of the expressed peptide transporter to internalize the specific peptide. Another method used for studying transporter specificity of Dal5, Ptr2 (35), and Fot (13, 14) is the Biolog Phenotype MicroArrays platform (Hayward, CA, USA). While studies on single peptide consumption have contributed to our general understanding of oligopeptide transporter specificities, it has not provided information on the kinetics of peptide utilization during fermentation in complex media containing peptides of varying length and amino acid composition. We recently implemented a screening methodology for studying peptide assimilation by yeast in a synthetic medium supplemented with a single-protein hydrolysate with a characterized peptide composition (10). Using this approach, the relative consumption of over one hundred di- to hexapeptides were monitored during fermentations with three commercial *S. cerevisiae* wine strains. The results demonstrated different peptide uptake kinetics by these strains.

In the present work, the same methodology was applied to further elucidate the peptide-length specificity of Opt and Fot from *S. cerevisiae* during fermentation. For this purpose, we have worked with CRISPR-Cas9-engineered strains expressing different sets of oligopeptide transporters genes (14). The impact of peptide uptake by Opt and Fot on fermentation kinetics in media containing different concentrations and sources of nitrogen was then investigated. Finally, we analyzed *FOT* and *OPT* gene expression during fermentation, with a particular focus on the *OPT1* gene and its expression pattern in sulfur-limited media. This work deepens the characterization on Opt and Fot transporters in *S. cerevisiae*, providing new insights on the important role of peptides during fermentation under enological conditions.

RESULTS

Effect of the different nitrogen sources on cell growth and fermentation kinetics

To characterize the different peptide transporters and their effect on the yeast performance in a peptide rich environment, a set of CRISPR-Cas9-engineered knockout strains for single or multiple peptide transporter genes (Table 1) were used in fermentation experiments. Fermentations were conducted in three media containing 220 g/L of fermentable sugars consisting of glucose and fructose in equimolar concentrations but with different conditions of nitrogen supply (Materials and Methods; Table 2). Medium NA100 contained ~100 mg/L YAN from NH_4^+ and FAA, medium NAP200 had an additional ~100 mg/L nitrogen added from peptides, and medium P200

TABLE 1 Oligopeptide transporter knockout/knockin strains created from *Saccharomyces cerevisiae* strain 59A (haploid derivative of wine strain EC1118) used in this study[a]

Strain name	Genotype	Purpose	Reference/source
59A (wt)	MATa ; ho; AMN1::loxP	Wild-type strain; control	Marsit et al. (7)
PepKO	MATa ; ho; AMN1::loxP ; FOT1-2::loxP-kanMx-loxP ; dal5; opt1; opt2	Control, complete KO for oligopeptide transport	Becerra-Rodríguez et al. (14)
Opt1	MATa ; ho; AMN1::loxP ; FOT1-2::loxP-kanMx-loxP ; dal5 ;opt2	Functionality of Opt1	This study
Opt2	MATa ; ho; AMN1::loxP ; FOT1-2::loxP-kanMx-loxP ; dal5 ;opt1	Functionality of Opt2	This study
opt1Δ	MATa ; ho; AMN1::loxP; opt1	opt1 KO	This study
opt2Δ	MATa ; ho; AMN1::loxP; opt2	opt2 KO	This study
Fot1	MATa ; ho; AMN1::loxP ; FOT1-2::FOT1 ; dal5; opt1; opt2	Functionality of Fot1	Becerra-Rodríguez et al. (14)
Fot2	MATa ; ho; AMN1::loxP ; FOT1-2::FOT2 ; dal5; opt1; opt2	Functionality of Fot2	Becerra-Rodríguez et al. (14)
Fot3	MATa ; ho; AMN1::loxP ; FOT1-2::FOT3 ; dal5; opt1; opt2	Functionality of Fot3 (from *S. cerevisiae* strain K1)	Becerra-Rodríguez et al. (14)
Fot1Fot2	MATa ; ho; AMN1::loxP ; dal5 ; opt1 ; opt2	Functionality Fot1 and Fot2 together	Becerra-Rodríguez et al. (14)
fot1fot2Δ	MATa ; ho; AMN1::loxP ; FOT1-2::loxP-kanMx-loxP	fot KO/functionality of Opt1 and Opt2 together	Becerra-Rodríguez et al. (14)

[a]Strains were selected to focus this study on Fot and Opt oligopeptide transporters.

contained ~200 mg/L nitrogen delivered solely from peptides. The biomass density (OD_{600}) and CO_2 production were monitored during 12 days of fermentation (see Materials and Methods).

In fermentations with NH_4^+ and FAA as the sole nitrogen source (NA100), all strains displayed similar profiles of cell growth and fermentation kinetics (Fig. 1). In general, doubling the YAN concentration by the addition of peptides (NAP200 and P200) increased the maximum cell population reached by the strains when compared to the NA100 medium (Fig. 1A and 2A). The exceptions were the PepKO, Opt1, and, to a lesser extent, fot1fot2Δ and Opt2 strains. In the case of the fot1fot2Δ and Opt2 strain, an increase in the maximum cell population by the addition of peptides was observed; however, it was not significantly different from the value reached in NA100. By contrast, the growth of the PepKO and Opt1 strains was not affected by the doubling of YAN with peptides. Indeed, both strains could not grow in the P200 condition (Fig. 1), which suggests that the Opt1 strain, like the PepKO strain, is not able to utilize peptides as a nitrogen source. Notably, both strains showed an atypical increase in the biomass density and CO_2 production rate at the endpoint of the experiment in the P200 medium (Fig. 1A and B) which may have been caused by the autolysis of part of the cell population and consequential release of assimilable FAA for growth. Due to this deviant behavior, the PepKO and Opt1 strains in the P200 condition were excluded from further statistical analysis of growth and fermentation parameters (Fig. 2).

TABLE 2 Nitrogen composition of the media used in the study[a]

	NA100: (NH_4^+ and AAs)	NAP200: (NH_4^+, AAs, and Peptides)	P200 and P200-SO_4: (Peptides only)
NH_4^+	30	30	–
Amino acids	78	82	8[b]
Peptides	–	100	200
Total	108	212	208

[a]The four media contained 220 g/L of glucose and fructose in equimolar concentrations. All values are displayed in milligram nitrogen per liter. NA100 and NAP200 contained another 17 mg/L nitrogen from proline; however, this amino acid was not taken into account as it is not assimilated by yeasts during fermentation (40).
[b]Nitrogen deriving from free amino acids in the bovine serum albumin hydrolysate.

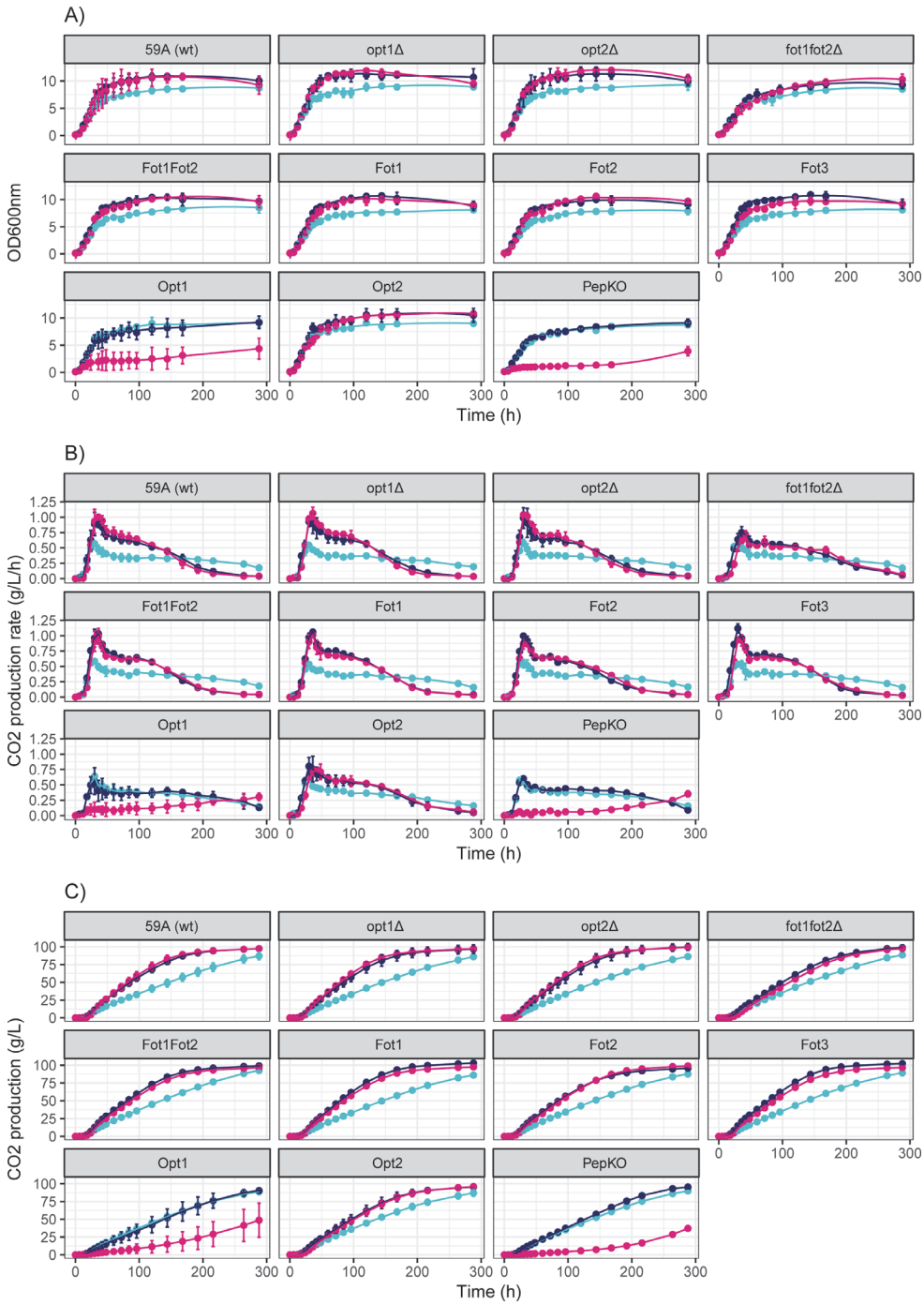

FIG 1 Growth and fermentation kinetics of the set of strains in different nitrogen conditions. Strain denominations can be found in Table 1. The NA100 condition is represented in light blue; NAP200 condition, in dark blue; and P200 condition, in pink. (A) Cell growth during fermentation. (B) Rate of CO_2 production over time. (C) CO_2 production over time.

The increase in the maximum cell population is negatively correlated with the time to reach 80% of attenuation, determined based on the maximum theoretical CO_2 production from glucose and fructose (2 mol CO_2/mol glucose/fructose) (Fig. 2B and C). This parameter was chosen as not all fermentations reached 100% of attenuation during the set experimental time. While all strains reached 80% of attenuation in the NA100 condition within ~250 hours on average, this point was reached within ~170 hours or less when the YAN content was doubled with the addition of peptides. Again, this tendency was more pronounced in those strains containing at least one *FOT*, which all

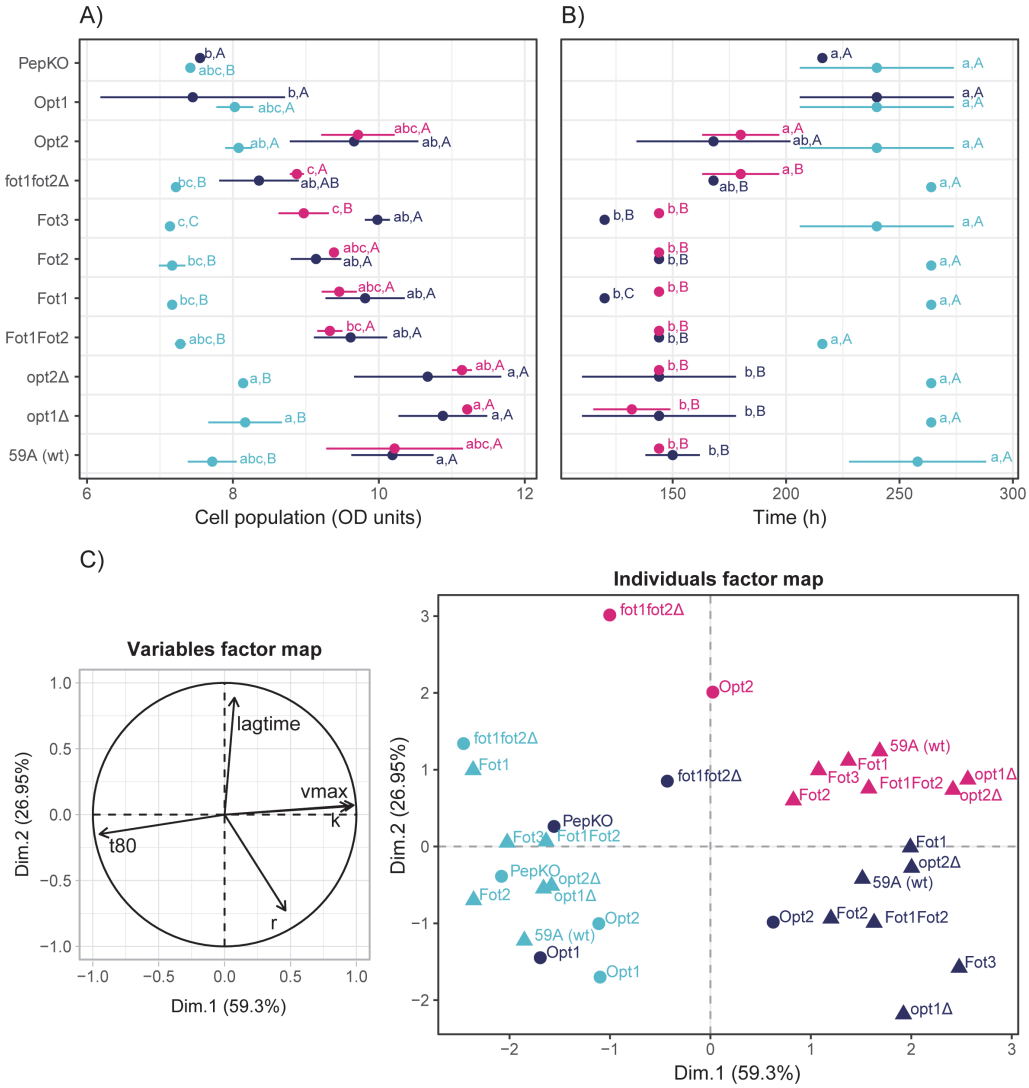

FIG 2 Growth and fermentation parameters with different nitrogen conditions. The NA100 condition is represented in light blue; NAP200 condition, in dark blue; and P200 condition, in pink. Letters in maximum cell population (A) and time to reach 80% of attenuation (B) denote the statistical groups from Tukey's tests ($P < 0.05$), with lower-case letters indicating different groups of strains per medium and upper-case letters indicating the groups of media for every strain. (C) Principal component analysis of growth and fermentation parameters from the different strains in the three media. t80, time to reach 80% of attenuation; vmax, maximum CO_2 production rate; lagtime, time to produce 1 g of CO_2; k, maximum cell population; r, maximum growth rate. Triangles and circles in the individuals factor map respectively represent those strains that have or do not have Fot.

