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FODMAPs in cereals, pseudo cereals and legumes: A systematic approach for the development of functional low FODMAP products

Thesis presented by

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Signature

Date: 27/07/2021

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Abbreviations

AcOH	Acetic acid
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
BM	Wickerham Basal Medium
CF-NTCyb	*Cyberlindnera fabianii* NTCyb
CFU	Colony forming units
CH_3CN	Acetonitrile
DM	Dry matter
DNA	Deoxyribonucleic acid
DP	Degree of polymerisation
DP_{av}	Average degree of polymerisation
E-AGLANP	Enzyme preparation α-galactosidase (*Aspergillus niger*) powder
E-AMGFR	Enzyme preparation amyloglucosidase (*Aspergillus niger*) powder
ED	Electrochemical detector
EF	Excess fructose
E-FRMXPD	Enzyme preparation fructanase mixture (*Aspergillus niger*) powder
ELSD	Evaporative light-scattering detection
EPS	Exopolysaccharide
FBD	Functional bowel disorder
FEH	Fructan exohydrolases
FFT	Fructan:fructan fructosyltransferase
FGID	Functional gastrointestinal disorder
FLAB	Fructophilic lactic acid bacteria
FODMAP	Fermentable oligo-, di, monosaccharides and polyols
FOS	Fructooligosaccharides
FosE	Exo-inulinase encoding gene in lactic acid bacteria
FP	Fagopyritol
FP-B1	Fagopyritol B1
FruA	Exo-inulinase encoding gene in lactic acid bacteria

FT	Fructosyltransferase
FU	Farinograph unit
GC/MS-TOF	Gas chromatography with time-of-flight mass spectrometry
GF	Gluten-free
GI-tract	Gastrointestinal tract
GOS	α-Galactooligosaccharides
H_2O	Water
H_2O_2	Hydrogen peroxide
HPAEC	High-performance anion-exchange chromatography
HPLC	High-performance liquid chromatography
IBS	Irritable bowel syndrome
K-BETA3	β-Amylase Assay Kit (Betamyl-3)
K-CERA	α-Amylase Assay Kit (Ceralpha Method)
K-FRUC	Fructan assay kit
K-FRUCHK	Fructan Hexokinase assay kit
LAB	Lactic acid bacteria
LF	Low FODMAP
LFD	Low FODMAP diet
LF-FST 5.1	*Lachancea fermentati* FST 5.1
LOD	Limit of detection
LOQ	Limit of quantification
MAL	Maltase encoding gene in *S. cerevisiae*
Man	Mannitol
MEBAK	Mitteleuropäische Brautechnische Analysenkommission
Mel	Melibiose
MeOH	Methanol
NaN_3	Sodium azide
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
OD	Optical density

PAD	Pulsed amperometric detection
PC	Protein concentrate
PdH	Palladium hydrogen reference electrode
PHGG	Partially hydrolysed guar gum
PI	Protein isolate
Raf	Raffinose
RD	Resistant dextrin
RFO	Raffinose family oligosaccharides
RS	Resistant starch
RSD	Relative standard deviation
S/N	Signal to noise ratio
SacA	ß-Fructosidase in lactic acid bacteria
SC-BY	*Saccharomyces cerevisiae* baker's yeast
SEM	Scanning electron microscopy
SFT	Sucrose:fructan fructosyltransferase
Sor	Sorbitol
SP	Single pump
SST	Sucrose:sucrose fructosyltransferase
SUC2	Invertase encoding gene in *S. cerevisiae*
SucP	ß-Fructosidase encoding gene in lactic acid bacteria
TOC	Total organic carbon
TTA	Total titratable acids
UV/DAD	ultraviolet light/ diode array detection
VI	Vacuolar invertase
XOS	Xylooligosaccharides
YPD	Yeast extract peptone dextrose

Symbols

DF	Dilution factor
G_A / F_A	Glucose/ fructose concentrations from hydrolysate A
G_B / F_B	Glucose/ fructose concentrations from hydrolysate B
G_f / F_f	Glucose/ fructose released from fructans
Hm	Maximal dough height
k	Water correction factor
M_S	Mass of sample
R^2	Correlation coefficient
S_A	Sucrose concentration from hydrolysate A
s_{y_1}	Residual standard deviation of linear equation
s_{y_2}	Residual standard deviation of quadratic equation
TV	Test variable
V_E	Volume of extract
V_{ret}	Volume of CO_2 retained in dough
V_{tot}	Total volume of CO_2

Abstract

FODMAPs (fermentable mono-, di, oligosaccharides and polyols) are dietary carbohydrates that have been identified as triggers of symptoms of irritable bowel syndrome (IBS); a diet with a reduced intake of FODMAPs is successfully alleviating symptoms in > 70 % of IBS patients. While whole grain cereals, pulses and products made from those are an essential part of a healthy plant-centered diet, they are also a major source of FODMAPs. Hence, with a lack of functional products with lowered FODMAP contents on the global market, the development of such is emerging in food science and industry. Firstly, an accurate and efficient analytical method for the quantification of FODMAPs, using one single analytical approach (high-performance anion-exchange chromatography coupled with pulsed amperometric detection; HPAEC-PAD), was developed, and served as analytical tool throughout further studies. The FODMAP-profiles of a broad range of cereal-product ingredients, including different cereals, pseudo cereals, gluten-free flours, pulses, pulse protein ingredients, commercial sprouts, and isolates were characterised. Two main classes of FODMAPs were found in the ingredients: fructans in gluten-containing cereals (wheat, spelt, barley, rye) and α-galactooligosaccharides (GOS) in pulses (peas, lentils, chickpeas, etc.). Isolates and fractions from different raw material (pulse protein ingredients, different starches, gluten) had varying GOS or fructan contents, depending on their production process. Gluten-free product ingredients (e.g., rice, millet, buckwheat) did not contain any of the FODMAPs commonly investigated. However, buckwheat accumulates other soluble indigestible carbohydrates (fagopyritols) that may act as FODMAPs. Six ingredients were selected to investigate the impact of malting on FODMAPs: wheat and barley (high in fructans), chickpeas and lentils (high in GOS), oat and buckwheat (‘low in FODMAPs’). In the pulses GOS levels diminished by 80 − 90 % upon the malting process; also, buckwheat fagopyritols were degraded. In contrast, fructan contents in barley and wheat malts were slightly elevated; 0.8 % fructans were even *de novo* synthesised in oat malt. Finally, aiming for the degradation of fructans and the production of a low FODMAP whole wheat bread, the application of yeast fermentation was investigated. The screening of the FODMAP degradation capability of various yeast isolates led to the selection of two promising strains: *Lachancea fermentati* FST 5.1 and *Cyberlindnera fabianii* NTCyb. While the latter revealed to be unsuitable for baking application (very low fermentation rate in wheat dough matrix), *L. fermentati* FST 5.1 outperformed conventional baker’s yeast (*Saccharomyces cerevisiae*), with a much more efficient fructan degradation and metabolism of the resulting excess fructose. Apart from low FODMAP contents, the resulting bread had optimal quality characteristics (technological as well as sensory attributes) comparable to the baker’s yeast fermented bread. Overall, the fundamental and systematic work of this thesis provides comprehensive and applicable knowledge essential for developing of low FODMAP products.

Chapter 1
Introduction

1.1 Introduction

The acronym FODMAPs (fermentable oligo-, di-, monosaccharides and polyols) is an umbrella term, comprising ubiquitous dietary carbohydrates that are small, osmotically active, poorly absorbed in the small intestine and rapidly fermented by colonic bacteria (Figure 1-1). While these physiological effects occur in all humans, including healthy individuals, the ingestion of FODMAPs has been associated with the induction of various gastrointestinal symptoms in individuals with functional gastrointestinal disorders, which severely impairs the individuals' quality of life. The "low FODMAP diet" has received much attention in recent years, and it certainly deserves the recognition; the dietary therapeutic approach has been shown to successfully alleviate symptoms, especially in irritable bowel syndrome (IBS) patients (Halmos *et al.*, 2014).

The commonly cited list of FODMAPs comprises α-galactooligosaccharides (GOS), fructans and fructooligosaccharides (FOS), lactose, fructose in excess of glucose and polyols, but is not limited to these (Gibson *et al.*, 2020; Halmos & Gibson, 2019). The identification of other carbohydrates with similar chemical and physiological characteristics and the respective dietary sources may extend this list. The work undertaken in this thesis focused on the commonly cited FODMAPs, but also discussed potential FODMAP-attributes of other carbohydrates, where relevant.

The oligosaccharides ***GOS*** are derivatives from sucrose with α (1 → 6) linked galactose units; the homologues include raffinose, stachyose, verbascose, and ajugose. Humans do not possess a digestive enzyme to cleave α-galactose linkages in GOS (α-galactosidases). Hence, GOS are not absorbed in the small intestine; the intact molecules are delivered to the colon, fermented by colonic bacteria, inducing gas formation (Gibson & Shepherd, 2005). These indigestible saccharides are typically found in pulses (up to ~ 10 − 16 %; most abundantly stachyose), where they are often referred to as the "flatulence causing factor" of the seeds (Martínez-Villaluenga *et al.*, 2008). The second group of oligosaccharides are ***fructans***; they are composed of fructose chains with one glucose moiety. Five types of fructans are classified by the type of linkages between the fructose residues and the position of the glucose molecule: (1) & (2) inulin- and levan-type fructans are linear, β (2 → 1) or β (2 → 6) linked, fructose chains with a terminal glucose residue; (3) graminan-type fructans are branched and contain both types of linkages with the glucose monomer at the end; (4) & (5) in neoseries-type (inulin or levan) fructans the glucose moiety is internally

linked. While inulin- and levan-type fructans are typically found in chicory and timothy grass, respectively, it is characteristic for cereals to contain graminan-type but also neoseries-type fructans (Livingston *et al.*, 2009; Verspreet *et al.*, 2015).

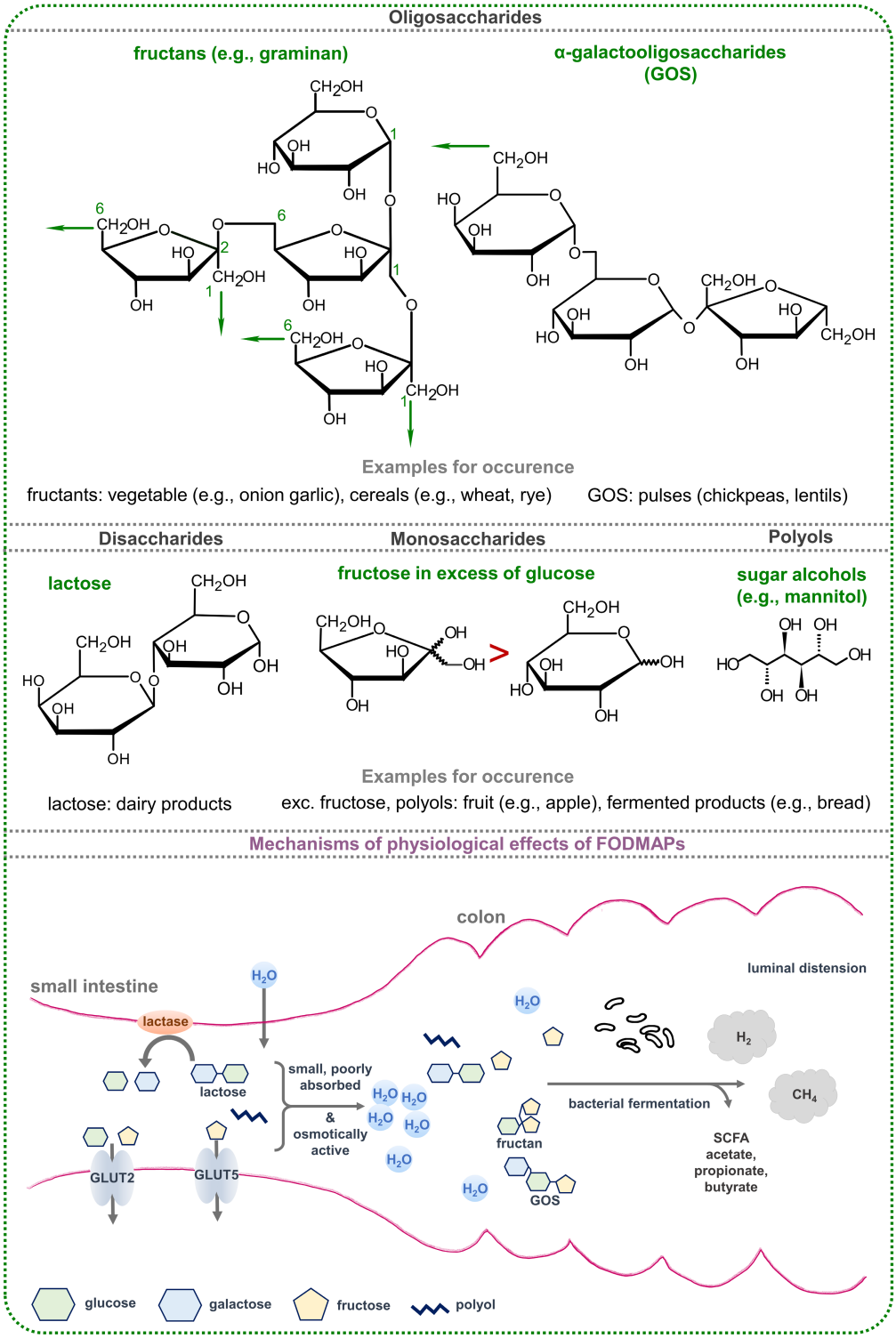

Figure 1-1. Chemical structures and physiological effects of common FODMAPs

Like GOS, fructans are indigestible and readily fermentable by the gut bacteria. Due to this fact, prebiotic health beneficial properties of fructans have received much attention, leading to the fortification of food products with fructans (mostly partially hydrolysed chicory-inulin, FOS, with different chain lengths); this further complicates the food choice for IBS patients with a pronounced sensitivity towards fructans. Furthermore, the ß (1→ 4) linked galactosyl-glucose disaccharide ***lactose*** is not tolerated by individuals lacking the brush border enzyme lactase. Lactose is found in mammalian milk, including cow's, sheep's, and goat's milk and thus the major FODMAP in dairy products (Gibson & Shepherd, 2010). In contrast to fructans and GOS, fructose can be absorbed in the small intestine. Fructose absorption is enhanced via the GLUT2 transporter in the epithelial membrane of the small intestine if it is present in an equimolar concentration to glucose. However, if ***fructose*** appears ***in excess of glucose***, it will be ingested through the less effective, glucose independent, GLUT5 transporter (cf. Figure 1-1). Because of the transporter's lower capacity, fructose is not sufficiently absorbed, transported to the colon, and exerts osmotic activity driving high amounts of water in the intestinal lumen (Gibson *et al.*, 2007; 2010). Fructose in excess of glucose, is found in honey or different fruit; it can also appear in fermented cereal-based products (Muir *et al.*, 2007; Ziegler *et al.*, 2016). Lastly, ***polyols***, the reduced forms of sugars in their chemical structure (sugar alcohols), are also poorly absorbed in the small intestine, osmotically active and delivered to the colon. Although erythritol seems to be more completely absorbed than other polyols (such as xylitol or mannitol) by healthy individuals, its physiological effect on IBS patients remains unknown (Arrigoni *et al.*, 2005; Lenhart & Chey, 2017). Sugar alcohols are commonly used as alternative sweeteners and occur naturally in different fruit and vegetables; also, fermented cereal-based products may contain polyols (Loponen & Gänzle, 2018; Muir *et al.*, 2009).

However, because FODMAPs (especially fructans) and dietary fibres share the characteristic of the fermentability by beneficial gut bacteria, reasonable criticism was directed towards eliminating healthy dietary fibres, particularly those found in whole grain cereals. Indeed, when adhering to the low FODMAP diet, it is crucial to replace high FODMAP foods by nutritionally equivalent low FODMAP alternatives (Muir *et al.*, 2019). The following cutoff levels classify *one serving* of a food product as low FODMAP: < 0.3 g oligosaccharides (sum of GOS and fructans) in cereal-based

products and pulses and < 0.2 g in vegetables and fruit, < 0.2 g of sorbitol or mannitol, < 0.4 g of total polyols, < 0.15 g of excess fructose, < 0.4 g of excess fructose if it is the only FODMAP present, < 1 g of lactose; < 0.5 g of total FODMAPs excluding lactose.

The work of thesis represents a systematic, fundamental approach, targeting the development of cereal-based functional products with a lowered FODMAP content.

An in-depth analysis of the global market situation of food products with a low FODMAP claim, contrasted with the status quo of knowledge in food science to produce such, is presented and thoroughly discussed in ***Chapter 2***.

In the first experimental chapter, ***Chapter 3***, the development and validation of a sensitive and accurate analytical method for the quantification of FODMAPs is shown. High-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD), an advanced and extremely sensitive technique for the analysis of carbohydrates, has been used as a single analytical approach for the quantification of all FODMAPs. The established method served as analytical tool for FODMAP quantifications throughout the work of this thesis. ***Chapter 4*** follows with a dry-matter based characterisation of the FODMAP profile in a wide variety of cereal-product ingredients, which is the foundation for further research, allowing a targeted selection of ingredients, depending on the application. Biochemical changes observed in cereals, pseudo cereals, and pulses (selected on the basis of Chapter 4) during the malting process are presented in ***Chapter 5***. The final experimental chapter of this thesis, ***Chapter 6***, demonstrates the application of yeast mediated FODMAP reduction for the production of a low FODMAP whole wheat bread and highlights the great potential of an unconventional yeast strain, *Lachancea fermentati* FST 5.1, that has not been previously used for bread-making.

The objectives, methods, and main findings of each chapter of this thesis are summarised in Table 1-1.

Table 1-1. Overview of chapters including the objectives, methods, and main findings

Chapter 2	***Chapter 3***	***Chapter 4***	***Chapter 5***	***Chapter 6***
FODMAP modulation as a dietary therapy for IBS: Scientific and market perspective	Optimisation and validation of a HPAEC-PAD method for the quantification of FODMAPs in cereals and products	Characterisation of the FODMAP profile in cereal product ingredients	Fundamental study on changes in the FODMAP profile of cereals, pseudo cereals, and pulses during malting	*Lachancea fermentati* FST 5.1: an alternative to baker's yeast to produce low FODMAP whole wheat bread
		Objectives		
Overview of current state of knowledge on strategies for the development of functional low FODMAP products and their use on the global market	Development of a suitable analytical method for a sufficiently accurate quantification of FODMAPs in diverse food matrices	Analysis of 35 cereal product ingredients and commonly consumed cereal-based products and gluten-free alternatives from Irish market	Elucidation of the impact of malting on fructan accumulating cereals barley/ wheat, GOS accumuating pulses chickpea/lentils, 'low FODMAP' grains oat/ buckwheat	Identification of potent alternative to baker's yeast for the production of low FODMAP bread with high consumer acceptance
		Methods		
Market analysis on functional products with low FODMAP claim, classification of products with FODMAP reduction approach contrasted with literature on researched techniques	Hot aqueous extraction of FODMAPs, HPAEC-PAD direct quantification of polyols, GOS, mono- and disaccharides with external reference standards and total fructans after enzymatic hydolysis to glucose and fructose	HPAEC-PAD analysis of FODMAPs in ingredients and products; dry-matter based categorisation of FODMAPs in ingredients and fresh weight serving size based categorisation of commercial products	Malting of barley, wheat, chickpeas, lentils, oat and buckwheat and quantification of FODMAPs throughout the malting process.	Screening of 96 potent yeast strains' ability to utilise different carbohydrates; identification of strains from *L. fermentati* and *C. fabianii* as most promising; comparative baking application of selected strains and baker's yeast
		Main findings		
Promising FODMAP reduction technologies were shown in scientific literature but have very limited application in food industry; global market of functional low FODMAP products has a high regional imbalance (lack of products in Europe) and is dominated by gluten-free products with inferior consumer acceptance.	Validated method, based on one single analytical technique using ion chromatography with electrochemical detection; separation of carbohydrates on two columns, CarboPac PA1 and CarboPac PA200; method suitable for the quantification of FODMAPs in divers matrices.	Two main sources of FODMAPs in ingredients: fructan accumulating cereals, GOS accumulating pulses; gluten-free cereals low in FODMAPs; varying contents in isolates/ fractionated ingredients; in products polyols, lactose and excess fructose present originating from other ingredients or processing; gluten-free products all low FODMAP.	Malting (i.e., germination) leads to further enrichment of fructans, especially oat resulted in 0.8 % DM *de novo* synthesised fructans: fructan synthesis during germination, followed by degradation during kilning. GOS in pulses are reduced throughout the malting process; buckwheat accumulates potential FODMAPs fagopyritols, but also these are degraded during malting.	*L. fermentati* FST 5.1 fermentation resulted in whole wheat bread with low FODMAP content, while high quality characteristcs were maintained, compared to baker's yeast fermented bread with higher FODMAP contents. *C. fabianii* NTCyb showed overall poor fermentation rate in wheat dough matrix: bread high in FODMAPs and poor quality characteristics.

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Chapter 2

FODMAP modulation as a dietary therapy for IBS: Scientific and market perspective

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2.1 Abstract

A diet low in fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs) is a promising therapeutic approach to reduce gastrointestinal symptoms associated with irritable bowel syndrome (IBS). However, a shift towards a more sustainable, healthy diet with higher inclusion of whole grain cereals (i.e., wheat, rye, barley) and pulses, naturally rich in FODMAPs, poses a severe challenge for susceptible individuals. Dietary restriction of fermentable carbohydrates (commonly called the 'low FODMAP diet') has received significant consideration. Hence, the development of functional low FODMAP products is emerging in food science and the food industry. Here, we evaluate the most promising yet neglected (bio)-technological strategies adopted for modulating the FODMAP contents in complex food systems and the extent of their uptake in the global food market. We extensively investigate the low FODMAP market (total ~ 800 products) and demonstrate that less than 10 % of the products with a low FODMAP claim used any of the FODMAP reduction techniques. The global market is currently dominated by gluten-free products, which are naturally low in FODMAPs and characterised by inferior sensory attributes.

2.2 Introduction

Poorly absorbed, osmotically active, and rapidly fermentable carbohydrates comprised under the acronym FODMAPs (fermentable oligo-, di-, monosaccharides and polyols) have increasingly gained attention in both scientific literature and the global food market in the last few years. This is due to the finding that the ingestion of FODMAPs by individuals with functional gastrointestinal disorders (FGIDs), particularly irritable bowel syndrome (IBS), was associated with the onset of severe and painful gastrointestinal symptoms (Gibson & Shepherd, 2005; 2010). It was shown that the avoidance of FODMAPs as a dietary therapy, the low FODMAP diet (LFD, cf. Figure 2-1), is a successful therapeutic approach to alleviate symptoms in > 70% of IBS patients (Halmos *et al.*, 2014). Palsson *et al.* (2020) recently re-evaluated the prevalence of functional bowel disorders (FBDs, subtype of FGIDs, all characterised by various severe gastrointestinal symptoms and substantially impaired quality of life). They found that > 25 % of the adult population in the US, Canada, and the UK suffers from FBDs, of which ~ 5 % were identified as IBS. While IBS and other FBD are more prevalent in women than in men, the overall prevalence seems to be decreasing after mid-life (older than 50 years) (Palsson *et al.*, 2020). A recent global study (evaluated the prevalence of FGIDs based on surveys in 33 countries) demonstrated that IBS is in fact a worldwide spread disorder (Sperber *et al.*, 2021).

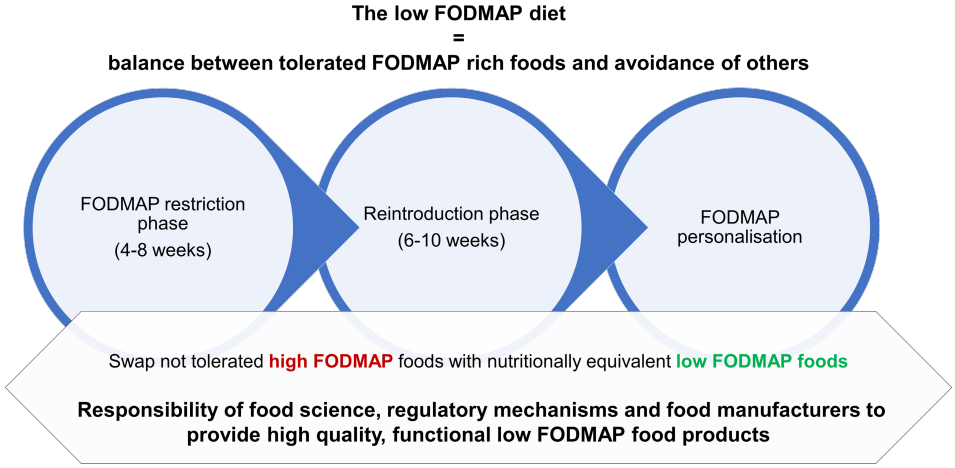

Figure 2-1. Low FODMAP diet and importance of nutritious functional low FODMAP products

Despite the solid scientific evidence that the LFD can effectively reduce symptoms in IBS patients, the diet is also facing criticism, particularly regarding avoiding healthy dietary fibres (Brouns *et al.*, 2019; Halmos & Gibson, 2019). It is crucial that a LFD is well educated to the medical sector and dieticians, who should fully support and lead

the correct protocol of the LFD (restriction phase > reintroduction phase > personalised LFD, cf. Figure 2-1) as a dietary therapy for IBS (Halmos & Gibson, 2019; Whelan *et al.*, 2018). The LFD does not mean a complete avoidance of any fermentable dietary fibre, but solely those that are easily fermented and lead to gastrointestinal symptoms in individuals with a pronounced sensitivity towards those. The same principle applies to low FODMAP product development. Atzler *et al.* (2021a) recently reviewed and identified characteristics of dietary fibres suitable for a LFD (e.g., low fermentation rate, low osmotic activity). Indeed, a key principle of the LFD is the replacement of non-tolerated high FODMAP foods with nutritionally equivalent low FODMAP foods (Figure 2-1). In this matter the availability of appealing, high quality and nutritious functional low FODMAP products to facilitate the food choice of IBS patients in the replacement of the conventional high FODMAP products is essential. Particularly whole grain cereals, pulses and products made from those are a substantial source of energy, dietary fibre, and micronutrients in a healthy and sustainable plant-centred diet; solely wheat and wheat-based products account for one fourth of the daily calorie intake in the European population (FAOSTAT, 2021). However, due to naturally occurring FODMAPs in cereals such as wheat or rye, and pulses (i.e., fructans and α-galactooligosaccharides, GOS), products made from those have to be largely avoided by IBS patients (Biesiekierski *et al.*, 2011; Ispiryan *et al.*, 2020; Muir *et al.*, 2019). Besides fructans and GOS, other FODMAPs, mostly found in dairy (lactose), and various fruit, vegetables, and fermented foods (fructose in excess to glucose, polyols, melibiose) can be found in cereal- and pulse-based products, derived from other ingredients or the processing (Ispiryan *et al.*, 2020; Ispiryan *et al.*, 2021a). The following FODMAP cutoff levels were validated and established based on the findings of clinical studies: per serving of food < 0.3 g of oligosaccharides e.g., sum of fructans and GOS in pulses and core grain-products; < 0.15 g of excess fructose; < 0.4 g of polyols; < 1 g of lactose; < 0.5 g total FODMAP excl. lactose (Barrett *et al.*, 2010; Halmos *et al.*, 2014; Ong *et al.*, 2010; Varney *et al.*, 2017). These should be the benchmark for targeted low FODMAP product development. Several studies investigated FODMAP reduction strategies, including the use of low FODMAP ingredients, and biotechnological FODMAP degradation through the action of purified enzymes, microbial enzymes during fermentation (specific yeast and lactic acid bacteria), and activation of seed-endogenous enzymes during germination (Atzler *et al.*, 2020; 2021b; Fraberger *et al.*, 2018; Ispiryan *et al.*,

2021b; 2021a; Li *et al.*, 2020; Longin *et al.*, 2020; Loponen *et al.*, 2017; Schmidt & Sciurba, 2021; Struyf *et al.*, 2017b; Ziegler *et al.*, 2016). The fundamental key principles of biotechnological FODMAP reduction have been recently reviewed by Nyyssölä *et al.* (2020).

Ultimately, this review aims to systematically reveal and interconnect the current state of knowledge of the most effective and applicable targeted FODMAP reduction strategies in scientific literature and their actual application to produce functional low FODMAP products on the global market, with a focus on bakery products and pasta. It is shown which market segments may be sufficiently covered and where currently known technologies should be more applied, while concomitant limitations are highlighted. Furthermore, the importance of a targeted approach for producing low FODMAP products is demonstrated, whether for products based on primarily low FODMAP ingredients or for biotechnological reduction strategies.

2.3 Global market on functional low FODMAP products

The availability of functional low FODMAP food products on the global market was systematically assessed for this review (up until 19th April 2021). All food products, recipe-developers and ingredients certified by the two Australian programs, the "Monash University Low FODMAP certification Program" and the "FODMAP friendly Program" were included in the global market analysis. Both programs certify foods according to the FODMAP cutoff values per serving of food and list the certified products in their mobile phone applications or websites (https://www.monash-fodmap.com/; https://fodmapfriendly.com/). Furthermore, products, which the manufacturer claimed to be suitable for a LFD, were included in the analysis. Regardless of the wording of certifying organisation or the manufacturers' FODMAP claim, all products included in the study are referred to as low FODMAP products (LF products). Products that were presented in specialised online shops (e.g., fodmarket.co.uk, fodshopper.com.au, foodoase.de) as suitable for a LFD, but were neither analytically tested by the two certification programs nor had a manufacturer's claim, were not included.

The analysis resulted in 791 products with a FODMAP related claim, available on the global market. Thereof, 97 % were certified by either of the two Australian certification programs. Furthermore, 18 products (2 %) were found, which were claimed by the manufacturer to be low in FODMAPs or suitable for a LFD. Nine products (1 %) which were claimed to be based on a "low FODMAP wheat" were included in the overall figure of available products (Figure 2-2, products with FODMAP claim) but were excluded from further evaluations, as a low FODMAP content of the actual products was not stated and potentially not given.

Since the introduction of the two certification programs in 2013/ 2014 (Lederman, 2014), an increasing number of food manufacturers joined the programs, with 127 companies offering their range of certified functional low FODMAP products today (Figure 2-2, companies certified from 2013 − 2021). Corresponding to the origin of the FODMAP concept, founded in 2005 by the research group of Prof. Peter Gibson (gastroenterology dept., Monash University Australia) (Gibson & Shepherd, 2005) and the certification programs, the majority of the certified products (42 %) was from Australian food manufacturers. Products originating from the US accounted for 33 % of all low FODMAP products. With 8 %, the UK had the third most significant share

of LF products on the global market. The remaining 17 % were from few EU countries (Sweden, Spain, Poland, Germany, France, Finland, Austria), and Norway, India, Brazil, Canada, and New Zealand.

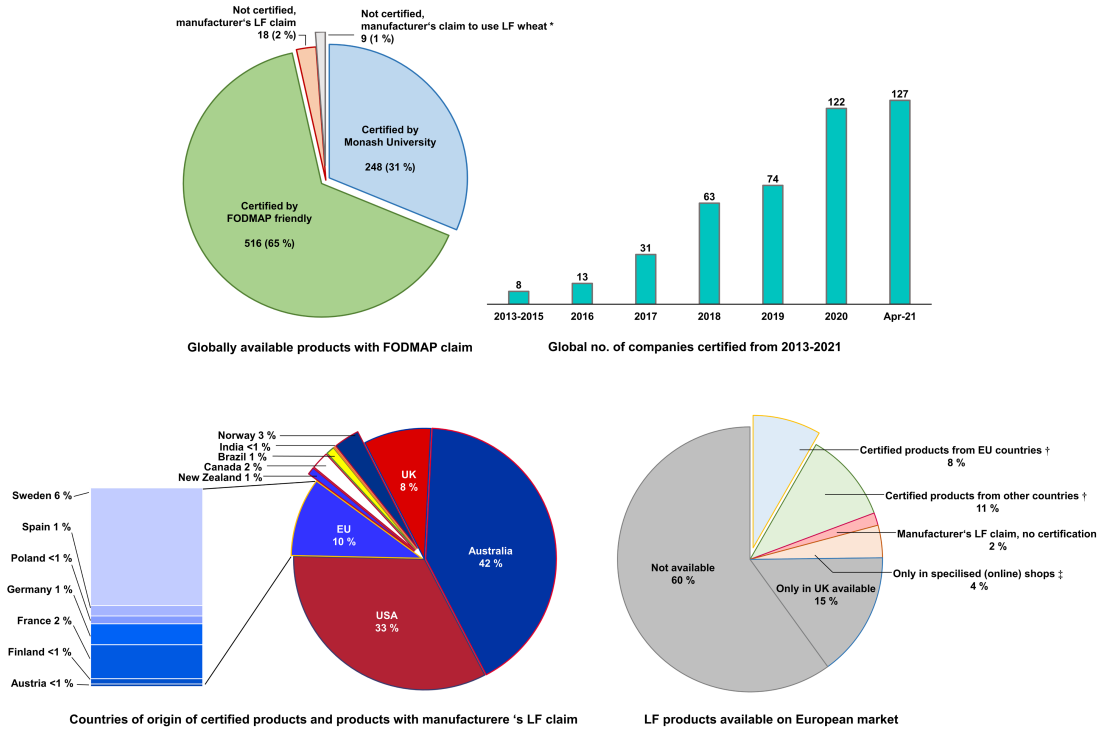

Figure 2-2. Globally available products with FODMAP claim: * not certified products with manufacturer's claim to use low FODMAP wheat as ingredient (2 ab wheat) are not included into further evaluations as those products are not necessarily low FODMAP. Products available on European market: † From all certified products from EU-based manufacturers, and certified products from other than EU-countries, which are also commonly available on EU-market > 90 % do not seem to carry the certification label on the product packaging. ‡ Specialised online shops such as foodoase.de or specific local health food stores offer imported, certified products, e.g., from Australia.