reached 80% of attenuation before 150 hours in NAP200 and P200 media. By contrast, the PepKO and Opt1 strains reached 80% of attenuation in the NAP200 medium within similar time as the NA100 condition, which supports the assumption that these two strains are unable to utilize peptides. Similar results were obtained for the maximum rate of CO_2 production (Vmax), as this parameter positively correlated with the maximum cell population and negatively correlated with the time to reach 80% of attenuation (Fig. 2C).

In addition to the maximum cell population, the time to reach 80% of attenuation and Vmax, the maximum growth rate, and the time to produce 1 g of CO_2 (Table S1 through S5) were considered to further investigate the data set by a principal component analysis (Fig. 2C). Strains across different media clearly grouped according to the time to reach 80% of attenuation and Vmax, although there were some outliers. The PepKO and Opt1 strains in the NAP200 medium clustered with the NA100 group, supporting the assumption that the Opt1 transporter does not contribute to the uptake of peptides in the 59A strain. The fot1fot2Δ strain in the NAP200 medium did not cluster with the rest of the NAP200 group. The Opt2 strain in NAP200 was the only strain not containing *FOT* that clustered together with the *FOT*-containing strains in the same medium. The Opt2 and fot1fot2Δ strains were more distinguishable in the P200 medium. These results suggest that Opt2 is the only peptide transporter able to compensate with its activity for the lack of Fot in the 59A strain. Yet, the peptide import by Opt2 did not have the same impact on growth and fermentation kinetics as Fot.

Uptake of oligopeptides by Opt and Fot transporters

The consumption of peptides by the yeast strains in the NAP200 and P200 media was monitored during the first 72 hours of fermentation (Fig. 3; Fig. S1 to S6). In addition, the changes in the FAA concentration in the NAP200 medium were measured during the same time period (Fig. 3; Fig. S6). The consumption of FAA in the NAP200 medium was similar in most strains, indicating that the presence or absence of the different peptide transporters did not influence amino acid consumption. Only the PepKO strain showed higher consumption levels for FAA. This strain is incapable of peptide transport, and faster FAA consumption might have occurred to compensate for the lack of oligopeptide transport.

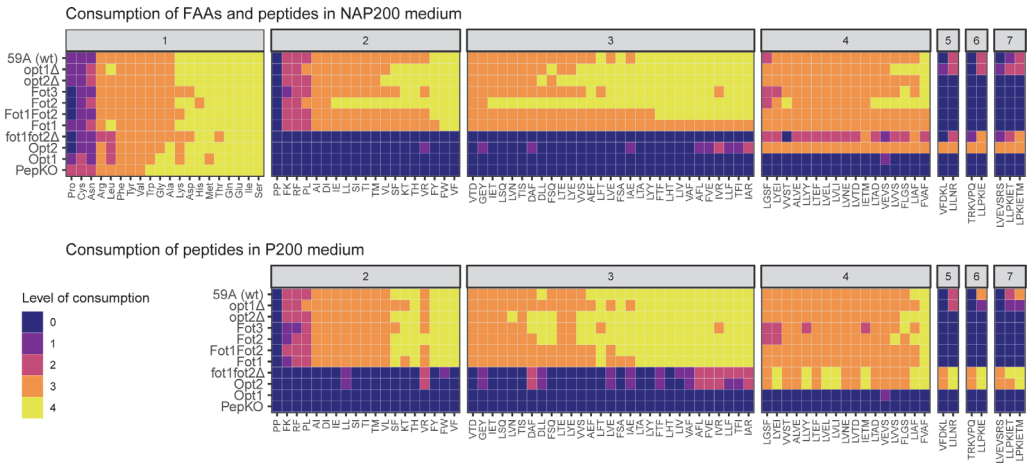

FIG 3 Consumption of FAA and peptides in NAP200 and P200 media. Peptides are grouped according to their number of amino acids residues. 1, FAAs; 2, dipeptides; 3, tripeptides; 4, tetrapeptides; 5, pentapeptides; 6, hexapeptides; 7, heptapeptides. The area under the curve (AUC) was calculated for the relative abundance curve of each peptide and FAA (Fig. S1 to S4). The AUC values were compared to a virtual negative control (100% abundance over 72 hours). The consumption of a particular peptide or FAA is level 0 when its abundance AUC is equal or higher than 80% of the negative control AUC; level 1, 60%–80%; level 2, 40%–60%; level 3, 20%–40%, and level 4 when the peptide abundance AUC is lower than 20% of the control AUC.

There was a clear distinction in the consumption of peptides based on their length. Those strains containing *FOT* (59A, opt1Δ, opt2Δ, Fot1Fot2, Fot1, Fot2, and Fot3) were all able to consume di-, tri-, and tetrapeptides (Fig. 3; Fig. S1 to S3; Fig. S5 and S6). Most di- to tetrapeptides were fully consumed between 18 and 36 hours of fermentation, even in presence of FAA and NH_4^+. The consumption of dipeptides with amino acid composition FK, RF, and PL by *FOT*-containing strains only started after depletion of the other dipeptides (at 18–24 hours) and were fully consumed after 72 hours in both media. Conversely, the strains containing *OPT2* and not *FOT* (fot1fot2Δ and Opt2) consumed tetra-, penta-, hexa-, and heptapeptides and only a few di- and tripeptides, with generally higher consumption levels in the P200 medium than in the NAP200. The wild-type strains 59A and opt1Δ, both containing *OPT2*, were also able to consume penta- to heptapeptides, although to a lesser extent than the fot1fot2Δ and Opt2 strains. As was expected from the fermentation kinetics analysis of the PepKO and Opt1 strains on NAP200 and P200 medium, neither of the strains were able to consume peptides. The only peptide that was not consumed by any of the strains in any condition was the dipeptide PP. These results re-define the current knowledge on the peptide length specificity of Fot, which goes up to tetrapeptides, and Opt2 in the wine strain 59A as the main transporter of peptides with a chain length containing four and more amino acids.

Strains expressing only Fot displayed similar profiles of di-, tri-, and tetrapeptide consumption in both the NAP200 and P200 conditions (Fig. 3; Fig. S5 and S6). However, the Fot2 expressing strain showed a more active consumption of di-, tri-, and tetrapeptides in the NAP200 condition than the other single-Fot strains, although the profile of consumption remained very similar (Fig. S5). This result indicates that the availability of FAA/NH_4^+ had limited influence on the specificity and activity of Fot.

Compared to Fot, the uptake of di- and tripeptides by the Opt2 transporter was limited. Out of the 19 dipeptides and 29 tripeptides of which the consumption was followed in this work, the Opt2 strain consumed only 1 or 2 dipeptides and 9 or 13 tripeptides in the NAP200 and P200 media, respectively (Fig. 3). Moreover, none of these di- and tripeptides were fully consumed during the first 72 hours of fermentation. By contrast, all tetra-, penta-, hexa-, and heptapeptides were fully consumed after 24–36 hours in both NAP200 and P200 media by the Opt2 strain. These results were corroborated by a similar peptide consumption profile observed with the fot1fot2Δ strain (containing *DAL5*, *OPT1*, and *OPT2*) when peptides were used as the sole nitrogen source (P200). However, in the NAP200 medium, the Opt2 and fot1fot2Δ strains showed a different uptake of peptides. First, di- and tripeptides were not consumed at all by the fot1fot2Δ strain (Fig. 2). Second, the consumption of tetra- to heptapeptides by the fot1fot2Δ strain only started after 24 hours when ~75% of FAA had been consumed (Fig. S3 and S4). This was different from the Opt2 strain, which took up these peptides much earlier. A similar activity of penta- to heptapeptide uptake by Opt2 as in the fot1fot2Δ strain was observed in the wild-type strain (59A) and opt1Δ strain. In all these strains, the uptake of penta- to heptapeptides by Opt2 was slower and started after depletion of most of the di-, tri-, or tetrapeptides. Thus, the uptake of longer peptides by Opt2 might be secondary to the uptake of FAA and smaller peptides in the presence of other peptide transporters.

Fermentations under SO_4^{2-} limitation and expression of oligopeptide transporter genes

A quantitative real-time polymerase chain reaction (RT-PCR) analysis was performed to study oligopeptide transporter gene expression during fermentation with different sources of nitrogen. To test the effect of the sulfur concentration on the expression of peptide transporter genes, as it was described for *OPT1* (34), fermentations were carried out on a version of the P200 medium with a reduction of 87% in SO_4^{2-} salts (medium P200-SO4; Table 2). The strains selected for this experiment were those containing the *OPT1* gene (59A, opt2Δ, fot1fot2Δ, and Opt1 strains). Yeast growth and CO_2 production rate in the P200-SO4 medium were monitored for 168 hours and compared to those in

the P200 medium (Fig. 4A). Strains 59A and opt2Δ had lower CO_2 production rates in the P200-SO4 medium in comparison to P200. This difference between the two media was also observed in growth, with lower biomass formation in the P200-SO4 medium by the end of the experiment. While the growth of the fot1fot2Δ strain in the P200-SO4 medium was also lower than in P200, the fermentation rate was not as affected as it was for the *FOT*-containing strains (59A and opt2Δ). No differences were detected between the P200 and P200-SO4 media for the Opt1 strain, due to the growth defect of this strain with peptides as the sole nitrogen source.

Gene expression was analyzed in NAP200, P200, and P200-SO4 at two different points of fermentation: 18 hours, which roughly corresponds to the beginning of the exponential growth phase, and 48 hours, which coincides with the beginning of the stationary phase (Fig. 4B; analysis of variance by Tukey's tests can be found in Table S6). The expression of all peptide transporter genes was higher at 48 hours than at 18 hours of fermentation. This was the case for all the strains and media tested, with values of normalized expression at 18 hours not higher than 0.7 (AU). Therefore, differences in

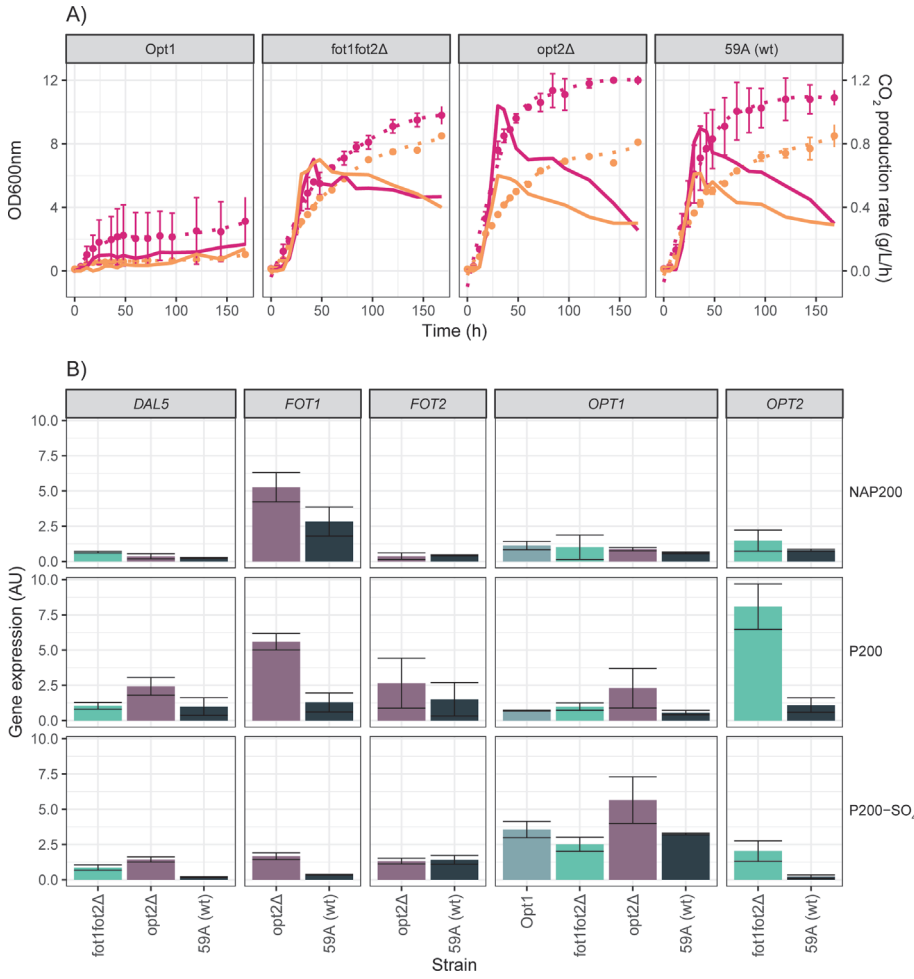

FIG 4 Influence of sulfur limitation on fermentation kinetics and the expression of peptide transporter genes. (A) Comparison of biomass production (OD_{600}, dashed line) and CO_2 production rate (straight line) between the strains grown in P200 (pink) or P200-SO_4 (orange). (B) Expression of *DAL5*, *FOT1*, *FOT2*, *OPT1*, and *OPT2* in the *OPT1*-containing strains grown in the NAP200, P200, and P200-SO_4 media, measured at 48 hours of fermentation.

gene expression were further analyzed at 48 hours (Fig. 4B). As expected, the expression of *OPT1* was higher in the medium with a reduced concentration of SO_4^{2-} in all the strains, which indicates that the gene is subjected to mechanisms of expression control as it has been previously reported (34). Moreover, the overexpression of *OPT1* was even more evident in the opt2Δ strain, suggesting that *OPT1* expression is under the control of *OPT2*. However, the overexpression of *OPT1* in the absence of *OPT2* was not observed in the Opt1 strain, probably due to the growth defect of this strain when peptides were the sole nitrogen source (Fig. 4A).

The *OPT1* gene in the 59A and Opt1 strains was sequenced to verify the possible gene variant in 59A and its peptide transporter KO derivatives. The *OPT1* gene sequence was identical in 59A and its derivative Opt1 strain (GeneBank accession number: OR468328). Likewise, the *OPT1* gene sequence in 59A and Opt1 strains was identical to the sequence in the genome of EC1118 (NCBI entry: FN393075.2, positions 35292 to 37691). The *OPT1* gene in 59A/EC1118 shares 99.25% of sequence identity with the reference strain S288C (NM_001181645.1) (Fig. S7), which translates to 100% identity at the protein sequence level. Therefore, this result confirmed that the *OPT1* gene in 59A/EC1118 does not contain any mutation that could turn the protein non-functional.

The *OPT2* gene was more highly expressed in the P200 medium in comparison to NAP200 or P200-SO4, both in the 59A and fot1fot2Δ strains. As peptides were the only source of nitrogen in P200, it seems that *OPT2* in this strain was repressed by the presence of NH_4^+ and FAA. This corroborates with lower peptide consumption levels by the fot1fot2Δ strain in NAP200 (Fig. 3). *OPT2* expression was also affected by the absence of sufficient SO_4^{2-}. Interestingly, the expression level of *OPT2* in the fot1fot2Δ strain was generally higher compared to that in the wild-type strain 59A. As Opt2 was the only active peptide transporter in this strain, higher expression of the gene might compensate for the lack of *FOT*. A similar trend was observed for *FOT1*, which was more expressed in the opt2Δ strain than in 59A. Interestingly, *FOT1* was significantly lower expressed in the P200-SO_4, which indicates that *FOT1* expression may be affected by the concentration of SO_4^{2-}. In contrast, *FOT2* did not seem to respond to the absence of *OPT2* or the low SO_4^{2-} concentration in the same way as *FOT1*. Similar to *OPT2*, higher expression levels of *FOT2* were observed in the absence of NH_4^+ and FAA (P200).

Despite the experimental setup not being focused on the dipeptide transporter *DAL5*, its expression was monitored along with *FOT* and *OPT* genes. The expression of *DAL5* was higher when peptides were the only source of nitrogen and for strains where either *OPT2* or *FOT* was missing.

DISCUSSION

In this work, the role of yeast oligopeptide transporters of the Opt and Fot families in the assimilation of peptides during fermentation under different nitrogen conditions were studied. For this purpose, we worked with engineered strains derived from 59A, a haploid version of the commercial *S. cerevisiae* wine strain EC1118, which contains functional genes for Opt1, Opt2, Dal5, Fot1, and Fot2 oligopeptide transporters. The strains were engineered to either express or lack the expression of single peptide transporter genes (14). 59A and most of its derivative strains were able to complete fermentation with peptides as the sole nitrogen source, demonstrating that peptides can support yeast growth during fermentation without requiring NH_4^+ nor FAA. These results reinforce the assumption that peptides can represent a powerful resource to cope with problems during fermentations associated with nitrogen deficiency.

Nonetheless, not all the 59A-derivative strains could complete fermentation with peptides as the sole nitrogen source. These include the complete peptide transporter knockout strain (PepKO) and the strain with Opt1 as the sole peptide transporter. Opt1 was originally characterized as the tetra- and pentapeptide transporter in descendants of the reference strain *S. cerevisiae* S288C (18, 21). In those strains, the given transporter was overexpressed within a plasmid (18, 21). Opt1 is also a glutathione transporter, and it has been hypothesized that this function might be primary to peptide transport

(20, 34). A potential incapacitating mutation of *OPT1* in 59A was excluded by Sanger sequencing, obtaining a translated protein sequence identical to the Opt1 sequence in S288C. In this study, the Opt1 strain did not consume any peptides, and the experiments did not include glutathione. Thus, the role of Opt1 in 59A and consequently EC1118 remains unclear. Nonetheless, the fot1fot2Δ strain has previously shown a growth defect in fermentations with glutathione as the sole nitrogen source in comparison with 59A (7), which in that work served as a validation of Fot as glutathione transporters but now indicates that the uptake of glutathione and other peptides by Opt1 may have a more regulatory role, e.g. by responding to oxidative stress or other stress-related events in the cell, rather than nutrients import.

Although the main factor contributing to the improvement of growth and fermentation kinetics was the increase in the nitrogen concentration, regardless of the type of nitrogen source, those strains containing *FOT* responded better when peptides were added to the medium. Fot, therefore, seem to be the most important peptide transporters in 59A, which supports previous characterization of these transporters in synthetic conditions, as well as with natural peptides from grape must (7, 14). Peptide analysis at different points of fermentation showed that 59A was able to consume a wide variety of peptides, ranging from di- to heptapeptides. Fot1, Fot2, and Fot3, previously characterized as di-tripeptide transporters (7, 13, 14), were also able to import tetrapeptides, while they did not transport penta-, hexa- or heptapeptides. Kinetics of peptide consumption during fermentation suggests that Fot are effective di-tetrapeptide transporters already active at the early stages of fermentation, even when preferable nitrogen sources (NH_4^+ and FAA) are also present. The strains expressing a single *FOT* also had different levels of peptide consumption in our experimental conditions, corroborating the results of Becerra-Rodríguez et al. (14). However, in contrast to the previously mentioned work, here, the Fot2 strain had the most active consumption of peptides instead of the strain containing both *FOT1* and *FOT2*. Discrepancies between the previous work and the present study could be due to the distinct types of peptides utilized. While Becerra-Rodríguez et al. monitored the consumption of single peptides through a colorimetric detection of yeast growth in laboratory conditions, this study carried out a semi-quantitative and qualitative analysis of the consumption of a complex mix of peptides derived from a bovine serum albumin (BSA) hydrolysate during model alcoholic fermentation under enological conditions. Therefore, the comparison of substrate specificity between the two studies is not pertinent.

As for Opt2, the strains expressing this gene while lacking *FOT* were also able to grow and carry out fermentation with peptides as a nitrogen source, although not to the same extent as *FOT*-containing strains. The distribution of nitrogen over the different peptide length groups is unknown, as only the relative decrease in abundance of the identified peptides could be followed. Therefore, it is not possible to state if the better response to the addition of peptides by the *FOT*-containing strains was caused by a higher efficiency of Fot or by a higher nitrogen content in the Fot-consumed peptide-length groups. We also determined a broader length specificity for Opt2, so far defined as a tetrapeptide transporter in *S. cerevisiae* (19). The strain with Opt2 as the sole peptide transporter gave preference to consuming tetra- to heptapeptides, while only partially consuming a few tripeptides and dipeptides. Indeed, the consumption of tetra- to heptapeptides by the Opt2 strain occurred within the first 36 hours of fermentation, regardless of the presence or absence of NH_4^+ and FAA. In contrast, the fot1fot2Δ strain, whose sole functional peptide transporter was proven to be Opt2, consumed tetra- to heptapeptides slower in the NAP200 medium compared to the P200. This result suggests that contrary to Fot, Opt2 oligopeptide transport activity may be affected by the presence of NH_4^+ and FAA when other peptide transporter genes are also present. Fot-mediated peptide transport occurred simultaneously, albeit with a slight delay, with FAA consumption. Most FAA and di- to tetrapeptides were completely consumed during the first 18–36 hours of fermentation by all strains containing *FOT*. By contrast, Opt2-associated peptide transport in both the 59A and opt1Δ strains occurred after most FAA and di-

to tetrapeptides were consumed, which suggests a preference for the consumption of di-tetrapeptides by Fot over the uptake of longer peptides by Opt2. We hypothesize that there is a balance between Fot and Opt2 activity, where Fot-mediated di-tetrapeptide consumption is the main system for oligopeptide acquisition during the first stages of fermentation. Due to the broad peptide length specificity of Fot, yeast can take up a wide range of nitrogen sources for fast growth, which is needed to become the dominant organism in a competitive environment such as grape must. We suggest that Opt2 activity is then used to obtain additional nitrogen from larger peptides when more easily assimilable nitrogen sources are depleted.

The peptide mapping strategy applied in this study allows for reliable peptide identification at the level of peptide length and amino acid composition but not the sequence (10). On this level, peptides that were not taken up or taken up poorly by either Fot or Opt2 shared similar chemical properties with those that were fast consumed and could, therefore, not be differentiated. A more in-depth analysis of sequence-based uptake preferences would still require experiments with synthetic peptides in this case. Another possibility to infer peptide sequences would consist in using a protease of known cutting site specificity to digest BSA, such as trypsin or chymotrypsin. Peptide mass data can then be used for peptide fingerprint identification through *in silico* approaches.

The expression of peptide transporter genes during fermentation was influenced by the available nitrogen source(s), potentially via the NCR system. Notably, expression levels of *FOT2* in the 59A and opt2Δ strains were higher in the medium containing only peptides (P200) than in the medium which contained both peptides and FAA/NH_4^+. Similarly, *OPT2* in the fot1fot2Δ strain also had a lower expression level in the presence of NH_4^+ and FAA. This corresponded with the earlier-mentioned lower consumption levels of peptides by the fot1fot2Δ strain in the presence of NH_4^+ and FAA. This effect was absent in the Opt2 strain, where Opt2 was the sole peptide transporter, which raises the question whether either *OPT1* or *DAL5* was involved in the regulation of *OPT2*. For example, such regulation has been shown on *OPT1* by *OPT2* (34, 38). Wiles et al. (34) speculated that in the absence of one of the *OPT*, yeast might up-regulate the other *OPT* to compensate for weaker oligopeptide uptake potential (34). However, the lack of peptide consumption by the Opt1 strain does not support this hypothesis. Furthermore, *OPT2* was more highly expressed in the absence of *FOT1* and vice versa, which is consistent with our hypothesis of an interplay between Fot1 and Opt2 functionality.

When the concentration of sulfur, known to repress *OPT1* (34), was reduced by 87%, a more pronounced expression of *OPT1* could be observed in all strains. However, this did not lead to improved growth of the Opt1 strain in the P200-SO4 medium, while other strains were still able to grow. The fact that *OPT1* gene expression responded positively to sulfur limitation indicates that Opt1 inability to support growth as peptide transporter is not due to alterations associated with gene expression.

Interestingly, *FOT*-containing strains (opt2Δ and 59A) displayed lower fermentation rates and reached a lower biomass formation under sulfur-limiting conditions. Sulfur and nitrogen metabolism are linked, since sulfate assimilation is required for the biosynthesis of the sulfur-containing amino acids methionine and cysteine (41). Marsit et al. (9) observed an up-regulation of the genes involved in the synthesis of cysteine and methionine in the strain 59A when compared to fot1fot2Δ (9). In this work, they concluded that peptides consumed by Fot were incorporated into the glutamate node, increasing *de novo* amino acid and glutathione biosynthesis. Based on these results, we hypothesize that peptide consumption by Fot induces the up-regulation of genes involved in the synthesis of sulfur amino acids, but due to the lack of SO_4^{2-} in the medium, there might be a metabolic imbalance that leads the cell to not grow or carry out fermentation properly. A deeper investigation into the link between peptide and sulfur metabolism is required to fully understand these mechanisms but was not within the scope of the current work.

This work has highlighted the importance of peptides as a nitrogen source for *S. cerevisiae* during fermentation. We demonstrated a broader peptide length specificity of Fot and Opt2 than previously reported. The results also showed that Opt2, not Opt1, was the main tetra- to heptapeptide transporter in the *S. cerevisiae* wine strain 59A. Furthermore, the complementary peptide uptake specificities of Fot and Opt2 and expression of the two at different phases of fermentation allowed yeast to consume preferred nitrogen sources in an orderly fashion. However, the fact that Opt1 as single available peptide transporter could not support growth, together with the inactivity of Ptr2 in this strain due to a gene truncation is indicative of the variability of peptide transporter functionality in yeast. Studies including different strains of *S. cerevisiae* are required to comprise the high intraspecific variability of *S. cerevisiae* and build a more global picture of peptide transport within the species.