2.3.1 Lack of products on European market and legal situation

While 60 % of the certified products were not at all available on the European market (Figure 2-2, availability of LF products in Europe), 21 % were only available in the UK, in specialised shops or had no certification (only manufacturer's FODMAP claim). Only 19 % of all functional LF products were commonly available in EU countries, while > 90 % of these did not actually carry any FODMAP related statement or claim (e.g., certification logo) on the packaging. For food manufacturers in the EU to label products safely and distinctly as a product with a low FODMAP content, a definition of the term and the necessary criteria, such as an EFSA approved nutrition claim (Regulation (EC) No 1924/2006; Regulation (EU) No 1169/2011), is entirely missing. A precise specification of the low FODMAP criteria, applied for the certifications in Australia, must be incorporated in the EU food regulations, such as it

already exists for gluten-free or gluten-reduced foods (Commission implementing regulation (EU) No 828/2014; Regulation (EU) No 1169/2011). The establishment of the necessary regulatory framework would increase the legal clarity for food manufacturers and consumers while ensuring correct application of the low FODMAP criteria and allow for a facilitated food choice for individuals adhering to the LFD.

Finally, the lack of regulations also comes with an increased risk of false information patients may receive from food producers or non-scientific articles, limiting the potential success of a correctly conducted LFD. In this context, the common classification of a product's FODMAP content solely based on the list of ingredients may not always be accurate. The following factors strongly limit the correctness of such classification: (1) The food production process may alter the FODMAP profile in an unknown manner, e.g., yeast or sourdough fermentation or the use of a specific type of wheat such as spelt do not guarantee a low FODMAP content. (2) The product may contain technical additives which are not listed in the ingredients but influence the FODMAP profile. (3) The FODMAP contents of ingredients may vary substantially and not allow for an estimation of the product's FODMAP content. For instance, protein ingredients from pulses or sprouted grain material can have highly variable FODMAP contents (Ispiryan *et al.*, 2020; 2021b; Joehnke *et al.*, 2021; Tuck *et al.*, 2018; Vogelsang-O'Dwyer *et al.*, 2020b), which are not predictable unless detailed information on the production process of the ingredient was provided, or the contents were analytically tested. Hence, to allow for food producers to exploit the scientifically proven approaches for low FODMAP food production, shown in the following sections, clear regulations defining the clinically tested cutoff levels and suitable analytical techniques for the FODMAP quantification are necessary.

2.3.2 Categories of available functional low FODMAP products

Among all products with a LF claim, 70 products (9 %) were breads, rolls, bagels, and wraps. Furthermore, 6 pizza bases (1 %), 24 pasta products (3 %), 24 baking mixes (3 %), 73 breakfast cereals (9 %), 135 sweet snacks, muesli bars, and pastry (17 %), 75 savoury snacks and crisp breads (10 %), 21 dairy alternative products (3 %), and 1 beer were certified as LF products; 44 ingredients (6 %) had a LF claim (Figure 2-3).

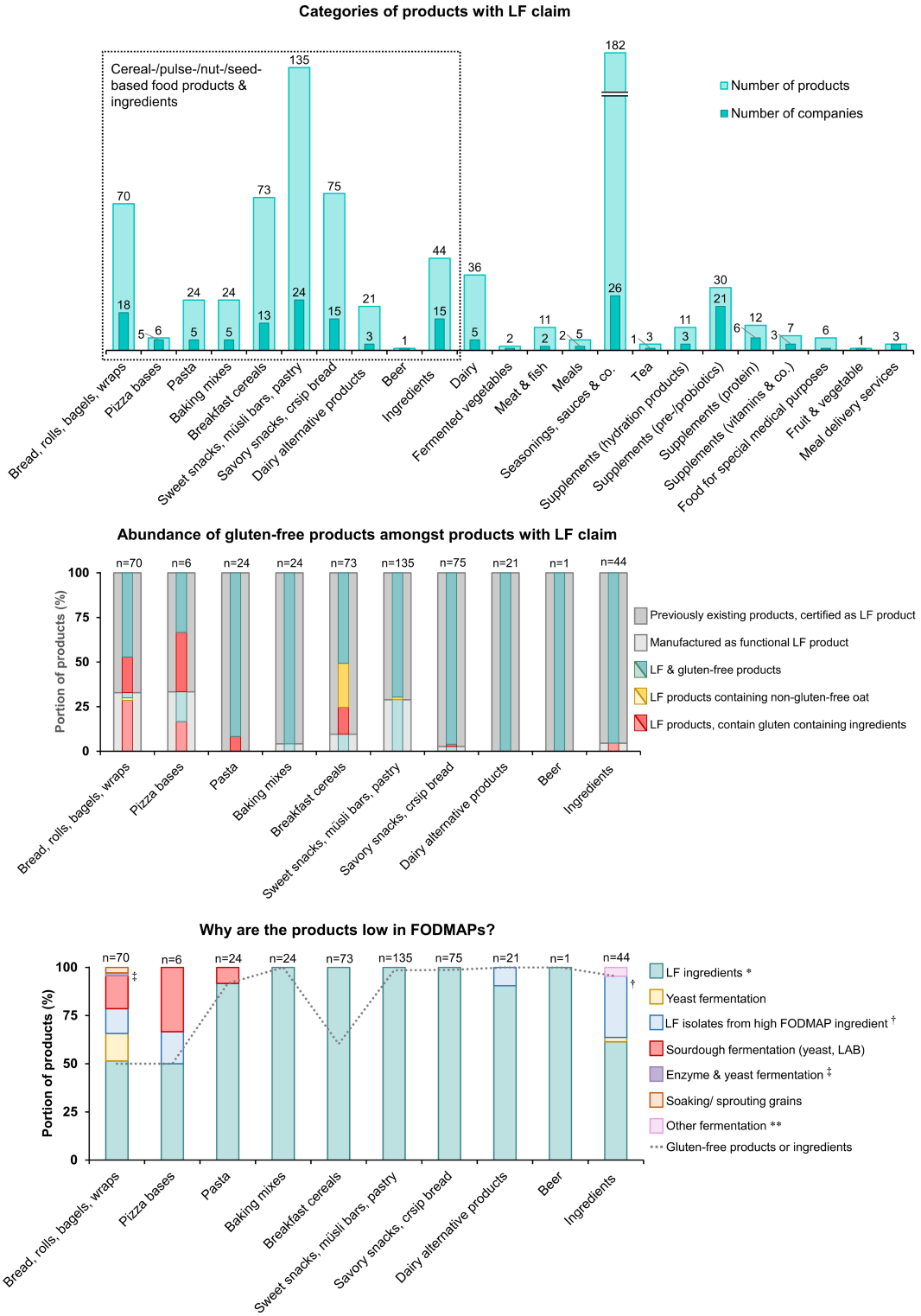

Figure 2-3. Categories of products with LF claim, abundance of gluten-free products among those and underlying FODMAP reduction technique. * Products or ingredients, based on solely low FODMAP ingredients, contain only low levels of high FODMAP ingredients/ high FODMAP ingredients with a very low serving size. † Gluten and starch-based ingredient from LO-FO pantry, based on patented technology (Pearce & Barrie, 2015). ‡ Bread from Fazer based on patented FODMAP reduction technology with LOFO™ enzyme-ingredient (Loponen *et al.*, 2017). ** Cocoa bean fermentation; includes different yeast, lactic acid bacteria, acetic acid bacteria that ferment GOS (Megías-Pérez *et al.*, 2018; Schwan & Wheals, 2004).

These cereal-/ pulse-/ nut-and seed-based products and ingredients comprised 60 % of all products with a LF claim and will be further discussed concerning their "FODMAP reduction" approaches and their base ingredients or the types of ingredients. Furthermore 23 % of the certified products were seasonings, sauces, and condiments. The remaining 17 % were distributed between dairy products, fermented vegetables, meat, and fish, ready to eat meals, tea, dietary supplements, food for special medical purposes, fruit (kiwifruit), and 3 meal delivery services.

2.3.3 Abundance of gluten-free products

Ingredients from gluten-free products also often have a low FODMAP content (Ispiryan *et al.*, 2020). Unsurprisingly, > 80 % of the cereal-/ pulse-/ nut- and seed-based products and ingredients were also gluten-free (the majority of which were rather previously existing products than produced as targeted functional LF product) (Figure 2-3). Correspondingly, the sweet and savoury snacks, muesli bars, pastry, crisp breads, baking mixes, breakfast cereals, dairy alternative products and the beer were all solely based on gluten-free ingredients or non-gluten-free oat (8 % of the breakfast cereals contained low amounts of barley malt extract and were thus not gluten-free, Figure 2-3). From the breads, rolls, bagels, and wraps, 33 products were based on gluten-free flours or flour composites (e.g., rice flour, maize-, tapioca-, potato starch). Pulse flours, such as soy flour, are also often found in gluten-free formulations but with very low addition levels; hence, their impact on the FODMAP contents is irrelevant. Similarly, 2 of the 6 pizza bases, 12 pasta products, and 12 ingredients were based on gluten-free flours or flour composites (Figure 2-4). Furthermore, 2 wraps, 1 pizza base, 1 pasta product, and 1 ingredient were based on buckwheat. However, despite its high nutritional value and general health benefits (Wijngaard & Arendt, 2006), the classification of buckwheat as low FODMAP grain may be debateable as previously discussed (Ispiryan *et al.*, 2020; 2021b). Buckwheat contains indigestible, fermentable carbohydrates, namely fagopyritols, with 0.2 – 3 % found in the groats' milling fractions (Steadman *et al.*, 2000). These compounds have similar structural and biochemical properties to GOS and are suspected to have a similar effect on IBS patients. Even though fagopyritol concentrations in buckwheat are lower than GOS in pulses (up to > 10 %), they may be sufficient to trigger IBS symptoms (cutoff level for oligosaccharides is 0.3 g per serving). To our knowledge no clinical studies are available on the tolerability of buckwheat by IBS patients; the IBS-symptom induction

by buckwheat should be further investigated and the necessity of an inclusion of fagopyritols into routinely analysed FODMAPs elucidated.

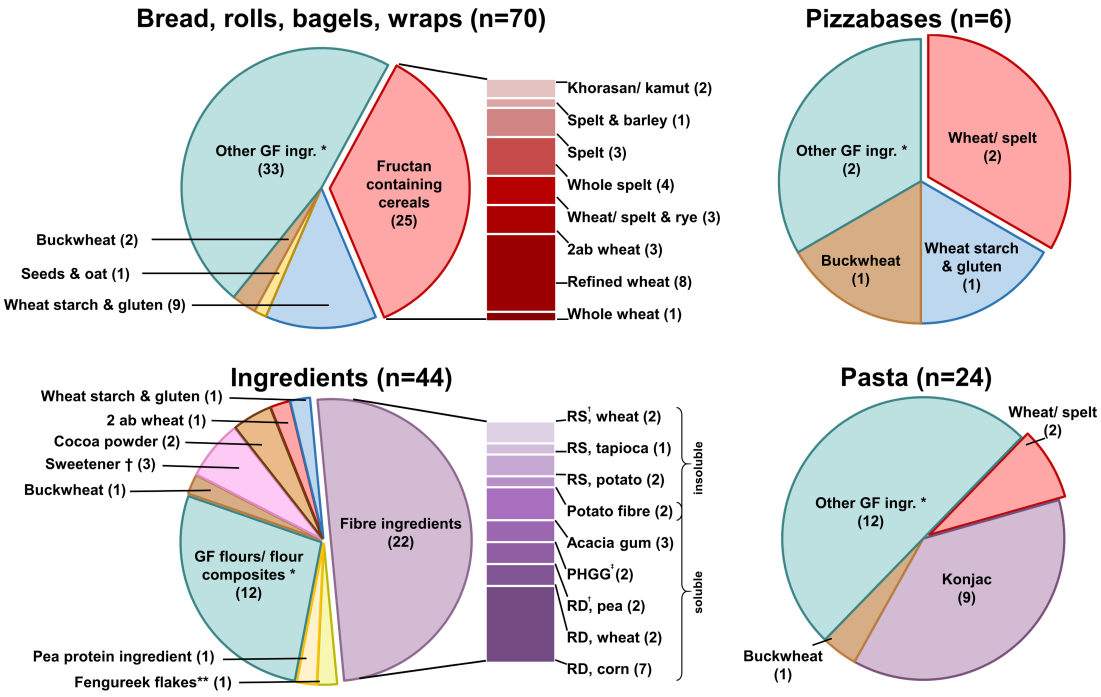

Figure 2-4. Base ingredients of low FODMAP bread, rolls, bagels, wraps, pizza bases, pasta, and types of raw ingredients with low FODMAP claim. * Gluten-free (GF) flours and flour composites include rice flour, maize and potato flour/ starch, tapioca starch, quinoa flour. † RS: resistant starch, RD: resistant dextrin. ‡ PHGG partially hydrolysed guar gum. ** Fenugreek seeds are legume seeds and accumulate GOS (Campbell & Reid, 1982), low FODMAP certification is for a small serving of 20 g.

2.4 Strategies to produce low FODMAP products and their current application

Low FODMAP products can be either produced by avoiding high proportions of high FODMAP ingredients in the product formulations or through biotechnological tools to degrade FODMAPs during the production process (Table 2-1, Figure 2-5). Regardless the approach used, the choice of ingredients should ensure that products are, as far as possible, rich in vitamins, minerals, complex non-FODMAP carbohydrates and protein, to enable the replacement of conventional FODMAP rich food products with low FODMAP alternatives of equal or greater nutritional and sensory quality. Targeted FODMAP reduction techniques are particularly relevant for bakery products, pasta, and their ingredients. This agrees with the unexploited potential of the current market situation and the prognosis for a high growth opportunity seen for the still "science-based niche" of low FODMAP bakery products (Mellentin, 2020). In this section, for each approach, the state of knowledge of the principle, the challenges, the current application on the market and potential associated patents are shown.

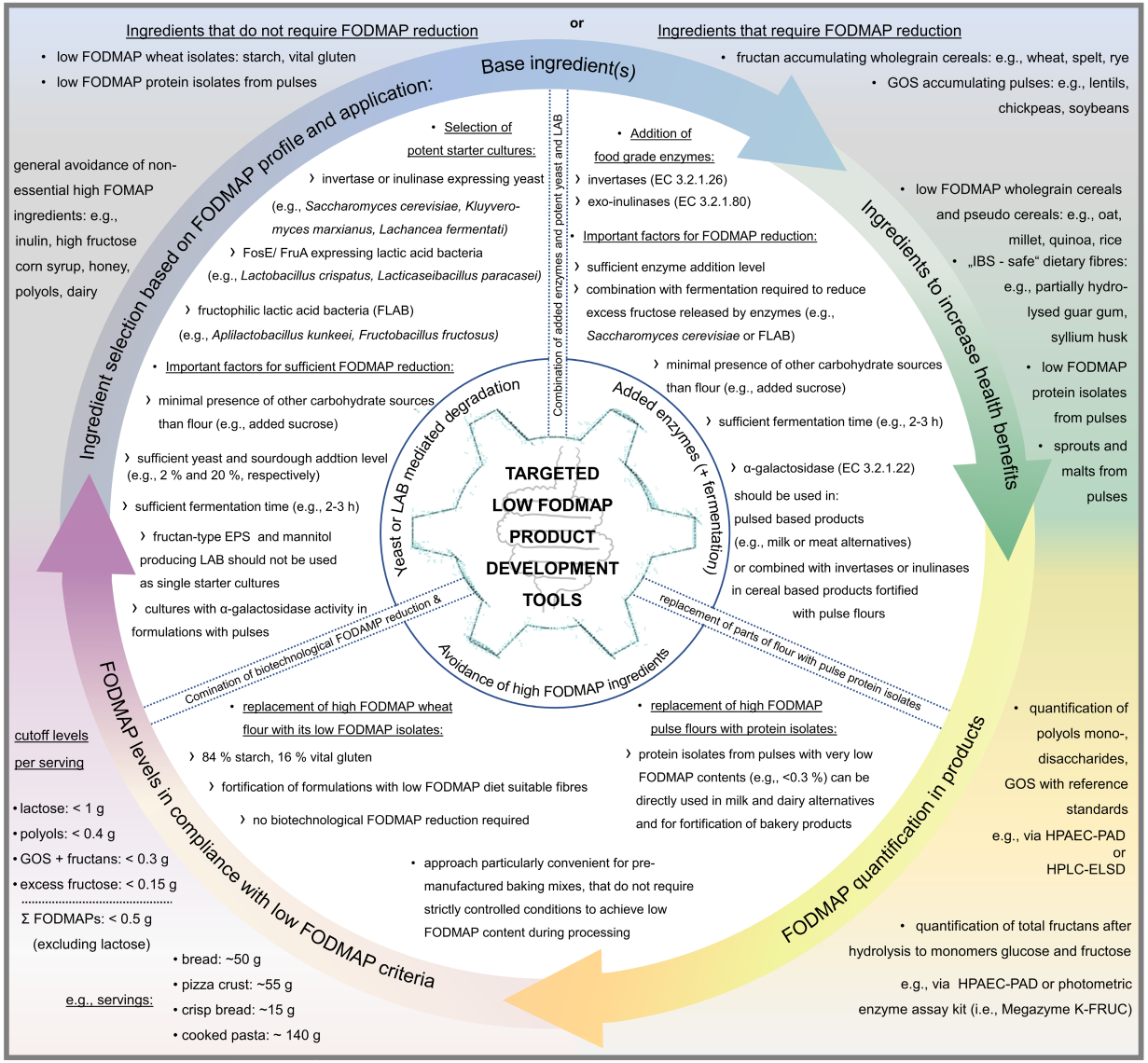

Figure 2-5. Targeted low FODMAP products development strategies suitable for cereal- and pulse-based bakery products, pasta, and beverages

Table 2-1. Summary of studied targeted FODMAP reduction approaches in ingredients and products and current application on the global market

Approach [references]	Products/ ingredients studied	FODMAPs reduced	Mechanism of FODMAP removal	Application on current market [a]
Physical FODMAP removal				
LF ingredients isolated from high FODMAP material [1-6]	Bread or ingredient based on starch- & vital gluten from wheat	Fructans	Fractionation of high FODMAP ingredient (starch, protein, fibre) and removal of FODMAPs as supernatant from isoelectric point precipitates from proteins or via aqueous washing and membrane filtration techniques	1 'plain flour', 10 products: breads, rolls, pizza base; 4 fibre ingredients
	Protein ingredients (lentils, fababean, lupin), plant-based milk alternative	GOS		2 dairy-alternative plant drinks, 1 protein ingredient, 7 fibre ingredients
Cooking [7-11]	Pasta (durum wheat semolina)	Fructans	Soluble carbohydrates leach into cooking water	-
	Various pulses	GOS		
Biotechnological FODMAP reduction during processing				
Enzyme addition (combined with fermentation) [12-15]	Wheat and rye breads	Fructan/ excess fructose/ mannitol	Added exo-inulinase FruA from *L. crispatus*, or invertase from *Aspergillus niger* combined with baker's yeast or *L. reuteri* or *L. frumenti* or *A. kunkeei* or *F. fructosus* fermentation: synergistically hydrolyse fructans, baker's yeast and/ or LAB metabolise excess fructose and mannitol	1 whole wheat-based bread
	Yellow pea protein-based crackers, meat analogue, spoonable product	GOS	Added commercial and recombinantly produced α-galactosidases from *Neosartorya fisherii* degrade GOS to sucrose and galactose	-
Yeast-fermentation (combined with enzyme addition) [14, 16-24]	Whole and refined wheat (different varieties) bread	Fructan, excess fructose	Yeast-invertase and/ or inulinase (*S. cerevisiae, K. marxianus, L. fermentati)* mediated hydrolysis of fructans, yeast metabolises excess fructose	10 breads made from predominantly refined flours
	Whole wheat and whole wheat - whole rye breads	Excess fructose	Baker's yeast metabolises excess fructose resulting from raffinose/ sucrose/ fructan-hydrolysis by LAB mediated or added inulinases	1 whole wheat-based bread

	Ingredient (ancient wheat flour, *Triticum turgidum* forma *sanus*)	fructan, excess fructose	Yeast- mediated (species unknown) enzymes invertase and/ or inulinase hydrolyse fructans and yeast metabolises excess fructose	1 ingredient: '2 ab wheat flour'
LAB-/ sourdough fermentation (combined with enzyme addition) [12-14, 21, 25-27]	Whole wheat, refined wheat, whole wheat - whole rye breads	Fructan, excess fructose	Exo-inulinase expressing LAB (FruA: *L. crispatus*; FosE: *L. paracasei*) hydrolyse fructans; LAB and yeast metabolise excess fructose without mannitol production	-
	Refined wheat bread	Excess fructose, mannitol	Strictly fructophilic lactic acid bacteria (FLAB; *A. kunkeei* or *F. fructosus*) efficiently metabolise excess fructose resulting from raffinose/ sucrose/ fructan-hydrolysis by added invertases and mannitol produced from fructose	-
		Fructan, excess fructose	Conventional sourdough cultures (*L. plantarum*, *L. citreum*, *F. sanfranciscensis*, *L. fermentum* etc.) synergistically with baker's yeast and other sourdough yeast (*C. milleri*) hydrolyse fructans and reduce excess fructose levels without production of high mannitol levels	16 products made from predominantly refined flours: breads, bagel, pasta, pizza base
Germination/ sprouting/ malting [11, 28]	Fresh sprouts (lentils, mung beans, buckwheat); malted ingredients (lentils, chickpeas, buckwheat)	GOS, FP [b]	Activation of endogenous α-galactosidases during germination, hydrolyse GOS; released galactose is utilised by growing embryo	-
Sprouting [11]	Fresh sprouts (rye, barley, wheat)	fructan	Dilution of present fructans in grains due to soaking with water; activation of endogenous fructan hydrolases during germination not reported [c]	2 breads from sprouted spelt and kamut

[a] Only products claimed to be low FODMAP by manufacturers or products certified by FODMAP friendly or Monash University. [b] Fagopyritols (FP) are major fraction of soluble carbohydrates of buckwheat, indigestible and suspected to act as FODMAP. [c] Apparent impact of sprouting contradicts different reports (Cozzolino *et al.*, 2016; Ispiryan *et al.*, 2021b; Krahl *et al.*, 2008; MacWilliam *et al.*, 1956) on impact of malting causing fructan accumulation throughout germination phase; potential shift fructan metabolism with alterations in germination conditions not yet studied.

References: [1] Atzler *et al.* (2021b), [2] Joehnke *et al.* (2021), [3] Pearce & Barrie (2015), [4] Vogelsang-O'Dwyer *et al.* (2020a), [5] Vogelsang-O'Dwyer *et al.* (2020b), [6] Vogelsang-O'Dwyer *et al.* (2021), [7] El-Adawy (2002), [8] Gélinas *et al.* (2016), [9] Han & Baik (2006), [10] Ispiryan *et al.* (2020), [11] Tuck *et al.* (2018), [12] Acín Albiac *et al.* (2020), [13] Li *et al.* (2020), [14] Loponen *et al.* (2017), [15] Nyyssölä *et al.* (2021), [16] Courtin *et al.* (2019), [17] Ispiryan *et al.* (2021a), [18] Kautz (2017), [19] Laurent *et al.* (2020), [20] Longin *et al.* (2020), [21] Schmidt & Sciurba (2021), [22] Struyf *et al.* (2017b), [23] Struyf *et al.* (2018), [24] Ziegler *et al.* (2016), [25] Fang *et al.* (2021), [26] Menezes *et al.* (2019), [27] Menezes *et al.* (2021), [28] Ispiryan *et al.* (2021b)

2.4.1 Avoidance of high FODMAP ingredients – use of ingredients naturally low in FODMAPs

Ingredients, that mainly contribute to high FODMAP contents of conventional products are typically fructan accumulating cereals, including all types of wheat (e.g., bread wheat, spelt, emmer, kamut, etc.), rye and barley, and GOS accumulating pulses (e.g., soy, lentils, peas, chickpeas, lupin, fababean, etc.) (Gélinas *et al.*, 2016; Ispiryan *et al.*, 2020). Fructan levels between 1 % and 5 % have been reported in different cereals, with a substantial part being stored in outer grain layers (Haskå *et al.*, 2008; Ispiryan *et al.*, 2020). Hence particularly products made from wholegrain flours contain high FODMAP levels (Biesiekierski *et al.*, 2011; Ispiryan *et al.*, 2019; Varney *et al.*, 2017). Pulses accumulate GOS (raffinose, stachyose, verbascose, ajugose; predominantly stachyose) at levels from 1 % up to 16 % (Ispiryan *et al.*, 2020; Martínez-Villaluenga *et al.*, 2008). In contrast, ingredients naturally low in FODMAPs comprise different flours and starches from gluten-free cereals, pseudo cereals or vegetables that do not accumulate fructans, GOS or other FODMAPs (e.g., oat, millet, rice, maize, quinoa, potato, etc.) (Ispiryan *et al.*, 2020). Hence, if the formulation of gluten-free products does not contain high amounts of other FODMAP-rich ingredients (e.g., dairy as a source of lactose, high fructose corn syrup, honey, polyol sweeteners), those are often found to also be very low in FODMAPs (Biesiekierski *et al.*, 2011; Ispiryan *et al.*, 2020).

As shown in the previous section, > 80 % of the commercially available products and ingredients with a LF claim were based on naturally low FODMAP and gluten-free ingredients (Figure 2-3 and Figure 2-4). Consequently, gluten-free products, also certified as low FODMAP, currently dominate the small market of functional low FODMAP products. However, gluten as an essential functional component does not have to be eliminated from the ingredients. Thus, the research of more targeted approaches has been of great interest and revealed the potential of different methods (Table 2-1, Figure 2-5).

Among the LF certified ingredients, there were 3 sweeteners, based on allulose, stevia and thaumatin, and erythritol, respectively. The latter is a polyol, and even though it is known that erythritol is not causing the same effect in healthy individuals as other polyols (better absorbed and less fermented by gut bacteria), its effect on IBS patients

was not investigated (Arrigoni *et al.*, 2005; Lenhart & Chey, 2017). While the Monash University certification criteria state, that foods with added FODMAPs, among which erythritol is included, are not even eligible for certification, an erythritol based sweetener is certified by the second certification program (FODMAP friendly). Such discrepancies underline the urgency of a clearly defined FODMAP concept incorporated in the respective legal framework. Finally, in the context of alternative non-FODMAP sweeteners, the chocolate manufacturer Cavalier holds a patent on the production of low sugar/ sugar-free products sweetened with mogroside enriched monk fruit extract, isomaltulose or the sweet tasting proteins thaumatin, monellin, mabinlin, pentadin, brazzein, curculin or miraculin (Table 2-2) Vergedem (2016).

2.4.2 Use of low FODMAP ingredients, isolated from high FODMAP raw material

Protein- and starch isolates from high FODMAP raw material, such as wheat or pulses, can have significantly lower fructan or GOS contents in the obtained functional ingredients, depending on their production process. Such ingredients may serve as base ingredients in different LF product formulations, which do not require any further FODMAP reduction techniques during the food processing (Figure 2-5).

Joehnke *et al.* (2021) demonstrated that the isolation of lentil protein by ultrafiltration was highly effective in removing compounds with a molecular weight below 10 kDa (this includes GOS), resulting in an ingredient with only ~ 0.4 % GOS obtained from lentils with ~ 4 % GOS. They also reported that the isolation of a protein ingredient from the same raw material, using isoelectric point precipitation, resulted in ~ 2 % residual GOS in the ingredient. In contrast, slightly different isoelectric point precipitation processes for the isolation of fababean and lupin protein ingredients have been shown to also remove most native GOS effectively (max. 0.1 % of residual GOS) (Vogelsang-O'Dwyer *et al.*, 2020a, 2020b). However, not all types of protein ingredients may be suitable in LF formulations without additional FODMAP reduction during the processing of the product. Several commercial pulse protein ingredients and a fababean protein-rich ingredient obtained by dry fractionation were shown to contain comparable GOS levels such as native pulses (~ 5 − 11 %) (Ispiryan *et al.*, 2020; Vogelsang-O'Dwyer *et al.*, 2020b). Pulse protein ingredients with low FODMAP contents could serve as base ingredients in dairy alternative products, as it was recently shown for milk alternatives based on lupin protein isolates (Vogelsang-O'Dwyer *et al.*,

2021). Among the commercial LF products, two soy protein-based milk alternatives and a pea protein ingredient were available (Figure 2-3 and Figure 2-4). The use of pulse protein ingredients in milk alternatives not only serves the LFD, because nowadays the environmental, ethical, and health-related concerns of consumers are generally increasing the demand for plant-based dairy alternatives.

For the production of LF bakery products, the replacement of wheat flour with its isolates, which are essential to the baking process, wheat starch and vital gluten, supplemented with non-FODMAP fibres, is an elementary yet beneficial, broadly applicable, and reliable approach. The wheat isolates were reported to contain only a fraction of the wheat-derived fructans (traces in starch and ~ 0.6 % in vital gluten (Ispiryan *et al.*, 2020). Hence, an 84 % starch- and 16 % gluten-based formulation (according to the composition of wheat) would only account for ~ 0.1 % wheat-derived fructans, in contrast to ~ 2 % fructans from whole wheat flour. Atzler *et al.* (2021b) applied this approach and investigated the potential enrichment of such starch- and gluten-based LF bread formulations with supposedly non-FODMAP, soluble (guar gum, psyllium) and insoluble fibres (bamboo, cellulose), at concentrations of the EU nutrition claims “source of fibre” (3 %) and “high in fibre” (6 %) (Atzler *et al.*, 2021b; Regulation (EC) No 1924/2006). They evaluated the consequences of incorporating fibres on technological and nutritional bread quality characteristics and highlighted the benefits of the LF bread fortification with psyllium at 3 %. The complete or partial replacement of wheat flour with its functional isolates is currently also applied in several commercially available LF products, including bread, rolls, a pizza base, and an ingredient (Figure 2-3 and Figure 2-4). The latter is a reconstituted wheat “plain flour” made from wheat starch, vital gluten, and guar gum. Pearce & Barrie (2015) (company Shoalhaven starches Pty Ltd; product marketed as LO-FO pantry) patented their technology to produce low FODMAP wheat isolates, including extensive aqueous washing and membrane filtration steps to effectively remove the FODMAPs (Table 2-2). Particularly vital gluten obtained through their technology is superior to conventionally available vital gluten, which still contains low levels of fructans. However, as explained above, with relatively low addition levels of gluten to product formulations, residual fructans in conventional gluten ingredients are unlikely to contribute to FODMAP levels in the final products, which would exceed a 0.3 g cutoff level for oligosaccharides per food serving.

Table 2-2. Overview of FODMAP related patents

Invention/ Claim	Approach to achieve low FODMAP content	Associated commercial product(s)	Assignee/ applicant	Inventor
Easily digestible chocolate product (WO 2016/097189 A1; published: 23/06/2016)				
Composition for manufacturing low sugar/ sugar-free chocolate products (bars, spreads, fillings, powders, beverages) sweetened with non-FODMAP sweeteners and replacement of whole milk to reduce lactose contents in milk chocolate products	Replacement of sucrose, sugar alcohols, FOS, inulin, with mogroside enriched monk fruit extract, isomaltulose and sweet tasting protein (taumatin, modellin, pentadin, brazzein, curculin, miraculin). Replacement of whole milk with milk protein extract or isolate, casein/ whey mixtures, soy protein. Potential addition of insoluble and/ or slowly fermentable fibres (cellulose, lignin), nuts.	-	Cavalier	Vergedem, F.
Wheat-based products in foods for the wheat intolerant (WO2015117182A1; published: 13/08/2015)				
Production of reconstituted low FODMAP wheat gluten protein-based flour, including other LF isolates from wheat or non-wheat origin and products made from such ingredients result in LF products.	Wheat gluten and other constituent of the reconstituted flour are extracted from wheat and other source material. FODMAPs are removed by washing insoluble material with water or separated from soluble higher molecular weight constituent by membrane filtration.	LO-FO pantry Plain flour	Shoalhaven Starches Pty Ltd	Pearce, R. J.; Barrie, A. L.
Low-fructan grain material and a method for producing the same (WO2016113465A1; published: 21/07/2016)				
Technology allowing efficient removal of fructans from grain material, to be used for LF products.	Seed or purified microbial starter produced with grain material with low damaged starch content (e.g., cut rye kernels) to promote spontaneous formation of microflora of lactobacilli able to efficiently utilize fructans (*L. ultunensis, L. crispatus, L. amylovorus, L. amylovorans, L. sobrius* and/or *L. acidophilus*). Use of the starter for fermentation of high fructan grains (rye, barley, wheat) and various LF products.	Fazer Stomach Friendly Rye Bread (product discontinued)	Oy Karl Fazer Ab	Loponen, J.
Methods of providing FODMAP friendly food items (US2020/0229481 A1; published: 23/07/2020)				
Substitution of onion and garlic in product formulations/ recipes to obtain 'FODMAP diet friendly' food while maintaining the flavour.	Onion and garlic in conventional recipes are replaced with garlic scapes, spring garlic tops, green tops of leeks, green spring onion tops, chives, green tops of scallions.	Gourmend Foods garlic scape and garlic chive powders, chicken broth	Ketan Vakil	Ketan Vakil
An enzyme exhibiting fructan hydrolase activity (WO2017220864A1; US 2019/0174773 A1; published: 28/12/2017; 13/06/2019)				
Enzyme allowing significant reduction of fructan content in grain and vegetable material/ products containing those.	Extracellular fructanase FruA from *L. crispatus* DSM 29598, isolated from rye sourdough (with low damaged starch), recombinantly produces in host cells; enzyme efficiently degrades fructans. Use of enzyme e.g., in combination with baker's yeast to produce LF products.	Fazer Stomach friendly Soft Wheat-Oat Flax Bread, LOFO™	Oy Karl Fazer Ab	Loponen, J.; Mikola, M.; Sibakov, J.
Wholemeal bread with reduced FODMAP content (WO2019034630A1; published: 21/02/2019)				
Production of wheat wholemeal bread with a significantly lowered FODMAP content.	Use of inulinase secreting yeast, *K. marxianus* strains, which efficiently degrade fructans in a co-culture with *S. cerevisiae* to achieve sufficient CO_2 production.	-	Katholieke Universiteit Leuven	Courtin, C.; Struyf, N.; Thevelein, J.; Verstrepen, K.

Moreover, several fibre ingredients with a LF claim, which are also isolates from high FODMAP raw material, were available. This included resistant starches and dextrins from pea and wheat, but also partially hydrolysed guar gum (PHGG) and acacia gum are isolates from GOS accumulating legume seeds (Figure 2-4) (Singh *et al.*, 1990). These fibre ingredients are LF certified based on the low content of the routinely tested FODMAPs. Furthermore, they are not or only slowly fermentable fibres and could therefore classify as IBS-suitable fibre; however, data on their tolerability in IBS clinical studies is incomplete (Atzler *et al.*, 2021a; So *et al.*, 2020). The fermentability of PHGG, acacia gum and different resistant starches (derived from maize and tapioca) was recently tested in an *in vitro* screening compared to rapidly fermentable short chain fructans, which are known to cause symptoms. With a moderate to very slow fermentability, these fibres were identified as potentially safe fibres to be tested *in vivo* in IBS patients (So *et al.*, 2020).