MATERIALS AND METHODS

Strains

The oligopeptide transporter knockout strains derived from the *S. cerevisiae* strain 59A (haploid variant of the commercial wine strain EC1118) were produced in an earlier study (14). Since the purpose of that previous work was to characterize Fot, a strain expressing the *FOT3* gene from another wine strain was also included (Table 1).

The fot1fot2Δ strain was created by replacing the tandem genes *FOT1–FOT2* by a *KANMX4* cassette. Genes *OPT1*, *OPT2*, and *DAL5* were then deleted sequentially from the fot1fot2Δ strain using the CRISPR/Cas9 system. *PTR2* is not functional in 59A, and it was, therefore, not required to delete this gene (13). Deletion of *OPT1*, *OPT2*, and *DAL5* oligopeptide transporter genes in fot1fot2Δ resulted in the strain *opt1Δopt2Δdal5Δfot1fot2Δ::KANMX4*, which is a full knockout strain for oligopeptide transport (PepKO). Using CRISPR/Cas9, each *FOT* gene (*FOT1* and *FOT2* from EC1118 and *FOT3* from *S. cerevisiae* strain K1) was knocked-in as a substitution for the *KANMX4* cassette in the PepKO strain. In this way, all *FOT* genes were individually located in the original *FOT1–FOT2* locus and, therefore, were under the regulation of the *FOT2* promoter and *FOT1* terminator.

Synthetic grape must

Fermentation experiments were carried out on synthetic grape must (SGM) (42). The initial SGM (NA100, Table 2) contained 220 g/L of glucose and fructose in equimolar concentrations and 108 mg/L of YAN from NH_4^+ and amino acids [except proline, which is not assimilated by yeasts during fermentation (40)]. The amount of nitrogen in the NA100 medium was deliberately low for the given concentration of fermentable sugars, since it subsequently served as a basis for the addition of 100 mg/L of nitrogen in the form of peptides from the BSA hydrolysate, constituting the NAP200 medium. The nitrogen source in the P200 medium contained only peptides from the BSA hydrolysate, added to reach c.a. 200 mg/L of nitrogen. To study the role of sulfur in the expression of *OPT1*, the P200-SO_4 medium was prepared with the same peptide composition as in P200 but with 87% reduced concentration of sulfur (from K_2SO_4 and $MgSO_4$). To compensate for the lack of potassium and magnesium in the P200-SO_4 medium, KCl and $MgCl_2$ were added to match the concentrations of these elements in NA100, NAP200, and P200. All media were filter sterilized using a 0.22-µm Steritop Vacuum Driven Disposable Filtration System (Merck-Millipore, Burlington, MA, USA) prior to yeast inoculation.

Preparation of BSA hydrolysate

A BSA enzymatic hydrolysate was used as a source of peptides in the fermentation media. The hydrolysate was prepared according to a previously reported method (10).

In addition to ultrafiltration of the hydrolysate using a Vivaflow 200 10,000 MWCO Hydrosart crossflow cassette (Sartorius, Göttingen, Germany), the hydrolysate was subsequently filtered using a vivaflow 200 2,000 MWCO Hydrosart crossflow cassette (Sartorius, Göttingen, Germany) to further concentrate the smaller MW peptide fraction (<2 kDa), potentially assimilable by yeast. The permeate fraction of the hydrolysate was then freeze dried and stored at −20°C until further use.

Fermentation experiments

Each yeast inoculum was prepared from a single colony that was pitched to a shake flask containing yeast peptone dextrose medium (1% yeast extract, 2% bacteriological peptone, and 2% glucose). The shake flasks were incubated overnight at 30°C and 150 rpm. Cells were washed twice with equal volumes of sterile 0.9% NaCl prior to inoculation into fermenters to deliver 5×10^6 cells/mL in a final volume of 100 mL of SGM. Fermentations were performed in duplicate at 24°C in 100-mL Pyrex bottles equipped with a GL45 open top PBT screw cap and PYREX Media Bottle Septum (Corning, Inc., Corning, NY, USA). A gas outlet was installed to prevent overpressure by piercing the septum with a Sterican Ø 0.8 × 40 mm single-use hypodermic needle (B. Braun, Melsungen, Germany) attached to a Millex-FG 0.2-µm hydrophobic PTFE filter (Merck KGaA, Darmstadt, Germany).

Samples (2 mL) were collected every 6 hours for the first 48 hours, then every 12 hours until 96 hours and finally every 24 hours until 168 hours. Monitoring of the CO_2 production continued every 24 hours until the end point (288 hours). Two additional OD_{600} measurements were performed at 240 and 288 hours, and a final sample was taken at 288 hours. The biomass density of the samples was assessed by measuring the optical density at 600 nm using an Ultrospec 10 Cell Density Meter (Biochrom Ltd., Cambridge, UK). The specific production rate of CO_2, monitored gravimetrically, was used as the main indicator of fermentation progress. After centrifugation at 9600 × *g* for 10 min at 4°C in a MicroCL 21R Microcentrifuge (Thermo Fisher Scientific, MA, USA), the supernatant and biomass pellet were separately stored at respectively −20°C and −80°C, until further analysis.

Amino acid analysis

The amounts of free amino acids were analyzed on a Waters ACQUITY UPLC system (Waters Corporation, Milford, MA, USA) that was coupled to a TUV detector after derivatization using Waters AccQ-Tag chemistry (43).

Peptide analysis

Sample preparation

For peptides analysis, the fermentation samples were first mixed (1:1) with methanol in BRANDplates pureGrade 96-Well Microplates (BRAND GMBH + CO KG, Wertheim, Germany). The plates were then centrifuged at 560 × *g* in a BioSan LMC-3000 centrifuge (Biosan) to remove the precipitate. Then, 20 µL from each well was transferred to a Waters Round well Polypropylene 350-µL 96-well Sample Collection Plate (Waters Corporation, Milford MA, USA) and diluted with MilliQ water (160 µL) to a final volume of 180 µL. All samples were spiked with 0.5 ppm caffeine (20 µL) to a final volume of 200 µL. Caffeine was used as an internal standard (reference housekeeping ion) during subsequent analysis.

Liquid chromatography mass spectrometry (ultra-high-pressure liquid chromatography ion mobility separation-enabled high-resolution mass spectrometry [UHPLC-IMS-HRMS])

Peptides were analyzed using the methodology described in reference (10). Briefly, Waters I-Class Plus (SM-FL) UPLC system (Waters Corporation, Milford, MA, USA) was used

coupled with a Waters Vion IMS-QTof Mass Spectrometer equipped with a LockSpray II Exact Mass source enclosure and MKII tool-free ESI probe assembly directly connected to the column outlet. Nitrogen was used as collision gas. The instrument was controlled by Waters UNIFI 1.9.4 (3.1.0, Waters Corporation, Milford, MA, USA).

The instrument was operated in positive polarity, sensitivity mode (32,000 FWHM at 556.2766 *m/z*), and labile ion mobility tune. The analysis type was set as Peptide Map (IMS), and the experiment type was set to MSe. Data were acquired in HDMSe mode with a scan time of 0.165 s. The following manual quadrupole profile was used: mass 150/250/450 (*m/z*), dwell time 60/20 (% scan time), ramp time 10/10 (% scan time).

The injection volume was 5 µL. Full loop (5 µL) injection mode was used with 3 vol overfill. Samples were analyzed using an Acquity UPLC HSS T3 Column (1.8 µm, 1 × 150 mm, Waters Corporation, Milford, MA, USA) kept at 45°C. The initial flow rate was 0.2 mL/min. The gradient was as follows: a 0–0.5 min hold at 3% B; 0.5–5.5 min linear gradient, 3%–28% B; 5.5–7 min linear gradient, 28%–95% B; 7–7.5 min hold at 95% B accompanied by a linear flow rate increase of 0.2–0.3 mL/min; 7.5–7.6 min linear gradient, 99%–3% B accompanied by a linear flow rate decrease of 0.3–0.2 mL/min; and 7.6–10 min hold at 3% B.

Data processing of amino acid and peptide data

The peptide mapping for peptide identification in the purified BSA hydrolysate fraction was conducted according to the method used by reference (10). Consumption of peptides up to seven amino acids in length was studied. A reduction from the initial peptide signal response of at least 20% was regarded as consumption, while peptides with an increase of >20% from the initial peptide signal response were excluded. A list with the mass spectrometric data of the final selection of di-heptapeptides included in this study can be found in Table S7. All subsequent peptide signal responses exceeding the response of the peptide at the starting point were normalized to an abundance of 100% with respective relative standard deviations. Relative consumption curves for FAAs were also calculated based on their abundance at the start of fermentation. The area under the curve (AUC) was calculated for the relative abundance curve of each peptide and FAA (Fig. S1 through S4). The AUC values were compared to a virtual negative control (100% abundance over 72 hours). The consumption of a particular peptide or FAA is in level 0 when its abundance AUC is equal or higher than 80% of the negative control AUC; level 1, 60%–80%; level 2, 40%–60%; level 3, 20%–40%; and level 4 when the peptide abundance AUC is lower than 20% of the control AUC.

Gene expression analysis

Expression levels of peptide transporter genes *DAL5*, *OPT1*, *OPT2*, *FOT1*, and *FOT2* were analyzed in technical duplicates in the 18- and 48-hour samples from fermentation experiments in NAP200, P200, and P200-SO4. RNA was extracted using a phenol-chloroform method. For this, RNA buffer (120 µL, 50 mM TRIS-HCL, pH 7.4; 100 mM NaCl; 10 mM EDTA) and glass beads were added to frozen biomass samples. Cells were then disrupted in a bead mill Disruptor Genie (Scientific Industries, Inc., Bohemia, NY, USA) for 3 minutes with 1-minute intervals for cooling on ice. RNA buffer + 1.3% SDS (450 µL) and acid phenol (450 µL, pH 5) were subsequently added to the samples, followed by another cell disruption cycle. Samples were then centrifuged for 10 min at 21,100 × *g*. The upper phase was transferred to a fresh Eppendorf tube. Acid phenol (300 µL) and chloroform (300 µL) were added, after which the samples were vortexed and centrifuged for 10 minutes at 21,100 × *g*. The upper layer (500 µL) was then transferred to a new 1.5-mL Eppendorf tube. RNA was allowed to precipitate by adding 4 M NaCl (20 µL) and 96% ethanol (1 mL) and subsequently letting it stand for 30 min at −20°C. A pellet of RNA was obtained after centrifugation at 21,100 × *g* for 10 minutes and subsequent washing with 70% ethanol (150 µL). The RNA pellet was then resuspended in RNase/DNase-free water (30–50 µL) to obtain the final RNA extract. Total RNA was treated with a gDNA

TABLE 3 Real-time polymerase chain reaction (RT-PCR) primers used in this study

Primer name	Sequence
ACT1-FW	CCACCATGTTCCCAGGTATT
ACT1-RV	CCAATCCAGACGGAGTACTT
DAL5-FW	ATCACCCAACGGTAAAATTG
DAL5-RV	ATCTTCTTCTGGTGTCACTT
OPT1-FW	CCACCAAGCACACCTTATAAC
OPT1-RV	ATTGCCACACCTGCTTCAACA
OPT2-FW	CCTGATGCTGTGACCTACTAT
OPT2-RV	ATGCATGCGCCTATCAACCAA
FOT1-FW	GCGGTTGGTTGTTGAACTTT
FOT1-RV	GGGCAGTGCTCAGAAGAATC
FOT2-FW	CGAGGGCTTATGACGAGGTA
FOT2-RV	GATCCCAGCGTAGTGGACAT

Removal Kit (Jena Bioscience, Jena, Germany) and quantified using the Invitrogen Qubit RNA Broad Range (BR) Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was obtained by RT-PCR from 1 µg of RNA using the FIREScript RT cDNA synthesis KIT (Solis BioDyne, Tartu, Estonia). For RT-PCR, 5× HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) was used. RT-PCR was conducted on a qTOWER G^3 (Analytik Jena AG, Jena, Germany) system. A list of the primers used can be found in Table 3. Gene expression values were normalized to those of the housekeeping gene *ACT1*.

Data treatment and statistical analysis

Data were treated and analyzed using R v4.2.1 (R Core Team 2022) and RStudio (RStudio Team 2022). The growth rate and maximum cell population were estimated by fitting the optical density curves with the summarizegrowth R function from the Growthcurver package (44). Significance of the analysis of variance between mean values was assessed using Tukey HSD method.

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H.Y.B., G.A., and I.N. conceptualized the project. H.Y.B. performed the experiments. H.Y.B., C.B.-R., and G.A. analyzed the data. C.B.-R. visualized the results. H.Y.B. and C.B.-R. wrote the manuscript. I.N., V.G., and G.A. reviewed and edited the manuscript. I.N. supervised the project. H.Y.B. and I.N. acquired the funding. All authors have read and agreed to the published version of the manuscript and declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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AUTHOR CONTRIBUTIONS

Hidde Yaël Berg, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft | Georg Arju, Conceptualization, Data curation, Formal analysis, Methodology, Writing – review and editing | Carmen Becerra-Rodríguez, Data curation, Formal analysis, Investigation, Software, Writing – original draft, Visualization | Virginie Galeote, Writing – review and editing | Ildar Nisamedtinov, Conceptualization, Funding acquisition, Supervision, Writing – review and editing

DATA AVAILABILITY

Data files and the R script for data treatment and analysis can be found at https://zenodo.org/record/8278654.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental Tables and Figures (AEM01141-23-s0001.docx). Tables S1-7 and Figures S1-7.

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Appendix 4

Publication III

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Nitrogen Availability and Utilisation of Oligopeptides by Yeast in Industrial Scotch Grain Whisky Fermentation

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ABSTRACT

Scotch grain whisky is produced with a substantial proportion of unmalted grains, which can result in nitrogen deficiency for yeast in the fermentable grain mash. This study examined nitrogen source availability and utilisation by three commercial whisky strains (*Saccharomyces cerevisiae*) during Scotch grain whisky fermentation, focusing on oligopeptides. Peptide uptake kinetics in synthetic whisky mash with defined peptides showed that oligopeptides of up to nine amino acids were taken up by the strains, albeit with some variability between the strains. The study found that peptides with appropriate molecular weights could replace free amino acids without negatively affecting fermentation kinetics. Moreover, fermentation performance improved when additional nitrogen was provided *via* peptides rather than diammonium phosphate. Analysis of industrial grain mash indicated that despite low initial yeast assimilable nitrogen, residual proteolytic activity from malt increased nitrogen availability during fermentation. Approximately 30% of the nitrogen consumed by yeast during grain mash fermentation was derived from peptides. LC-HRMS peptide analysis revealed complex dynamics of peptide formation, degradation, and utilisation. This study highlights the importance of oligopeptides in ensuring optimal fermentation efficiency in Scotch grain mash and similar substrates.

KEYWORDS

Oligopeptides; *Saccharomyces cerevisiae*; Scotch grain whisky; yeast assimilable nitrogen (YAN)

Introduction

A readily available supply of easily assimilable nitrogen compounds, including amino acids and low-molecular-weight oligopeptides, is required for *Saccharomyces cerevisiae* to attain optimal fermentation efficiency during whisky fermentation.[1–3] Assimilated amino acids and peptides can play both anabolic and catabolic roles in yeast, for example, through the biosynthesis of structural and functional proteins and nucleic acids [4,5] or the formation of higher alcohols and their associated esters that impart flavour properties of the final product.[6,7] A prevalent belief is that most of the assimilable nitrogen for yeast in wort is produced during the malting process.[8,9] Unlike the production process of malt whisky, which the whole mash bill is composed of malted grains, Scotch grain whisky worts are primarily derived from unmalted grains.[10] Consequently, it is crucial to consider a potential limitation of nitrogenous compounds in this process.

Scotch grain whisky is usually produced from mash containing approximately 90% (w/w) unmalted grains, most commonly wheat.[8,10] The unmalted grains are mechanically milled to release starch from the grain's protein matrix and then mixed with process liquor, which is a combination of process water, backset, distillation condensate, and weak worts.[9] The resulting grain slurry is subsequently cooked to gelatinise the starch. The cooking temperature varies based on the specific gelatinisation temperature of the starch. Nevertheless, most Scotch grain distilleries typically use much higher temperatures (95–145 °C) for starch gelatinisation.[9] The cooked grain slurry is then combined with milled malt slurry in a conversion tank and maintained at mashing temperatures (63–65 °C) for up to 30 min.[9] The primary objective of this process, termed conversion, is to transform gelatinised starch into fermentable sugars (mostly maltose, glucose, and maltotriose).[11] Traditionally, wort has been separated from the grains to provide a clear fermentable liquid. However, the wort separation step has been largely eliminated in modern grain whisky distilleries; instead, the entire mash is transferred to the fermenter after cooling and pitching with yeast.[9]

As mentioned above, it is widely considered that a significant proportion of the yeast-assimilable nitrogen present in the final wort is derived from the malt. During the malting process, proteolytic enzymes are released into the endosperm to degrade grain storage proteins (i.e., hordeins, glutelins, albumins, and globulins) into peptides and free amino acids.[12] Many of these enzymes, including

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aminopeptidases and some endoproteinases, are sensitive to heat and are largely inactivated at temperatures above 55 °C, used during the conversion step.[11,13] However, studies have described the formation of soluble nitrogenous compounds during conversion by heat-stable endoproteinases and carboxypeptidases.[11,14] These proteases are inactivated only at temperatures above 70 °C.[11] Unlike brewing, the malt stream in the Scotch grain whisky production process does not reach this temperature, nor does it contain a separation and boiling step.[9,10,15] Consequently, in addition to the conversion step, the proteolytic activity can also be present during fermentation. In the context of Scotch grain whisky production, where the malted substrate constitutes on average 10% of the mash bill, this may be crucial for supplying sufficient nitrogen for yeast to ensure good fermentation kinetics, especially as the addition of exogenous proteolytic enzymes or nitrogen supplements is prohibited.[16]

The utilisation of oligopeptides as a yeast nitrogen source has received limited attention in the field of Scotch grain whisky production. Our previous study on wine yeast showed that oligopeptides can supply yeast with sufficient nitrogen to complete fermentation without the need for additional amino acids.[16] Thus, oligopeptides may also be an important secondary source of nitrogen for yeast during Scotch grain whisky fermentation.

In this study, we aim to investigate the availability, production, and utilisation of different yeast-assimilable nitrogen sources during Scotch grain whisky fermentation. Three commercial strains of *Saccharomyces cerevisiae*, commonly used in whisky production, were selected for analysis. The focus was on the role of oligopeptides as a secondary source of nitrogen for yeast. Our findings provide insights into nitrogen availability in Scotch grain whisky fermentation and highlight the importance of oligopeptides for ensuring optimal fermentation efficiency.

Experimental

Strains

Three commercial *S. cerevisiae* whisky yeast strains (denoted as SC1, SC2 and SC3) were provided by Lallemand Inc. (Montreal, QC, Canada). The strains were stored as cryocultures in 25% glycerol at −80 °C. The relevant strain information is presented in Table 1.

Each yeast inoculum was prepared by streaking the cryoculture on a yeast peptone dextrose (YPD) agar plate (1% yeast extract, 2% bacteriological peptone, and 2% glucose) and pitching a single colony into a shaker flask containing YPD broth. The flasks were incubated overnight at 30 °C, with shaking at 150 rpm. Cells were washed twice with equal volumes of sterile 0.9% NaCl prior to inoculation into fermenters to deliver 5×10^6 cells/mL, based on OD_{600} measurements.

Table 1. Whisky strains used in this study.

Strain acronym	Type of whisky recommended for	Relative nitrogen requirement*
SC1	Grain Whisky	Low
SC2	Grain and malt whisky	Low
SC3	Malted grain whisky	High

The information displayed in the table has been summarised from the provided technical data sheets of the respective strains (Lallemand Inc., Montreal, QC, Canada).
*Nitrogen requirement relative to the other strains used in this study.

Fermentations

Synthetic whisky mash fermentations

Fermentation experiments were performed on synthetic whisky mash (SWM) to confirm the importance of peptides as a nitrogen source to whisky yeast. In addition, the potential benefits of nitrogen supplementation, either through organic or inorganic means, were investigated.

Preparation of the bovine serum albumin hydrolysate

A bovine serum albumin (BSA) enzymatic hydrolysate was used as a source of peptides in a synthetic whisky mash (SWM). The hydrolysate was prepared according to a previously reported method.[16,17]

The hydrolysate was produced from BSA (40 g/L) in a 1 L benchtop fermenter (Applikon, Delft, The Netherlands) using the industrial protease COROLASE®7089 (AB Enzymes, Darmstadt, Germany). The protease dose rate was 0.5% w/w BSA, and hydrolysis was performed at pH 7 (maintained by titration using 2 M NaOH) for 20 h at 50 °C. After completion of BSA hydrolysis, the hydrolysate was filtered with a 10 kDa Vivaflow® 200 10,000 MWCO Hydrosart crossflow cassette (Sartorius, Göttingen, Germany) to remove the protease and larger peptides. To further concentrate the smaller MW peptide fraction (≤2 kDa), another filtration step was performed using a Vivaflow® 200 2,000 MWCO Hydrosart crossflow cassette (Sartorius, Göttingen, Germany). The hydrolysate was then freeze-dried and stored at −20 °C until further use.

Synthetic whisky mash (SWM) composition

The composition of SWM is an adaptation of the synthetic grape must described by Salmon and Barre.[18] Citric and malic acids were excluded from the medium, and the sugar concentration, free amino acid ratios (Table S1), and pH were adjusted to mimic whisky mash. These values were based on measurements taken from a commercial whisky production process.

The base SWM contained 150 g/L maltose and glucose (9:1 w/w) and was modified by adding different nitrogen sources (Table 2). Thus, in the first experiment (AP230), 130 mg/L of nitrogen was added with amino acids, and 100 mg/L was added in the form of small molecular weight peptides (smaller than 2 kDa) from the BSA hydrolysate. In the second experiment, all nitrogen (200 mg/L) was supplied in the form of small-MW peptides from the BSA hydrolysate (P230). To study the effect of additional nitrogen supplementation with (in)organic sources, 70 mg/L of nitrogen was added to the P230 medium from either the BSA hydrolysate (PP300) or diammonium phosphate (PD300). The pH of all the media was adjusted to 5.2. The media were then filter-sterilised using a 0.22 µm Steritop® vacuum-driven disposable filtration system (Merck-Millipore, Burlington, MA, USA) prior to yeast inoculation.

Table 2. Nitrogen composition of the synthetic media used in this study.

	AP230 (Amino acids + Peptides)	P230 (Peptides)	PP300 (Additional peptides)	PD300 (Additional ammonia)
Amino acids	130	30*	40*	30*
Peptides	100	200	260	200
Ammonia	-	-	-	70
Total	**230**	**230**	**300**	**300**

All media contained 150 g/L maltose and glucose (9:1, w/w). All values are displayed as milligrams of nitrogen per litre. Free proline was not taken into account as it is not or poorly assimilated by yeasts during fermentation [4, 19].
*Nitrogen deriving from free amino acids in the BSA hydrolysate.

Table 3. Temperature, pH, and ethanol concentration gradients used in uninoculated grain mash to study free amino acid and peptide production by native malt proteases.

Time (h)	Temperature (°C) (Gradient)	Ethanol (g/L) (Manual every 12h)	pH (Gradient)
0	25	0	5.2
12	29.5	20	4.75
24	34	50	4.3
36-60	34	70	4.3

The ethanol concentration was increased stepwise by injecting 96% ethanol into the bioreactor.

Fermentations

All fermentations on synthetic media (100 mL) were performed in triplicate at 24 °C in 100 mL Pyrex™ bottles equipped with a GL45 open-top PBT screw cap and PYREX™ Media Bottle Septum (Corning Inc., Corning, NY, USA). A gas outlet was installed to prevent overpressure by piercing the septum with a Sterican® Ø 0.8 × 40 mm single-use hypodermic needle (B. Braun, Melsungen, Germany) attached to a Millex-FG 0.2 µm hydrophobic PTFE filter (Merck KGaA, Darmstadt, Germany).

Samples (2 mL) were collected every 6 h for the first 48 h, every 24 h until 120 h, and every 48 h until 192 h. Monitoring of CO_2 production was continued until the endpoint (288 h), after which a final sample was collected. The biomass density of the samples was assessed by measuring the optical density at 600 nm using an Ultrospec® 10 Cell Density Meter (Biochrom, Ltd., Cambridge, UK). The specific production rate of CO_2, monitored gravimetrically, was used as the main indicator of the fermentation progress. After centrifugation at 9600 × g for 10 min at 4 °C in a MicroCL 21 R Microcentrifuge (Thermo Fisher Scientific, MA, USA), the supernatant was filtered through Minisart RC 0.2 µm syringe filters (Sartorius, Göttingen, Germany) and stored at −20 °C until further analysis.

Grain mash fermentations

Unfiltered grain mash, produced from approximately 10% malted barley and 90% unmalted wheat, was obtained from a commercial Scotch grain whisky distillery. Fermentation was performed in 500 mL of grain mash in 1 L Biobundle benchtop fermenters (Applikon, Delft, the Netherlands) using the cultivation control software BIOEXPERT V2 v2.96 (Applikon, Delft, the Netherlands). The fermenters were equipped with pH and temperature sensors. The production of CO_2 was measured with an HPR–20 R&D Benchtop Gas Analysis System (Hiden Analytical Ltd., Warrington, UK) using MASsoft 10 Professional software (Hiden Analytical Ltd., Warrington, UK). To allow for stable measurement of CO_2 production, the headspace was flushed at a rate of 100 mL/min (5% CO_2, 95% N_2).

The fermentation temperature was raised from 25 °C to 34 °C in the first 24 h to mimic the heat production that occurs during the factory process due to yeast activity, and then maintained at 34 °C for 60 h. All fermentations were performed in triplicate. Samples (10 mL) were collected every 12 h from the bottom of the fermenter using a sample port and a 10 mL sterile syringe (B. Braun, Melsungen, Germany).

The formation of free amino acids and peptides due to residual proteolytic activity in the grain mash was analysed in an uninoculated grain mash by simulating the changes in temperature, pH, and ethanol concentration during fermentation. The change gradients of the latter parameters were based on the actual measurements of grain mash fermentation with strain SC2 and are shown in Table 3. The uninoculated mash contained 200 µg/mL G418 (InvivoGen, Toulouse, France) and 2 ppm Lactoside 247™ (Lallemand, Montreal, QC, Canada) to prevent microbial growth.