Ultimately, the principle of the wheat flour replacement with gluten and starch isolates, fortified with "IBS-safe" fibres, could be a cornerstone for the development of beneficial LF bakery products and pasta; it could provide a simple, efficient, and reliable approach for LF products, without the need of any further FODMAP reduction techniques. This is particularly interesting for premanufactured baking mixes which do not require any controlled conditions to reduce FODMAPs at home (i.e., enzymatic, yeast or sourdough FODMAP reduction during strictly controlled incubation or fermentation conditions). However, further *in vitro* and *in vivo* studies are needed to validate the tolerability of different dietary fibres by IBS patients and studies to investigate the fibres' techno-functional and nutritional properties when incorporated in food products.

2.4.3 Impact of cooking during food preparation

Oligosaccharides, including fructans and GOS, but also di- and monosaccharides (glucose, fructose, sucrose, maltose) are well soluble in water and have been shown to diffuse into the hot water during the cooking process of pasta and pulses (El-Adawy, 2002; Gélinas *et al.*, 2016; Ispiryan *et al.*, 2020; Stone *et al.*, 2020).

A reduction of GOS (mostly referred to as raffinose family oligosaccharides, RFO or α-galactosides in relevant literature) after soaking and subsequent cooking has been reported in number of studies in a wide variety of pulses, e.g., chickpeas, lentils, yellow

and green peas, soybeans, or kidney beans. However, the effectivity of the reduction was highly variable (~ 10 − 80 %) (El-Adawy, 2002; Han & Baik, 2006; Stone *et al.*, 2020; Tuck *et al.*, 2018). This approach is particularly interesting for the direct consumption of the cooked pulses in meals, where they serve as a rich source of plant protein, dietary fibre, and micronutrients. Nevertheless, because the cooked pulses often still contain GOS levels exceeding the cutoff level of 0.3 g per serving (Tuck *et al.*, 2018), satiating servings of pulses still have to be avoided by GOS-sensitive IBS patients (according to serving size recommendations of Monash University mobile phone application). Further targeted studies could focus on identifying optimal soaking and cooking conditions for different types of pulses to achieve a potential reduction resulting in < 0.3 g of GOS per serving, which could be either indicated on the product packaging for consumers or used for the production of canned products.

Furthermore, ~ 40 % of durum wheat fructans in pasta are lost in the cooking water (Gélinas *et al.*, 2016; Ispiryan *et al.*, 2020). Nonetheless, a 140 g serving of the resulting cooked pasta (Edwards, 2017) usually still contains > 0.3 g of fructans (Biesiekierski *et al.*, 2011; Gélinas *et al.*, 2016; Ispiryan *et al.*, 2020). Hence, > 90 % of the currently commercially available LF pasta products are wheat- and gluten-free products (Figure 2-3 and Figure 2-4). However, functional LF pasta could also be produced by partial replacement of the durum wheat semolina with low FODMAP ingredients. For example, for a semolina containing ~ 1.2 % fructans (Ispiryan *et al.*, 2020) the replacement of 20 % with alternative ingredients would be sufficient to obtain a cooked pasta with < 0.3 g fructans per serving. The substitution of semolina with other nutritionally beneficial ingredients, including fibres or protein-rich ingredients, is an approach that has been of great research interest, primarily aiming an increase in nutritional value compared to conventional pasta (Bustos *et al.*, 2015; Mercier *et al.*, 2016). However, to our best knowledge, the FODMAP content has not been considered in any of the researched approaches. Even though the incorporation of some ingredients revealed to be challenging in terms of sensory and technological quality characteristics, advances in pasta research have demonstrated different possibilities. For instance, Hoehnel *et al.* (2020) have recently shown the benefits of wheat pasta fortification (semolina replaced at a level of ~ 24 %) with a mixture of protein-rich ingredients from buckwheat, fababean, and lupin and achieved particularly an improved high-quality protein profile. As the substitution of semolina

with other low FODMAP ingredients (e.g., LF grains, LF protein isolates from pulses, dietary fibres) has not been investigated to date for the production of LF pasta, studies are required on the incorporation of different ingredients and the concomitant consequences. This will aid to identify optimal wheat based low FODMAP pasta formulations, which do not require further FODMAP reduction techniques.

2.4.4 Biotechnological FODMAP reduction

Besides technologies based on the physical removal or avoidance of FODMAPs, functional products can also be obtained with biotechnological FODMAP reduction techniques. Thereby, the main FODMAPs of cereals and pulses, fructans and GOS, can be degraded by added enzymes, microbial enzymes during a fermentation process, or activated endogenous seed-enzymes during a germination process. Biotechnological FODMAP reduction techniques allow for targeted FODMAP degradation and provide products with a good source of wholegrain-intrinsic, beneficial dietary fibres (Laatikainen *et al.*, 2016).

While some fructan accumulating plants, such as chicory and cocksfoot (*Dactylis glomerata*) contain linear fructan molecules composed of ß (2 → 1), inulin-type, or ß (2 → 6), levan-type, linked fructose chains with a terminal glucose moiety, it is characteristic for cereals to contain fructans composed of branched molecules with both types of linkages and a terminal glucose residue (graminan-type) or linear molecules with an internal glucose residue (neo-series-type) (Livingston *et al.*, 2009; Verspreet *et al.*, 2015; Verspreet *et al.*, 2017). Cereal fructans can be degraded with ß-fructofuranosidases, including invertases (EC 3.2.1.26), fructan exo- (EC 3.2.1.80) and endohydrolases (EC 3.2.1.7; fructan hydrolases are commonly referred to as inulinases or fructanases; the term inulinases will be used in this review). Both invertases and exo-inulinases catalyse the hydrolysis of terminal non-reducing moieties, while fructan chains are hydrolysed to fructose monomers and the glucose unit. Endo-inulinases cleave internal glycosidic bonds and thereby produce several shorter fructan chains. Whereas invertases have a higher specificity towards shorter fructan chains (average degree of polymerisation, DP, ~ 5), inulinases also quickly degrade long-chain fructans (Nilsson *et al.*, 1987; Struyf *et al.*, 2017b). With average DPs of 5 − 7 in wheat (Ispiryan *et al.*, 2019; Verspreet *et al.*, 2015), both enzymes are capable to

hydrolyse the majority of wheat fructans under optimal conditions (Struyf *et al.*, 2017b).

The indigestible α-galactosyl linkages in GOS (raffinose, stachyose, verbascose) are cleaved by α-galactosidases (EC 3.2.1.22), which results in the release of $1-3$ galactose units and sucrose. Even though ß-fructofuranosidases also technically reduce the amount of raffinose, stachyose, and verbascose through the action of the enzyme on the sucrose-end of the saccharides, their indigestible linkages remain intact (Atzler *et al.*, 2020). The resulting degradation products, besides the released fructose (namely melibiose, manninotriose and manninotetraose), are still accounted as FODMAPs.

An important key element of this approach is that the oligosaccharide degradation should always be linked to a strategy for reducing the resulting degradation products in order to avoid an accumulation of other FODMAP carbohydrates. And notwithstanding that some of the principles of these methods have been applied for food production for centuries (i.e., yeast leavening and sourdough fermentation for breadmaking), it is important to approach them now as targeted FODMAP reduction strategies. Strictly controlled and defined process parameters and product formulations are essential to achieve the required FODMAP reduction.

2.4.4.1 Addition of purified enzymes

The purified enzyme preparations can be of plant or microbial origin (Nyyssölä *et al.*, 2020). Food grade ingredients of the enzymes, specifically produced for food and beverage applications, are available for instance from the company Creative Enzymes ®: endo-/ and exo inulinases from *Aspergillus niger* (NATE 1245 and 1246), invertases from baker's yeast (NATE-0357) or *Candida* sp. (DIA-205), and α-galactosidase from *Aspergillus niger* (DIS-1012).

The most relevant enzymes for bakery products and pasta are invertases and inulinases. These will degrade cereal fructans to fructose and glucose (Atzler *et al.*, 2020; Struyf *et al.*, 2017b). Even though GOS (i.e., raffinose) levels are also expected to be quickly reduced, the resulting melibiose and fructose still account towards the total FODMAP content, such as the fructose from sucrose, raffinose and fructans (Atzler *et al.*, 2020; Ispiryan *et al.*, 2021a). Hence, linked purely enzyme- or fermentation-based solutions are required. For bakery products, combining baker's yeast fermentation in a simple yeast-leavened bread or using sourdough technology are the most favourable

approaches (CO_2 production in glycolysis by microorganisms essential for the leavening). Yeast and specific lactic acid bacteria (LAB) utilise the fructose, released from the enzymatic degradation of the saccharides, during the fermentation and proofing of the products. The synergistic effect of added enzymes, yeast and LAB is an effective strategy to lower total FODMAP contents. Acín Albiac *et al.* (2020) have recently demonstrated the potential of the combination of a commercial invertase preparation (added at a level of 1 U/ 10 mg fructan) in a wheat sourdough with potent strains of fructophilic lactic acid bacteria. These microorganisms were capable of utilising the fructose released from the fructan degradation without distinct accumulation of mannitol, a common LAB metabolite in sourdough. Moreover, Li *et al.* (2020) applied a commercial exo-inulinase containing baking ingredient (Fazer LOFO^{TM}, 1 % based on flour, activity not reported) in a wheat-rye sourdough and straight dough yeast-leavened bread and achieved complete fructan degradation and very low fructose and mannitol levels. Alternatively, enzymes such as amyloglucosidase and amylases to release glucose from starch and decrease the amount of fructose present in excess to glucose could be used (Melim Miguel *et al.*). However, increased levels of free glucose and fructose in products may have several disadvantages concerning sensory (too sweet), and nutritional (increased glycaemic index) attributes. Pasta is possibly the only product, that may not require additional techniques for excess fructose removal; it is expected that the free fructose will largely leach into the cooking water (Gélinas *et al.*, 2016). If a sufficient fructan- and fructose reduction is achieved via enzyme-based fructan degradation and fructose consumption by baker's yeast or LAB, low levels of melibiose resulting from the raffinose degradation have a comparably minor impact on the total FODMAP content (if wheat is the only GOS containing ingredient). Nevertheless, it is still essential to consider melibiose levels when calculating the total FODMAP content, e.g., melibiose from a complete degradation of ~ 0.2 % raffinose from the flour would already account for ~15 % of the maximal tolerated oligosaccharide level of 0.3 g/ 50 g of bread (Ispiryan *et al.*, 2021a). An additional strategy for the degradation of the α-galactosyl linkages in GOS may be necessary if the fortification of cereal-based products with pulses is considered. Fortification of wheat-based products with pulse flours is emerging, as it offers, among other advantages, an excellent possibility to increase the nutritional quality of the plant protein composition (Boukid *et al.*, 2019). Such products do not have to be avoided in low FODMAP applications, as a combination of inulinases/

invertases and α-galactosidases can be applied to degrade fructans as well as GOS. A recent study demonstrated the potential of α-galactosidase application in different pulse-based product prototypes (meat analogues, crackers, spoonable products) to lower GOS levels by up to > 90 % (Nyyssölä *et al.*, 2021). Furthermore, the use of α-galactosidases has been described in soy-based milk alternatives to lower GOS contents (Nyyssölä *et al.*, 2020).

Among the commercially available LF products, there was one bread where the above-mentioned exo-inulinase containing ingredient LOFO^{TM} was used in combination with baker's yeast fermentation to produce a wheat-based LF bread. According to manufacturer's claim, the bread has 0.2 g/ 100 g FODMAPs. The production of the LOFO^{TM} ingredient is based on a patented technology by Loponen *et al.* (2017) (company Fazer Mills, Finland; Table 2-2). It contains an exo-inulinase (FruA), isolated from *Lactobacillus crispatus* DSM 29598 and recombinantly produced in host cells such as *Pichia pastoris.* The inulinase expressing *L. crispatus* was in turn isolated from a sourdough and has been shown to efficiently degrade fructans in a sourdough system (Li *et al.*, 2020; Loponen, 2016; Loponen *et al.*, 2017). In an application example of the patent, the inventors describe a dosage of 0.18 % enzyme based on flour (e.g., 1 U/ 18 mg fructan for 500 U/ g activity of enzyme preparation stated in the patent) in a wheat bread that was made in a straight dough process with 3 % baker's yeast and 1.8 % sucrose. The enzyme was added to the formulation suspended in the water of the recipe, and the dough was left to rise for 2 h at 37 °C. The resulting bread had a fructan concentration of 0.05 %, which was reduced by ~ 90 % compared to the flour. However, the fructose concentration in the bread was 0.4 % which may exceed the cutoff value of 0.15 g/ 50 g of bread for excess fructose, if glucose concentrations in the bread were lower than 0.1 % (levels not reported). In such cases, a higher dosage of the enzyme, may allow for a faster fructan degradation, and hence more efficient fructose consumption by the yeast over the fermentation period. Atzler *et al.* (2020) reported an almost complete inulinase mediated degradation of wheat fructans in an aqueous solution already after 1 h with 1 U enzyme per 0.001 mg fructans. To fully validate the application of purified enzymes in low FODMAP baking, further studies are required to identify optimal process parameters (i.e., enzyme dosage, fermentation time and temperature, product formulations) using commercially available food grade enzyme preparations.

2.4.4.2 Yeast and lactic acid bacteria mediated FODMAP reduction

Specific strains of yeast and lactic acid bacteria (LAB) can express invertases, inulinases or α-galactosidases which degrade the oligosaccharides during the fermentation of a production process. Yeast and LAB are powerful tools for FODMAP reduction in bakery products, but not a guarantee, unless targeted methods with selected starter cultures and defined process parameters are used, rather than short fermentation industrial bread-making methods or conventional sourdoughs.

Baker's yeast (*Saccharomyces cerevisiae)*, the most commonly used leavening agent in industrial baking, can express invertase (Nilsson *et al.*, 1987; Sainz-Polo *et al.*, 2013). It was shown that yeast invertase mediated release of fermentable carbohydrates (from sucrose, raffinose and fructans), plays an essential role in the bread-making process, apart from maltose, providing glucose and fructose as substrates for CO_2 production, particularly in the first hour of fermentation (Struyf *et al.*, 2017a). Yeast invertase has a higher affinity towards short chain fructans and quickly degrades up to DP ~ 5 fructans in the first hour of fermentation. In contrast, the degradation of higher DP fructans is slower (Nilsson *et al.*, 1987). Nonetheless, several studies reported that ~ 40 − 90 % of the initially present fructans in wheat flour are degraded by yeast invertase during the bread-making process (Gélinas *et al.*, 2016; Knez *et al.*, 2014; Laurent *et al.*, 2020; Longin *et al.*, 2020; Nilsson *et al.*, 1987; Pejcz *et al.*, 2019; Schmidt & Sciurba, 2021; Struyf *et al.*, 2017b; 2018; Ziegler *et al.*, 2016). For example, a white wheat bread made with flour that contains ~ 1.2 % fructans would require only ~ 30 % fructan degradation to result in a bread with fructan levels below the cutoff of 0.3 g/ 50 g bread (considering a moisture of 40 % in bread). A wholemeal bread with ~ 2 % initial fructans from the flour would require ~ 60 % fructan degradation. However, even though the yeast partly metabolises the released fructose, insufficient fermentation times can result in excess fructose levels exceeding 0.15 g/ 50 g bread, despite the fructans being degraded. Ziegler *et al.* (2016) have first shown the significance of extended fermentation and proofing times for low FODMAP baking with baker's yeast. After 1 h of fermentation and proofing they had already achieved a fructan degradation by 60 %, but fructose released from raffinose, sucrose and fructans, led to a level of ~ 1 % excess fructose in the bread. An extended fermentation time of 2.5 h, in contrast, allowed for a reduction of the excess fructose by ~ 70 % and overall low FODMAP levels (4.5 h of fermentation even resulted in

> 90 % fructan degradation and only 0.03 % excess fructose). However, fermentation times of industrial bread making (e.g., Chorleywood process commonly used in UK (Delcour & Hoseney, 2010)) may often be too short for achieving sufficiently low FODMAP levels, especially in wholemeal bread (Schmidt & Sciurba, 2021). Besides the fermentation and proofing times, other factors such as the yeast addition level (Struyf *et al.*, 2017b), the extraction rate of the flour (i.e., refined flour vs. wholemeal flour) (Schmidt & Sciurba, 2021), and other sources of fermentable sugars in the ingredients (e.g., added sucrose) (Struyf *et al.*, 2017b) are decisive for the final FODMAP content. Furthermore, Li *et al.* (2020) demonstrated the limitation of baker's yeast to achieve sufficiently low FODMAP levels in rye- and wheat- (in equal parts) based bread, as rye contains significantly more fructans than wheat (~ 4 % vs. ~ 2 % in wheat). Despite a 56 % fructan degradation and relatively low fructose levels after 3 h of fermentation and proofing, the remaining fructan levels still exceeded 0.3 g/ 50 g bread. Hence, although conventional baker's yeast has the potential to be applied in low FODMAP baking, a targeted approach considering the described factors is essential. Currently, only 10 of the commercially available breads with a LF claim could be categorised as low FODMAP due to the effect of baker's yeast fermentation (Figure 2-3). Furthermore, one wheat flour was available, marketed as '2ab wheat' (made from an ancient wheat species *Triticum turgidum* forma *sanum*), claimed to have a lower FODMAP content than common bread wheat flour due to the pre-fermentation of the flour with yeast (Kautz, 2017).

As an alternative to conventional baker's yeast, different non-*Saccharomyces* yeast species have proven their potential to efficiently lower FODMAP contents in a wholemeal wheat bread (Courtin *et al.*, 2019; Ispiryan *et al.*, 2021a; Struyf *et al.*, 2017b; 2018). Strains belonging to *Kluyveromyces marxianus* species have been shown to degrade fructans much more efficiently due to cell-wall associated inulinases and the expression of those into the fermenting dough, in contrast to *S. cerevisiae* species, which degrade fructans solely with cell-wall invertases. As *K. marxianus* species are unable to ferment maltose, they need to be used either in a co-culture with *S. cerevisiae* or require an alternative carbohydrate source (e.g., added sucrose or amyloglucosidase to release glucose from starch) to achieve an appropriate dough rise. But even with the additional sources of carbohydrates, resulting breads were shown to have fructan contents by far below the cutoff levels, owing to the yeast's unique ability

to express extracellular inulinases (Struyf *et al.*, 2017b; 2018). Furthermore, another non-*Saccharomyces* yeast strain, originating from a Kombucha culture, *Lachancea fermentati* FST 5.1, has also been shown to degrade fructans more efficiently than conventional baker's yeast and result in bread with high quality characteristics (volume, texture, aroma), comparable to baker's yeast. The underlying fructan degradation mechanism remains to be elucidated (Ispiryan *et al.*, 2021a). Moreover, a *Torulaspora delbrueckii* strain, isolated from a sourdough culture, appeared promising with a higher fructan reduction rate than baker's yeast in a wheat flour slurry (Fraberger *et al.*, 2018). A targeted application of the strain in products, however, has not been reported yet. Also, the potential of different *S. cerevisiae* strains from bakery and other industrial applications with pronounced high invertase activities and fructan substrate specificities for their use in low FODMAP baking applications has been recently demonstrated (Laurent *et al.*, 2020; 2021).

In sourdough baking, lactic acid bacteria represent an additional group of microorganisms besides yeast. Bread formulations typically contain approximately 20 % sourdough. Industrial sourdough bread production often also includes baker's yeast as a leavening agent. The added sourdoughs can be produced either by spontaneous fermentation (forming natural microflora through repeated reinoculation of the dough with water and flour) or by propagating with starter cultures. Different types of sourdough (i.e., type I, II) are characterised by their dough yield (amount of dough obtained from 100 parts of flour), fermentation conditions, and the prevalent microflora (Arendt *et al.*, 2007; Gobbetti & Gänzle, 2013; Loponen & Gänzle, 2018). As Loponen & Gänzle (2018) reviewed, the application of sourdough technology for low FODMAP baking can be compelling but is much more complex than yeast fermentation due to the high diversity of LAB microflora under different conditions and their concomitant metabolic processes. Several factors accentuate the necessity for targeted sourdough fermentation rather than conventional sourdough techniques, especially for the production of whole wheat or rye-based sourdough bread (Pejcz *et al.*, 2020; Schmidt & Sciurba, 2021). Firstly, fructan degradation by LAB is often limited to intracellular hydrolysis of short chains (DP < 4) with ß-fructosidases (SacA, SucP), as oligosaccharides with higher DP cannot be transported into the intracellular area (Gänzle, 2020; Loponen & Gänzle, 2018). Furthermore, heterofermentative lactic acid bacteria do not utilise fructose as a carbon source but reduce it to mannitol;

thereby fructose serves as an electron acceptor for the regeneration of reduced cofactors. Another undesired attribute of LAB for low FODMAP baking, is that certain species can produce exopolysaccharides (EPS) composed of fructose monomers (levan, inulin, fructooligosaccharides). Both, mannitol, and EPS production in relevant levels, however, are linked to the presence of endogenous or added sucrose in the formulation (Loponen & Gänzle, 2018). Furthermore, LAB can hydrolyse GOS through the action of levansucrase, α-galactosidase and sucrose phosphorylase activity. However, as not all species express α-galactosidase activity, the fermentation of GOS rich material (i.e., pulses) can lead to the accumulation of melibiose and its higher oligosaccharides. Levansucrases (glycosyltransferases) in turn, which release fructose from the sucrose end of GOS, can also catalyse the synthesis of fructans (Teixeira *et al.*, 2012; Tieking *et al.*, 2003).

Research over the past few years has proposed highly efficient sourdough based LF baking approaches. Even though extracellular exo-inulinases (mostly referred to as fructanases in relevant literature) are rare in LAB, few species have been identified to express two different exo-inulinases: FruA and FosE. The aforementioned strain *L. crispatus* DSM 29598 (obligate homofermentative) expresses FruA and was isolated from a sourdough described in a patent application. According to the inventor, the growth of fructan-degrading LAB could be promoted by choice of flour with low damaged starch content, as in such case, fructan rather than starch is readily available as a source of fermentable carbohydrates (Loponen, 2016). Wheat- and rye-based bread prepared with 20 % *L. crispatus* fermented sourdough (16 h, 37 °C; bread dough fermentation 3 h, 37 °C) and 2 % yeast was shown to contain very low FODMAP levels, far below all individual cutoff levels. The use of heterofermentative *Limosilactobacillus reuteri* or *Limosilactobacillus frumenti*, either in co-culture with *L. crispatus* or with 1 % addition of FruA containing LOFO^{TM} ingredient resulted in higher mannitol concentrations, yet still FODMAP levels below the cutoff levels. This synergistic effect of the highly efficient fructan degrading LAB or the FruA containing ingredient combined with baker's yeast was capable of degrading the majority of the wheat and rye derived fructans, without the accumulation of fructose or mannitol (Li *et al.*, 2020). Two clinical trials comparing the symptom induction of a conventional rye sourdough bread and a sourdough bread made with the *L. crispatus* strain provided evidence for a better tolerability of the sourdough bread made with the potent strain.

The exact process parameters and product formulations were unknown, but only *L. crispatus* fermented bread had FODMAP contents below the cutoff levels (Laatikainen *et al.*, 2016; Pirkola *et al.*, 2018). Phylogenetic analysis of exo-inulinases in LAB showed the presence of FruA in the genomes of *Ligalilactobacillus salivarius*, *Ligalilactobacillus equi*, *Latilactbacillus curvatus*, *Lb. amylovrus* and *Lb. delbrueckii.* The second exo-inulinase FosE was characterised in *Lacticaseibacillus paracasei* and homologues were found in the genomes of *Lacticaseibacillus casei*, *Lactiplantibacillus plantarum*, *Pediococcus pentosaceus*, *Pediococcus acidilactici* and *Liquorilactobacillus* spp. (taxonomy according to recent reclassification of genus lactobacillus (Zheng *et al.*, 2020)) (Li *et al.*, 2020). Another concept that has been recently described for low FODMAP sourdough baking is the use of strictly fructophilic lactic acid bacteria (FLAB). In contrast to other LAB, these prefer fructose over glucose as growth substrate and utilise it as a carbon source and an electron acceptor while producing mannitol, which in turn can be used too. The authors identified a potent combination of two FLAB strains with maximal fructose (*Aplilactobacillus kunkeei* B23I) and mannitol (*Fructobacillus fructosus* MBIII5) consumption rates, which were applied in a preliminary baking experiment in combination with commercial invertase. The resulting bread made from refined wheat flour had very low total FODMAP contents of ~ 0.3 % DM (based on dry matter; corresponds to ~ 0.08 g/ 50 g of fresh bread), which is far below the cutoff levels. An application of this concept in a more challenging bread system (e.g., based on whole rye and wheat) has not been described yet, but seems promising. The production of a low FODMAP bread from refined wheat flour using conventional bread-making techniques (with or without sourdough) seems rather not challenging. Different studies reported low fructan, mannitol, and fructose levels in refined wheat-based sourdough breads prepared with varying combinations of homo- and heterofermentative LAB (Fang *et al.*, 2021; Menezes *et al.*, 2019; 2021; Schmidt & Sciurba, 2021). In contrast, the synergistic effect of *Lactiplantibacillus plantarum* and baker's yeast was shown to efficiently lower fructan contents in whole wheat sourdough breads (< 0.3 % DM). When rye flour was used, however, remaining fructan levels where > 2 % DM (other FODMAPs were not reported but may have been high in both sourdough breads). Also different whole wheat- or rye-based sourdough breads made with baker's yeast and commercial sourdough cultures (with the yeast *Candida milleri* and hetero-fermentative *F. sanfranciscensis*) were reported to contain fructan and/ or excess

fructose and mannitol levels exceeding the cutoff levels (Schmidt & Sciurba, 2021). Another recent study reported particularly high mannitol levels > 2 % in sourdoughs prepared with a commercial starter culture (Pitsch *et al.*, 2021).

Further studies should focus on applying the above-described targeted concepts, investigate synergistic effects of purified enzymes and potent yeast and LAB strains, and explore possibilities for their application in different formulations of bakery products, especially those containing high portions of whole rye flour or fortified products with pulses. Currently available commercial products with a LF claim, which were categorised as LF due to the use of sourdough technology, included 12 breads, 2 pizza bases, and 2 pasta products, while only 3 of the products contained a small portion of rye flour (Figure 2-3 and Figure 2-4).

2.4.4.3 Activation of endogenous enzymes

Endogenous seed enzymes can act to hydrolyse storage carbohydrates and are activated or synthesized during the germination process of seeds. Low temperatures and high moisture (condition achieved by soaking of grains, called imbibition) initiate the germination process and stimulate the production of plant hormones, which in turn stimulate the production of endogenous enzymes (Bewley *et al.*, 2013). These modify the seeds' composition and nutritional value, which is important for malted or sprouted ingredients, used for brewing purposes, as functional ingredients in bakery products, and fresh sprouts for direct consumption. The germination process positively contributes to the flavour and increases bioactive compounds and minerals in cereals, pseudo cereals, and pulses (Kaukovrita-Norja *et al.*, 2004; Mäkinen & Arendt, 2015). Although with the correct use of terminology, the germination is terminated with the protrusion of the seedcoat by the radicle (Bewley *et al.*, 2013), most literature includes observations made during the following stage of the seedling growth when referring to the germination process. Hence, the impact of germination on FODMAP carbohydrates discussed in the following includes both the actual germination and the following seedling growth stage.

During the germination process, α-galactosidase activities, which act to hydrolyse the indigestible linkages in raffinose, stachyose and verbascose, are increased (Blöchl *et al.*, 2008; Lien *et al.*, 2018; Reddy & Salunkhe, 1980). The potential of germination to decrease GOS levels in a wide variety of pulses has been extensively studied and

proven to be effective in up to the complete removal of these saccharides, as reviewed by Nyyssölä *et al.* (2020). While germination under light and dark conditions does not seem to affect the GOS degradation (Vidal-Valverde *et al.*, 2002), the extent of the degradation directly correlates with the germination time, as the content of oligosaccharides constantly decreases (Wang *et al.*, 1997). Germination has also been shown to be effective in the reduction of GOS in cereals and pseudo cereals, as well as other α-galactosides, such a fagopyritols in buckwheat (Gamel *et al.*, 2006; Harris & MacWilliam, 1954; Horbowicz *et al.*, 1998; Jia *et al.*, 2015; MacWilliam *et al.*, 1956). Despite a large number of studies reporting a significant reduction of GOS upon 2 − 6 days of germination, Tuck *et al.* (2018) observed elevated GOS levels in sprouted chickpeas. Germinating seeds contain anabolic as well as catabolic enzymes, and the GOS-catabolism typically predominates. But if the germination process is impaired, for example due to desiccation, GOS biosynthesis may be initiated (Blöchl *et al.*, 2008). This highlights the importance of controlled germination conditions to achieve the desired degradation. Ultimately, sprouted or malted pulse ingredients may serve as valuable ingredients in low FODMAP formulations to increase the nutritional value of cereal-based products, but also for the direct consumption as fresh sprouts on salads or cooked pulses in meals. The enzymatic degradation during germination combined with the physical removal of GOS during cooking is particularly powerful for GOS reduction. However, the latter applications require further studies to clearly define the germination conditions needed to achieve sufficiently low GOS levels in different kinds of pulses, which could then be indicated on the product packaging for the consumers.

In contrast to the well-studied metabolism of GOS during the germination of different seeds, biochemical changes concerning the fructan metabolism during the germination of cereals have received much less attention. Still, a few studies reported a clear trend of fructan development during the malting process (Cozzolino *et al.*, 2016; Harris & MacWilliam, 1954; Ispiryan *et al.*, 2021b; Krahl *et al.*, 2008; MacWilliam *et al.*, 1956), supposedly also controlled by different anabolic and catabolic enzymes of the fructan metabolism in plants (invertases, fructan hydrolases, fructosyltransferases), as discussed in our recent study (Ispiryan *et al.*, 2021b). An initial, slight decrease during the imbibition (steeping) is followed by a significant increase in fructans upon 3 to 9 days of germination, followed by partial decomposition during the kilning (final drying

step of the process). Green malts (sprouted grains before kilning) from barley, wheat and spelt were shown to contain 30 – 300 % higher fructan levels than the raw grains, depending on the duration of the germination time (Harris & MacWilliam, 1954; Krahl *et al.*, 2008; MacWilliam *et al.*, 1956). Germinated oat contained even 1.4 % DM *de novo* synthesized fructan, while the raw grains contained only traces (Ispiryan *et al.*, 2021b). Despite the decrease during the kilning step (up to ~ 50 %), the fructan content of the malts from barley, spelt and wheat were reported to be still 10 − 60 % higher compared to the raw material, and oat malt still contained 0.8 % DM fructans (Cozzolino *et al.*, 2016; Ispiryan *et al.*, 2021b; Krahl *et al.*, 2008; MacWilliam *et al.*, 1956). Although Krahl *et al.* (2009) reported no significant changes in the fructan content in malted barley, this may be explained by the impact of kilning which may have compensated for an increase during the germination. In clear contrast to this stand the findings of Tuck *et al.* (2018); they reported lowered fructan levels in sprouted wheat, barley, and rye grains. However, as the results are reported based on the fresh weight, and sprouts contain approx. 50 % water, compared to dry seeds which contain 10 – 15 % water, this effect might be, at least partly, explained by dilution of the fructans. Nonetheless, further studies should investigate whether variations in the germination process (e.g., imbibition time and conditions, exposure to light) can shift the metabolism of fructans towards a prevalence of the catabolism. Indeed, two commercially available breads with a LF claim were solely made from sprouted grains from spelt and kamut (Figure 2-3). Both types of grains have been shown to contain comparable fructan levels to bread wheat (Gélinas *et al.*, 2016; Longin *et al.*, 2020; Ziegler *et al.*, 2016). Hence a degradation of the fructans during the sprouting process is likely and deserves more scientific attention.

2.5 Food science and regulatory mechanisms facing limitations with current view of FODMAPs and future perspectives

Over the past few years, substantial research efforts have already revealed promising and powerful tools for the production of low FODMAP functional products. Those are based either on avoiding high portions of FODMAP-rich ingredients in product formulations or on biotechnological FODMAP reduction strategies mediated by added enzymes, microbial enzymes during a fermentation process, and endogenous enzymes during a germination process. Further studies should focus on combined approaches (i.e., replacement of parts of high FODMAP ingredients, added enzymes, potent yeast,

and LAB species) and investigate the different synergistic effects in a larger variety of products and product formulations (i.e., including different whole grains and pulses in bakery products, pasta, extruded products). Moreover, an industrial-scale production of low FODMAP processing ingredients, including affordable commercial food-grade enzyme preparations with required functionalities, as well as potent yeast and LAB starter cultures, is necessary. Ultimately, alongside the effort to identify effective and reliable FODMAP reduction strategies, it should be emphasised that complete removal of FODMAPs is not desirable. Particularly fructans are important prebiotics and should only be reduced as much as required (Muir *et al.*, 2019). Varney *et al.* (2017) reported cutoff levels that were set conservatively, and their reliability was tested in a number of studies (Barrett *et al.*, 2010; Halmos *et al.*, 2014; Ong *et al.*, 2010).

Furthermore, it seems inevitable to address several factors that complicated the interpretability of current scientific literature. Studies investigating FODMAP reduction strategies often solely report results on the "main FODMAP". An in-depth understanding of the (FODMAPs) degradation mechanisms, the degradation products formed, and their consequences is the golden rule of any targeted biochemical modifications during food processing. Moreover, studies refer to various cutoff levels resulting from older studies where the currently known levels were not standardised and published yet. However, if the FODMAP concept is to be accepted based on scientific evidence and implemented in regulatory mechanisms and food production, the adaptation of the most recent and valid cutoff levels (Muir *et al.*, 2019; Varney *et al.*, 2017) must be acknowledged and applied consistently to analytical results based on the fresh weight of products as they would be consumed (not the dry weight). Importantly, accurate and suitable analytical approaches are necessary with clearly defined target analytes, minimal required detection limits and concomitant interferences and errors. Especially the sufficiently accurate analysis of fructans is challenging. To name a few factors: (1) Studies where fructan analysis was conducted using photometric enzyme assays tend to report very low values; it should be noted that those methods have relatively high detection limits (0.2 − 1 % depending on the assay used). This means that any values reported below those limits are somewhat not meaningful. (2) Several studies used the Megazyme Fructan HK assay kit (K-FRUCHK) to quantify fructans in cereals, pulses and products made from those. However, because high sucrose, fructose, glucose, and maltose levels in sample

extracts (such as it is the case for cereal-based products) result in high blank values, the accuracy of low fructan levels is strongly impaired. Hence, the Fructan assay kit (K-FRUC) which eliminates absorbance from non-fructan derived reducing sugars with the inclusion of a borohydride reduction step, is better suitable for such samples. (3) Regardless of which photometric enzyme assay kit or other enzymatic approaches are used, it important to consider that the fructan degrading enzyme preparation (exo-, and endo-inulinases) also releases fructose from raffinose, stachyose and verbascose (Ispiryan *et al.*, 2019); hence, as advised in the manufacturer's protocols for the assay kits, an additional α-galactosidase treatment of material that contains these sugars is essential to not overestimate the fructan content.

Lastly, in close cooperation of biomedical and food scientists, further research is necessary to elaborate on carbohydrates, comprised under the acronym FODMAPs. As highlighted in two recent reviews, the typically listed group of FODMAP carbohydrates includes the vast majority of such but is not limited to them (Gibson *et al.*, 2020; Halmos & Gibson, 2019). So *et al.* (2020) recently investigated the *in vitro* fermentability, and hence "FODMAP-potential" of xylo-oligosaccharides (XOS) derived from corn and almond shell and reported a high fermentability of the corn-derived XOS (similar to fructans) and a low fermentability of the almond shell derived XOS. Furthermore, they also identified a rapid fermentability of a carrot peel derived mixture of cellulose, hemicellulose, and pectin. Further *in vitro* and *in vivo* studies are needed to identify other carbohydrates with FODMAP potential. Relevant carbohydrates should be included into the routinely analysed list of FODMAPs, to enable the broadening of the knowledge on their natural occurrence and potentially incorporate them into food testing programs. Also the relevance of other passively absorbed monosaccharides and brush-border hydrolase deficiencies within the FODMAP concept, and a potential inclusion of affected highly abundant carbohydrates (such as sucrose) should be elucidated. The identification of other potential triggers may lead to an even higher success rate of the low FODMAP diet.

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Chapter 3

Optimisation and validation of a HPAEC-PAD method for the quantification of FODMAPs in cereals and cereal-based products

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3.1 Abstract

This study presents an analytical method for the quantification of fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs) in cereals and cereal-based products, considering diverse ingredients, such as different cereals in addition to wheat, pulses, or pseudo cereals. All carbohydrates have been separated, identified, and quantified with a high-performance anion-exchange chromatographic system coupled with pulsed amperometric detection (HPAEC-PAD). The total fructan content and the average degree of polymerisation (DP_{av}) have been determined after enzymatic hydrolysis to the monomers glucose and fructose, based on the principle of the official method for fructan quantification in food products, AOAC 997.08. The methods for extraction, separation, and detection as well as fructan determination are based on several other studies and were modified to minimise interferences in the analysis. The method has been validated regarding the limits of detection and quantification, the linearity, the repeatability, and the accuracy, as well as the DP_{av} of the fructans.

3.2 Introduction

Fermentable oligo-, di-, monosaccharides and polyols (FODMAPs) are carbohydrates often associated with symptoms of irritable bowel syndrome (IBS). They comprise α-galactooligosaccharides (GOS), fructans and fructooligosaccharides (FOS), lactose, fructose in excess of glucose and polyols. GOS (often in non-FODMAP related literature referred to as raffinose family oligosaccharides, RFO) are α-galactose derivatives (1 → 6 linked) from sucrose (α-glucose 1 → 2 linked to ß-fructose) and found especially in pulses, but also in different grains such as wheat, barley or rye (Biesiekierski *et al.*, 2011; Muir *et al.*, 2009). Fructans are composed of fructose-chains, containing one glucose residue. Depending on the fructan-source different types are classified. Inulin- and levan-type fructans are linear β (2 → 1) or β (2 → 6) fructosyl-fructose chains with one final glucose residue. The branched group, called graminan-type fructans, contains both types of linkages (β (2 → 1) and β (2 → 6)) and is commonly found in cereals. As humans do not possess the enzymes α-galactosidase or fructanases, fructans as well as GOS are not digested in the small intestine, delivered to the large intestine and fermented by colonic bacteria, inducing gas formation (Gibson & Shepherd, 2005). Also the β (1 → 4) linked galactosyl-glucose disaccharide lactose is not tolerated by some individuals because of the lack of the enzyme lactase. Lactose is the major FODMAP in dairy products. If the monosaccharide fructose appears in excess to glucose, it is not absorbed sufficiently (Gibson & Shepherd, 2010). This may be relevant in fermented cereal-based products, depending on the fermentation conditions (Ziegler *et al.*, 2016). Finally, polyols, which are reduced forms of sugars in their chemical structure, also called sugar alcohols, are poorly absorbed and may also be found in fermented cereal-products (Loponen & Gänzle, 2018).

Studies have shown that a reduction in the intake of FODMAPs (the low FODMAP diet) is an effective therapeutic approach for reducing IBS-symptoms (Halmos *et al.*, 2014; Staudacher *et al.*, 2011). Hence, current research has increasingly focused on the development of functional food products with lowered FODMAP contents; although a standardised analytical tool for the determination of FODMAPs in diverse food matrices is required (Loponen & Gänzle, 2018; Méance *et al.*, 2017; Menezes *et al.*, 2018). This study aimed to develop an analytical method for the quantification of FODMAPs in cereals and cereal-based products, considering the composition of the

products may be very diverse, particularly owing to the use of specific novel ingredients with beneficial techno-functional or nutritional properties.

The successfully applied analytical methodology described by Muir *et al.* (2009) comprises liquid chromatographic separation coupled with evaporative light-scattering detection (HPLC-ELSD) for the quantification of the smaller FODMAPs; this is combined with the photometric determination of glucose and fructose after enzymatic hydrolysis of fructans with an enzyme assay (K-FRUCHK from Megazyme), a modified version of the original AOAC method 999.03 (K-FRUC from Megazyme) (McCleary *et al.*, 2000). However, these two photometric methods do not deliver information about the average degree of polymerisation (DP_{av}) of fructans. Knowledge about chain length is important as putative beneficial properties or induction of IBS symptoms may relate to the DP of fructan molecules (Rumessen & Gudmand-Høyer, 1998). Muir *et al.* (2007) proposed a modification of the K-FRUCHK assay, that enables an estimation of the DP_{av}. However, the photometric determination has a low analytical sensitivity and thus high detection limits, leading to unreliable results if fructan levels are below 1 % on dry weight basis. Furthermore, the combination of different analytical methods may be laborious for many samples. Also, both fructan assays must be amended with the additional GOS correction step (incubation of the sample extracts with α-galactosidase) as fructose released from GOS by inulinases, included in the assay, leads to an overestimation of the total fructan content (McCleary *et al.*, 2019), thus increasing the complication of the assays. Another study conducted by Chumpitazi *et al.* (2018) laboriously quantified all FODMAPs using a number of different enzyme assay kits.

High-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) has increasingly gained popularity for the analysis of carbohydrates because of its ability to separate different classes of carbohydrates (sugar alcohols, mono-, di- and oligosaccharides as well as polysaccharides) and its high sensitivity (Corradini *et al.*, 2012).

Ziegler *et al.* (2016) proposed an analytical method based on HPAEC-PAD for the quantification of FODMAPs in wheat. However, their approach for the quantification of fructans is not suitable if fructan sources other than wheat or a combination of different sources (diverse recipes of cereal-based products) are considered. Generally,

there are two approaches for quantifying fructans, classified as “direct” and “indirect” methods by Stöber *et al.* (2004). The direct method comprises the evaluation of each peak in the chromatogram belonging to fructan molecules of different chain lengths and linkages. The applicability of this approach is limited due to several factors. On the one hand, only few reference standards of fructans are available to ensure an accurate quantification. On the other hand, the identification and assignment of peaks as fructans for different matrices may be very laborious and have interference from oligo- and polysaccharides other than fructans. Thus, for example, Haskå *et al.* (2008) hydrolyzed sample extracts with the enzyme amyloglucosidase, to remove coeluting malto-dextrins from the fructans’ fingerprint. However, the fingerprint may be very different for varying fructan sources and dependent on storage conditions or processing of the fructan containing material; this requires a tedious characterisation of each material.

Hence, the indirect approach for the determination of the total fructan content is simpler and considered as more reliable. Thereby, fructans are hydrolysed to their monomers fructose and glucose. The amounts of those sugars are used for the calculation of the total fructan contents and the DP_{av}. Two different approaches are described: the mild acid and the enzymatic hydrolysis of the fructans (AOAC 997.08; Haselberger & Jacobs, 2016; Huynh *et al.*, 2007; Stöber *et al.*, 2004; Verspreet *et al.*, 2012). This study applied an adapted version of the method described by Huynh *et al.* (2007) for the quantification of fructans after extraction and separation of all FODMAPs via HPAEC-PAD using a modified version of the method described by (Ziegler *et al.*, 2016).

3.3 Materials and methods

3.3.1 Materials

Ultrapure water, with a resistivity of 18.2 MΩ·cm and a total organic carbon (TOC) content < 5 ppb (ASTM Type I), used for the preparation of HPAEC-PAD eluents, all standard solutions, and sample preparation, was obtained from a Thermo Scientific™ Dionex™ IC Pure™ Water purification system (Sunnyvale, CA, USA). Extra pure, 50 % (w/w) sodium hydroxide (NaOH) solution (in water) was purchased from Thermo Fisher Scientific (ACROS Organics™; Dublin, Ireland). Electrochemical-grade sodium acetate (NaOAc) was purchased from Thermo Scientific™ (Dionex™ AAA-Direct Reagents; Dublin, Ireland). HPLC-grade acetonitrile (CH_3CN) as well as methanol (MeOH) were purchased from Sigma-Aldrich (Darmstadt, Germany) and glacial acetic acid (AcOH) was from Fisher Scientific (J.T. Baker™; Loughborough, UK). Sodiumazide (NaN_3) was obtained from Thermo Fisher Scientific (Alfa Aesar; Lancashire, UK). Potassium hexacyanoferrate (II) trihydrate (Carrez I, $K_4[Fe(CN)_6]\cdot 3H_2O$) and zinc acetate dihydrate (Carrez II, $Zn(OAc)_2\cdot 2H_2O$) were purchased from Sigma-Aldrich (Darmstadt, Germany). D-Chiro-inositol was purchased from Carbosynth (Compton, UK). The fructooligosaccharides 1-kestotriose (1-kestose), 1,1-kestotetraose (nystose), and 1,1,1-kestopentaose (1F-fructofuranosyl-nystose) were purchased from FUJIFILM Wako Pure Chemicals (Neuss, Germany); verbascose was from Megazyme (Bray, Ireland); glucose, galactose, fructose, sucrose, melibiose, lactose monohydrate, raffinose pentahydrate, stachyose tetrahydrate, and the sugar alcohol kit (arabitol, dulcitol, erythritol, mannitol, maltitol, adonitol, xylitol, sorbitol) were from Sigma-Aldrich (Darmstadt, Germany). All carbohydrate reference standards were of > 98 % purity, except for 1,1,1-kestopentaose (80 % purity). Amyloglucosidase (E-AMGFR), α-galactosidase (E-AGLANP), inulinase (E-FRMXPD), the fructan assay kit (K-FRUC), and the α-amylase assay kit (K-CERA) were purchased from Megazyme (Bray, Ireland).

3.3.2 HPAEC-PAD

Separation and quantification of all carbohydrates was performed on a Dionex™ ICS-5000^+ system (Sunnyvale, CA) equipped with a SP Single Pump (analytical gradient pump), AS-AP Autosampler, a 10 µL injection loop (full loop injection used) and ED Electrochemical Detector cell with a conventional gold working electrode and a PdH

reference electrode. The gold carbo quad waveform, which was shown to be most suitable for reproducible results in the analysis of carbohydrates, was applied (Rohrer, 2013). The pulsed potential starts with a period (0.2 s) that allows the charging current to decay at +1.05 V, followed by a detection period (0.2 s) measuring the current from the analyte oxidation at 1.05 V. This is followed by reductive cleaning at –1.05 V (0.01 s), activation and further cleaning of the working electrode surface by Au-oxide formation at +1.55 V (0.01 s), and reduction at +0.85 V (0.06 s).

The eluents, ultrapure water (line A), 225 mM NaOH (line B) and 500 mM NaOAc (line C; vacuum filtered through a 0.2 µm filter), and the syringe wash solution 5 % CH_3CN (line D) were kept under N_2 atmosphere using a direct connection to a Peak Scientific (Inchinnan, UK) Corona Air Compressor and Corona Nitrogen Generator (constant pressure of 4.5 – 6 psi). The separation of mono- and disaccharides, fructans and GOS was performed on a Thermo Scientific™ Dionex™ CarboPac™ PA200 analytical column (3 × 250 mm) with the corresponding guard column applying gradient elution, according to Ziegler *et al.* (2016) with some modifications. This column is specifically applied for the separation of oligo- and polysaccharides; thus it does not fully separate some simple sugars (Corradini *et al.*, 2012). Sugar alcohols; glucose and galactose; and melibiose and fructose coeluted on that column. Their separation was achieved using a Thermo Scientific™ Dionex™ CarboPac™ PA1 analytical column (2 × 250 mm) with the corresponding guard column (hereafter referred to as CarboPac PA1 or CarboPac PA200, respectively), applying an isocratic elution with 18 mM NaOH. The compositions of the mobile phases for the chromatographic methods on both columns are presented in Table A-1 (Appendix A-1). Separation and detection were carried out at 25 °C and 0.25 mL/ min flow rate. The columns were washed with 500 mM NaOAc followed by 225 mM NaOH after each separation run; the latter condition also promoted the removal of potential carbonate contamination from the column and a clean-up of the working electrode surface, avoiding a loss of reproducibility in peak area due to oxidised products on the detector surface.

3.3.3 Sample preparation and FODMAP extraction

Whole wheat flour (Odlums, Ireland), rye flour (Thylmann, Germany) and wheat starch (Roquette, France) were used for experiments as supplied. Whole wheat grains

(locally sourced) were ground with a QIAGEN Tissue Lyser II (Hilden, Germany) to a particle size of ≤ 0.5 mm. Baked products, including bread and biscuits, and cooked pasta were freeze-dried and ground to a fine powder using the Tissue Lyser. The extraction of the carbohydrates was based on the method described by Ziegler *et al.* (2016) with different changes and supplementations deduced from the official method for fructan analysis in food products (AOAC 997.08) and Huynh *et al.* (2007). An aliquot of 400 ± 0.5 mg of flour or powder from the lyophilised product was mixed thoroughly with 1 mL of MeOH and left for 5 min in a closed reaction tube, to inactivate interfering native enzymes from the samples, such as α-amylases from cereals. Subsequently, 100 µL of internal standard rhamnose (9 mg/ mL) and 20 mL of 80 °C H_2O containing 50 mg/ L NaN_3 were added, and the mixture was subjected to the first extraction step using a BANDELIN Sonoplus HD 3100 homogeniser (Berlin, Germany) equipped with a MS73 microtip and operated at 75° amplitude for 2 × 15 s. Hot H_2O was used to enhance the solubilisation of fructans and to denature native cereal enzymes. The intermixture of NaN_3 prevented the microbial degradation of carbohydrates during sample preparation, storage, and analysis at room temperature. After centrifugation at 1520 *g* for 5 min, the supernatant was transferred into a 100 mL volumetric flask and the extraction was repeated with 20 mL 80 °C H_2O (containing 50 mg/L NaN_3). The supernatants were combined, cooled to room temperature, and the proteins were precipitated by adding 200 µL Carrez I (15 g/ 100 mL) and Carrez II (23 g/ 100 mL), respectively. After adjustment to 100 mL, the extract was centrifuged at 3000 *g* for 10 min and filtered through 0.2 µm syringe driven polyamide filter (Chromafil AO-20/25, Machery Nagel, Düren, Germany). If extracts were frozen prior to analysis, they were reheated to 80 °C in a Stuart Scientific SHT 1D test tube heater (UK) to redissolve precipitated fructans. Samples were extracted in at least duplicates, unless otherwise stated.

3.3.4 Identification of Carbohydrates

The retention times of the reference standards and the peaks in the chromatograms were compared. Additionally, sample extracts were spiked with reference standards. Furthermore, enzymatic degradation trials with α-galactosidase, inulinase, and amyloglucosidase were conducted to confirm the identification and the purity of peaks (not all data shown).

3.3.5 Quantification of mono-, di-, oligosaccharides and polyols

Extracts were diluted and analysed via HPAEC-PAD. The quantification of the carbohydrates was conducted using mixtures of the reference standards in the ranges between 0.1 – 1 mg/ L and 1 – 20 mg/ L. The software Chromeleon 7.2 was used for data acquisition and processing. Analytical results were calculated to g analyte per 100 g dry matter of the sample. Therefore, moisture contents were determined according to AACC 44-15.02.

3.3.6 Determination of total fructan content and DP_{av}

The procedure for the enzymatic hydrolysis for fructan quantification was based on the method described by Huynh *et al.* (2007) and the principle of AOAC 997.08 Official Method for fructan analysis in food products. Two 500 µL aliquots of the diluted sample extract (usually 5- to 20- fold dilution, taking into consideration the substrate-to-enzyme ratio and the high glucose amounts resulting from the glucose released by amyloglucosidase from coextracted starch and dextrins) were subjected to two separate enzymatic treatments (with enzyme mixtures A and B). The enzyme mixtures (150 µL) were added to the samples. Enzyme mixture A contained a 1:1:1 mixture of amyloglucosidase, α-galactosidase, and 0.1 M NaOAc-buffer; the latter was replaced by inulinase in mixture B. The lyophilised enzymes were diluted in 0.1 M NaOAc-buffer at pH 4.5 (prepared according to AOAC 997.08) to 220 U/ mL, resulting in 11 U/150 µL for each enzyme in the mixtures A and B. The reaction mixtures were incubated in a water bath in 2 ml screwcap microtubes for 30 min at 60 °C. Subsequently, the enzymes were inactivated in a test tube heater at 100 °C for 40 min. After the solution cooled to room temperature, 350 µL H_2O were added to the hydrolysate to bring the volume to 1 mL. The precipitated enzymes were removed by centrifugation at $10{\cdot}000\ g$ for 2 min. The hydrolysates were measured via HPAEC-PAD with the CarboPac PA200 column. The total fructan content and the average degree of polymerisation (DP_{av}) were calculated on the basis of the results of free (hydrolysate A) and released (hydrolysate B) glucose, fructose and sucrose, based on Huynh *et al.* (2007). The concentrations of glucose (G_f) and fructose (F_f) released from fructans were calculated according to equations eq 1 and eq 2, where $G_{A/B}$, $F_{A/B,}$ and S_A are the determined glucose, fructose, and sucrose concentrations from hydrolysates A and B (µmol/ L); 180.16 is the molecular weight of glucose or fructose; DF is the dilution factor (i.e., dilution factor from extract dilution before hydrolysis multiplied

by 2 from dilution of hydrolysate to 1 mL); V_E is the extract volume (100 mL) and M_S is the sample mass (400 ± 0.5 mg).

$$G_f[\%] = \frac{(G_B - G_A - S_A) * 180.16 * DF * V_E}{10\,000 * M_S} \quad \text{eq 1}$$

$$F_f[\%] = \frac{(F_B - F_A - S_A) * 180.16 * DF * V_E}{10\,000 * M_S} \quad \text{eq 2}$$

The DP_{av} is calculated according to eq 3. The fructan content is finally calculated according to eq 4, and k (eq 5) is the water correction factor (water uptake during hydrolysis), which is dependent on the chain lengths of fructan molecules. This calculation is suitable for native cereal fructans, such as graminan- or neolevan-type fructans, with one glucose residue in each fructan molecule.

$$DP_{av} = \frac{F_f}{G_f} + 1 \quad \text{eq 3}$$

$$k = \frac{180 + 162 * (DP_{av} - 1)}{180 * DP_{av}} \quad \text{eq 4}$$

$$Fructan\ [\%] = k * (G_f + F_f) \quad \text{eq 5}$$

The calculation of fructan concentrations for partially hydrolysed fructans is slightly different and does not deliver information about the average degree of polymerisation. If longer chains of fructans are partially hydrolysed not every shorter chain will contain a glucose residue, as the native fructans contain only one glucose moiety. However, eq 3 presumes one glucose residue per fructan molecule. Thus, the average degree of polymerisation would be overestimated if most molecules are FOS without glucose. Subsequently, the total amount of fructan would be underestimated. This error can only be eluded if the average degree of polymerisation is known or can be estimated, enabling a more accurate calculation of the water correction. As fructan (inulin or FOS) isolates are often food additives, their structures are well-known. A FOS standard with a DP_{av} of $2-8$ was analysed in three different concentrations in duplicates and supported this hypothesis (data not shown). If information about the average degree of polymerisation is not available, this can be obtained using the fructan fingerprint in the chromatogram of a sample hydrolysed with amylo-glucosidase, as described by Haskå *et al.* (2008) and Nemeth *et al.* (2014).

3.3.7 Validation

The HPAEC-PAD method for the quantification of FODMAPs has been validated regarding the limits of detection and quantification (LOD and LOQ), linearity, repeatability, and accuracy. Furthermore, the determination of the average degree of polymerisation of the fructans in the wheat matrix was validated according to Verspreet *et al.* (2012). Therefore, different spiking experiments and replications of extractions with wheat wholemeal flour and wheat starch were conducted.

Wholemeal flour was spiked with the reference standards in five different concentrations (0.5 – 12 mg/ L). Each level was spiked and extracted in a triplicate. The LOD and LOQ were determined with signal to noise ratios (S/N) of 3 and 10, respectively, from the analytes in the matrix. The wholemeal flour was extracted and analysed in six replicates and in two additional duplicates by three different analysts, indicating the repeatability of the method. For the validation of the DP_{av} determination of fructans, wheat starch was spiked with the reference standards kestose (DP 3) and nystose (DP 4) in four different concentrations in triplicates (0.1 − 0.7 % based on the weight of wheat starch); additionally, 0.2 % raffinose and 0.4 % sucrose were added to mimic interfering components from wheat.

3.3.8 Total fructan quantification via enzyme assay

The Megazyme fructan assay kit, K-FRUC, was used as a reference method for the determination of the total fructan content. The interference of GOS (mentioned in the assay procedure as raffinose family oligosaccharides, RFO) was taken into account by incubation of the sample extracts with α-galactosidase prior to the degradation of starch, malto-dextrins, and sucrose, as described in the controls and precautions of the assay procedure.

3.3.9 Determination of α-amylase activity

The impact of the extraction temperature and of the initial addition of MeOH (cf. the procedure for FODMAP extraction) on the activity of the native enzyme α-amylase in wheat was investigated using the Ceralpha Method with the Megazyme α-amylase assay kit. The enzyme extraction procedure was modified to obtain indications about the impact of the extraction temperature and the addition of MeOH. The α-amylase activity was determined after enzyme-extraction according to the assay manual.

Furthermore, the activities were determined after extraction with heated (80 °C) extraction buffer, with a mixture of extraction buffer and MeOH at room temperature, and finally with a heated (80 °C) mixture of MeOH and buffer.

3.3.10 Statistical analysis

The statistical evaluation of the validation experiments and the investigation of the suitability of linear and quadratic regression as calibration models, applying Mandel's fitting test, were carried out with MS excel 2010. For comparison of the fructan contents obtained via HPAEC-PAD determination and enzyme assay kit, an independent t-test was carried out ($p = 0.05$) with SPSS Statistic 26 (IBM Corp.,323 Armonk, NY, USA).

3.4 Results and discussion

3.4.1 Sample preparation- extraction of FODMAPs

For the extraction of carbohydrates from cereals, different extraction media are described in literature. For instance, Haskå *et al.* (2008), Pico *et al.* (2015), and Verspreet *et al.* (2012) tested H_2O and EtOH as pure extraction agents and in different combinations. Although, the use of 80 % EtOH is known to be advantageous in minimising the coextraction of starch, it leads to an incomplete extraction of fructans (Haskå *et al.*, 2008). Also 10 % EtOH and 90 % EtOH have been shown to have a lower extraction efficiencies for different mono-, di-, and oligosaccharides in comparison to 100 % H_2O (Pico *et al.*, 2015). To achieve complete extraction of carbohydrates, especially the higher-DP fructans, a second extraction step of the remaining pellet from the EtOH extraction, using H_2O as extraction medium is necessary (Haskå *et al.*, 2008; Verspreet *et al.*, 2012). Before the supernatants can be combined, the EtOH must be fully evaporated, and the residues must be recovered in aqueous solution, particularly if enzymatic hydrolysis is applied for fructan analysis. The simplest efficient extraction medium appears to be 100 % H_2O. Verspreet *et al.* (2012) showed no significant difference between the fructan results obtained from EtOH/ H_2O supernatants compared to those from only H_2O supernatants. However, their method applies mild acid hydrolysis for the quantification of fructans, which is less sensitive to the interference of coextracted starch. Stöber *et al.* (2004) showed a strong interference from starch in fructan analysis, leading to an overestimation of the glucose amount released from fructans (G_f, cf. eq 1 and eq 5) as commercial fructan degrading enzyme preparations release glucose from non fructan compounds, such as starch and malto-dextrins (Stöber *et al.*, 2004). This inaccuracy can be conquered if a starch-degrading enzyme (amyloglucosidase) is included, as described in the method of this study.

Another important parameter is the temperature of the extraction medium. Although smaller carbohydrates are easily extracted at room temperature, fructans, especially those with longer chains, solubilise better at higher temperatures (Haskå *et al.*, 2008). Most methods described for fructan analysis extract at 80 °C (AOAC 997.08; Haskå *et al.*, 2008; Huynh *et al.*, 2007; McCleary *et al.*, 2000; Rakha *et al.*, 2010; Stöber *et al.*, 2004; Verspreet *et al.*, 2012). Extractions with heated and room-temperature H_2O

were compared in preliminary work for this study, using the K-FRUC assay. Only 70 % of the fructans extracted with hot H_2O could be extracted with room-temperature water (data not shown). Furthermore, the use of hot water inactivates interfering native enzymes of the sample (Verspreet *et al.*, 2012; Ziegler *et al.*, 2016). This effect could be enhanced with the suspension of the sample in MeOH prior to extraction. The analysis of the native α-amylase activity (K-CERA) in commercial whole wheat flour and milled wheat grains, after extraction with a MeOH/ extraction buffer mixture at room temperature and heated (80 °C) extraction buffer and the mixture, confirmed this assumption (Figure A-1, Appendix A-1).

The determined α-amylase activities were in line with the glucose amounts in the commercial whole wheat flour, obtained from the HPAEC-PAD profiles of the extracts with the different conditions. Furthermore, the amounts of fructose, glucose, and sucrose gave indication of sucrose-hydrolysing invertase activity (potentially from microbial contamination, as preliminary trials did not include NaN_3 addition). Samples extracted at room temperature, without the addition of MeOH contained the highest amounts of glucose and fructose and the lowest amounts of sucrose, whereas the addition of MeOH and extraction with H_2O heated to 80 °C led to the lowest levels of glucose and fructose and the highest sucrose levels. The values obtained from the extraction at room temperature with the addition of MeOH were in between the amounts described above. This indicates that the hot extraction medium enhances enzyme inhibition. The high difference in the glucose amounts derived from the different extraction conditions compared with the differences in the sucrose and fructose amounts evidenced the higher amylase activity in samples extracted at room temperature without the addition of MeOH (Figure A-2, Appendix A-1). This overestimation leads to misinterpretation of the ratio of glucose to fructose, which is important for the determination of FODMAPs, as fructose can act as a FODMAP if it occurs in higher levels than glucose in the consumed product (Gibson & Shepherd, 2005). However, it should be kept in mind that using H_2O as extraction medium for samples which are high in starch, will lead to coextraction of the starch; the partial hydrolysis can be minimised by the actions undertaken in this study but not fully excluded.

3.4.2 Identification of FODMAPs

The identification of the carbohydrates separated on both columns (CarboPac PA200 and CarboPac PA1) is shown in Figure 3-1 with wheat wholemeal extract as an example.

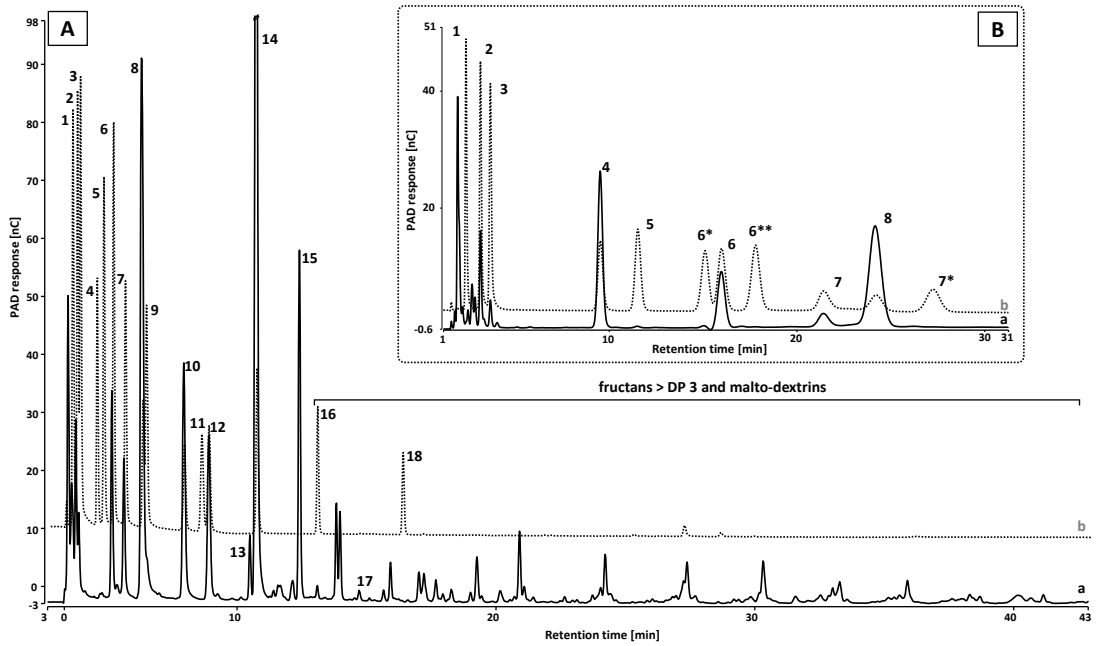

Figure 3-1. HPAEC-PAD (A) CarboPac PA200 and (B) CarboPac PA1 profiles of (a) wheat wholemeal extract (b) standard mixture: (1) xylitol, (2) sorbitol, (3) mannitol, (4) rhamnose, (5) arabinose, (6*) galactose, (6) glucose, (6**) xylose, (7) fructose, (7*) melibiose, (8) sucrose, (9) lactose, (10) raffinose, (11) verbascose, (12) 1-kestose, (13) fructosylraffinose, (14) maltose, (15) unidentified fructan of DP3, (16) nystose, (17) maltotriose, and (18) 1,1,1-kestopentaose. Numbers marked with an asterisk (* or **) in the CarboPac PA1 profile are not separated from the corresponding numbers without the asterisk on the CarboPac PA200 profile.

All compounds in the HPAEC-PAD profiles were either identified by comparing the retention times of reference standards (pure and added to the sample matrix) or, if commercial standards were not available, by comparison with other studies and hydrolysis of sample extracts with different enzymes. Peaks in the chromatogram, which disappeared after incubation of the sample extracts with inulinase, could be assigned as fructans, sucrose, fructosylraffinose and GOS. The hydrolysis led to an increase of fructose and glucose and the appearance of the degradation products from fructosylraffinose, raffinose/ stachyose (not separated on CarboPac PA200, separation on CarboPac PA1 not shown) and verbascose, resulting in melibiose (confirmed with reference standard; separated on CarboPac PA1, coeluted with fructose on CarboPac PA200), manninotriose and manninotetraose, respectively (Figure 3-2). Incubations with α-galactosidase degraded GOS and fructosylraffinose and led to an increase of

glucose/ galactose (only separated on CarboPac PA1 column), sucrose, and kestose (Figure 3-2).

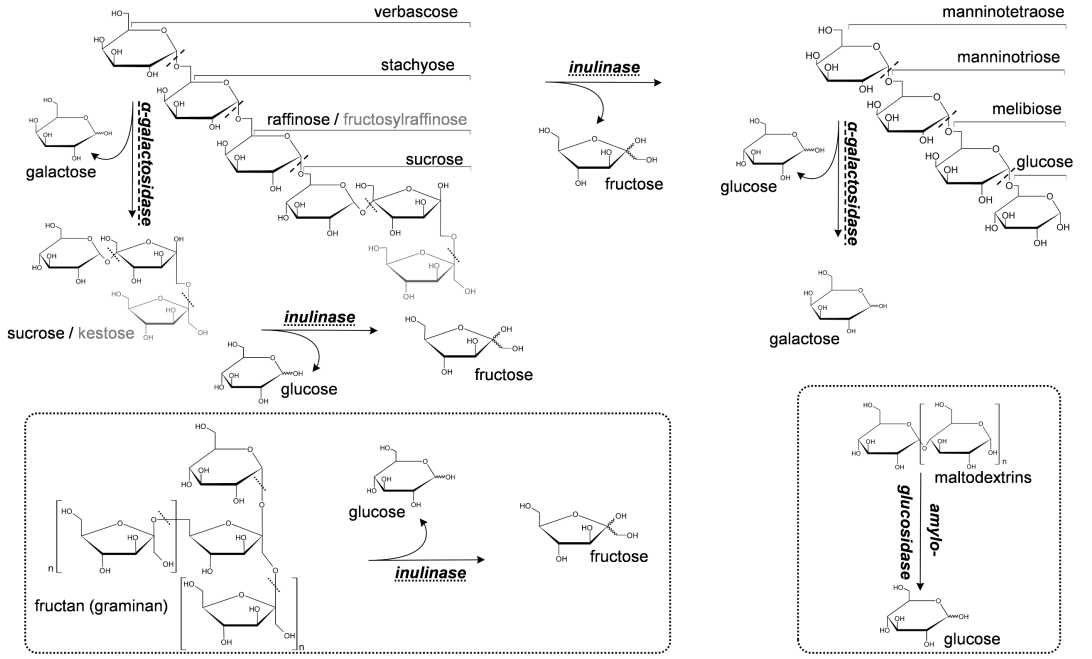

Figure 3-2. Enzymatic hydrolysis of fructans, GOS, and malto-dextrins; conducted with wheat extract and standard solutions to obtain additional information for peak identification.