Analysis

Preparation of grain mash samples for amino acids and peptide analysis

Grain mash samples (10 mL) were centrifuged at 13000 × g for 15 min at 20 °C in a Hettich® ROTANTA 460/460 R centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) to separate the solids. The liquid phase was then filtered using Minisart RC 0.2 µm syringe filters (Sartorius, Göttingen, Germany) to remove any residual biomass.

Sugars were removed from the grain mash by fermentation with a peptide transporter knockout (PepKO) strain.[16, 20] The latter was necessary to avoid the loss of amino acids due to Maillard reactions when total amino acid analysis was carried out under high temperature and acidic conditions. First, the samples were heated at 75 °C for 20 min to remove any residual proteolytic activity. In addition, the uninoculated grain mash samples were freeze-dried in a Heto PowerDry PL3000 Freeze Dryer (Thermo Fisher Scientific, MA, USA) and reconstituted in MilliQ water to remove ethanol, which would otherwise prevent fermentation. To ensure proper fermentation with the PepKO stain, 100 µL of a nutrient stock (Table S2) containing minerals, vitamins, and 100 mg/L nitrogen from NH_4^+ was added to the samples. Because the PepKO strain used was a descendant of a wine stain, the nutrient stock was also supplied with 0.1% glucoamylase (MEGA PACIFIC TECHNOLOGY INC, Arcadia CA, USA) to break down maltose and maltotriose into more easily assimilable glucose.[21] In addition, because the PepKO strain contained KanMX as a marker for the deletion of *FOT* peptide transporter genes,[22] 200 µg/mL G418 (InvivoGen, Toulouse, France) was added to prevent microbial contamination. Fermentation with the PepKO strain was performed in 15 mL conical tubes (Eppendorf AG, Hamburg, Germany) at 32 °C and at 150 rpm. The lids of the tubes were slightly opened to prevent overpressure. The inoculum (20 µL) was prepared as

previously described. The end of the fermentation process was indicated by the cells dropping from the culture medium. As the samples contained variable concentrations of residual sugars, the fermentation time was sample dependent. Cells were separated from the samples by centrifugation at 9600 × g for 10 min at 4 °C in a MicroCL 21 R Microcentrifuge (Thermo Fisher Scientific, MA, USA) and filtered using Minisart RC 0.2 µm syringe filters (Sartorius, Göttingen, Germany).

The resulting samples were filtered to obtain $a \leq 2$ kDa fraction using Vivaspin 2 centrifugal concentrators (Sartorius AG, Göttingen, Germany) according to the manufacturer's instructions. The retentate was washed twice with 2 mL of MilliQ water. The permeate was freeze-dried and reconstituted in Milli-Q water to obtain the final sample.

Analysis of free and bound amino acids

Concentrations of free and peptide-bound amino acids were analysed on a Waters ACQUITY UPLC® system (Waters Corporation, Milford, MA, USA) coupled to Waters ACQUITY UPLC tuneable ultraviolet (TUV) detector (Waters Corporation, Milford, MA, USA) after derivatisation using Waters AccQ-Tag chemistry, as described by Fiechter and Mayer.[22] For total amino acids, the samples were first hydrolysed with 6 M HCl + 1% (v/v) phenol at 105 °C for 22 h in vacuum using an Eldex H/D workstation (Eldex Laboratories Inc., Napa, CA, USA). The peptide-bound amino acids were calculated by subtracting the measured free amino acids from the total amino acids.

Peptide analysis

Sample preparation. The SWM samples were first mixed (1:1) with methanol in BRANDplates™ pureGrade™ 96–Well Microplates (BRAND GMBH + CO KG, Wertheim, Germany). The plates were then centrifuged at 560 × g using a BioSan LMC–3000 centrifuge (Biosan, City, Company) to remove the precipitate. Then, 20 µL from each well was transferred to a Waters™ round well polypropylene 350 µL 96–well sample collection plate (Waters Corporation, Milford, MA, USA) and diluted with Milli-Q water (160 µL) to a final volume of 180 µL. All samples were spiked with 0.5 ppm caffeine (20 µL) to a final volume of 200 µL. Caffeine was used as an internal standard (reference housekeeping ion) during subsequent analysis.

Ice-cold grain mash samples (100 µL) were first mixed with 900 µL of ice-cold methanol in 1.5 mL Eppendorf® tubes (Eppendorf, Hamburg, Germany) and centrifuged at 11,200 × g at 4 °C for 20 min in a MicroCL 21 R Microcentrifuge (Thermo Fisher Scientific, MA, USA) to remove the precipitate. The samples were then freeze-dried in a Heto PowerDry PL3000 Freeze Dryer (Thermo Fisher Scientific, MA, USA) and reconstituted in 100 µL Milli-Q water. All samples were spiked with 0.5 ppm caffeine to a final volume of 110 µL.

Liquid chromatography-mass spectrometry (ultra-high-pressure liquid chromatography ion mobility separation-enabled high-resolution mass spectrometry (UHPLC-IMS-HRMS)). The peptides were analysed using the methodology described by Arju et al. (2022). Briefly, the Waters I-Class Plus (SM-FL) UPLC system (Waters Corporation, Milford, MA, USA) was coupled with a Waters Vion IMS-QTof Mass Spectrometer equipped with a LockSpray II Exact Mass Source Enclosure and MKII tool-free ESI probe assembly directly connected to the column outlet. Nitrogen was used as collision gas. The instrument was controlled using Waters UNIFI 1.9.4 (3.1.0, Waters Corporation, Milford, MA, USA).

The instrument was operated in positive polarity, sensitivity mode (31,000 FWHM at 556.2766 m/z), and labile ion mobility tuning mode. The analysis type was set as Peptide Map (IMS), and the experiment type was set to MSe. Data were acquired in the HDMSe mode with a scan time of 0.165 s for synthetic and 0.250 s for grain mash. The following manual quadrupole profile was used for SWM: mass 150/250/450 (m/z), dwell time 60/20 (% scan time), ramp time 10/10 (% scan time); and for grain mash: mass 162/300/500 (m/z), dwell time 35/35 (% scan time), ramp time 15/15 (% scan time).

The injection volume was 5 µL. Full loop (5 µL) injection mode was used with a 3–volume overfill. The samples were analysed using an Acquity UPLC HSS T3 Column (1.8 µm, 1 × 150 mm, Waters Corporation, Milford, MA, USA) maintained at 45 °C. The initial flow rate was 0.15 mL/min. The gradient for synthetic mash was as follows: a 0–0.5 min hold at 1% B; 0.5–10.5 min linear gradient, 1–30% B; 10.5–12 min linear gradient, 30–95% B accompanied by a linear flow rate increase of 0.15–0.2 mL/min; 12–12.5 min hold at 95% B accompanied by a linear flow rate increase of 0.2–0.25 mL/min; 12.5–12.6 min linear gradient, 95–1% B accompanied by a linear flow rate decrease of 0.25–0.15 mL/min; and 12.6–15 min hold at 1% B. The gradient for grain mash was as follows: a 0–1 min gold at 1% B; 1–16 min linear gradient, 1–30% B; 16–17 min linear gradient, 30–95% B accompanied by a linear flow rate increase of 0.15–0.2 mL/min; a 17–17.5 min hold at 95% B accompanied with a linear flow rate increase of 0.2–0.3 mL/min; 17.5–17.6 min gradient, 95–1% B accompanied with linear flow rate decrease of 0.3–0.15 mL/min; a 17.6–20 min hold at 1% B.

Data treatment and statistical analysis

Data was treated and analysed using R v4.3.2 (R Core Team 2023) and RStudio (RStudio Team 2023). The growth rate and maximum cell population in SWM were estimated by fitting the optical density curves with the "summarize-growth" R-function from the GrowthCurver package.[23] The significance of ANOVA between the mean values was assessed using Tukey's HSD method.

Peptide mapping for peptide analysis was conducted according to the method described by Arju et al.[24] The time window for peptide detection was extended to 14 min

to accommodate the extended gradient used to analyse the grain mash samples. Peptide mapping in the grain mash samples of peptide masses was performed against five barley storage proteins and nine wheat storage proteins (Table S3).

Data processing of amino acids and peptides from the strain characterisation in SWM was performed, as described by Berg et al.[16] A list of the mass spectrometric data of di- to nonapeptides identified in the BSA hydrolysate in this study is shown in Table S6.

All data were visualised using ggplot2.[24]

Results

Effect of oligopeptides on the fermentation kinetics in synthetic whisky mash

Fermentation experiments were performed on synthetic whisky mash (SWM) to study the uptake of peptides as a nitrogen source by the whisky yeast strains used in this study (Table 1) under different conditions of nitrogen supply (Table 2). In the first experiment, the fermentation medium (AP230) contained 130 mg N/L from free amino acids (FAA) and 100 mg N/L from peptides with a molecular weight (MW) ≤ 2 kDa. In the second medium (P230) the FAA were deficient (30 mg N/L), and peptides were the only substantial source of nitrogen (200 mg N/L). Finally, the effect of additional nitrogen supplementation (70 mg N/L) with either diammonium phosphate (DAP) or peptides was studied (media PP300 and PD300, respectively). The effect on the fermentation kinetics is shown in Figure 1.

In the experiments with a total N content of 230 mg N/L, containing either free amino acids (56.5% of N) and peptides (43.5% of N) (AP230) or only peptides (P230), the three strains displayed no significant differences in maximum cell population (Figure 1A), maximum CO_2 production rate (Vmax, Figure 1B), and fermentation time (Figure 1C). Strain SC1 was the only strain that exhibited a significantly higher Vmax value in the P230 medium, resulting in a slight, yet statistically insignificant reduction in overall fermentation time (80% attenuation, determined based on the maximum theoretical CO_2 production from 150 g/L maltose and glucose (9:1). These findings corroborate previous results from the experiments we conducted with a wine yeast and suggest that peptides individually can also provide the necessary nitrogen to these whisky strains.[16]

Among the three strains, strain SC3 exhibited the lowest fermentation performance (Vmax and fermentation time) at 230 mg N/L (Figure 1), which could be attributed to its higher nitrogen demand than that of the other strains. Interestingly, with additional nitrogen supplementation (70 mg N/L) with diammonium phosphate (DAP, medium PD300), the fermentation kinetics did not improve, whereas a positive impact was observed with an increased peptide concentration (medium PP300). Thus, in the PP300 medium, the maximum cell population and Vmax increased by 52% and 62%, respectively, while the fermentation time decreased by 42%, compared to the P230 medium. Similarly, strain SC1 in the PP300 medium showed a 28% increase in Vmax and a 36% decrease in fermentation time, although the biomass density remained similar to that in the P230 medium. In the case of strain SC2, the maximum cell population and Vmax increased by 17% and 11%, respectively,

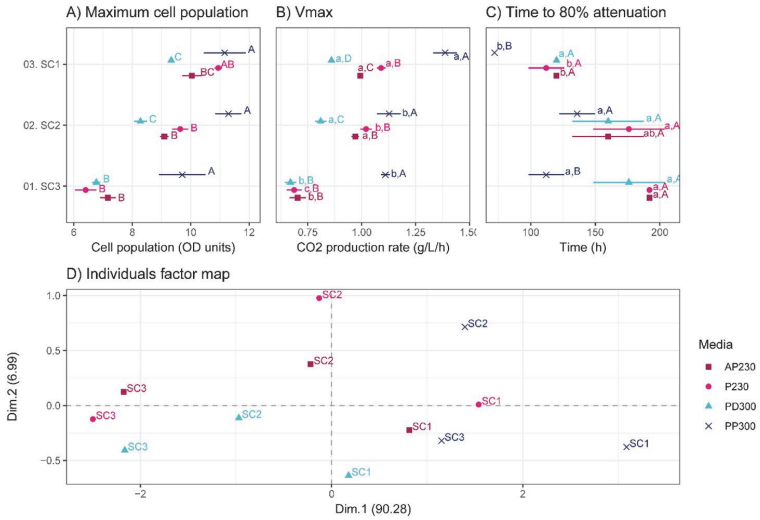

Figure 1. Cell growth and fermentation kinetics in synthetic whisky mash with different nitrogen conditions. Letters denote the statistical groups from Tukey's tests ($p < 0.05$), with lower-case letters indicating different groups of strains per medium and upper-case letters indicating the groups of media for each strain. The "time to 80% attenuation" was chosen as not all fermentations reached 100% of attenuation during the set experimental time. Principal component analysis (PCA) (D) was performed using the three fermentation parameters depicted in A, B, and C.

resulting in a 23% decrease in fermentation time, albeit not statistically different from the fermentation time in the lower nitrogen-containing P230 medium. Interestingly, DAP supplementation (PD300 medium) had a negative effect on the maximum cell population and Vmax by strain SC1 and SC2. Nevertheless, despite variations in the maximum cell population and Vmax, the fermentation time remained unaffected.

A principal component analysis plot was constructed to support these observations (Figure 1D). The experiments in the AP230 and P230 media clustered together by strain, indicating that peptides can provide the necessary nitrogen to these whisky strains when FAA are limited, without affecting fermentation efficiency. For strain SC3, the experiments conducted on the PD300 medium cluster with the AP230 and P230 lower nitrogen-containing media, showing no or minimal effect of ammonium supplementation on this strain. The negative effect of DAP supplementation on the other strains can be observed by their respective PD300 medium clustering away from their respective AP230 and P230 clusters. The positive effect of increased nitrogen content from peptide supplementation (PP300) on strain SC3 can be seen by its clustering with strain SC1 in the AP230 and P230 media. Strain SC3 in the PP300 medium also moved closer to this cluster, whereas strain SC1 in the PP300 medium formed its own group.