Furthermore, incubation with amyloglucosidase degraded maltose, maltotriose and higher malto-dextrins from coextracted and partially hydrolysed starch (retention times on corresponding columns summarised in Table A-2, Appendix A-1).

These hydrolysis trials also gave indications about the purity of the peaks, excluding on the one hand coelution of other compounds with those of interest. On the other hand, it could be seen, in accordance with other studies, that higher-DP fructans and malto-dextrins, eluting in the same area on the CarboPac PA200 (after 13 min onward), are very difficult to assign clearly to one of those oligo- and polysaccharide groups (Haskå *et al.*, 2008; Rakha *et al.*, 2010). Hence, any quantitative or semiquantitative evaluation, including the fingerprint of the fructan profile or the integration of peaks assigned to one DP, can only be carried out if the sample extracts were initially incubated with amyloglucosidase, as described by Rakha *et al.* (2010). Furthermore, in various matrices from products containing different cereals, pseudo cereals and pulses as ingredients, the chromatographic profiles, especially in the higher-DP area, may be very difficult to identify (cf. Figure A-3, Appendix-A1, showing profiles from different typical cereal product ingredients). To evaluate peaks from that area further studies focused on the characterisation of the specific ingredients

would be required, such as those conducted by Haskå *et al.* (2008) or Rakha *et al.* (2010) for wheat and rye. Thus, this study approached the fructan quantification and determination of the DP_{av} after enzymatic hydrolysis to the monomers.

3.4.3 Validity of method

The method presented for the quantification of specific carbohydrates, considered as FODMAPs, was checked, and validated regarding its linearity, accuracy, repeatability, and limits of detection and quantification.

The linearity of a method refers to the whole method, including all steps of sample preparation. Hence, replicates of reference standard dilutions would not indicate the linearity of a method (Kromidas & Ermer, 2008). It describes the relation of analytical signal and concentration of analytes. However, the term linearity itself is misleading and might suggest a linear relation is required. In reality, the impact of sample matrix and the principle of analytical measurement often lead to the fact that a regression other than linear is required to describe the mathematical correlation of signal and concentration (Kromidas, 2006; Kromidas & Ermer, 2008). Regarding existing HPAEC-PAD methods for the quantification of carbohydrates, a quadratic function may reveal a better fit in a calibration model, depending on analytical system, sample type, and analytes investigated (Haselberger & Jacobs, 2016; Huang & Rohrer, 2016). In this study, the calibration model was evaluated using the Mandel's fitting test. The correlation coefficient of the calibration curve and visual comparison of linear and quadratic regression functions as well as residual plots (Figure 3-3 and Table 3-1) give first indication about the suitability of a regression model. If the mathematical model is chosen correctly, the residuals should be distributed normally around the zero level; in contrast, the pattern of residuals of the linear function is a sign for an incorrect model. Moreover, the Mandel's fitting test allows better evidence, determining a potential significant difference in residual variances from linear and quadratic calibration (Brüggemann *et al.*, 2006; Kromidas & Ermer, 2008).

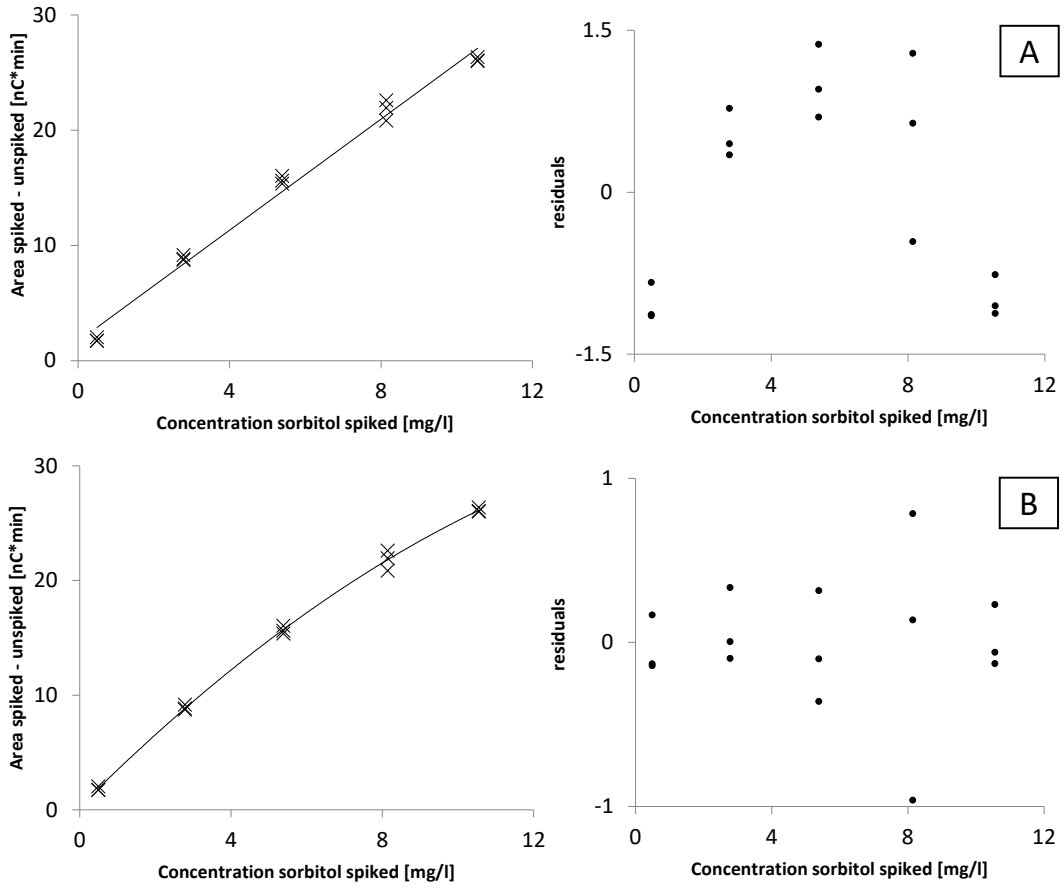

Figure 3-3. (A) Linear and (B) quadratic regressions with corresponding residual plots on the example of sorbitol spiked in the wheat matrix.

According to eq 6, the test variable (TV) is calculated and compared with the corresponding value of the F-distribution with 1 and n – 3 degrees of freedom at a significance level of $\alpha = 0.05$ ($F_{0.05,\,1,\,n-3}$).

$$TV = \frac{(n-2) * s_{y_1}^2 - (n-3) * s_{y_2}^2}{s_{y_2}^2} \qquad \text{eq 6}$$

$$s_{y_1} = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{n-2}} \qquad \text{eq 7}$$

$$\hat{y}_1 = a + bx$$

$$s_{y_2} = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{n-3}} \qquad \text{eq 8}$$

$$\hat{y}_2 = a + bx + cx^2$$

Hypothesis H_0 (i.e., no significant difference existed between the residual variances) was rejected if $TV > F_{0.05, 1, n-3}$. Residual standard deviations of linear and quadratic regressions (s_{y_1} and s_{y_2}) are calculated according to eq 7 and eq 8, respectively. As presented in Table 3-1, for all analytes of interest, a second-order polynomial equation was more suitable for describing the correlation of signal and analyte concentration (always TV > 4.6).

Table 3-1. Validation of the analytical method regarding LOD, LOQ, recovery, repeatability (RSD) and fit to a regression model (Mandel's test)

	LOD/ LOQ [a] [µg/ L]	$RSD_{interday}$/ $RSD_{intraday}$ [%] (n = 6) [b,c]	recovery [a] [%] (n = 3, m = 5) [c]	fit regression model [a]		
				R^2 linear/ R^2 quad [d]	s_{y_1}/s_{y_2} [e]	TV (0.05,1,12) [f]
xylitol	2 / 7	-	100.7 ± 1.5	0.979 / 0.998	1.15 / 0.34	131.67
sorbitol	2 / 3	6.6 / 4.6	100.1 ± 3.7	0.989 / 0.998	0.99 / 0.40	68.43
mannitol	1 / 2	3.8 / 4.0	97.4 ± 3.7	0.992 / 0.999	0.97 / 0.43	55.45
rhamnose	1 / 3	3.9 / 6.9	100.1 ± 1.2	0.995 / 0.999	0.57 / 0.30	33.93
glucose	1 / 3	2.3 / 10.3	96.3 ± 3.9	0.994 / 0.998	1.12 / 0.59	34.77
fructose	0.5 / 2	3.0 / 5.5	94.1 ± 4.5	0.997 / 0.998	0.27 / 0.19	13.13
lactose	1 / 6	-	100.8 ± 2.4	0.997 / 0.997	0.38 / 0.33	4.96
raffinose	$4 / 4 \times 10^1$	3.3 / 4.5	97.6 ± 5.3	0.989 / 0.990	0.28 / 0.24	5.91
verbascose	$1 \times 10^1 / 4 \times 10^1$	-	94.5 ± 2.9	0.992 / 0.992	0.15 / 0.13	4.91
kestose	$5 / 5 \times 10^1$	2.3 / 7.7	101.4 ± 5.7	0.989 / 0.991	0.38 / 0.30	8.71
nystose	$3 / 4 \times 10^1$	4.7 / 6.0	97.7 ± 2.2	0.998 / 0.998	0.13 / 0.11	5.28
kestopentaose	$1 \times 10^1 / 5 \times 10^1$	-	97.6 ± 1.8	0.998 / 0.998	0.09 / 0.08	5.79

[a] The limits of detection and quantification (LOD and LOQ), recovery (± standard deviation) and fit to a regression model were determined with data obtained from spiked wheat whole meal flour. Each compound was added at five levels between (0.5 – 12 mg/ L) in triplicate. [b] Replications of whole wheat flour extractions on the same day were used to determine the intraday repeatability; duplicates on three different days were used to determine the interday repeatability. [c] n, number of replications; m, number of spiking concentrations. [d] R^2, correlation coefficient. [e] s_{y_1} and s_{y_2}, residual standard deviations from the linear and the quadratic regressions, respectively. [f] TV, test variable by means of the Mandel's test. TV is compared to 4.6 resulting from the F-distribution, at α=0.05, for 1 and 12 degrees of freedom, to reject or accept the H_0 (no significant difference between the residual variances of the linear and quadratic regressions).

The LOD and LOQ of the method were in the ranges of 0.5 – 10 µg/ L and $2 – 5 \times 10^1$ µg/ L, respectively. In comparison with other analytical methods, HPAEC-PAD is known to be very sensitive; for instance, Muir *et al.* (2009) determined LOD of 0.05 – 0.1 g/ L for FODMAP quantification via HPLC-ELSD.

However, the main advantage is the more accurate and sensitive electrochemical fructan determination in contrast to photometric enzyme assays (Muir *et al.*, 2009; Verspreet *et al.*, 2012). Carbohydrates spiked in wheat wholemeal flour were recovered at 94.1 – 101.4 %, which indicated the good accuracy and selectivity of the method. The analysis of wheat flour replicates on the same day and on different days revealed an acceptable reproducibility of the method, with RSDs in the range of 2.3 – 10.3 %. The highest variation in the results was observed for the analysis of glucose in the replicates on different days, conducted by different analysts. As discussed above, this deviation is due to partially hydrolysed, coextracted starch.

To estimate the validity of the method for determining the DP_{av} of fructans, wheat starch was spiked with different concentrations of a kestose (DP 3) – nystose (DP 4) mixture, sucrose, and raffinose (to mimic the natural interference in wheat).

The DP_{av} determined for the rye flour, whole wheat flour, and for the inulin reference standard from the K-FRUC assay (Table 3-2) were in accordance with those from other studies (Rakha *et al.*, 2010; Verspreet *et al.*, 2012). The DP_{av} resulting from the wheat starch spiking experiment was close to the actual DP_{av} of kestose and nystose (Table 3-2), indicating a good suitability of the method for calculating the DP_{av} after enzymatic hydrolysis. However, this method is limited to the determination of the DP_{av} of fructans containing one glucose residue, as explained in the calculation in the Materials and methods section 3.3.6.

Table 3-2. Validation of DPav determination after enzymatic hydrolysis

	DP_{av} ± standard deviation	
	experimental	expected
rye flour [a]	9.1 ± 0.1	
whole wheat flour [a]	5.5 ± 0.1	
inulin [a]	28.4 ± 0.6	> 25
spiked wheat starch [b]	3.3 ± 0.1	3.5

[a] Rye flour, whole wheat flour, and inulin were analysed in duplicates. [b] Kestose and nystose were added to wheat starch in 4 levels (0.1 – 0.7 % of sample weight) in triplicate.

3.4.3.1 Enzymatic fructan determination

The determination of the total fructan content via HAPEC-PAD was compared with the results obtained from the photometric fructan assay (K-FRUC). Both methods are based on the measurement of enzymatically released glucose and fructose monomers from fructans. In accordance with other studies (Haskå *et al.*, 2008; Verspreet *et al.*, 2012), the total fructan contents obtained from the enzymatic assay, are overestimated because of GOS if the additional α-galactosidase is not included in the procedure (data not shown), because inulinases release fructose from those non fructan compounds (Figure 3-1). Thus, this correction was included in the enzymatic assay.

In contrast to the total fructan determination via HPAEC-PAD after acid hydrolysis, as described by Verspreet *et al.* (2012), the calculation after enzymatic hydrolysis did not require any further corrections as the inclusion of α-galactosidase in both enzyme mixtures (cf. section 3.3.6) avoided any GOS interferences. The calculation is based on the difference between hydrolysates A and B. In hydrolysate A, GOS are degraded to galactose and sucrose; because B contains additionally inulinase, GOS are fully degraded to the monomers galactose, glucose and fructose (Figure 3-2), whereas fructose and glucose released from GOS are considered in the calculation as sucrose from A (eq 1 and eq 2). Finally, the determination via HPAEC-PAD resulted in comparable fructan values to the photometric determination; differences between both methods were not significantly different (Table 3-3, $p > 0.05$).

Table 3-3. Enzymatic total fructan determination via HPAEC-PAD and the assay kit

	Fructan [g/ 100 g] ± standard deviation	
	K-FRUC	HPAEC-PAD
inulin [a]	26.82 ± 0.13	27.84 ± 0.34
plain wheat flour [a,b]	1.28 ± 0.03	1.38 ± 0.03
whole wheat flour [a,b]	1.83 ± 0.00	1.92 ± 0.03

[a] Analyses carried out in duplicates. [b] Results based on dry matter. Means ± standard deviation within each row were not significantly different ($p > 0.05$).

3.4.4 Application of the method

This method has been applied for FODMAP quantification in different commercial cereal-based products; the results obtained were assessed as reasonable on the basis of the product's ingredients and comparable literature data (data not shown) (Biesiekierski *et al.*, 2011; Muir *et al.*, 2009). Further studies will concentrate on the FODMAP characterisation of cereal-product ingredients and the development of products with lowered FODMAP contents using the presented method as an analytical tool.

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Chapter 4
Characterisation of the FODMAP profile in cereal product ingredients

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4.1 Abstract

Cereal-based products, such as bread and pasta, are staple foods in the western diet. Due to the nature of their basic ingredients and the diversity of recipes, the amount of fermentable short-chain carbohydrates (FODMAPs) in those products may be high. This study characterised the FODMAP-profiles of a broad range of cereal product ingredients, serving as a basis for low FODMAP product development. Different cereals, pseudo cereals, gluten-free flours, pulses, pulse protein ingredients, commercial sprouts, and other cereal product ingredients were analysed, using anion-exchange chromatography with electrochemical detection. Wheat and related cereals were high in fructans. Pulses, such as peas contained high galactooligo-saccharides (GOS) amounts. Whereas GOS levels in pulse protein ingredients varied, depending on their production. Gluten-free flours, for instance rice-flour, showed low FODMAP-profiles. Amongst those, buckwheat, which does not contain any of the FODMAPs investigated, contained high amounts of other soluble indigestible carbohydrates, namely fagopyritols; these may have a similar effect on a sensitive gut as GOS. Finally, ingredients contained mainly high levels of fructans and GOS. Yet, the analysis of commonly consumed commercial cereal products, including bread, pasta, crackers, and biscuits, highlighted the relevance of lactose, fructose in excess of glucose and polyols. These products serve as benchmarks for further product development.

4.2 Introduction

Small dietary carbohydrates, which are not digested in the human intestine, and fermented by bacteria in the colon, are entitled with the well-recognised acronym FODMAPs (fermentable oligo-, di-, and monosaccharides and polyols). These carbohydrates can have beneficial or adverse effects on the human health. For individuals with functional gastrointestinal disorders, such as irritable bowel syndrome (IBS), the ingestion may be problematic, leading to different symptoms, for instance, bloating and abdominal pain or an altered bowel habit. IBS can be a severe condition and thus, highly implicate on the patient's quality of life. Research over the past two decades has shown that dietary therapy with a reduced intake of FODMAPs (the low FODMAP diet) is successful in the treatment of IBS. Due to this fact the low FODMAP diet has been largely in focus lately. This often led to misinterpretation, incorrect application of the diet and thus, criticism in terms of lack of nutrients and long-term effects on the health. As emphasized in the recent review by Halmos & Gibson (2019) the correct application of a personalized, individual low FODMAP diet for each patient is crucial for a successful treatment of IBS (Halmos and Gibson, 2019).

The often quoted, and exclusively investigated, list of FODMAPs comprises the most abundant dietary indigestible, osmotically active, and readily fermentable carbohydrates with α-galactooligosaccharides (GOS), fructans and fructooligosaccharides (FOS), lactose, fructose in excess of glucose and polyols. Due to the nature of the basic cereal product ingredients and the diversity of product recipes, cereal-based products can contain high levels of FODMAPs.

GOS (also named raffinose family oligosaccharides, RFO, or α-galactosides) are known to be storage carbohydrates with protective plant-physiological functions in seeds of pulses (legumes). These oligosaccharides are α (1 → 6) linked galactosyl-derivatives from sucrose with the most common homologues raffinose, stachyose, and verbascose. Due to the absence of the enzyme α-galactosidase, GOS are not digested in the human gut and fermented by the microflora in the colon. This leads to gastrointestinal discomfort and to symptoms, such as bloating and abdominal pain, in IBS patients as well as in healthy individuals. The metabolism of GOS in plants also involves cyclitols, such as inositol or pinitol and their galactosides such as galactinol

and ciceritol (Martínez-Villaluenga *et al.*, 2008). Besides proposed health benefits of galactosides such as ciceritol (prebiotic potential), their contribution to the flatulence-causing effect has received little attention and is not fully understood (Horbowicz & Obendorf, 1994; Zhang *et al.*, 2017). Similarly, indigestible fructans are storage carbohydrates in different plants, including cereals and serve the plant with energy during drought and other extreme conditions (Verspreet *et al.*, 2015). Cereal fructans are predominantly composed of branched β (2 → 1) and β (2 → 6) linked fructose chains with a terminal glucose (graminan-type). Stems and leaves of the oat plant also accumulate neo-levan type fructans with β (2 → 1) and/or β (2 → 6) linked fructose chains, with the glucose residue linked internally (Livingston *et al.*, 1993). The disaccharide lactose, which is the main FODMAP in dairy products, may also be found in cereal-based products depending on their formulation, as later highlighted in this study. Fructose may occur in high excess to glucose in some fermented cereal-based products (Ziegler *et al.*, 2016). Likewise, polyols (sugar-alcohols), such as mannitol, the reduced form of fructose, may be produced during fermentation in cereal products (Sahin *et al.*, 2019).

Due to the lack of definition and regulations of FODMAPs in the EU legislation, very few products with a low FODMAP labelling are available on the European market. Only gluten-free products, predominantly made from ingredients naturally low in FODMAPs serve as an alternative for individuals following the low FODMAP diet. Most products, labelled by the official Monash University certification program (Monash University, 2020) or other organisations, are mainly gluten-free products. However, these products are often lacking sensory appeal and nutritional value. Thus, the development of palatable functional low FODMAP products with a high nutritional value is an emerging area of research.

This is a fundamental study on the characterisation of the FODMAP profiles of a broad range of cereal product ingredients. The gained knowledge serves as a basis for the development of products with a lowered FODMAP content using different (bio-) technological approaches. Furthermore, this study aimed to highlight relevant FODMAPs in cereal products, other than fructans and GOS deriving from the ingredients or the processing. Therefore, commonly consumed products of different categories, which also serve as benchmarks for further product development, were analysed. Different studies have been conducted, characterising the FODMAP content

in a wide range of food, as it is consumed (Biesiekierski *et al.*, 2011; Muir *et al.*, 2009). However, these studies serve as dietetic guide for patients following the low FODMAP diet. A dry matter-based characterisation of the FODMAP profiles of raw ingredients as a tool for product-development remains scarce. The HPAEC-PAD method for the quantification of FODMAPs, applied in this study allowed a detailed characterisation of the ingredients and their respective products (Ispiryan *et al.*, 2019).

4.3 Materials and Methods

4.3.1 Ingredients and food products

All ingredients for analysis were commercially sourced, except for the fababean protein rich flour and the protein isolates from lupin and fababean, which were developed as part of an EU project (Protein2Food project, grant no. 635727) by Fraunhofer Institute (IVV), Germany. In total 35 ingredients from five different categories were analysed: (I) wheat-based ingredients (wholemeal flour, baker's flour, biscuit flour, semolina, vital gluten, starch, bran), spelt, rye and barley; (II) gluten-free cereals and pseudo cereals (whole oat flour, quinoa, millet, buckwheat, brown rice), other gluten-free ingredients (oat bran, corn starch, potato starch); (III) pulses (lentils, chickpea flour, soy beans, green peas, yellow peas, proteinrich fababean flour); (IV) protein ingredients from pulses (carob protein, pea protein, abovementioned lupin and fababean protein isolates, commercial lupin protein); (V) sprouted ingredients from quinoa, pea, lupin, buckwheat, lentils. The suppliers of all ingredients are compiled in Table A-3 (Appendix A-1). Food products, available on the Irish market, represent examples of commonly consumed cereal products of different categories, including bread, pasta, biscuits and crackers, and their gluten-free alternatives, respectively (Table 4-1).

Table 4-1. Selected commercial cereal products available on the Irish market

Product	Ingredients on packaging	Nutritional value/ 100g
	Bread	
White wheat loaf	wheat flour (wheat, calcium carbonate, iron, thiamin, niacin), water, yeast, salt, vegetable oil (rapeseed), soya flour, emulsifier: E472e, flour treatment agent: ascorbic acid (vitamin C), vegetable fat (palm).	energy 219 kcal, protein 8.7 g, carbohydrate 43 g, sugars 2.42 g, fat total 1.4 g, saturated 0.4 g, dietary fibre 2.8 g, sodium 1.1 g
Brown Soda bread	wheatmeal, buttermilk (38%), wheat flour, sugar, rapeseed oil, raising agent: sodium hydrogen carbonate, salt.	energy 239 kcal, protein 8.5 g, carbohydrate 43 g, sugars 4.2 g, fat total 2.5 g, saturated 0.3 g, dietary fibre 5.7 g, sodium 1.7 g
Wholewheat Soda bread	fresh buttermilk (36%), wholemeal wheat flour (36%), wheat flour, raising agents: sodium hydrogen carbonate, diphosphates, salt, wheatgerm.	energy 219 kcal, protein 7.9 g, carbohydrate 41 g, sugars 1.8 g, fat total 1.0 g, saturated trace, dietary fibre 6.5 g, sodium 1.4 g
Wheat sourdough bread (San Franciscan style sourdough)	wheat flour, water, salt, yeast	energy 245 kcal, protein 9.2 g, carbohydrate 49 g, sugars 1.3g, fat total 1.0 g, saturated 0.2 g, dietary fibre 3 g, sodium 1.4 g

Product	Ingredients on packaging	Nutritional value/ 100g
Gluten free white loaf	water, potato flour, corn starch, tapioca starch, white rice flour, buckwheat flour, thickening agent (xanthan gum, cellulose, agar), rice bran, pea protein, yeast, sourdough (fermented quinoa, rice, and maize flour), psyllium husk, salt, rapeseed oil, flour treatment agent (ascorbic acid), acidifier (glucono-delta-lactone), acids (citric acid, malic acid, tartaric acid).	energy 200 kcal, protein 7.7 g, carbohydrate 35.7 g, sugars 0.6 g, fat total 1.1 g, saturated 0.3 g, dietary fibre 8.3 g, sodium 1.05 g
	Crackers	
Wheat-based garlic crackers	wheat flour (wheat flour, niacin, iron, thiamin, riboflavin, folic acid), sunflower oil, garlic powder (3.5%), palm oil, salt, sugar, rice flour, inactive yeast (wheat, barley), cane sugar syrup, flavouring, yeast.	energy 488 kcal, protein 8.1 g, carbohydrate 63 g, sugar 2.4 g, fat, total 21.5 g, saturated 3.4 g, dietary fibre 4.4 g, sodium 1.0 g
Wheat-based plain crackers	flour (what flour, calcium, iron, niacin, thiamin), vegetable oil (palm), salt, raising agent (sodium bicarbonate), yeast.	energy 440 kcal, protein 10 g, carbohydrate 67 g, sugars 1.4 g, fat total 13.5 g, saturated 6.2 g, dietary fibre 3.8 g, sodium 1.3 g
Gluten free oat-crackers	wholegrain oats (86%), sustainable palm fruit oil, maize starch, sea salt, raising agent: ammonium bicarbonate, honey.	energy 460 kcal, protein 10.6 g, carbohydrate 59 g, sugars 1.8 g, fat total 16.8 g, saturated 6.6 g, dietary fibre 7.6 g, sodium 1.8 g
	Biscuits	
Wheat-based biscuits	flour (54%) (wheat flour, calcium, iron, niacin, thiamin), vegetable oil (palm), wholemeal wheat flour (16%), sugar, partially inverted sugar syrup, raising agents (sodium bicarbonate, malic acid, ammonium bicarbonate), salt, dried skimmed milk	energy 473 kcal, protein 7.3 g, carbohydrate 69 g, sugars 16.7 g, fat total 20.7 g, saturated 2.0 g, dietary fibre 3.3 g, sodium 1.3 g
Gluten free biscuits	gluten free oat flour (oat flour), vegetable margarine, muscovado sugar, cornflour, partially, inverted sugar syrup, raising agent (sodium bicarbonate), flavouring, vegetable margarine: palm oil, rapeseed oil, water, salt, emulsifier (mono-, diglycerides of fatty acids), muscovado sugar: sugar, molasses, colour (plain caramel).	energy 476 g, protein 6.4 g, carbohydrate 67 g, sugars 24.8 g, fat total 19.4 g, saturated 7.6 g, dietary fibre 4.3 g, sodium 0.4 g
	Pasta	
Wheat spaghetti	durum wheat semolina	energy 176 kcal, protein 5.8 g, carbohydrate 36 g, sugars 1.1 g, fat total 0.7 g, saturated 0.2 g, dietary fibre 2.2 g, sodium 0.1 g
Gluten free spaghetti	corn flour 79.8%, rice flour 19.7%, emulsifier: mono-, diglycerides of fatty acids.	energy 356 kcal, protein 6.5 g, carbohydrate 79 g, sugars 0.5 g, fat total 1.5 g, saturated 0.5 g, dietary fibre 1.2 g, sodium 0.02 g

4.3.2 Sample preparation and FODMAP quantification

Commercial flours of the different ingredients were used for analysis as supplied. Whole grains, seeds and the raw pasta were milled with a Bühler laboratory disc mill (Braunschweig, Germany) or ground using a QIAGEN Tissue Lyser II (Hilden, Germany), to a particle size of ≤ 0.5 mm. The breads, biscuits, crackers as well as cooked pasta (cooked according to instructions on packaging), were freeze-dried and ground to a fine powder. Three packets of each product were purchased, and equal amounts of each packet pooled, disrupted into small pieces and 10 – 15 g freeze-dried for 3 days.

The quantification of mono-, disaccharides, galactooligosaccharides, fructans, and polyols was conducted via high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD), performed on a Dionex™ ICS-5000+ system (Sunnyvale, CA, USA) as described by Ispiryan *et al.* (2019). All carbohydrates, except the fructans, were quantified using authentic reference standards, as specified in the previous study (Ispiryan *et al.*, 2019). Raffinose and stachyose were determined as the sum of both sugars using raffinose pentahydrate from Sigma-Aldrich (Darmstadt, Germany) as a reference standard, performed on the Thermo Scientific™ Dionex™ CarboPac™ PA200 column. A qualitative separation and analysis of raffinose and stachyose was achieved on the Thermo Scientific™ Dionex™ CarboPac™ PA1 column. The total fructan content and the average degree of polymerisation were determined after enzymatic hydrolysis with two enzyme mixtures A and B, where only B contained fructan degrading inulinases. The calculation was based on the quantification of the monomers glucose and fructose released from fructan molecules (Ispiryan *et al.*, 2019). The significance of the fructose released from sucrose and the fructose released from the hydrolysis with the enzyme mixture B was determined for samples in which the levels of sucrose exceeded the theoretically calculated fructan levels. Samples in which no significant difference ($p > 0.05$) was determined and all levels below 0.1 g/ 100 g are referred to as n.d. (not detected) in further discussions. A flow chart summarising the extraction procedure and the fructan determination according to Ispiryan *et al.* (2019) is illustrated in Figure A-4 (Appendix A-1).

All extractions were carried out in duplicate. The results of the ingredients are presented in g analyte per 100 g sample on a dry weight basis (g/ 100 g DM), whereas

the results of the products are additionally presented on a fresh weight basis ("as is"). The weight before and after freeze-drying was recorded and used for the calculation of the FODMAP contents on the "as is" basis. The dry matter of the ingredients and products after freeze-drying was determined according to AACC 44-15.02.

4.3.3 Reference analysis of fructans in oat

The fructan assay kit K-FRUC (Megazyme, Bray, Ireland), last updated in October 2018, was applied for the determination of fructans in oat flour and oat bran, as reference to the determination via HPAEC-PAD. The optimised assay contained in addition to exo- and endoinulinases also levanases; the latter are specifically applied to cleave levan type fructans as well as highly branched fructans. Not in the kit supplied, α-galactosidase (E-AGLANP, Megazyme, Ireland, Bray) was used to take into account interfering GOS. Samples extracts were incubated with the additional enzyme prior to the degradation with the first enzyme mixture, according to controls and precautions of the assay procedure.

4.3.4 Ash determination

The ash contents of the different wheat flours, the wheat starch and the semolina were determined according to AACC 08-01.01, 08-17.01 and 08-12.01, respectively.

4.3.5 Statistical analysis

Statistical analyses were performed with SPSS Statistic 24 (IBM Corp., Armonk, NY, USA). Within the fructan analysis via HPAEC-PAD, a significant difference of the sucrose content in the sample and the fructose released from sucrose and potentially additional fructans after incubation with inulinase was determined by means of an independent t-test ($p = 0.05$); cf. Ispiryan *et al.* (2019) for details on the fructan analysis. One-way ANOVA followed by Tuckey's test were applied to determine statistical significance between the fructan levels of the different wheat flours and wheat isolates and between wheat, spelt, rye and barley flours ($p < 0.05$).

4.4 Results and discussion

4.4.1 FODMAP levels in the ingredients

The FODMAP levels are reported in five groups of the ingredients. The categorisation is based on the plant-origin of the ingredients and their composition, the typical use, and the type of the ingredients.

4.4.1.1 Group I – fructan containing cereals

Wheat, spelt, rye and barley are commonly used ingredients in cereal products. Amongst them, wheat is the basic ingredient in a variety of staple foods being part of the western diet, such as bread and pasta. Depending on the product type also milling fractions or isolates from different components from the wheat grains are applied in formulations. Their HPAEC-PAD profiles and the FODMAP levels are presented in Figure 4-1 and Table 4-2, respectively.

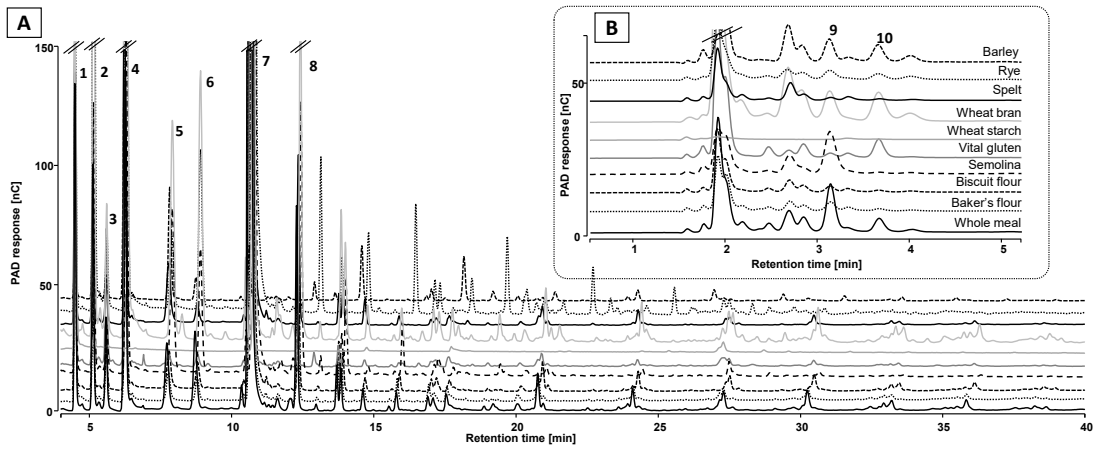

Figure 4-1. HPAEC-PAD (A) CarboPac PA200 and (B) CarboPac PA1 profiles of ingredients from Group I – fructan containing cereals with identical order of the profile in (A) and (B): (1) rhamnose [internal standard], (2) glucose, (3) fructose, (4) sucrose, (5) raffinose/stachyose, (6) kestose, (7) maltose, (8) DP3 fructan, (9) sorbitol, (10) mannitol. Peaks in (A) eluting after (8) are fructans with > DP3 and malto-dextrins

The abundant FODMAPs in wheat as well as in the other cereals of Group I were fructans, ranging from 0.85 − 1.88 g/ 100 g DM (Table 4-2).

Table 4-2. FODMAP contents of cereal product ingredients.

Ingredient	FODMAP contents ± standard deviation [g/100g DM] [a]											
	Mono-/Disaccharides [b, c]			Polyols [b]				Oligosaccharides				
	Glucose	Fructose	EF [d]	Xylitol (cyclitol) [e]	Sorbitol (cyclitol) [f]	Mannitol	∑	Raffinose/ Stachyose [b]	Verbascose (FP-B1) [b,g]	∑	Total fructan [h]	DP_{av}
Group I												
Whole meal	0.18 ± 0.02	0.07 ± 0.01	-	n.d.	0.04 ± 0.00	0.01 ± 0.00	0.05	0.14 ± 0.00	n.d.	0.14	1.88 ± 0.09 [A]	6.7
Baker's flour	0.06 ± 0.00	0.06 ± 0.00	-	n.d.	0.01 ± 0.00	n.d.	0.01	0.06 ± 0.00	n.d.	0.06	1.19 ± 0.00 [B, C]	5.6
Biscuit flour	0.07 ± 0.00	0.05 ± 0.00	-	n.d.	n.d.	n.d.	n.d.	0.09 ± 0.00	n.d.	0.09	1.48 ± 0.03 [B]	5.4
Semolina	0.13 ± 0.02	0.04 ± 0.00	-	n.d.	n.d.	n.d.	n.d.	0.31 ± 0.00	n.d.	0.31	1.20 ± 0.02 [C]	4.3
Vital gluten	0.14 ± 0.00	0.18 ± 0.01	-	n.d.	n.d.	n.d.	n.d.	0.03 ± 0.00	n.d.	0.03	0.60 ± 0.00 [D]	4.4
Wheat starch	n.d.	n.d.	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d. [E]	-
Wheat bran	0.41 ± 0.03	0.15 ± 0.01	-	n.d.	0.02 ± 0.00	0.04 ± 0.00	0.06	0.41 ± 0.01	n.d.	0.41	3.40 ± 0.15 [F]	5.0
Spelt	0.07 ± 0.00	0.05 ± 0.00	-	n.d.	n.d.	n.d.	n.d.	0.13 ± 0.00	n.d.	0.13	0.85 ± 0.01 [D]	4.1
Rye	0.64 ± 0.02	0.08 ± 0.01	-	n.d.	n.d.	0.01 ± 0.00	0.01	0.13 ± 0.00	n.d.	0.13	3.61 ± 0.08 [F]	8.9
Barley (whole grains)	0.56 ± 0.02	0.05 ± 0.00	-	n.d.	n.d.	n.d.	n.d.	0.56 ± 0.02	n.d.	0.56	1.38 ± 0.09 [C]	3.7
Group II												
Whole oat flour	0.04 ± 0.00	0.03 ± 0.00	-	n.d.	n.d.	n.d.	n.d.	0.29 ± 0.00	0.02 ± 0.00	0.31	n.d.	-
Oat bran	0.02 ± 0.00	0.03 ± 0.00	0.01	n.d.	n.d.	n.d.	n.d.	0.29 ± 0.00	0.04 ± 0.00	0.33	n.d.	-
Quinoa	0.26 ± 0.00	0.13 ± 0.00	-	n.d.	0.28 ± 0.01 [f]	n.d.	0.28	0.09 ± 0.00	n.d.	0.09	n.d.	-
Millet	0.06 ± 0.00	0.03 ± 0.00	-	n.d.	n.d.	n.d.	n.d.	0.15 ± 0.00	n.d.	0.15	n.d.	-
Buckwheat flour	0.09 ± 0.00	0.05 ± 0.00	-	0.07 ± 0.00 [e]	0.17 ± 0.00 [f]	n.d.	0.24	0.01 ± 0.00	0.89 ± 0.00 [g]	0.01	n.d.	-
Brown rice	0.09 ± 0.01	0.02 ± 0.00	-	n.d.	n.d.	n.d.	n.d.	0.13 ± 0.01	n.d.	0.13	n.d.	-
Corn starch	n.d.	n.d.	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-
Potato starch	n.d.	n.d.	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-

Group III												
Lentil (whole seeds)	0.24 ± 0.01	0.04 ± 0.00	-	n.d.	0.95 ± 0.03 [f]	n.d.	0.95	2.54 ± 0.02	1.44 ± 0.01	3.98	n.d.	-
Chickpea flour	0.05 ± 0.00	n.d.	-	0.01 ± 0.00 [e]	n.d.	n.d.	0.01	2.05 ± 0.06	0.05 ± 0.00	2.11	n.d.	-
Soy (whole seeds)	0.11 ± 0.00	0.02 ± 0.00	-	0.04 ± 0.00 [e]	0.06 ± 0.01 [f]	n.d.	0.10	3.37 ± 0.04	0.19 ± 0.00	3.55	n.d.	-
Green pea (whole seeds)	0.14 ± 0.00	0.01 ± 0.00	-	n.d.	0.01 ± 0.00 [f]	n.d.	0.01	1.87 ± 0.05	2.61 ± 0.09	4.48	n.d.	-
Yellow pea (whole seeds)	0.13 ± 0.00	0.01 ± 0.00	-	n.d.	0.02 ± 0.00 [f]	n.d.	0.02	2.12 ± 0.05	2.63 ± 0.06	4.75	n.d.	-
Fababean (prot. rich flour)	0.13 ± 0.00	0.09 ± 0.00	-	n.d.	0.03 ± 0.00 [f]	n.d.	0.03	1.42 ± 0.01	3.45 ± 0.01	4.87	n.d.	-
Group IV												
Fababean prot. ** (85%)	0.01 ± 0.00	0.02 ± 0.00	0.01	n.d.	n.d.	n.d.	n.d.	0.03 ± 0.00	0.06 ± 0.00	0.08	n.d.	-
Carob prot. * (≥ 48%)	0.02 ± 0.00	0.05 ± 0.00	0.03	n.d.	n.d.	n.d.	n.d.	4.15 ± 0.02	1.36 ± 0.00	5.51	n.d.	-
Pea prot. * (≥ 83%)	0.02 ± 0.00	n.d.	-	n.d.	n.d.	n.d.	n.d.	0.57 ± 0.01	0.59 ± 0.00	1.16	n.d.	-
Lupin prot. ** (94%)	0.01 ± 0.00	0.01 ± 0.00	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-
Lupin prot. * (≥ 38%)	0.05 ± 0.00	0.06 ± 0.00	-	n.d.	n.d.	n.d.	n.d.	9.55 ± 0.02	1.19 ± 0.04	10.74	n.d.	-
Group V												
Quinoa sprouts	0.57 ± 0.05	n.d.	-	n.d.	n.d.	n.d.	n.d.	0.20 ± 0.01	n.d.	0.20	n.d.	-
Pea sprouts	0.19 ± 0.07	0.02 ± 0.00	-	n.d.	n.d.	n.d.	n.d.	2.59 ± 0.04	1.1 ± 0.01	3.69	n.d.	-
Lupin sprouts	0.09 ± 0.01	0.01 ± 0.00	-	0.15 ± 0.00 [e]	n.d.	n.d.	0.15	3.44 ± 0.05	n.d.	3.44	n.d.	-
Buckwheat sprouts	0.59 ± 0.04	0.08 ± 0.00	-	0.10 ± 0.00 [e]	0.01 ± 0.00 [f]	n.d.	0.11	n.d.	0.29 ± 0.01 [g]	n.d.	n.d.	-
Lentil sprouts	0.06 ± 0.00	0.17 ± 0.00	0.11	n.d.	n.d.	n.d.	n.d.	1.35 ± 0.02	0.68 ± 0.01	2.03	n.d.	-

[a] extractions carried out in duplicates and measured via HPAEC-PAD, results referred to dry matter (DM). [b] n.d., not detected or levels below 0.005 g / 100 g DM. [c] no lactose detected in any of the ingredients. [d] EF, excess fructose = glucose – fructose. [e f] unidentified cyclitols suspected to be for instance chiro-inositol, myo-inositol or pinitol, estimated as xylitol or sorbitol, respectively. [g] FP-B1, fagopyritol B1, estimated as sucrose. [h] n.d., not detected in means of no significant difference in sucrose values and fructose values determined from difference of assay A and B in fructan determination ($p > 0.05$), or levels below 0.1 g / 100 g DM. (*) commercial protein ingredients, (**) protein ingredients delivered from research projects. Group I fructan means ± standard deviations with different superscript capital letters are significantly different ($p < 0.05$).

The analysis of the different wheat flours, containing different fractions of the grains, revealed similar values as reported by Haskå *et al.* (2008). Cereal fructans, such as those found in wheat, spelt, rye or barley are predominantly of the branched, graminan-type and contain β (2 → 1) as well as β (2 → 6) linked fructose monomers to the terminal sucrose. They are mainly located in the outer layers of the wheat grain (Haskå *et al.*, 2008). Thus, the bran contained the highest amounts of fructans (3.40 g/ 100 g DM), whereas the lowest level was detected in the baker's flour with 1.19 g/ 100 g DM, followed by the whole meal (1.88 g/ 100 g DM). The difference in these two flours is resulting from the lower extraction rate of the baker's flour, which thus contains fewer outer parts from the grain. The ash content of a flour is characteristic for the extraction rate of the flour-product and correlates also with the fructan levels in the flour. Both components of the wheat grain, the minerals as well as the fructans, are mainly located in the outer layers of the grains (Figure A-5, Appendix A-1). Thus, the fructan content of commercial flours has a strong dependence on the extraction rate of the flour-product.

Biscuit flour, baker's flour and semolina are produced from different varieties of wheat, namely soft, hard and durum wheat, respectively. The differentiation of soft wheat and hard wheat for non-bread-making and bread-making wheat is according to the North American terminology. Wheat, which is easier to crush (soft wheat) is used for biscuits, while wheat which is harder to crush (hard wheat) is used for bread-making (Delcour & Hoseney, 2010).The fructan contents of baker's flour from hard wheat and durum wheat semolina were similar (1.19 and 1.20 g/ 100 g DM), whereas biscuit flour produced from soft wheat had slightly higher fructan levels (1.48 g/ 100 g DM). Spelt, which also represents a wheat species, contained lower amounts of fructans (0.85 g/ 100 g DM) than the flours from soft wheat, hard wheat, and the durum wheat semolina. Ziegler *et al.* (2016) determined the fructan contents in a number of different varieties of bread wheat, spelt as well as durum wheat. In contrast to the results in this study, no significant difference in the fructan contents in spelt and bread wheat (corresponding to hard wheat in Northern American terminology) was determined, while fructans in durum were slightly lower. However, the fructan levels of the different varieties of each species varied significantly. Thus, as Ziegler *et al.* (2016) stated, a general categorisation of the different species of wheat to contain higher or lower levels of fructans is not reasonable. Furthermore, as mentioned above

in the context of baker's flour and whole meal, commercial flour-products have different extraction rates. The spelt flour analysed in this study was 'Type 630'. The type-number corresponds to the extraction rate of a flour. It reflects the ash content (%) multiplied by 1000 (Belitz *et al.*, 2009). Hence, a flour of 'Type 630' contains less parts of the outer layer of the grain than flours from the whole grain which have a Type-number of > 1000 (Belitz *et al.*, 2009).

The analysis of the isolates from wheat, revealed that about one third of the wheat fructans were detected in the gluten isolate (0.60 g/ 100 g DM), unlike the wheat starch which did not contain any soluble carbohydrates. Thus, the contribution of the gluten ingredient to the total FODMAP content should be considered for applications in low FODMAP products.

Nemeth *et al.* (2014) reported that the content of fructans in barley is dependent on breeding and cultivar of the plant, with levels ranging from 0.9 − 4.2 g/ 100 g DM. The barley grains of the variety Beatrix (brewer's barley), analysed in this study, contained 1.38 g fructans in 100 g DM. The fructan levels in rye (3.61 g/ 100 g DM) were comparable to those found in other studies and significantly higher than the levels in wheat, spelt and barley (Karppinen *et al.*, 2003; Rakha *et al.*, 2010). The average degrees of polymerisation in the cereals investigated were 4 – 9 with the shortest chain-lengths in spelt and barley and the longest chain-lengths in rye. Previous studies reported similar degrees of polymerisation for these cereals (Nemeth *et al.*, 2014; Rakha *et al.*, 2010; Verspreet *et al.*, 2012).

Furthermore, GOS were found in the ingredients of Group I. The main representative of those oligosaccharides is the trisaccharide raffinose. Stachyose occurs only in low levels or in traces (Henry & Saini, 1989). The next higher saccharide in this series of oligosaccharides, verbascose, was not found in any of these ingredients. The GOS levels ranged from 0.06 g/ 100 g DM in the baker's flour to 0.56 g/ 100 g DM in barley. No other FODMAPs were found in considerable amounts in any of the Group I ingredients. The disaccharide lactose was not detected in any of the ingredients of Group I - V (Figure 4-2) and will not be further discussed for the following groups. Only very low amounts of fructose were determined, which did not exceed the levels of glucose in any of the samples. The sugar alcohols sorbitol and mannitol were detected in very low levels only (0.01 – 0.04 g/ 100 g DM). This would not be

clinically relevant in food products, according to the cutoff level of 0.4 g total polyols per serving, defined by Varney *et al.* (2017).

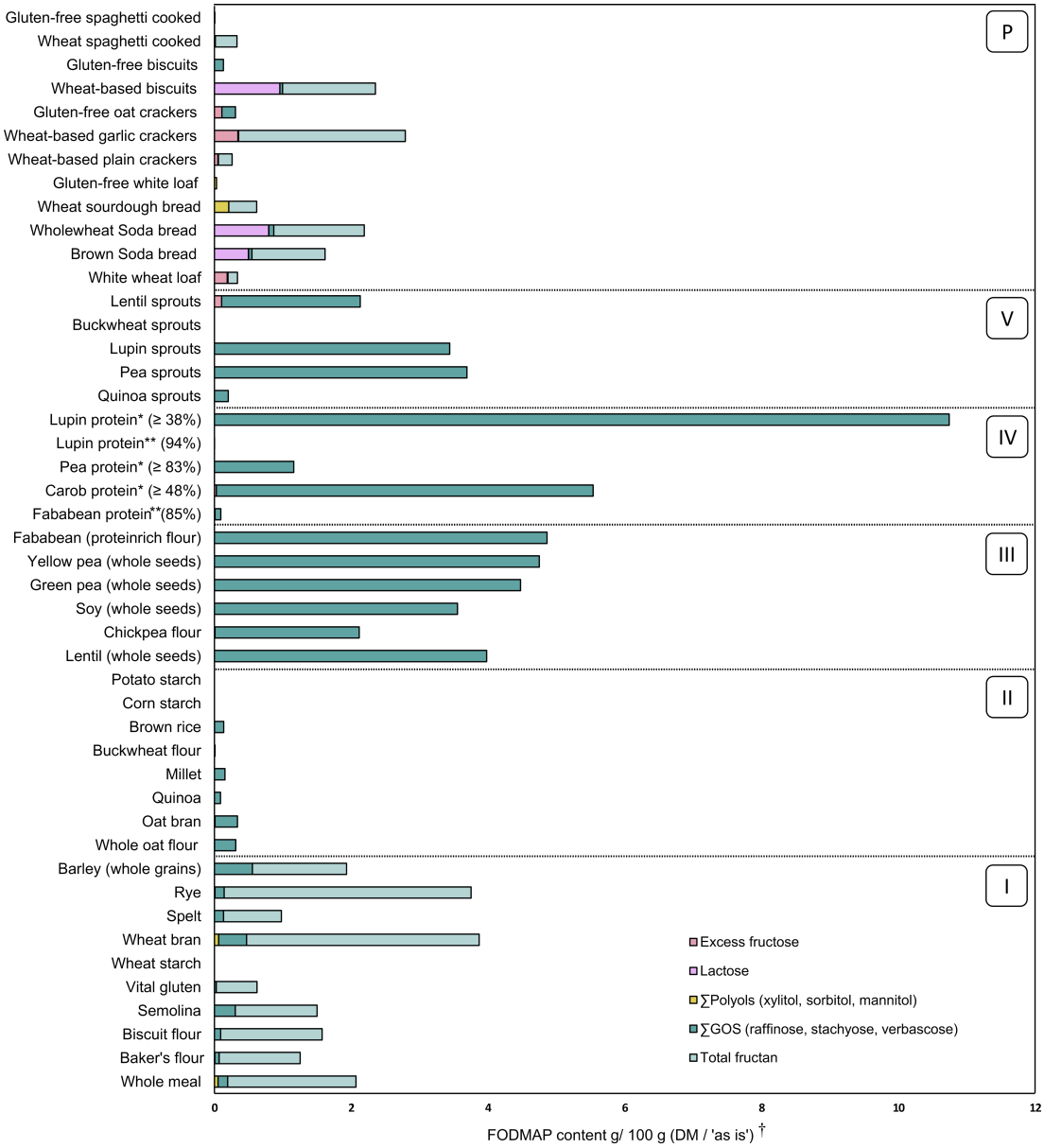

Figure 4-2. FODMAP contents of cereal-product ingredients divided into Group I − V. (Group I) − fructan containing cereals, (Group II) - low FODMAP and gluten-free cereals and pseudo-cereals, (Group III) − seeds and flours from pulses, (Group IV) − pulse protein ingredients, (Group V) − commercial sprouts and FODMAP levels of commercial products (P) quantified via HPAEC-PAD. Protein ingredients marked with one asterisk (*) are commercial protein ingredients, those marked with two asterisks (**) are protein ingredients delivered from EU project, provided by Fraunhofer IVV. (†) FODMAP levels of ingredients are referred to the dry matter, whereas levels in products are referred to the fresh weight 'as is'.

4.4.1.2 Group II – low FODMAP and gluten-free cereals and pseudo cereals

The ingredients of this group, the cereals oat, millet and rice, the pseudo cereals quinoa and buckwheat, and the fractions and isolates oat bran, corn starch and potato starch are basic ingredients of most gluten-free products.

Apart from being gluten-free, all these ingredients are also very low in FODMAPs (cf. HPAEC-PAD profiles in Figure 4-3 and values in Table 4-2). Only low to moderate levels of GOS were detected, with the lowest levels in quinoa and the highest levels in oat bran (0.09 and 0.33 g/ 100 g DM, respectively).

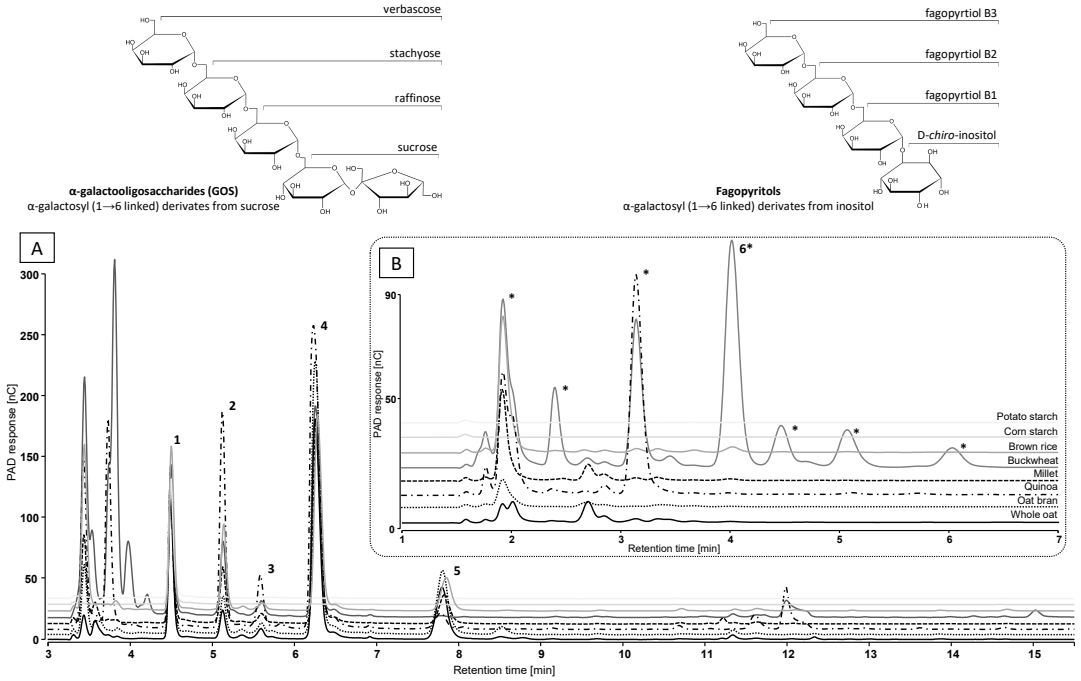

Figure 4-3. Chemical structures of α-galactooligosaccharides (GOS) and fagopyritols and HPAEC-PAD (A) CarboPac PA200 and (B) CarboPac PA1 profiles of ingredients from Group II - low FODMAP and gluten-free cereals and pseudo cereals in identical order of the profile in (A) and (B): (1) rhamnose [internal standard], (2) glucose, (3) fructose, (4) sucrose, (5) raffinose/ stachyose, (6*) fagopyritol B1. All peaks marked with an asterisk are unidentified compounds suspected to be cyclitols and fagopyritols.

None of these ingredients contained fructans at levels above 0.1 g/ 100 g DM. However, fructan levels in oat found in literature were contradictory to the findings in this study. Dodevska *et al.* (2013) as well as Biesiekierski *et al.* (2011) determined 0.35 % and 0.32 % fructans in oat flakes (as eaten, moisture not excluded from total weight), respectively. Given that oat contains ~ 0.3 % GOS, additional 0.3 % fructans would add up to 0.6 %; this could exceed the cutoff level for oligosaccharides, depending on the product and serving size (0.3 g per serving; (Varney *et al.*, 2017). Both studies used the K-FRUCHK Megazyme assay without correction of the GOS-

interference with α-galactosidase (Biesiekierski *et al.*, 2011; Dodevska *et al.*, 2013). Since inulinases used in the assay to cleave fructans also cleave the terminal fructose from GOS, fructan results from the assay, obtained without the addition of α-galactosidase, are overestimated. However, high levels of fructans were reported in other parts of the oat plant, such as stems and leaves (Livingston *et al.*, 1993). These fructans have in contrast to other cereal fructans a different structure. As described in section 4.4.1.1, most cereals contain graminan-type fructans where fructose units are β (2 → 1) and β (2 → 6) linked to the terminal sucrose. Fructans found in oat hay are neolevan-type fructans; they are branched or linear and consist of β (2 → 1) and/ or β (2 → 6) linked fructose chains with an internal glucose residue. Fructan molecules consisting of exclusively β (2 → 6) bound fructose chains were also reported in parts of the oat plant (Livingston *et al.*, 1993). The enzymes supplied in the K-FRUCHK assay as well as in the inulinases applied for the fructan determination via HPAEC-PAD in this study, are specifically cleaving inulin- and graminan-type fructans but underestimate levan-type fructans (McCleary *et al.*, 2019). The neolevan-type fructans with exclusively β (2 → 6) bound fructose are similar to levan-type fructans. An updated version (October 2018) of the K-FRUC Megazyme assay contains, in addition to the inulinases also levanases in the fructan cleaving enzyme mixture. Thus, in order to exclude an underestimation of potential fructans in oat, the optimised assay was applied. However, the analysis of the whole oat flour and the oat bran with that assay, in line with the HPAEC-PAD determination, revealed no fructans detectable.

No other carbohydrates which are considered as FODMAPs were found in these ingredients. Yet, the FODMAPs determined in this study, as well as in other FODMAP-literature, are not the only indigestible and readily fermentable dietary sugars. Other carbohydrates such as passively absorbed monosaccharides other than fructose, the disaccharide lactulose, or oligosaccharides other than fructans and GOS may also be considered as FODMAPs (Halmos & Gibson, 2019).

Based on the findings of this study the classification of buckwheat may change from a low FODMAP to a high FODMAP ingredient. To date, there is no other scientific study where the FODMAP profile of buckwheat was determined. As shown in Table 4-2, buckwheat did not contain any of the FODMAPs commonly analysed. Also, according to the mobile phone application from the Monash University, buckwheat is listed as a low FODMAP grain (Monash University, 2020). However, oligosaccharides

called fagopyritols represent the majority of soluble carbohydrates in buckwheat. These compounds are α-galactosides from D-*chiro*-inositiol and occur in levels up to ~5 % in different buckwheat fractions, with fagopyritol B1 as the most abundant representative (Horbowicz *et al.*, 1998). An estimation of the peak in the HPAEC-PAD profile suspected to be fagopyritol B1 (Figure 4-3) as sucrose revealed an approximate concentration of ~1 g/ 100 g DM; a reference standard was not available. These oligosaccharides have similar structural properties to GOS (cf. chemical structures in Figure 4-3) and require the enzyme α-galactosidase for the hydrolysis and absorption into the intestinal lumen; it is well known that the human gut lacks this enzyme. Fagopyritols are, thus, indigestible, fermentable carbohydrates. On the one hand, there have been studies indicating a beneficial, blood glucose lowering effect from inositol and fagopyritols for diabetes patients (Kawa *et al.*, 2003). On the other hand, there is evidence, that these carbohydrates may have a similar impact on a sensitive gut, such as in IBS patients, as GOS found in pulses (Horbowicz *et al.*, 1998). In addition to fagopyritols and GOS, buckwheat and pulses contain so called cyclitols and their α-galactosides. These are precursor and intermediate compounds of the biosynthesis of fagopyritols and GOS (Horbowicz *et al.*, 1998; Martínez-Villaluenga *et al.*, 2008). Although these small carbohydrates may cause an altered bowel habit, similar to polyols, *in vitro* and *in vivo* studies are required to support this hypothesis.

4.4.1.3 Group III – seeds and flours from pulses

Pulses have traditionally been consumed along with cereals. Nowadays, cereal products, including bakery products and beverages based on, or with the addition of pulses, have gained significant importance. This trend appeared due to their composition, particularly the high protein content. Pulse ingredients are applied in bakery products, provide beneficial techno-functional properties, and contribute to high nutritional values. However, pulses generally reveal in their native composition a high FODMAP content. Thereby GOS are the main saccharides in pulses, described as FODMAPs, with up to 10 − 16 % (Martínez-Villaluenga *et al.*, 2008).

In accordance with literature, stachyose was the predominant GOS detected in all ingredients of this group. Raffinose appeared in lower levels, whereas the levels of verbascose ranged from 0.05 g/ 100 g DM in chickpea flour up to 3.45 g/ 100 g DM in fababean flour. These two ingredients also represented the two extremes of the total

GOS levels, with 2.11 g/ 100 g DM in the chickpea-flour and 4.87 g/ 100 g DM in the fababean flour (Table 4-2); the amounts analysed in all pulses were within ranges described in other studies (Kannan *et al.*, 2018; Martínez-Villaluenga *et al.*, 2008).

No other FODMAPs were determined in substantial amounts. No fructans were found in any of the pulses. However, besides the fact that very little information on the quantification of fructans in raw pulses is available, some studies described moderate to very high fructan levels in cooked pulses. Dodevska *et al.* (2013) determined 0.8 − 1.5 g/ 100 g DM fructans in beans and lentils, respectively. As discussed in section 4.4.1.2 and a previous study (Ispiryan *et al.*, 2019), this overestimation is due to the interference of GOS in the analysis using the enzyme assay without the appropriate correction. In accordance with the findings in this study, Huynh *et al.* (2007) did not determine any fructans in fababeans and pea, applying a similar method of fructan determination via HPAEC-PAD after enzymatic hydrolysis and with the inclusion of α-galactosidase. McCleary *et al.* (2019) conducted different trials, presenting the GOS interference in the Fructan Assay Kits. They presented an apparent fructan content of 2.85 − 3.05 % in mung beans (contain 3.05 % GOS). The analysis with the inclusion of α-galactosidase revealed values below the limit of quantification of the assay (0.1 − 0.11 %) (McCleary *et al.*, 2019).

Furthermore, as discussed for Group II, the precursor and intermediate compounds of GOS, cyclitols and their α-galactosides, are also found in pulses, and may have a similar effect on the GI-tract as GOS, thus contributing to even higher FODMAP levels.

4.4.1.4 Group IV – pulse protein ingredients

Because pulses are a very good sources of protein, they are often used as raw material to produce protein-isolates (PI; ~ 90 % protein) or protein concentrates (PC; 40 − 75 % protein) as ingredients for the food industry; these are used for instance for protein fortification to increase the nutritional value (e.g., in gluten-free products), and as techno-functional ingredients to improve the rheology properties of the end-products (Singhal *et al.*, 2016).

The FODMAP levels, in particular GOS, in the different PI and PC varied highly. The PI from fababean (85 % protein content) and lupin (94 % protein content), provided by Fraunhofer IVV, contained only traces of GOS. In contrast, the commercial protein

ingredients from carob (48 % protein content) and lupin (38 − 42 % protein content) contained very high amounts of GOS, 5.51 g and 10.74 g/ 100 g DM, respectively. Carob (locust bean) also belongs to legumes and is rich in GOS (Table 4-2). The occurrence of raffinose, stachyose and verbascose in carob seeds was identified qualitatively (Amuti & Pollard, 1977), quantitative data was not available. In lupin seeds, similarly high values between 6.5 −11.7 g/ 100 g DM were determined in another study (Andersen et al., 2005). The lupin PC had the highest levels of GOS in all pulses (Figure 4-2). Due to the high amount of GOS in both PC, presumably the preparation of these commercial protein ingredients did not involve a separation of the soluble carbohydrates. In contrast to this, the PI from fababean and lupin were obtained by isoelectric point precipitation. Within that preparation process, soluble carbohydrates, including GOS were removed. The commercial PI from pea (85 % protein content) also contained moderate levels of GOS (1.16 g/ 100 g DM); the production process is not known.

These results indicate that different approaches to produce pulse protein ingredients have a significant impact on the FODMAP contents and should thus be investigated for low FODMAP applications. The use of the PC from carob and lupin or the PI from pea, may result in high FODMAP products, depending on the proportion of the ingredient in the formulation. The PI from fababean and lupin, in contrast, may be directly used in any product formulation.

4.4.1.5 Group V – commercial sprouts

Sprouted grains and seeds are also applied in bakery products. High enzyme activities favour techno-functional properties and a higher bioavailability of nutrients increases the nutritional value. Horstmann *et al.* (2019) for instance, applied different sprouts in gluten-free bread systems. These ingredients displayed functional attributes relating to higher dough quality as well as improved nutritional value of the bread.

The flours from the commercial sprouts from buckwheat and quinoa did not contain substantial amounts of any carbohydrate, currently considered as FODMAPs (Figure 4-2). The semi quantitatively determined amount of fagopyritol B1 in the buckwheat sprouts was lower in comparison to the buckwheat flour (0.29 g vs. 0.89 g/ 100 g DM; Table 4-2).

In pulses, such as in lupin, lentil or pea, germination or sprouting have been often reported to be effective for the removal of GOS; depending on the germination conditions the levels of α-galactosides can be diminished to different extents. This effect has been identified to occur due to increased activities of the native enzyme from the pulses, α-galactosidase, which cleaves the α (1 → 6) linkages in GOS molecules (Kannan *et al.*, 2018; Martínez-Villaluenga *et al.*, 2008). The GOS levels in the commercial lentil and lupin sprouts (2.03 g and 3.44 g/ 100 g DM, respectively) were markedly lower than in the lentil flour and the commercial lupin PC (3.98 g and 10.74 g/ 100 g DM, respectively). Also the flour from sprouted peas contained less GOS (3.69 g/ 100 g DM) than the flour from raw green and yellow peas (4.48 g and 4.75 g/ 100 g DM, respectively). Nevertheless, the lower amounts were still relatively high, considering the clinical cutoff level of 0.3 g oligosaccharides per serve (Varney *et al.*, 2017). However, if only small levels (5 %) are added to the formulations (Horstmann *et al.*, 2019), resulting bakery products may still be considered as low in FODMAPs, depending on other components of the recipes.

4.4.2 FODMAP contents in commercial cereal products and their gluten-free alternatives

The FODMAP profiles of commonly consumed, representative cereal products from the Irish market from different categories, including bread, pasta, biscuits and crackers and their gluten-free alternatives, were determined and serve as benchmarks for further low FODMAP product development.

The FODMAP levels of all products are presented in Table 4-3. Furthermore, Table 4-3 shows whether the products meet the low FODMAP criteria reported by Varney *et al.* (2017). The white wheat loaf, the Irish soda breads as well as the sourdough bread were all based on wheat as the basic ingredient (cf. Table 4-1). As demonstrated in section 4.4.1.1, the main FODMAP in wheat are fructans (1.19 − 1.88 g/ 100 g DM). However, the FODMAP profiles of the breads differed greatly. The white wheat loaf contained only 0.14 g/ 100 g (0.22 g/ 100 g DM) of fructans. From the list of ingredients, it can be seen that the bread was produced from a yeast fermented dough. Different studies have shown that yeast fermentation is capable of degrading the fructans in the wheat flour (Struyf *et al.*, 2017; Ziegler *et al.*, 2016).