Oligopeptide uptake by whisky yeast strains in synthetic whisky mash

In addition to the fermentation kinetics, peptide consumption by the three whisky yeast strains was monitored during the first 72 h of fermentation (Figures 2, S1–4). Intensive uptake of peptides with different chain lengths (up to nine amino acids) simultaneously with FAA was observed in both AP230 and P230 media for all strains (Figure S4). The conserved ability of the three strains to take up peptides from the environment was also evident from their similar peptide consumption profiles in these two media.

In the medium where additional nitrogen was added in the form of DAP (PD300), ammonia was consumed first, which resulted in delayed uptake of di- and tripeptides and limited the uptake of peptides with longer chain lengths in all strains (Figures 2, S1–4). In addition, the strains revealed distinct patterns of peptide uptake in the medium with increased peptide content (PP300), which may be attributed to their individual nitrogen requirements. For example, strain SC1 showed similar consumption levels for the di- and tripeptides in the PP300 medium as in the P230 medium (Figure 2), while peptides with a longer chain length were taken up more slowly. In strain SC2, this effect was less pronounced, whereas in strain SC3, all peptides were taken up simultaneously. As the methodology utilised in this study does not enable the exact quantification of individual peptides, it was impossible to determine the exact quantity of nitrogen contributed by each peptide length group to the overall nitrogen intake.

Together with the observed effects of peptides on fermentation performance (Figure 1), these results support our hypothesis that peptides can serve as a substantial alternative source of nitrogen for yeast.

Nitrogen availability and the role of peptides in Scotch grain whisky fermentation

To study the role of peptides as a yeast nitrogen source in Scotch grain whisky fermentation, a grain mash sample from an industrial Scotch grain whisky production process was obtained, and fermentation was conducted in one-litre benchtop fermenters using the three strains previously characterised for their peptide uptake capability in SWM.

All strains completed fermentation in the grain mash, albeit with distinct fermentation kinetics (Figure 3). Strain SC1 showed the fastest fermentation, ceasing active fermentation by 24 h. After this point, the CO_2 production rate significantly decreased, and fermentation was complete after approximately 48 h. In contrast, with strain SC2 and SC3 the CO_2 production rate started to cease at 36 h with fermentation completion at 48 h. The ethanol content of the fermentation with strain SC2 was the highest at 8.8 ± 0.2%(v/v), followed by strain SC3 at 8.7 ± 0.2%(v/v), and strain SC1 at 8.5 ± 0.4%(v/v), which were not significantly different from each other ($\alpha = 0.05$).

The initial grain mash contained 90.2 ± 6.73 mg N/L from FAA (Figure 4). According to previously published studies, this amount does not meet the minimum requirement for satisfactory fermentation efficiency (150–250 mg N/L) for the given fermentable sugar content.[5,25] However, the grain mash also contained oligopeptides, some of which could be a potential source of N for yeast. In total, 70.4 ± 9.9 mg N/L was determined to be derived from peptides with a MW ≤ 2 kDa. Thus, the sampled grain mash initially contained up to 160.6 ± 12.0 mg N/L of potentially yeast-assimilable nitrogen.

The production of grain mash for Scotch grain whisky fermentation does not involve boiling, which could result in residual proteolytic activity during fermentation originating from the malt. To determine the amount of additional potentially yeast-assimilable nitrogen liberation, an experiment was conducted using an uninoculated grain mash, where the actual grain mash fermentation conditions were simulated by gradually changing the pH and ethanol concentration (Table 3, Figure 4). The results showed an increase in nitrogen content in both FAA and, to a lesser extent, peptides ≤ 2 kDa. The increase of potentially available nitrogen content amounted to a total of 58.4 ± 6.28 mg N/L in 36 h, with 45.8 ± 4.1 mg N/L from FAA and 12.6 ± 4.7 mg N/L from peptides. Therefore, a total of 219.0 ± 13.5 mg N/L was available for fermentation, with 38% of it derived from peptides with a MW ≤ 2 kDa. Corroborating the observed fermentation kinetics and literature data, this quantity was sufficient to complete fermentation.[5]

Although the strains displayed varying fermentation and nitrogen uptake kinetics, the amounts of nitrogen consumed

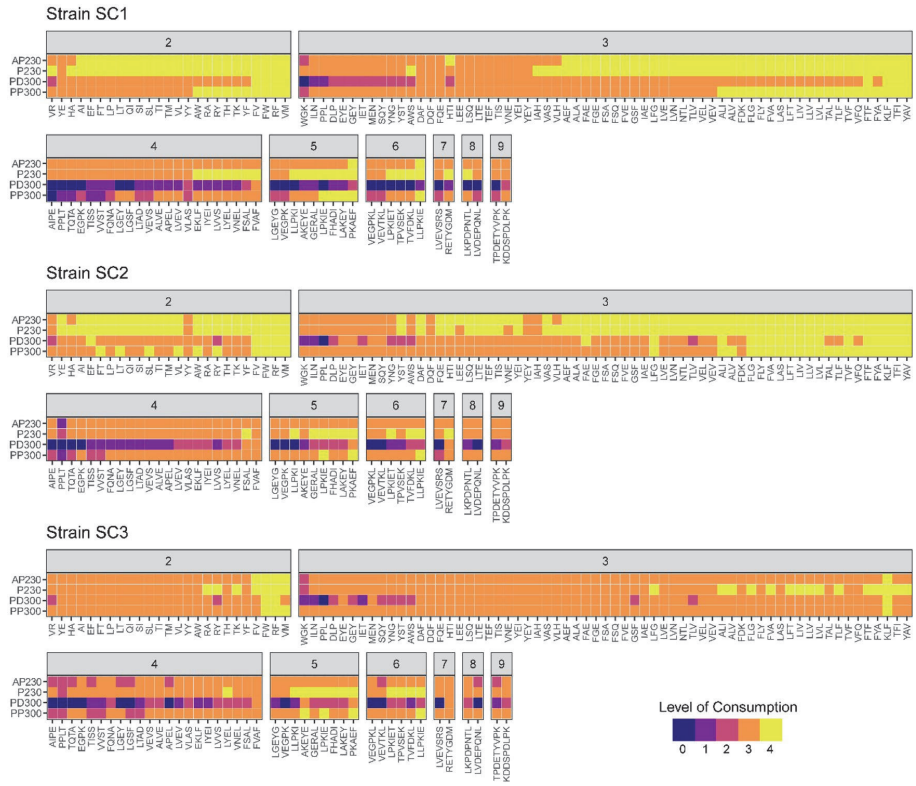

Figure 2. Consumption of peptides in synthetic whisky mash by the three whisky strains during the first 72 h of fermentation. Peptides were grouped according to the number of amino acid residues: 2, dipeptides; 3, tripeptides; 4, tetrapeptides; 5, pentapeptides; 6, hexapeptides; 7, heptapeptides; 8, octapeptides; and 9, nonapeptides. The area under the curve (AUC) was calculated from the relative abundance curve of each peptide. The AUC values were compared to those of a virtual negative control (100% abundance over 72 h). The consumption of a particular peptide was level 0 when its abundance AUC was equal to or higher than 80% of the negative control AUC; level 1, 60–80%; level 2, 40–60%; level 3, 20–40%; and level 4, when the peptide abundance AUC was lower than 20% of the control AUC.

from the grain mash were similar (Figure 4). Thus, strain SC1 consumed 186.8 ± 14.0 mg N/L, with 128.2 ± 7.9 mg N/L derived from FAA and 58.6 ± 11.5 mg N/L from peptides. Similarly, strain SC3 consumed 193.6 ± 14.5 mg N/L, with 134.5 ± 7.9 mg N/L derived from FAA and 59.1 ± 12.2 mg N/L from peptides. Strain SC2 consumed 175.3 ± 13.7 mg N/L, with 126.0 ± 7.9 mg N/L derived from FAA and 49.4 ± 11.2 mg N/L from peptides. Thus, peptide-derived nitrogen in these fermentations comprised ~30% of the total nitrogen assimilated by yeast.

Peptide production, degradation and consumption (based on the change in their MS peak intensities) at different sampling points were compared (Figure 5). In total, 153 peptides ranging from 2 to 23 AA in length could be followed. Peptide annotations were obtained by mapping the peptide masses to the six most abundant barley storage proteins (hordeins) and nine wheat storage proteins (glutenins) (Table S3).[26,27] However, this approach is subject to a degree of identification inaccuracy due to the numerous proteins that were included in peptide screening, as well as those that were not included. Consequently, we decided to attribute only the peptide length to the observed peptide candidate masses, as suggested in another study.[27]

Analysis of peptides in the uninoculated mash revealed complex dynamics of peptide production and degradation in the grain mash due to malt proteases (Figure 5A). During the first 12 h of incubation, degradation of peptides occurred, as shown by the decreased relative concentration of most peptides, as well as the increased concentration of free amino acids (Figure 4). The hydrolysis continued between 12 and 36 h, first with increasing relative concentrations of higher MW peptides (12 to 24 h), which were then further degraded into smaller ones between 24 and 36 h, suggesting an interplay between various proteases and peptidases. The simultaneous production and degradation of peptides also explains the limited change in the contribution of peptides to the overall increase in the potentially yeast-assimilable nitrogen content of the grain mash, as shown in Figure 4.

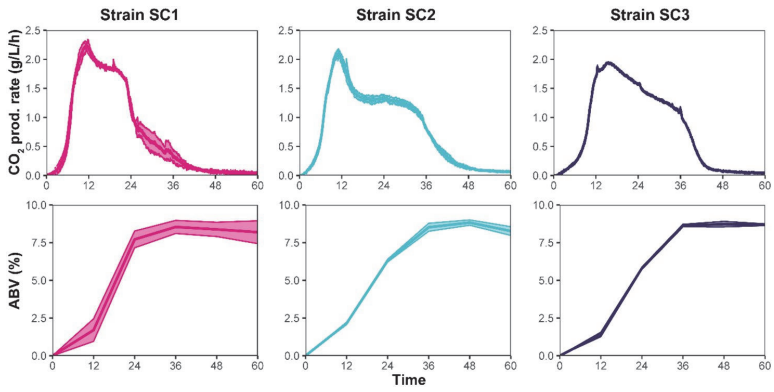

Figure 3. CO_2 Production rate and ethanol concentration by volume (ABV) during laboratory-scale simulated scotch grain whisky fermentation with three commercial whisky strains.

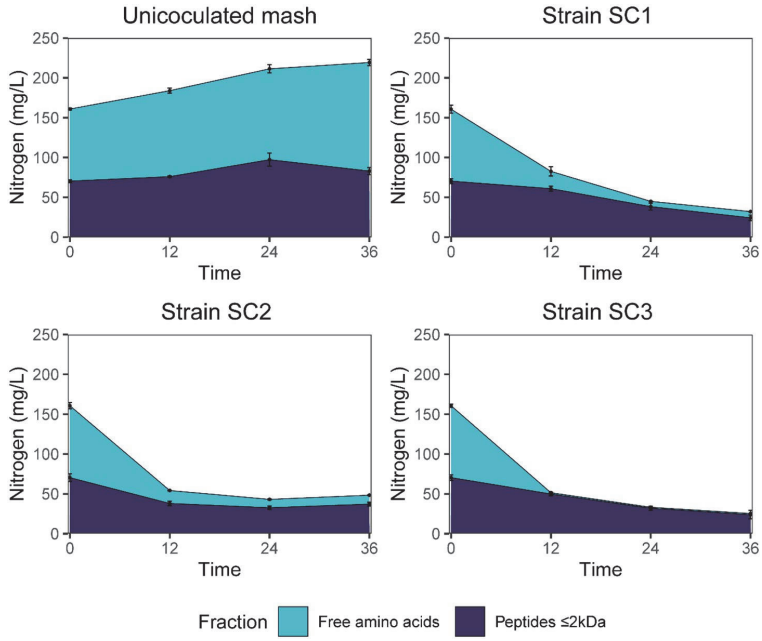

Figure 4. Changes in FAA and oligopeptide (≤ 2 kDa) content during fermentation of scotch grain mash and uninoculated grain mash. One experiment with uninoculated grain mash, simulating the fermentation process with changing pH and EtOH concentration, was conducted to determine the background production of potentially available nitrogen sources for yeast (Table 3). Free proline was not taken into account as it is poorly assimilated by yeast during fermentation.[4,19]

The complex kinetics of peptide formation and degradation due to proteolysis by malt-derived enzymes makes it difficult to precisely interpret the extent of their consumption by yeast (Figure 5B–D). Thus, the changes in the relative concentration of small MW peptides (two to five AA in chain length) during fermentation in the first 12 h were very similar to those observed in the uninoculated mash incubation experiment. In contrast, a higher number of peptides with a negative fold change in the relative concentration during later stages of fermentation when free amino acids were depleted suggests their consumption by yeast. In all three strains, the net change between the start and end of fermentation suggested the consumption of di-to-pentapeptides (average fold change of 0.6 compared to 0.9 in the uninoculated mash).