Table 4-3. FODMAP contents of commercial cereal-products from Irish market

Products	FODMAP contents ± standard deviation [g/100g 'as is'] [a]								
	Mono-/Disaccharides [b]				Polyols	Oligosaccharides			
	Glucose	Fructose	EF [c]	Lactose	∑Polyols [d]	∑GOS [d]	Total fructan [e]	Serve [g] [f]	Meets low FODMAP criteria [g]
Bread									
White wheat loaf	0.11 ± 0.00	0.30 ± 0.01	0.19	n.d.	n.d.	0.01	0.14 ± 0.01	50	Yes
Brown Soda bread	0.22 ± 0.02	0.20 ± 0.01	-	0.50 ± 0.03	n.d.	0.05	1.07 ± 0.01		No
Wholewheat Soda bread	0.23 ± 0.01	0.07 ± 0.00	-	0.79 ± 0.01	n.d.	0.07	1.32 ± 0.06		No
Wheat sourdough bread	0.10 ± 0.00	0.06 ± 0.00	-	n.d.	0.21	n.d.	0.41 ± 0.00		Yes
Gluten-free white loaf	0.10 ± 0.00	0.04 ± 0.00	-	n.d.	0.03	n.d.	n.d.		Yes
Crackers									
Wheat-based plain crackers	0.08 ± 0.00	0.13 ± 0.01	0.05	n.d.	n.d.	0.01	0.20 ± 0.01	30	Yes
Wheat-based garlic crackers	0.71 ± 0.01	1.05 ± 0.04	0.34	n.d.	n.d.	0.01	2.44 ± 0.05		No
Gluten-free oat crackers	0.36 ± 0.00	0.47 ± 0.00	0.11	n.d.	n.d.	0.20	n.d.		Yes
Biscuits									
Wheat-based biscuits	0.38 ± 0.02	0.31 ± 0.01	-	0.96 ± 0.08	n.d.	0.04	1.36 ± 0.09	30	No
Gluten-free biscuits	0.45 ± 0.01	0.45 ± 0.01	-	n.d.	0.01	0.13	n.d.		Yes
Pasta									
Wheat spaghetti uncooked	0.18 ± 0.00	0.10 ± 0.00	-	n.d.	0.01	0.08	1.34 ± 0.11	55	No
Wheat spaghetti cooked	0.07 ± 0.01	0.02 ± 0.00	-	n.d.	n.d.	0.01	0.32 ± 0.03	140	No
Gluten-free spaghetti uncooked	0.20 ± 0.00	0.19 ± 0.01	-	n.d.	0.03	n.d.	n.d.	55	Yes
Gluten-free spaghetti cooked	0.03 ± 0.00	0.03 ± 0.00	-	n.d.	n.d.	n.d.	n.d.	140	Yes

[a] extractions carried out in duplicates and measured via HPAEC-PAD, results referred to fresh weight ('as is'). [b] n.d., not detected or levels below 0.005 g/ 100 g DM. [c] EF, excess fructose = glucose – fructose. [d] sum of polyols includes xylitol, sorbitol, mannitol; sum of GOS includes raffinose, stachyose, verbascose. [e] n.d., not detected in means of no significant difference in sucrose values and fructose values determined from difference of assay A and B in fructan determination ($p > 0.05$), or levels below 0.1 g/100 g DM. [f] serving sizes according to (Edwards, 2017). [g] cutoff levels per serve according to Varney *et al.* (2017): 0.3 g oligosaccharides, 0.4 g polyols, 0.15 g excess fructose, 1 g lactose, 0.5 g total FODMAPs excluding lactose.

However, the hydrolysis of the fructans by the yeast invertase (in the case of baker's yeast) can lead to the release of fructose and thus fructose in excess of glucose, depending on the product fermentation conditions. The white bread analysed in this study contained 0.19 g/ 100 g (0.30 g/ 100 g DM) fructose in excess of glucose. A number of studies reported the degradation of fructans in wheat by different yeast species under different conditions (Fraberger *et al.*, 2018; Struyf *et al.*, 2017; Ziegler *et al.*, 2016). For instance, Ziegler *et al.* (2016) have shown, the amount of fructose remaining in bread after the fermentation with baker's yeast is dependent on the fermentation time. In contrast to this, soda breads are produced without any fermentation; chemical leavening with soda leads to the production of gas during the baking process and hence, the higher bread volume. Thus, high amounts of fructans, 1.07 g (1.81 g) and 1.32 g/ 100 g (2.30 g/ 100 g DM), in the brown soda bread and the whole wheat soda bread, respectively, were determined. The fructan content in the whole wheat soda bread was higher due to the additional wheatgerm, and, presumably, slightly different proportions of whole meal flour and wheat flour in the recipes. Furthermore, both breads contained lactose (0.50 g and 0.79 g/ 100 g), the main sugar found in milk; both formulations included ~ 40 % buttermilk. Also in the sourdough bread, only moderate levels of fructans (0.41 g [0.69 g] / 100 g [DM]) were found. This can be attributed to the fermentation with lactic acid bacteria (LAB) and yeast in sourdoughs (Loponen & Gänzle, 2018). In comparison to the yeast fermented white loaf, the sourdough bread contained more fructans. However, the extent of fructan degradation is dependent on the product formulation, different fermentation conditions, and most importantly the application of different species and strains. Low levels of mannitol were also detected (0.19 g [0.33 g] /100 g [DM]). Sourdough bread, is fermented with yeast and a range of LAB. This bread, called "San Franciscan Sourdough" was fermented amongst other LAB with *Lactobacillus sanfranciscensis*, which has an obligately heterofermentative metabolism (Gobbetti & Corsetti, 1997). Certain heterofermentative LAB possess the enzyme mannitol-dehydrogenase, which reduces fructose released from fructan-hydrolysis to mannitol. Depending on the fermentation conditions and the substrates available, some LAB are capable to produce high levels of mannitol (Sahin *et al.*, 2019). Lastly, the gluten-free bread did not contain any ingredient naturally high in FODMAPs (cf. Table 4-1), except for the pea protein, which may be high in GOS, depending on the production of that ingredient

(cf. section 4.4.1.4). However, the pea protein is only a small portion of the formulation. Thus, the bread had overall a very low FODMAP profile (Figure 4-2).

Two different wheat-based crackers were analysed; the plain crackers had an overall low FODMAP content with 0.20 g/ 100 g (0.21 g/ 100 g DM) fructans and 0.05 g/ 100 g (0.06 g/ 100 g DM) fructose in excess of glucose. According to the ingredients list (Table 4-1), this product was also produced from a yeast fermented dough. In contrast, the wheat-based garlic crackers had high amounts of fructans, deriving from the wheat flour and the garlic powder in the formulation; the latter is a rich source of fructans (Muir *et al.*, 2009). The garlic crackers contained additionally high levels of fructose in excess of glucose 0.34 g/ 100 g (0.36 g/ 100 g DM), since the dough of these crackers was also fermented with yeast. Similar to the gluten-free bread, the gluten-free oat-based crackers did not contain substantial amounts of any FODMAPs. Low levels of fructose in excess of glucose were found (0.11 g [0.12 g] /100 g [DM]), deriving from the honey in the formulation, and low levels of GOS (0.20 g [0.21 g] /100 g [DM]), which naturally occur in oats, were detected.

The wheat-based biscuits contained, besides high fructan levels (1.34 g [1.41 g] /100 g [DM]), also high amounts of lactose (0.96 g [1.00 g] /100 g [DM]), originated from milk as part of the formulation. The gluten-free oat-based biscuits did not contain any high FODMAP ingredients, and thus only low levels of GOS (0.13 g [0.14 g] /100 g [DM]) were detected.

The pasta was analysed before and after cooking. The durum wheat pasta contained 1.34 g/ 100 g (1.50 g/ 100 g DM) fructans before, and 0.32 g/ 100 g (0.92 g/ 100 g DM) after cooking; 40 % of the wheat fructans were lost in the cooking water. This corresponds to the findings of Gélinas *et al.* (2016). The gluten-free pasta, made from the low FODMAP ingredients corn flour and rice flour (Table 4-1), did not contain any FODMAPs (Figure 4-2).

4.4.3 Conclusion

This work is the first comprehensive study providing a dry matter-based characterisation of the FODMAP profiles of a wide range of cereal product ingredients. Existing FODMAP literature predominantly serves as nutritional guidance for individuals adhering to the low FODMAP diet. This study, on the contrary, serves as a tool for the development of functional food products with a lowered FODMAP

content. The extensive knowledge on compositional information of the raw ingredients enables a targeted application of (bio-) technological approaches to lower FODMAP levels.

In accordance with other studies, wheat, rye, spelt and barley were confirmed as rich sources of fructans, while pulses had high GOS levels (Biesiekierski *et al.*, 2011; Haskå *et al.*, 2008; Martínez-Villaluenga *et al.*, 2008; Nemeth *et al.*, 2014). Protein ingredients from pulses had varying levels of GOS, depending on their production process. Commercial sprouts from pulses contained moderately high amounts of GOS, despite the GOS degrading effect of the sprouting process (Martínez-Villaluenga *et al.*, 2008). The gluten-free cereals oat, millet, and rice as well as the pseudo cereals quinoa and buckwheat did not contain substantial amounts of any of the FODMAPs investigated. However, buckwheat, which is currently listed as low FODMAP grain, was outstanding due to the major fraction of soluble carbohydrates, fagopyritols. Those sugars are indigestible, fermentable, and structurally similar to GOS and may have a similar effect on a sensitive gut. Further studies are needed to identify the contribution of fagopyritols to the flatulence-causing effect in IBS-patients. In addition to the FODMAPs found in the ingredients, the relevance of fructose in excess of glucose, polyols and lactose was highlighted by means of the analysis of representative, commonly consumed cereal products of different categories. This study is the foundation for the development of nutritious and appealing functional cereal products with a lowered FODMAP content.

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Chapter 5

Fundamental study on changes in the FODMAP profile of cereals, pseudo cereals, and pulses during the malting process

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5.1 Abstract

Whole grains and pulses are rich in nutrients but often avoided by individuals with gastrointestinal disorders due to high levels of fermentable oligo-, di-, monosaccharides and polyols (FODMAPs). This study investigated the impact of malting as a natural delivery system for endogenous enzymes. Malts from barley and wheat (naturally high in fructans), lentils and chickpeas (high in α-galactooligosaccharides), oat and buckwheat (low in FODMAPs) were produced. While barley and wheat malts had slightly elevated fructan levels, in oat malt 0.8 g/100g DM fructans were de novo synthesized. In lentils and chickpeas, α-galactooligosaccharides diminished by 80 − 90 %. Buckwheat did not contain any FODMAPs commonly investigated, but fagopyritols which may have a similar physiological effect. Also, fagopyritols were degraded. While malted pulses and buckwheat are directly suitable for low FODMAP applications, using the combined approach of malting and fermentation, malted cereals could contribute to high nutritional values of such products.

5.2 Introduction

Whole grain cereals, pseudo cereals, and pulses (legumes) and food products made from those are an essential component of a healthy diet. They are rich in proteins, dietary fibre, vitamins, and minerals (Fardet, 2010). Nowadays these nutrients, especially dietary fibres, are lacking in the western diet. In this respect, whole grains and pulses can make an important contribution to a wholesome, healthy diet.

Many grains and pulses contain fructans and α-galactooligosaccharides (GOS, often referred to as raffinose family oligosaccharides, RFO), which are indigestible and readily fermentable carbohydrates. Fructans mainly occur in cereals and are structurally divided into five groups, classified by their linkages: the inulin-, levan-, graminan-, inulin neoseries-, and levan neoseries-type fructans. Cereals contain predominantly graminans, which are composed of β (2 → 1) and β (2 → 6) linked fructose chains with a terminal glucose. GOS are α (1 → 6) linked galactosyl derivatives from sucrose and occur in lower levels in cereals and in high levels in pulses (Ispiryan *et al.*, 2019; Livingston *et al.*, 2009). Because of the lack of digestive enzymes to hydrolyse GOS and fructans, the human intestine cannot metabolise those carbohydrates. They are part of the dietary fibres of cereals and pulses that can, on the one hand, contribute to positive health effects due to their indigestibility in the human intestine and fermentability by the gut microbiota (Van den Ende, 2013; Vijn & Smeekens, 1999). On the other hand, however, people suffering from functional gastrointestinal disorders such as irritable bowel syndrome (IBS) have to avoid pulses and grains high in these carbohydrates (Muir *et al.*, 2019). Together with a number of other short-chain carbohydrates, fructans and GOS comprise the well-known group of FODMAPs (fermentable oligosaccharides di-, monosaccharides, and polyols). Besides fructans and GOS, the milk sugar lactose, sugar alcohols (polyols) and fructose in excess of glucose belong to the list of FODMAPs. However, as recently highlighted by Halmos & Gibson (2019), this list covers the vast majority of dietary FODMAPs. Other indigestible carbohydrates, such as fagopyritols (FP) from buckwheat, may be included in this list. The ingestion of FODMAPs by individuals with IBS is often associated with gastrointestinal discomfort and symptoms, including an altered bowel habit, bloating and abdominal pain. Numerous studies over the last years, have shown the effectivity of a dietary therapy. An individualised diet low in the specific FODMAPs, towards which the individuals show sensitivities, is a successful

therapeutic approach in the treatment of IBS (Gibson & Shepherd, 2010; Halmos *et al.*, 2014).

However, the avoidance of grains and pulses due to their high FODMAP contents reduces the availability of satiating and healthy food choices drastically. Hence, the targeted reduction of FODMAPs while maintaining the nutritional value is a promising approach to enable a symptom-free consumption of grains and pulses and food products made from those. The investigation of (bio-) technological approaches to lower FODMAP levels in ingredients and food products is an emerging area of research. Firstly, cooking and canning have been described to lower GOS levels in pulses; likewise, fructans from pasta dissolved into the cooking water (Tuck et al., 2018; Ispiryan et al., 2020). The degradation of fructans in the production of bread by microbial enzymes, either achieved by fermentation with lactic acid bacteria and yeast, or the addition of purified commercial enzyme-sources, were reported to be effective (Loponen *et al.*, 2017; Loponen & Gänzle, 2018; Struyf *et al.*, 2017). Furthermore, the impact of native endogenous enzymes is known for a long time to diminish GOS levels in pulses. Those are activated during the germination process, leading to a reduction of GOS (Blöchl *et al.*, 2008; El-Adawy, 2002; Frias *et al.*, 1996; Martínez-Villaluenga *et al.*, 2008; Trugo *et al.*, 1999). This study investigated the impact of malting, which is a combined approach of controlled and limited germination, involving the preceding steeping, and subsequent kilning (Kunze *et al.*, 2004).

Malts can serve as a natural delivery system for enzymes. Hence, the main hypothesis of this study was that malting may serve as a tool to modulate FODMAP profiles due to endogenous enzymes of the seeds activated or synthesized throughout the malting process. Thus, this study examined changes in FODMAP contents of different raw ingredients observed during malting. Therefore, six different seeds were selected based on the findings of a previous study; two high fructan grains (barley and wheat), two high GOS seeds (lentil and chickpea) and two low FODMAP grains (oat and buckwheat) (Ispiryan *et al.*, 2020). Wheat and barley are the most commonly malted cereals, largely used for the production of beer and the distillation of whiskey. As malting or germination are known to reduce antinutritional compounds, increase bioavailability of minerals and vitamins, and improve protein digestibility, a more versatile application of malted ingredients is of interest (El-Adawy, 2002; Fardet, 2010). Malts can be applied for instance in non-alcoholic malt-based beverages or

baked products (Bewley *et al.*, 2013; Gobbetti *et al.*, 2019). Particularly, the malting of pulses is not common. However, malted pulses could serve as valuable ingredients in low FODMAP formulations as a rich source of plant protein. Also malted oat and buckwheat could contribute to the nutritional value of different products.

Malting, as a tool to modulate FODMAP levels has not been examined to date. Furthermore, studies investigating the developments of fructans and GOS in cereals and pulses are scarce and contradictory, as reported and discussed in this fundamental study.

5.3 Materials and Methods

5.3.1 Raw material used for malting

Spring malting barley (*Hordeum vulgare cv. beatrix*) grains were obtained from Saaten union (France). Wheat (*Triticum aestivum*) grains and chickpeas (*Cicer arietinum*) were purchased in local food stores (Cork, Ireland). Oat (*Avenia sativa*) grains were supplied by Societa' Agricola Zannini Daniele e Claudio (Jesi, Italy) and lentils (*Lens culinaris var. Itaca*) were obtained from Agroservice S.p.A., San Severino (Marche, Italy). Buckwheat (*Fagopyrum esculentum*) grains were purchased from Trouw B.V. (Rotterdam, Netherlands). The germinative energy according to MEBAK 1.4.2.5 of all seeds was at least 93 % (MEBAK, 2011).

5.3.2 Malting

To avoid microbial contamination, all raw seeds were disinfected with 10 % (v/v) hydrogen peroxide (H_2O_2) prior to malting, according to Oliveira *et al.* (2012). Therefore, perforated stainless-steel steeping boxes, filled with 300 g of seeds, were placed in plastic boxes (30 x 20 cm). The seeds were immersed in 2 L H_2O_2 and stirred for 10 min, followed by a washing step with 2 L distilled H_2O for 5 min, and another disinfection and washing cycle of 5 min each. The disinfected seeds were transferred to a sterilized batch of plastic boxes and air dried for 24 h under a vertical sterile laminar flow. Four boxes of each raw material were prepared for malting.

Malting of wheat and barley was carried out according to the standard micromalting method (MEBAK 1.5.3) from Mitteleuropäische Brautechnische Analysen-kommission, MEBAK (2011). Steeping and germination were conducted in a humidity-controlled chamber (KOMA SunRiser, Heinsberg-Dremmen Germany). The disinfected, air-dried grains were transferred into perforated stainless-steel steeping boxes, which were placed into plastic boxes. Four boxes for each raw material were prepared, and the grains covered with 2 L of 14 °C tempered drinking quality treated brewery water. For the alternation of wet and air rest steps during the steeping process, the perforated steel boxes were transferred into empty plastic boxes or covered with a fresh batch of tempered brewing water. The combination of wet steeps and air rests was carried out for a total of 3 days, at 14 °C and a humidity of 80 % until a final steeping degree of 45 % was reached. The grains were germinated for 3 days, at the same conditions, in the perforated steel boxes placed into dry plastic boxes. Finally,

the green malt was kilned in a Joe White malting machine (Australia) according to Krahl *et al.* (2009). Kilning of the malts was conducted for 14 h at 50 °C, followed by 1 h at 60 °C, 1 h at 70 °C, and finally 1 h at 80 °C for wheat and 3 h at 80 °C for barley. The rootlets were removed using a thresher (Wintersteiger LD180, Wintersteiger AG, Ried, Austria). Samples from different stages of the malting process were immediately frozen and freeze-dried for further analysis.

Malting of lentil, chickpea, oat, and buckwheat was conducted using the same facilities as described above. The malting parameters for all seeds are presented in Table 5-1.

Table 5-1. Malting parameters for the selected seeds

Raw material	Steeping	Germination	Kilning
Barley[1]	3 d, 14 °C, (45 %)	3 d, 14 °C	14 h, 50 °C; 1 h, 60 °C; 1 h, 70 °C; 3 h, 80 °C
Wheat[1]	3 d, 14 °C, (45 %)	3 d, 14 °C	14 h, 50 °C; 1 h, 60 °C; 1 h, 70 °C; 1 h, 80 °C
Chickpea[2]	7 h, 20 °C, (50 %)	3 d, 20 °C	20 h, 50 °; 1 h, 65 °C; 5 h, 75 °C
Lentil[2]	12 h, 25 °C, (55 %)	4 d, 25 °C	17 h, 50 °C; 1 h, 60 °C; 5 h, 65 °C
Oat[3]	35 h, 14 °C, (45 %)	5 d, 15 °C	5 h, 35 °C; 6 h, 50 °C; 6 h, 60 °C
Buckwheat[4]	12 h, 10 °C, (40 %)	4 d, 15 °C	5 h, 45 °C; 17 h, 50 °C

Malting methods on the basis of [1] MEBAK (2011) (with slight modifications), [2] internally developed unpublished malting methods, [3] Klose *et al.* (2010), [4] Wijngaard *et al.* (2007) (with slight modifications)

5.3.3 Sample preparation and FODMAP quantification

The raw seeds and the malts were disrupted using a QIAGEN Tissue Lyser II (Hilden, Germany), to a particle size of ≤ 0.5 mm (Ispiryan *et al.*, 2019). The samples from the malting process were freeze-dried for 3 days and disrupted in the same way.

The quantification of the carbohydrates was performed using high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD), performed on a Dionex™ ICS-5000+ system (Sunnyvale, CA, USA), equipped with Dionex™ CarboPac™ PA1 and PA200 columns (Thermo Fisher Scientific, Sunnyvale, CA, USA), as previously described (Ispiryan *et al.*, 2019; Ispiryan *et al.*, 2020). Briefly, 400 mg of sample were mixed with 1 mL of methanol and 100 µL internal standard rhamnose (9 mg/ mL) were added. The sample was extracted twice with 20 mL 80°C H_2O containing 50 mg/ L sodium azide, using a Sonoplus homogenizer. The combined extracts were cleared with Carrez I and Carrez II, brought to 100 mL and filtered through 0.2 µm syringe filters before HPAEC-PAD analysis.

The same equipment and chemicals as previously described by Ispiryan *et al.* (2019) were used. All carbohydrates except total fructans were quantified using authentic reference standards. D-pinitol was purchases from Sigma-Aldrich (Darmstadt, Germany), and galactinol hydrate from Carbosynth (Compton, UK). The remaining reference standards for xylitol, sorbitol, mannitol, rhamnose, galactose, glucose, fructose, sucrose, raffinose, stachyose, verbascose, kestose, nystose, and kestopentaose were the same as reported by Ispiryan *et al.* (2019). No reference standards for fagopyritol B1 and ciceritol were available. The amounts were estimated using the galactinol standard. The fructan content was determined via HPAEC-PAD after enzymatic hydrolysis as previously reported (Ispiryan *et al.*, 2019). In short, two 500 µL aliquots of appropriately diluted sample extract were mixed with 150 µL enzyme mixture A (1:1:1 mixture of α-galactosidase, amyloglucosidase, and 100 mM NaOAc-buffer) and enzyme mixture B (1:1:1 mixture of α-galactosidase, amyloglucosidase, and inulinase), respectively, and incubated for 30 min at 60 °C. After inactivation of the enzyme at 100 °C for 40 min and addition of 350 µL H_2O containing 50 mg/ L sodium azide, the hydrolysates were subjected to HPAEC-PAD analysis.

All extractions were carried out in duplicate. The results of the malts and grains are presented in g analyte per 100 g sample on a dry weight basis (g/ 100 g DM). The dry matter (DM) of the seeds, the malts, and the samples from the malting processes after freeze-drying was determined according to MEBAK 1.5.1.1 (MEBAK, 2011).

5.3.4 Reference analysis of fructans

The fructan assay kit K-FRUC (Megazyme, Bray, Ireland) was applied for the determination of the fructan development in oat grains during the malting process, as reference to the determination via HPAEC-PAD. This assay contained, in addition to the exo- and endoinulinases used in the HPAEC-PAD method, also levanases; the latter are specifically applied to cleave levan type fructans as well as highly branched fructans. Samples were treated with α-galactosidase (E-AGLANP, Megazyme, Ireland, Bray) before degradation of starch, maltodextrins, and sucrose, for the removal of interfering GOS.

5.3.5 Fructan development fingerprint during oat malting process

Undiluted extracts of each step from the oat malting process were incubated with the enzyme mixture A from the HPAEC-PAD fructan assay, including amyloglucosidase, α-galactosidase and NaOAc-buffer (Ispiryan *et al.*, 2019). After degradation of malto-oligosaccharides and GOS, peaks were assigned to fructans. As additional elucidation the same extracts were incubated with inulinases only, to confirm a correct assignment of the fructan peaks. The same enzyme preparations were used for these incubations as for the fructan assay according to Ispiryan *et al.* (2019).

5.3.6 Raw material and malt analyses

The raw seeds and malts were disrupted and prepared for analyses as described above. The moisture in the raw ingredients and the malts was determined in triplicate, according to MEBAK 1.5.1.1. The development of the activities of α- and β-amylases in the grains and malts was determined with the assay kits K-CERA and K-BETA3 (Megazyme), respectively. The sample-extracts for the Megazyme assays were prepared in duplicates and another duplicate of each of the replicate-extract further treated according to the assay procedures.

5.3.7 Scanning electron microscopy

Whole raw seeds and malts were freeze-dried and cross-sectioned using a scalpel. The specimens were mounted on aluminium stubs with double-sided adhesive carbon tape and sputter-coated with a 5 nm layer of gold/palladium (Au:Pd = 80:20) using a Quorum Q150R ES Sputter Coating Unit (Quorum Technologies Ltd., Sussex, UK). The samples were examined using a JSM-5510 scanning electron microscope (JEOL Ltd, Tokyo, Japan), operated at an accelerating voltage of 5 kV.

5.3.8 X-ray tomography

The analyses were performed using a Zeiss XRadia XRM520 equipment at the 4D Imaging Lab, Lund University, Sweden. The X-ray source was a polychromatic cone beam with a source voltage of 40 kV, a power of 3 W and exposure time of 60 min. Radiographs of 1601 pixels width and 1014 pixels height were measured during the 360° rotation. An optical magnification of 0.4x was used with the camera set 2 x 2 binning to give an image width and height of 1024 pixels. The field of view was adjusted for each studied sample in order to maximise the resolution and capture the

full sample width. This is a reason for the cubic voxels having different dimensions for each image in the final reconstructed images.

5.3.9 Statistical analysis

Statistical analyses were performed with SPSS Statistic 26 (IBM Corp., Armonk, NY, USA). An independent t-test ($p = 0.05$) was applied within the calculation of the HPAEC-PAD fructan analysis, as described by Ispiryan *et al.* (2020). One-way ANOVA followed by Tukey's test ($p = 0.05$) were applied to determine statistical significance for fructan, GOS and sucrose levels between raw seeds, samples from the malting process and the corresponding malts.

5.4 Results and discussion

5.4.1 General characteristics of raw material and malts

Malting of the seeds was conducted under strictly controlled conditions aiming to activate endogenous enzymes and degrade carbohydrates, storage proteins and cell walls. As part of the malting process seeds were dried and converted to storable ingredients with a significantly lowered moisture in the final malts. These contained 6 − 9 % moisture in comparison to the germinated seeds with approximately 45 % water content (Table 5-2).

Table 5-2. Characteristics of raw and malted seeds.

Ingredients	moisture (%) [a, b]	α-amylase (CU/ g DM) [a,c]	β-amylase (U/ g DM) [a,c]
high fructan seeds			
Raw barley	13.45 ± 0.06 [A]	0.03 ± 0.00 [A]	12.72 ± 1.21 [A]
Barley malt	6.21 ± 0.03 [B]	99.35 ± 4.60 [B]	12.57 ± 0.62 [A]
Raw wheat	12.40 ± 0.28 [A]	0.12 ± 0.00 [A]	33.55 ± 0.49 [A]
Wheat malt	6.91 ± 0.02 [B]	192.43 ± 0.92 [B]	43.16 ± 4.34 [A]
high GOS seeds			
Raw chickpea	11.06 ± 0.01 [A]	0.50 ± 0.00 [A]	n.d.
Chickpea malt	5.80 ± 0.01 [B]	1.71 ± 0.09 [B]	n.d.
Raw lentil	11.86 ± 0.06 [A]	1.32 ± 0.01 [A]	n.d.
Lentil malt	8.89 ± 0.04 [B]	1.45 ± 0.06 [A]	n.d.
low FODMAP seeds			
Raw oat	13.10 ± 0.02 [A]	0.06 ± 0.00 [A]	0.57 ± 0.03 [A]
Oat malt	7.24 ± 0.03 [B]	28.65 ± 1.46 [B]	1.25 ± 0.01 [B]
Raw buckwheat	13.90 ± 0.01 [A]	0.05 ± 0.02 [A]	0.12 ± 0.05 [A]
Buckwheat malt	7.45 ± 0.01 [B]	33.73 ± 0.71 [B]	0.50 ± 0.06 [B]

[a] DM, dry matter; means ± standard deviations of raw and corresponding malted seeds with different superscript capital letters are significantly different ($p < 0.05$).
[b] analyses carried out in triplicate. [c] Megazyme assay-extractions carried out in duplicate.
[d] n.d., not detected in means of levels below limit of quantification of the assay.

For the common application of cereal malts, the mobilisation of the grains' constituents is important for the availability of fermentable compounds during the brewing process. The most important enzymes hydrolysing carbohydrates for the malting and brewing application are α- and β-amylases, which are produced and activated with the initiation of the germination of cereals (Table 5-2). These amylolytic enzymes are responsible for the mobilisation of starch. This study investigated the impact of the malting process on other carbohydrates than starch, including fructans, GOS and fagopyritols (FP). These carbohydrates are a significantly lower proportion

of the grain than starch. Their development and potential role in the carbohydrate metabolism during the malting process is presented in the following sections.

The different tissues of the seeds storing the FODMAPs are highlighted in Figure 5-1. GOS and fructans in cereals are predominantly stored in the outer layer of the grain, the aleurone layer (Fardet, 2010). The seed coat in pulses is mainly composed of insoluble fibres. Soluble carbohydrates, including GOS, are stored in the cotyledon (Tosh & Yada, 2010). FP in buckwheat are mainly accumulated in the embryonic axis and the cotyledon of the achene (Figure 5-1; e, f, i) (Steadman *et al.*, 2000).

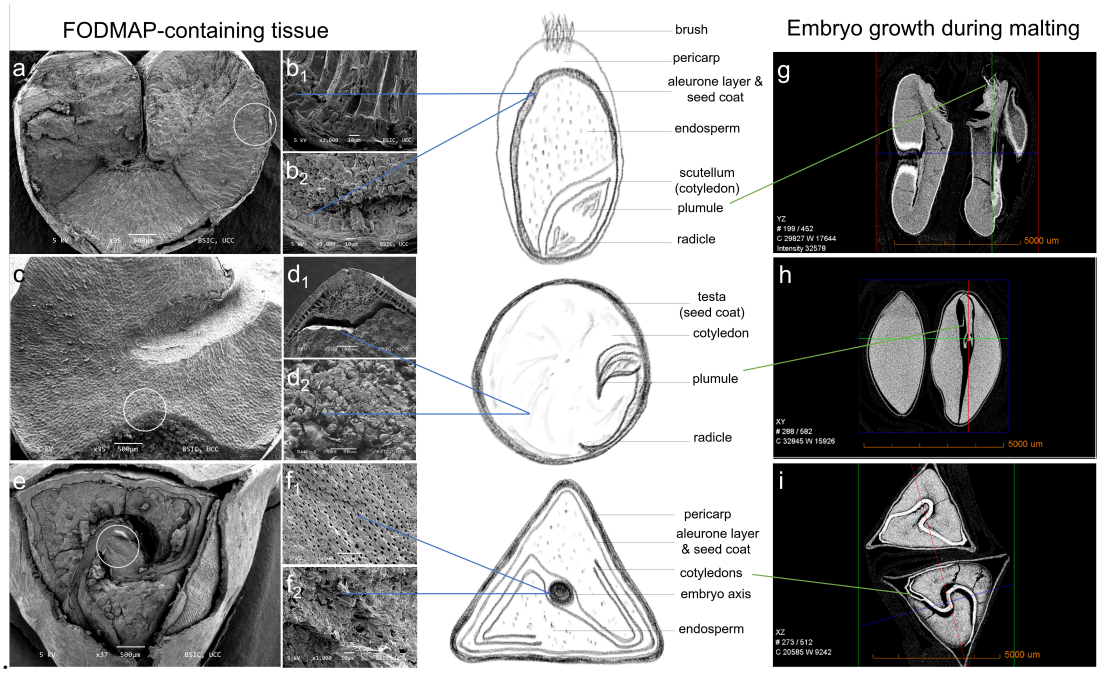

Figure 5-1. (a-f): SEM micrographs of wheat (a: whole grain cross section, b_1: raw grain, b_2: malted grain) highlighting GOS- and fructan-storing tissue (aleurone layer) in cereals-grains, lentil (c, whole seed cross section, d_1: raw seed, d_2: malted seed) highlighting GOS-storing tissue (cotyledons) in pulse-seeds, buckwheat (e: whole grain cross section, f_1: raw grain, f_2: malted grain) highlighting FP-storing tissue (embryo axis with cotyledons). (g-i): X-ray images of unmalted (left/ top) and malted (right/ bottom) wheat, lentil, buckwheat seeds showing different growth of embryo during the malting process.

The comparison of the SEM images of the raw seeds and their corresponding malts indicated compositional changes typically observed during the malting process. The SEM images of the malts showed cell-wall degradation, modification of the starch granules and mobilised layers of protein (Figure A-6, Appendix A-1). The irregular particles between, and on the starch-granules were the mobilised fragments of protein, resulting from the malting process. The growth of the embryo in the different kernels during germination is visualised on the X-ray images (Figure 5-1, g-i). The malts of the chickpeas and the lentils (dicotyledonous seeds) showed an equatorial split in the

cotyledon of the seed due to the growth of the shoot. In the cereals (monocotyledonous), barley, wheat, and oat the embryo is located at the dorsal side of the grains and the shoot partly grew into the starchy endosperm, leaving the whole seed intact. Also, the buckwheat (dicotyledonous) achene remained intact with the growth of the shoot from the embryonic axis at the top of the achene (Figure 5-1, i). The growth of shoots into storage tissues, such as the endosperm, may aid the absorption of products of storage reserve mobilisation in the germination process (Bewley *et al.*, 2013). However, physically observed changes in the micrographs did not directly relate to the changes in the FODMAP profiles in the seeds.

5.4.2 Changes in the FOMAP profile during the malting process

In accordance with other studies, fructans and GOS were the predominant FODMAPs in the seeds investigated in this study and are generally found in cereals, pseudo cereals, and pulses (Table 5-3) (Biesiekierski *et al.*, 2011; Ispiryan *et al.*, 2020). However, the biochemical effect of the malting process observed in the different raw material, which were naturally high in fructans, high in GOS or showed an overall low FODMAP profile was highly variable. Thus, the discussion of the FODMAP changes due to the malting process is held by groups of FODMAPs, the fructans and the GOS, rather than by high or low FODMAP categories of the raw ingredients.

Lactose and polyols such as xylitol, sorbitol, mannitol or maltitol did not naturally occur in any of the raw materials and their corresponding malts and are therefore not further discussed in this study. Fructose in excess of glucose was also not found in any of the malts. The raw seeds are generally very low in the monosaccharides (Cimini *et al.*, 2015; Ispiryan *et al.*, 2020). During the malting process of the cereals and pseudo cereals, the activities of starch-hydrolysing enzymes, such as α- and β-amylases are increased (Table 5-2), which leads to an increase of glucose to a much higher extend in the malts than fructose (Table 5-3). Also, in the malts of the pulses, where no amylase activities were determined, either equal amounts of glucose and fructose or higher glucose levels were found.