The only major difference among the three yeast strains was observed in the experiment with strain SC3 (Figure 5D), in which the apparent increase in peptides was much more pronounced during the first stage of fermentation

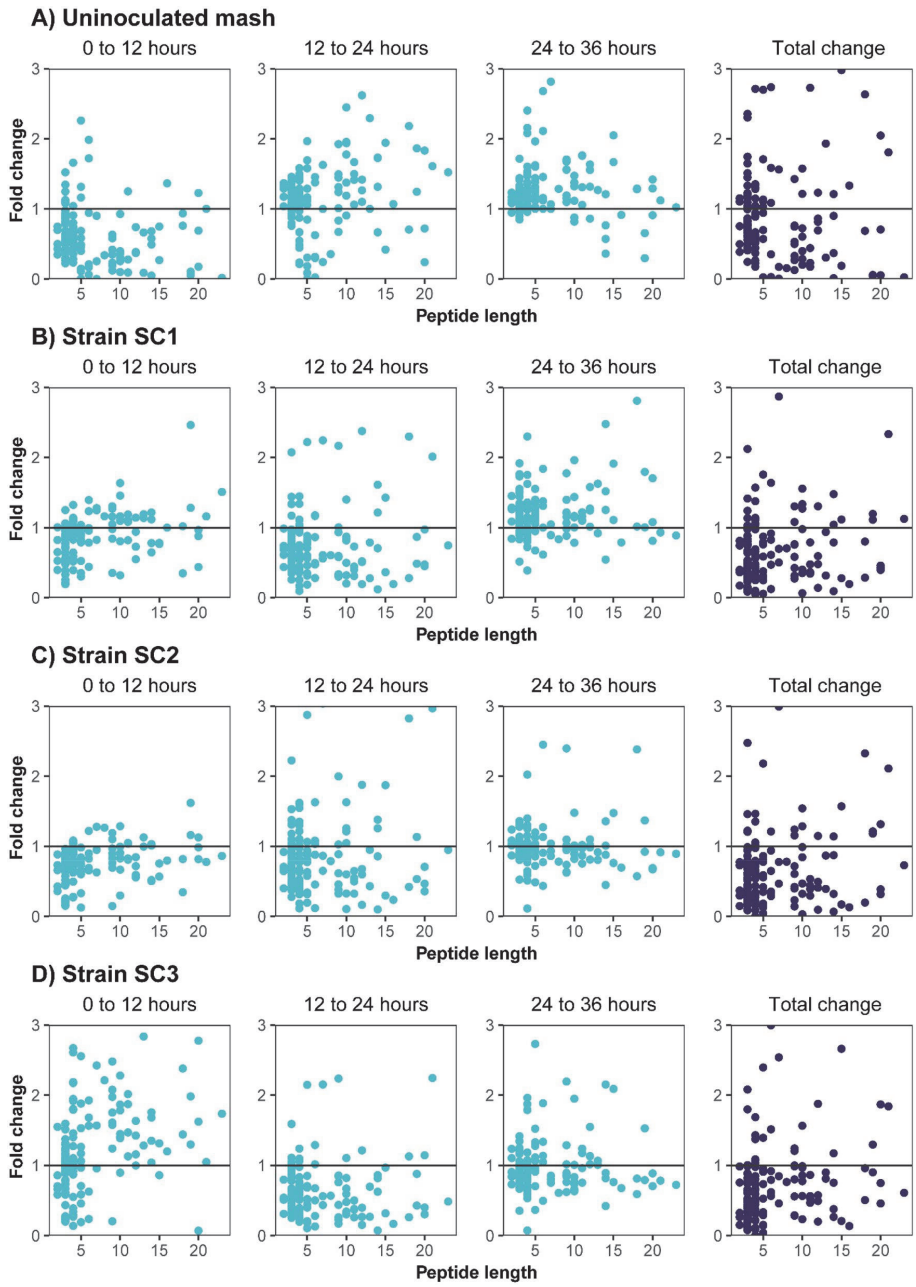

Figure 5. Fold changes in peptide abundance between samples of the uninoculated scotch grain mash and the fermentations of the same mash with strains SC1-3. The "total change" depicts the fold change between peptides candidates at the 0-h sample and the 36-h sample.

(0 to 12 h). The exact reasons for this are unknown but may be related to the longer lag phase observed for this strain compared to the other two strains during which the consumption of peptides did not take place yet. Bound amino acid analysis of the 2 kDa fraction in strain SC3 did not show a substantial increase in the nitrogen content of peptides between 0 and 12 h (Figure 4). Therefore, it was not possible to determine the significance of this observation without quantitative data on specific peptides at more frequent sampling points.

Overall, these findings indicate that the production, degradation, and consumption of peptides during Scotch grain whisky fermentation is a highly dynamic and complex process.

Discussion

Around 90% of the raw material used in the production of Scotch grain whisky consists of unmalted grains, which may lead to nitrogen deficiency for yeast in the fermentable grain mash. This study investigated the availability and utilisation of nitrogen sources by yeast during the fermentation of Scotch grain whisky, with a particular focus on the role of oligopeptides. First, three commercial *Saccharomyces cerevisiae* whisky strains were assessed for their ability to take up peptides from peptide-supplemented synthetic whisky mash, using various nitrogen sources. All three strains were able to complete fermentation with peptides as the sole nitrogen source, demonstrating that peptides with a suitable chain length can satisfy the nitrogen requirements of yeast. We previously characterised a *Saccharomyces cerevisiae* wine yeast (59 A, haploid variant of the commercial wine strain EC1118 [28]) for its ability to take up peptides with different chain lengths in a similar analysis and found that it could utilise peptides up to seven amino acids in length, which were the highest chain length peptides determined by the analyses used.[16] By further optimising the peptide mapping analysis, in the current study we suggest that the three whisky strains can take up peptides of up to at least nine amino acids in length. Like the wine strain 59 A, these whisky strains took up smaller peptides first, with the only exception being that the whisky strains preferred di- and tripeptides, whereas in 59 A, tetrapeptides were also preferred.[16] This can be explained by the activity of the Fot family of peptide transporters, which is specific to wine yeasts.[16,20,28,29] Ptr2 (previously characterised as a di- and tripeptide transporter [30]) is presumably the active di- and tripeptide transporter in whisky strains, explaining why tetrapeptides were taken up later with the other larger peptides.

The applied peptide mapping methodology in combination with using a single protein digest, allows for reliable peptide identification at the level of peptide length and amino acid composition but not the sequence.[17] Peptides with different consumption levels shared similar chemical properties and could not be differentiated. Further investigation of the sequence-specific intake of peptides by these whisky strains necessitates experiments with synthetic peptides. Alternatively, proteases with known cleavage sites, such as trypsin or chymotrypsin, could be used to produce the BSA hydrolysate. By employing in silico methods, peptide mass data can then be utilised for peptide fingerprint identification.

Measurements taken from the Scotch grain mash obtained from an industrial distillery indicate that when a significant proportion of the mash bill is unmalted (approximately 90% in this case), the initial YAN content of the grain mash (~160 mg N/L) is relatively low, considering that the minimum nitrogen requirement for satisfactory fermentation efficiency for the mash with the respective fermentable sugar concentration remains between 150 and 250 mg N/L [5] and that not all peptides in the ≤2 kDa molecular weight (MW) fraction might be fully assimilable by yeast. However, our study showed that the residual proteolytic activity that remained in the mash after conversion provided an additional 36% of potentially assimilable nitrogen to yeast during fermentation, of which the majority (80%) was in the form of free amino acids and 20% was contributed by peptides with MW ≤ 2 kDa. The fermentation experiments with three commercial whisky strains indicated that the final nitrogen content in the mash remained sufficient to support complete fermentation, with the strains taking up ~185 mg N/L on average, of which ~30% can be attributed to peptides. Thus, the remaining proteolytic activity in the mash seems essential for the fermentation process, and peptides form a considerable source of nitrogen for yeast during Scotch grain whisky fermentation.

Several protease families are involved in the germination process of barley, and are therefore present in mash.[31] Heat-stable endoproteinases and carboxypeptidases may retain their activity in the mash during subsequent fermentation because the temperature of the conversion process does not exceed 70 °C,[11,14,31] which was observed in our experiments. The LC-HRMS analysis of relative concentrations of individual peptides in uninoculated mash, in which the temperature, pH, and ethanol concentration were changed to simulate the fermentation process, suggested that these conditions favoured the activity of both proteolytic enzyme classes of malt, resulting in the production of peptides and FAA. However, this process is dynamic and depends on the fermentation stage. The formation of free amino acids and small peptides occurred mostly at the beginning of fermentation (0 to 24 h, $T = 24 - 34$ °C, pH= 5.2 – 4.3, EtOH= 0 – 50 g/L), whereas higher MW peptides started to accumulate in the later stages (12 h and further). Because of this highly dynamic process, involving both production and degradation of peptides, limited variation in the nitrogen concentration in the MW ≤ 2 kDa peptide fraction of the uninoculated mash was observed. The extent of peptide uptake in the fermenting mash during the first phases of fermentation (0 to 12 h) was difficult to estimate because of the co-occurring activity of different malt proteases; however, the consumption of di- to pentapeptides was more apparent between 12 and 36 h.

The current MS-based methodology for studying the composition and concentration of peptides in unelucidated matrices, such as Scotch grain mash, has limitations. First, not all protein sequences in barley and wheat have been fully sequenced.[32] Thus, in the present study, the obtained peptide masses were mapped only to the major (most abundant) known storage proteins in barley (hordeins) and wheat (glutenins) for annotation.[26,27] However, this approach is subject to a degree of identification inaccuracy due to the numerous protein sequences (total of 15) that were included for peptide mapping, as well as potentially unknown proteins that were not included but were also degraded into peptides. This limits the annotation of peptides to their size (number of AA residues in the chain length) instead of the exact

AA sequence. As the MS peak intensity of peptides can be highly dependent on the amino acid composition and sequence,[33] only the relative quantification of peptides by fold-change analysis of individual detected peptides was performed in this study. In combination with fractionation and subsequent AA analysis of the obtained molecular fractions, it was possible to explain these trends more quantitatively. However, some inaccuracies may have occurred in this case, for example due to retention of some smaller peptides on the cut-off filter. Ideally, the qualitative and quantitative analyses of peptides should be integrated into a single analysis. One method that currently exists makes use of the UV absorbance of peptides by coupling a photodiode array detector between LC and MS. The UV peak areas can be converted to absolute concentrations based on the law of Lambert-Beer, which requires the molar extinction coefficient of the peptide.[34] However, this analysis would require extended chromatographical times to resolve most UV peaks in complex media, in addition to being hindered by the highly variable ionisation efficiencies of short peptides,[34] which are required for the quantification of different peptides that co-elute under one UV peak.[35] Further research to develop qualitative and quantitative analyses of peptides by MS in complex fermentation samples is therefore required.

Although the addition of nitrogen nutrients to grain whisky fermentation is not allowed by regulation in Scotland,[35] it is allowed in other places. Therefore, the effects of additional nitrogen supplementation with either peptides or by the often industrially applied ammonium salt DAP on the fermentation performance in synthetic whisky mash were studied. In these experiments, supplementation with peptides resulted in higher biomass accumulation and CO_2 production rates, and consequently, shorter fermentation times, than with the medium with a lower nitrogen content. This is in stark contrast to the effect of ammonium supplementation on fermentation performance, which was neutral or even negative. These results seem counterintuitive considering the popularity of DAP in industry; however, they are in line with what has been observed in other studies. In one study, nitrogen supplementation with a peptone induced higher biomass and ethanol production than ammonium sulphate.[36] Another study revealed slower fermentation rates with ammonium compared to free amino acids, delivering equivalent amounts of nitrogen.[37] Gene expression has been shown to be differentially reprogrammed based on the nitrogen source added:[38] when ammonia was added, there was a higher expression of genes involved in amino acid biosynthesis, whereas the addition of amino acids resulted in a higher expression of genes related to protein biosynthesis. Thus, a possible explanation for the observed differences in the effect of nitrogen supplementation is that (peptide-derived) amino acids can be directly used in protein biosynthetic processes, while ammonium must first be incorporated into amino acids through *de novo* biosynthesis, requiring cellular resources including carbon and energy.[39] Under the conditions used in this study, ammonia delayed peptide uptake, possibly through nitrogen catabolite repression.[40] We hypothesised that this negative effect of ammonium supplementation on fermentation kinetics is due to the interplay between two factors: on the one hand, the cells need to *de novo* synthesise all the required amino acids, and on the other hand, the presence of ammonia hinders the uptake of peptides which could otherwise supply readily usable amino acids.

Strain SC3 is recommended for use in malted grain fermentation due to its higher nitrogen demand. However, our study suggests that this strain may also be suitable for grain fermentations with mostly unmalted grains, albeit with lower efficiency than strain SC1. To achieve optimal process efficiency, it is essential to consider the available nitrogen content of the mash and the method of nitrogen supply to the yeast. This was demonstrated by our results on synthetic whisky mash, where strain SC3 greatly benefitted from additional nitrogen supplementation in form of small peptides. In the production of Scotch grain whisky, the use of malts with higher proteolytic strength or increasing the proportion of malted to unmalted grains can accommodate a specific yeast strain with a higher nitrogen demand. Outside of Scotland, it is permissible to use exogenous proteases to liberate free amino acids and peptides from the protein fraction of the mash or to add external nitrogen sources to the process. Increasing the nitrogen content in grain fermentation with the appropriate source of nitrogen may open the door to other strains that can be applied in the production of grain whisky.

In conclusion, these results show that peptides can play a considerable role as nitrogen sources to yeast in Scotch grain whisky fermentation. The three whisky yeast strains were able to take up a wide variety of peptides and use them effectively to attain complete fermentation. In industrial Scotch grain mash, the concentration of yeast assimilable nitrogen, including that produced during fermentation owing to residual proteolytic activity proved to be sufficient, with peptides being a considerable source of nitrogen.

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Data availability statement

Data files and the R script for data treatment and analysis can be found at: https://zenodo.org/records/13118415
All data are contained in the manuscript and the supplemental material.

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