Table 5-3. FODMAP contents in raw and malted seeds.

r. = raw m. = malted	FODMAP contents ± standard deviation [g/100g DM] [a]											Fructan/ GOS/FP-B1
	Mono-/Disaccharides [b, c]			Polyols (cyclitols) [b,e]		Oligosaccharides						
	Glucose	Fructose	EF [d]	Xylitol (pinitol)	Sorbitol (inositol)	Raffinose/ stachyose [b]	Verbascose [b]	∑ GOS	Galactinol (FP-B1) [b,f]	Ciceritol [b, g]	Total fructan [h]	± raw to malt
high fructan seeds												
Barley r.	0.06 ± 0.01 [A]	0.06 ± 0.00 [A]	-	n.d.	n.d.	0.49 ± 0.01 [A]	n.d.	0.49 ± 0.01 [A]	n.d.	n.d.	1.09 ± 0.02 [A]	+0.18
Barley m.	1.88 ± 0.17 [B]	0.17 ± 0.00 [B]	-	n.d.	n.d.	0.17 ± 0.00 [B]	n.d.	0.17 ± 0.00 [B]	n.d.	n.d.	1.27 ± 0.01 [B]	
Wheat r.	0.05 ± 0.00 [A]	0.04 ± 0.00 [A]	-	n.d.	n.d.	0.34 ± 0.01	n.d.	0.34 ± 0.01	n.d.	n.d.	1.23 ± 0.00 [A]	+0.2
Wheat m.	1.25 ± 0.01 [B]	0.20 ± 0.01 [B]	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.43 ± 0.04 [B]	
high GOS seeds												
Chickpea r.	0.33 ± 0.00 [A]	0.05 ± 0.00 [A]	-	0.95 ± 0.04 [A]	n.d.	3.80 ± 0.01 [A]	0.06 ± 0.00 [A]	3.86 ± 0.01[A]	0.24 ± 0.01	3.45 ± 0.04 [A]	n.d.	-3.37
Chickpea m.	0.41 ± 0.04 [A]	0.28 ± 0.00 [B]	-	2.19 ± 0.02 [B]	n.d.	0.48 ± 0.00 [B]	0.01 ± 0.00 [B]	0.49 ± 0.00 [B]	n.d.	0.91 ± 0.01 [B]	n.d.	
Lentil r.	0.12 ± 0.01 [A]	0.06 ± 0.00 [A]	-	0.23 ± 0.01 [A]	n.d.	2.64 ± 0.00 [A]	1.25 ± 0.00 [A]	3.89 ± 0.00 [A]	0.01 ± 0.00	1.16 ± 0.05 [A]	n.d.	-3.05
Lentil m.	0.19 ± 0.00 [B]	0.21 ± 0.00 [B]	0.01	0.77 ± 0.04 [B]	n.d.	0.57 ± 0.00 [B]	0.27 ± 0.00 [B]	0.84 ± 0.00 [B]	n.d.	0.50 ± 0.03 [B]	n.d.	
‚low FODMAP' seeds												
Oat r.	0.04 ± 0.00 [A]	0.02 ± 0.00 [A]	-	n.d.	n.d.	0.24 ± 0.02 [A]	0.01 ± 0.00	0.25 ± 0.02[A]	n.d.	n.d.	n.d.	+0.77
Oat m.	0.75 ± 0.01 [B]	0.33 ± 0.01 [B]	-	n.d.	n.d.	0.07 ± 0.01 [B]	n.d.	0.07 ± 0.01 [B]	n.d.	n.d.	0.77 ± 0.01	
Buckwh. r.	0.09 ± 0.01 [A]	0.04 ± 0.00 [A]	-	n.d.	0.03 ± 0.00 [A]	n.d.	n.d.	n.d.	0.33 ± 0.01 [f]	n.d.	n.d.	-0.33
Buckwh. m.	1.84 ± 0.08 [B]	0.08 ± 0.00 [B]	-	n.d.	0.08 ± 0.00 [B]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	

[a] extractions carried out in duplicates and measured via HPAEC-PAD, results referred to dry matter (DM); means ± standard deviations of raw and corresponding malted seeds with different superscript capital letters are significantly different ($p < 0.05$). [b] n.d., not detected or levels below 0.005 g/ 100 g DM. [c] no lactose detected in raw or malted seeds. [d] EF, excess fructose = glucose – fructose. [e] cyclitols are products from GOS metabolism, relevance in context of FODMAPs not known. [f] FP-B1, fagopyritol B1 estimated as galactinol. [g] ciceritol estimated as galactinol [h] n.d., not detected in means of no significant difference in sucrose values and fructose values determined from difference of assay A and B in fructan determination ($p > 0.05$), or levels below 0.1 g/ 100 g DM (Ispiryan *et al.*, 2020)

5.4.2.1 Impact of malting on the fructan content in the seeds

Barley, wheat, and oat belong to the same family of grasses (*poaceae*) (Livingston *et al.*, 2009). Only in the grains of wheat and barley fructans were determined, with 1.23 g and 1.09 g/ 100 g DM, respectively. As shown in this study and a previous study, oat is categorised as a cereal grain, naturally low in FODMAPs (Ispiryan *et al.*, 2020). Only traces of fructans were found in raw oat grains. However, the malting process led to a significant increase of fructans in the oat malt. With 0.77 g/ 100 g DM fructans, oat malt is a high FODMAP ingredient, similar to the other cereals. Also, the fructan levels in wheat and barley were slightly elevated by 16 − 17 % to 1.43 g and 1.27 g/ 100 g DM, respectively (Table 5-3). To exclude an underestimation of neolevan-type fructans in oat, which are known to occur in other parts of the oat plant in high levels (Livingston *et al.*, 1993), an additional reference analysis of the oat-fructans was conducted using the K-FRUC Megazyme assay. No differences were seen between the different analytical approaches (Table A-4, Appendix A-1). Studies investigating the impact of malting or germination on fructans in cereals are scarce and contradictory. Similar findings to this study were reported by MacWilliam *et al.* (1956), with an increase of fructans by 60 % in barley malt. Also Cozzolino *et al.* (2016) and Krahl *et al.* (2008) saw an increase by 10 − 30 % in barley malt and 30 % in spelt malt. However, the same authors in a second study did not see any effect of malting on barley, wheat and rye, despite applying the identical malting regime they had used for spelt (Krahl *et al.*, 2009). Furthermore, Tuck *et al.* (2018) saw a significant decrease in fructans in sprouted wheat, barley and rye grains. The most essential difference between a sprouting and malting process is the final step of the malting, the kilning. The absence of this step contradicts the results in this study, rather than providing an explanation for the different outcomes. However, the study conducted by Tuck *et al.* (2018) reports FODMAP levels based on the fresh weight of the foods, in contrast to the dry-matter-based characterisation in this study. Hence, the fructan-lowering effect may be, at least partly, attributed to dilution of the fructans by the soaking of the grains with water rather than the germination process. The detailed impact of the entire malting process is discussed in section 5.4.3.1.

Finally, in the malts of chickpeas, lentils, and buckwheat, no fructans were detected (Table 5-3). None of these seeds naturally contains fructans (Ispiryan *et al.*, 2020). Contradictory values found in literature, reporting fructan levels in pulses, result from

an overestimation of the enzymatic fructan determination if GOS are not taken into account, as shown by McCleary *et al.* (2019) and previously discussed (Ispiryan *et al.*, 2019; 2020).

5.4.2.2 Impact of malting on the GOS content in the seeds

In accordance with a previous study, the raw grains of wheat, barley and oat contained moderate levels of GOS (predominantly raffinose) with 0.34 g, 0.49 g, and 0.24 g/ 100 g DM, respectively (Ispiryan *et al.*, 2020). Malting process has led to a complete disappearance of those sugars in wheat, and to a decrease by 65 − 72 % in barley and oat. This effect has also been reported by Harris & MacWilliam (1954) and MacWilliam *et al.* (1956). In both studies raffinose was fully decomposed in barley malts. Likewise, no GOS remained in the sprouted wheat, rye, and barley grains from Tuck *et al.* (2018).

The investigated pulses, chickpeas and lentils are known as high FODMAP seeds and contained in accordance with other studies high amounts of GOS (predominantly stachyose, but also raffinose and verbascose), 3.86 g and 3.89 g/ 100 g DM, respectively (Biesiekierski *et al.*, 2011; Ispiryan *et al.*, 2020). The process of malting has led to a significant reduction of those carbohydrates in the pulses. GOS in chickpea malt diminished by 87 % and in lentil malt by 78 %, to 0.49 g and 0.84 g/ 100 g DM, respectively. The effect of malting per se on pulses is scarcely studied. However, the beneficial effect of germination on the nutritional profile of pulses, including reduction of the flatulence causing factor GOS, has been widely investigated (El-Adawy, 2002; Frias *et al.*, 1996; Martínez-Villaluenga *et al.*, 2008). Tuck *et al.* (2018) likewise saw a decrease of GOS in sprouted mung beans and kidney beans, by 44 % and 47 %, respectively, based on the fresh weight. In contrast to this, the authors saw a significant increase of GOS in chickpeas, by 63 %. According to Bewley *et al.* (2013) GOS synthesis may occur in germinating peas when germination is slowed or impeded. Despite this, controlled germination and malting seem to be very effective ways to significantly reduce GOS in pulses, based on the findings of this and numerous other studies. The potential of germination to decrease GOS in different cereals and pulses has been discovered 40 − 50 years ago and has been continuously studied to this day (Kannan *et al.*, 2018; Martínez-Villaluenga *et al.*, 2008; Reddy & Salunkhe, 1980).

Lastly, the pseudo cereal buckwheat does not contain any of the commonly investigated FODMAPs. Thus, it is currently categorised as a low FODMAP food or ingredient (Ispiryan *et al.*, 2020; Monash University, 2020). However, as previously discussed by Ispiryan *et al.* (2020), FP are the major fraction of soluble carbohydrates in buckwheat. These are also indigestible, fermentable di- and oligosaccharides, and may be considered as FODMAPs. Buckwheat embryos accumulate up to 5 % FP during maturation; 0.2 % to 3 % are found in different milling fractions from the buckwheat groats (Horbowicz *et al.*, 1998; Steadman *et al.*, 2000). Those compounds are α-galactosides from the cyclitol D-*chiro*-inositol and are suspected to have a similar impact on the human digestive system as GOS. Their structural similarity (cf. Figure 5-3) explains the necessity of the enzyme α-galactosidase for an absorption into the intestinal lumen. It is known that this enzyme is not present in the human gut. Thus, there is evidence that FP may have a flatulence causing effect in susceptible individuals, deriving from the consumption of buckwheat, like the effect of GOS deriving from pulses (Horbowicz *et al.*, 1998). Also, concentration ranges of FP in buckwheat seeds could be sufficient to trigger symptoms in IBS patients (cutoff levels according to Varney *et al.* (2017): 0.3 g oligosaccharides per serving).

The main FP found in buckwheat is the mono-galactosyl cyclitol fagopyritol B1 (FP-B1) (Horbowicz *et al.*, 1998). No authentic reference standards of FP were available. In this study, the most abundant representative of the FP-series, FP-B1, was semi-quantitatively determined using a galactinol standard, with 0.33 g/ 100 g DM in raw buckwheat grains. Like GOS, FP-B1 was degraded during the malting process; only traces were found in the buckwheat malt. Even though no study has investigated the impact of malting on the carbohydrate profile of buckwheat, Horbowicz *et al.* (1998) and Jia *et al.* (2015) observed the same phenomenon during the germination process.

5.4.3 Metabolism of FODMAPs in germinating seeds

Fructans, GOS, and FP are storage carbohydrates in the seeds of the different plants. Their metabolic functions are complex and not entirely understood. However, all three groups of carbohydrates seem to play a role in plant-protection under abiotic stress conditions, such as drought or thermic stress (Horbowicz *et al.*, 1998; Livingston *et al.*, 2009; Van den Ende, 2013). Similarly, their synthesis and utilisation appear to serve the growth of the embryo and the seedling during the malting process.

5.4.3.1 Fructan synthesis and utilisation during germination in wheat, barley, and oat

The metabolism of fructans during the malting or germination process is very poorly studied. Observations on the fructan development during malting in this study, and knowledge on the fructan development during kernel maturation, provide evidence that the development is controlled by endogenous enzymes of the grains activated throughout the malting process.

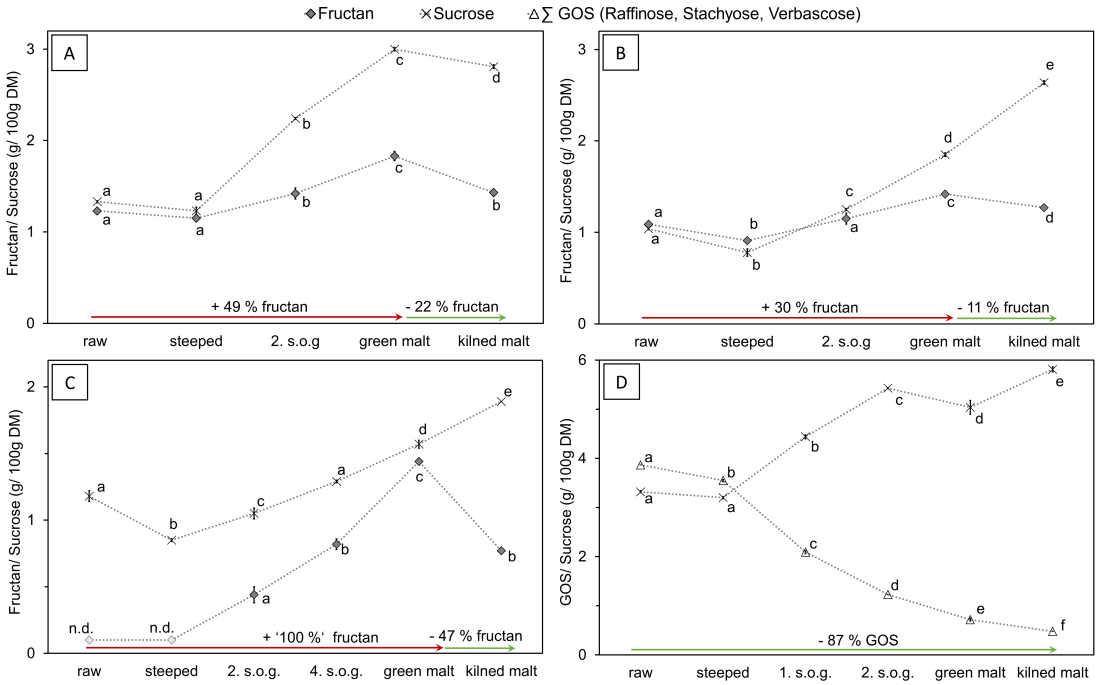

Figure 5-2. Development of GOS, fructan and sucrose throughout the malting process in high fructan grains (A: wheat, B: barley), low fructan grain (C: oat), high GOS seed (D: chickpea). s.o.g. stage of germination. Green malt corresponds to the end of the germination process. Data points with different letters were significantly different ($p < 0.05$). Fructan levels in raw and steeped oat grains: n.d., not detected in means of no significant difference in sucrose values and fructose values determined from difference of assay A and B in fructan determination ($p > 0.05$).

The malting process of wheat, barley, and oat showed the same trend of fructan development (Figure 5-2). In the first stage of malting, after steeping, a slight decrease was observed in barley and wheat. Raw oat contained only traces of fructans, thus no change was determined after the steeping process. MacWilliam *et al.* (1956) examined the changes during the steeping of barley in three points; they also observed after an initial increase a decline throughout the imbibition process. Also Krahl *et al.* (2008) saw a slight decrease in the pre-germination phase of spelt. In the following germination process, the fructan content increased continuously until at the last day of germination a fructan-maximum was reached in the green malts. Whereas in wheat

and barley the fructans increased by 49 % and 30 %, the green malt of oat contained 1.44 g/100 g DM of *de novo* synthesised fructans. MacWilliam *et al.* (1956) germinated barley grains for 9 days (in contrast to 3 – 5 d in this study). The more vigorous germination led to an increase of fructans in the green malt by ~300 %. Finally, kilning led to partial decomposition of the fructans in wheat, barley, and oat; 11 %, 22 % and 47 % of the fructans in the green malts, respectively, diminished during the kilning process (Figure 5-2). The same trend was again determined by MacWilliam *et al.* (1956); they observed a decline by 58 %. Krahl *et al.* (2008), reported the fructan development until the third day of in total 7 days germination. Thus, a decline in fructans due to the kilning process cannot be deduced from the given data. The peak suspected in the green malt, after a long germination time, was not recorded or shown. Furthermore, the fructan development was, in accordance with MacWilliam *et al.* (1956) , paralleled by an initial decline, followed by an increase in sucrose, which is produced from starch-catabolism products and serves as the main substrate for fructan synthesis (Bewley *et al.*, 2013; Cimini *et al.*, 2015).

The HPAEC-PAD profile with the *de novo* synthesised fructans in oat malt is shown in Figure 5-3. The peaks were assigned to fructans of the different degrees of polymerisation (DP) by comparing retention times to fructan reference standards and to characterised fructan profiles (e.g., wheat, barley, rye). As additional elucidation for the correct assignment of the peaks the fructan fingerprint of enzymatically treated extracts was obtained. Malto-oligosaccharides and GOS were degraded and only peaks remained in the chromatogram area of carbohydrates with DP ≥ 3 which could be assigned to fructans. The same peaks disappeared after incubation with the fructan degrading inulinases (cf. Figure A-7, Appendix A-1).

5.4.3.2 Development of fructans during malting linked to kernel maturation

The development of fructans in all three investigated cereal grains during the malting process has a similar trend to the development described for wheat kernel maturation. Immature wheat kernels accumulate fructans up to 35 g/ 100 g DM in the first week of maturation, which gradually decrease until the end of the maturation process to the approximate 2 g/ 100 g DM typically found in wheat (Cimini *et al.*, 2015). As during the maturation process, different exogenous and endogenous factors may influence the

activation of anabolic or catabolic enzymes of the fructan metabolism during the malting process (Figure 5-3).

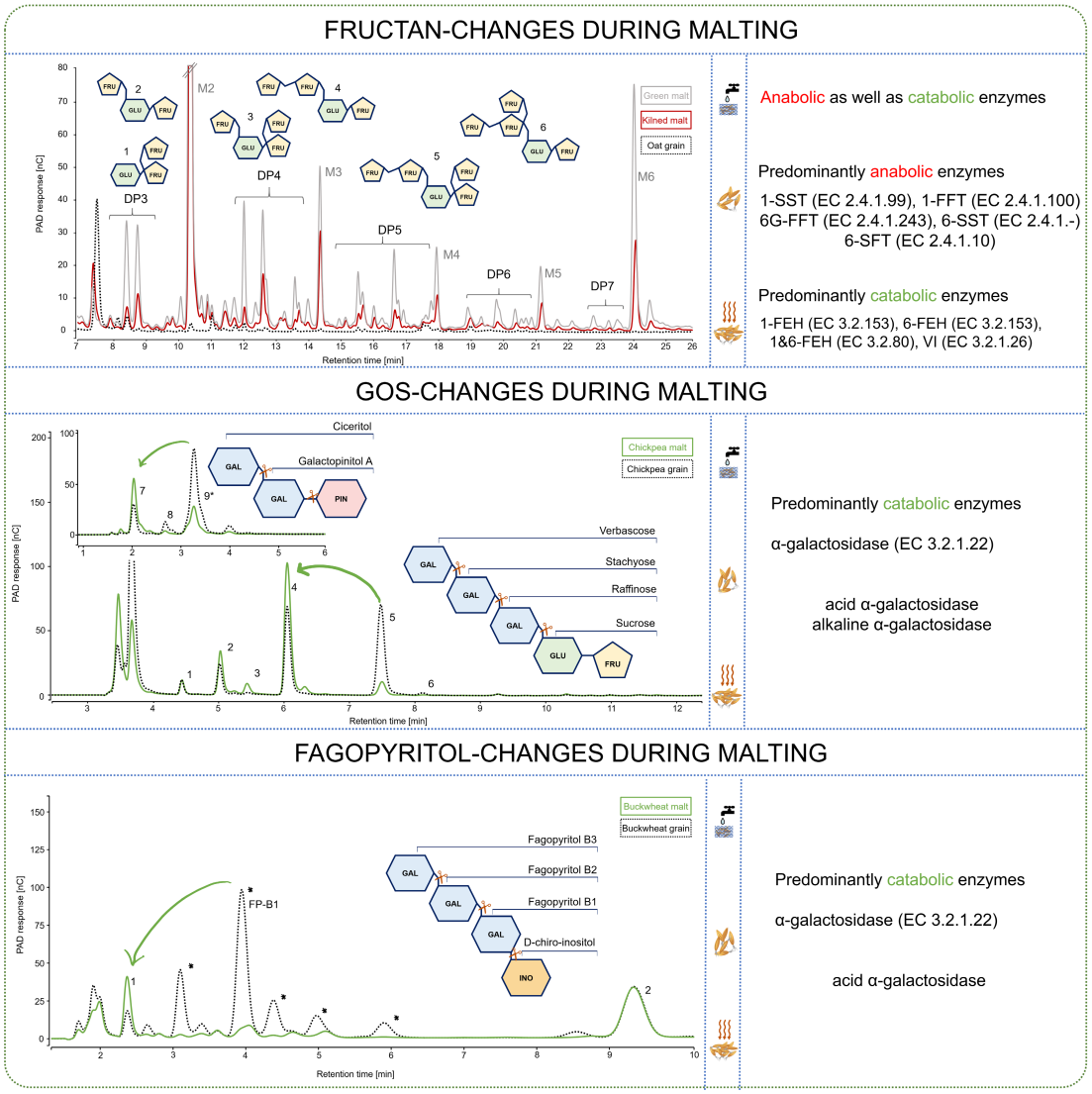

Figure 5-3. HPAEC-PAD profiles and FODMAP-alteration (left side) and involved endogenous enzymes (right side) during malting. Fructan changes: CarboPac PA200 profiles of oat grain, green malt, kilned malt with de novo synthesized fructans, (1) 1-kestose, (2) 6^G-kestotriose (neokestose), (3) 1&6^G-kestotetraose, (4) 6^G,6-kestotetraose, (5) 6^G,1&6-kestopentaose, (6) (1&6^G,6)-kestopentaose. Suggested fructan structures based on Livingston *et al.* (1993); suggested involved enzymes of fructan metabolism based on reported mechanisms during kernel maturation (Cimini *et al.*, 2015); sucrose:sucrose 1-fructosyltransferase (1-SST), fructan:fructan 1-fructosyltransferase (1-FFT), sucrose:fructan 6-fructosyltranferase (6-SFT), sucrose:fructan 1-fructosyltransferase (1-SFT), fructan:fructan 6G-fructosyltransferase (6G-FFT), sucrose:sucrose 6-fructosyltransferase (6-SST), fructan exohydrolases (FEH), vacuolar invertase (VI). GOS changes: CarboPac PA200 profiles of raw and malted chickpeas with GOS-degradation and CarboPac PA1 profiles with galactosyl cyclitol-degradation, (1) internal standard rhamnose, (2) glucose, (3) fructose, (4) sucrose, (5) raffinose/stachyose, (6) verbascose, (7) pinitol, (8) galactinol, (9*) suspected ciceritol; involved enzymes of GOS-metabolism during germination according to Blöchl *et al.* (2008). Fagopyritol changes: CarboPac PA1 profiles of raw and malted buckwheat with FP-degradation, (1) D-*chiro*-inositol, (2) internal standard rhamnose, all peaks marked with asterisk are suspected FP; involved enzymes of FP-metabolism during germination according to Jia *et al.* (2015)

Cimini *et al.* (2015) extracted enzymes from the fructan metabolism during different stages of kernel maturation, incubated substrates with those and determined via HPAEC-PAD the synthesis or degradation of fructans. Additionally, they determined the expression of fructan biosynthetic and degrading genes during different stages of kernel maturation. The authors had shown in early stages of maturation activities of several fructosyltransferases (FT), which mediate the biosynthesis of predominantly graminan-type but also neoseries-type fructans, found in wheat. Pronounced activities of sucrose:sucrose 1-fructosyltransferase (1-SST), fructan:fructan 1-fructosyltransferase (1-FFT), sucrose:fructan 6-fructosyltranferase (6-SFT), sucrose:fructan 1-fructosyltransferase, fructan:fructan 6G-fructosyltransferase (6G-FFT) and sucrose:sucrose 6-fructosyltransferase (6-SST) were determined. These enzymes may also be involved in the accumulation of fructans during the germination phase of barley, wheat, and oat (Figure 5-3). More prominent activities of 1-SST, 6G-FFT and 6-SFT in oat, may control the synthesis of neolevan-type fructans, which are found in oat stems and leaves and are suspected to be synthesised in oat malt (cf. suggested structures Figure 5-3) (Livingston *et al.*, 2009).

Cimini *et al.* (2015) did not find activities of fructan synthesising enzymes in mature wheat kernels. However, the germination process could initiate the activation and production of these enzymes in a similar way as amylase activities increased (Table 5-2). Furthermore, different activities of fructan exohydrolases (FEH) and vacuolar invertase (VI) are reported during the kernel maturation; 1-FEH, 6-FEH, and VI were shown to be active particularly in the beginning of the maturation process. The activity of 6&1-FEH remained stable until the end of the maturation accompanied by the constant fructan-degradation (Cimini *et al.*, 2015). Similarly, the decline in fructans during the kilning process could be controlled by dominating 6&1-FEH activity.

Such FEH mediated fructan degradation during the withering of the green malt may play a role in osmotic regulation of the seeds' drought resistance, as it was hypothesised for wheat kernel maturation (Cimini *et al.*, 2015). Livingston *et al.* (2009) discuss a potential relevance of fructans in membrane stabilisation under thermic and drought stress. The utilisation and synthesis of fructans provides evidence for a possible role of the fructans in fastening and optimising the membrane reorganisation during kernel hydration and germination. The breakdown of fructans may need less ATP than starch-breakdown. This would provide a metabolic advantage

in the pre-germination phase during imbibition (Cimini *et al.*, 2015). This study indicates a possible role of fructans in germination mechanisms and efficiency in cereals, particularly in oat, where the most significant development was observed.

Extensive investigations on the above described anabolic and catabolic enzyme activities throughout the malting process could provide further evidence for this hypothesis. Even though few studies mentioned the occurrence of invertases in barley malt, their role in the fructan metabolism is not considered. They are solely mentioned in the context of sucrose hydrolysis during the mashing in beer or whiskey production (Prentice, 1972; Vriesekoop *et al.*, 2010). However, diverse acid plant invertases or ß-fructosidases occur in the grains and can have different functions in terms of the fructan metabolism. While VI preferably hydrolyse the glycosidic bond between glucose and fructose in sucrose and in fructans, FEH hydrolyse the final fructose moieties in fructans and FT elongate sucrose molecules or fructans with an extra fructose residue. All these plant acid invertases act as fructose transporter either to sucrose, fructans or water (Van den Ende, 2013).

Ultimately the germination time may influence the fructan development substantially. A longer germination process seems to lead to a higher activity of anabolic as well catabolic enzymes. This is shown by the comparison of 3 days germinated barley malt in this study to 9 days germinated barley malt from MacWilliam *et al.* (1956). Likewise, the 3 days germinated barley and wheat malts compared to 5 days germinated oat malt show the same trend (Figure 5-2). As described above, larger amounts of fructans were synthesised in the green oat malt in this study and the green barley malt from the study by MacWilliam *et al.* (1956). Correspondingly, a higher decline was observed in the same malts during kilning.

The metabolism of fructans is a complex process and influenced by various genetic and exogenous factors. Environmental factors have a significant effect on the accumulation of fructans in cereals (Cimini *et al.*, 2015; Livingston *et al.*, 2009). This may be similar for the germination process. For instance, the exposure to light during the germination process, may influence the biosynthesis of fructans in the grains. In this context, lower fructan levels were found in wheat grains grown in a glasshouse compared to outdoor grown plants (Veenstra *et al.*, 2017). Moreover, different varieties of the respective cereal grains may accumulate significantly more fructans in

the germination process. For instance, barley can contain up to 4 % fructans in different varieties (Nemeth *et al.*, 2014). As in this study, for most malting and brewing applications spring varieties of barley are used, which have a lower resistance towards cold-stress and a lower fructan content. The effect of the germination process on the fructan content in wheat and barley observed in this study was marginal. However, this may be different for other varieties of the cereal grains, such as the effect observed in oat malt.

5.4.3.3 GOS and fagopyritol utilization during germination of pulses and buckwheat

As discussed in section 5.4.2.2, germination is well-known for its potential to decrease GOS levels in pulses (Martínez-Villaluenga *et al.*, 2008). This aligns with the effect of malting. Whether the utilisation of GOS is essential for the germination process is not fully understood. On the one hand the inhibition of GOS hydrolysis was shown to impair the germination of peas, on the other hand the germination efficiency of low-GOS soybean lines was not affected (Blöchl *et al.*, 2008). However, several studies have shown a significant decrease of GOS in pulses upon germination. These oligosaccharides are rapidly mobilised by the endogenous catabolic α-galactosidases and provide thereby energy during the seed germination (Blöchl *et al.*, 2008).

Unlike fructans, which had a variable development throughout the malting process, GOS gradually decreased with each step of the malting (Figure 5-2). While during the steeping process already a slight drop of the GOS in chickpeas was observed, during the following 3 days of germination GOS diminished by 81 %. Finally, the kilning led to further slight decrease and thus a total reduction by 87 %. This development agrees with other studies on the germination of lentils and chickpeas (El-Adawy, 2002; Frias *et al.*, 1996). Furthermore, Frias *et al.* (1996) showed that after an extended germination of 6 days, under dark conditions, GOS were undetectable in the germinated lentils. El-Adawy (2002) did not determine any GOS already after 3 days of germination of chickpeas in the dark. Frias *et al.* (1996) have shown a slightly faster degradation of GOS with periodical light exposure during the germination, whereas germination in the dark led to an accumulation of sucrose. The GOS degradation throughout the malting process in this study, which is conducted entirely under dark conditions, was accompanied by an increase in sucrose (Figure 5-2). However, other

studies have shown, that sucrose may also be metabolised during dark germination conditions of different pulses (El-Adawy, 2002; Lien *et al.*, 2018; Trugo *et al.*, 1999).

Whereas a number of studies reported a constant decrease of GOS in pulses as the germination progresses, Trugo *et al.* (1999), determined slightly higher raffinose and stachyose levels in soybean and chickpea malts obtained from a 48 h germination compared to the malts after 24 h germination. Similarly, Tuck *et al.* (2018) reported even increased GOS levels in sprouted chickpeas, compared to raw chickpeas. Blöchl *et al.* (2008) have shown that germinating seeds from peas contain anabolic as well as catabolic enzymes. While the GOS catabolism typically predominates during germination, biosynthesis of GOS may also be initiated if the germination process is impaired.

Different studies have demonstrated that the degradation of GOS during the germination, occurs due to increased levels of endogenous α-galactosidases (Blöchl *et al.*, 2008; Lien *et al.*, 2018; Reddy & Salunkhe, 1980). These cleave the terminal α-galactosyl-moieties from GOS, thus providing readily available energy for the germinating seed (Figure 5-3). While sucrose levels increased with progressing GOS-hydrolysis (Figure 5-2), galactose levels remained between 0.05 − 0.15 g/ 100 g DM since released galactose was constantly metabolised. Blöchl *et al.* (2008) extensively studied the mechanisms of the GOS breakdown in germinating peas and suggested a model for the GOS metabolism. Two types of α-galactosidases were identified. The acidic α-galactosidase was found to be present already in protein storage vacuoles next to GOS in ungerminated pea seeds. It was activated by a pH-shift during imbibition and proposed to be responsible for about 50 % of the GOS breakdown during early stages of germination. The resulting increase in sucrose was associated with the stimulation of alkaline α-galactosidase production and the progressing GOS degradation after radicle protrusion (Blöchl *et al.*, 2008). It is suspected that the same model applies to the malting process in pulses.

The activity of α-galactosidase is known to also hydrolyse α-galactosyl cyclitols (Figure 5-3). These compounds are described as precursor or co-products of the GOS-biosynthesis (Martínez-Villaluenga *et al.*, 2008). In contrast to pulses, buckwheat seeds accumulate only the α-galactosyl cyclitols FP. GOS are not found in buckwheat seeds. However, FP in buckwheat have a similar role in plant physiology to GOS in

pulses (Horbowicz *et al.*, 1998). The germination process has been shown to elevate acid α-galactosidase activities in buckwheat and lead to the hydrolysis of FP to galactose and D-*chiro*-inositol (Horbowicz *et al.*, 1998; Jia *et al.*, 2015). This coincides with the trend observed in this study. In Figure 5-3 the HPAEC-PAD profile from raw buckwheat shows different oligomers and isomers of FP. In the profile of malted buckwheat those compounds almost completely disappeared. The utilisation of FP during the germination of buckwheat may serve as readily available energy source, similar to GOS in pulses.

5.4.4 Conclusion

To our best knowledge, this is the first study to investigate the effect of malting on the FODMAP content of different seeds. Malting has been shown to be a powerful tool to modify FODMAP levels. The accumulation of fructans may be certainly interesting for other than low FODMAP applications, due to favourable effects of fructans for the plant (more resistant towards environmental stress) and for the human health (prebiotic). However, for applications where a low FODMAP content is needed, the malted cereal ingredients should be avoided or subjected to fermentations with inulinase- or invertase-possessing lactic acid bacteria and/ or yeast.

In contrast to malted cereals, malted, dry low FODMAP pulse ingredients may have a versatile application for the development of functional food products and thus contribute to a higher nutritional value of a diet low in FODMAPs. Sprouted pulses are suitable for the direct consumption such as on salads. The malted pulse ingredients, which deliver high amounts of protein, vitamins, and enzymes, could also be applied in recipes of cereal-based products, thereby improving technological properties, and fortifying the nutritional value. Pulse malts may also be used as base ingredients for plant-based beverages as milk replacement.

Lastly, as FP, the major fraction of soluble, indigestible, and fermentable carbohydrates in buckwheat seeds, are degraded during the malting process, buckwheat malt also serves as wholesome low FODMAP ingredient. However, the actual fate of FP and other α-galactosyl cyclitols in the human digestion is not well studied. Further investigations are necessary to understand the availability of α-galactosyl cyclitols to diverse gut bacteria, and thus their potential to trigger unpleasant gastrointestinal symptoms in IBS patients.

5.5 Acknowledgement

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Chapter 6

Lachancea fermentati FST 5.1: an alternative to baker's yeast to produce low FODMAP whole wheat bread

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6.1 Abstract

A diet low in fermentable oligo-, di-, monosaccharides and polyols (FODMAPs) is a successful therapeutic approach to alleviate symptoms of irritable bowel syndrome. However, wheat, as a fructan accumulating grain, is a major source of FODMAPs. Baker's yeast degrades fructans during fermentation, yet conventional whole wheat bread is often still high in FODMAPs. In this study, 96 yeast isolates from different environments were screened regarding their capability to metabolise FODMAPs. Two promising isolates were identified: *Lachancea fermentati* FST 5.1 and *Cyberlindnera fabianii* NTCyb, and their potential to produce low FODMAP whole wheat bread was compared to baker's yeast (*Saccharomyces cerevisiae*). A comprehensive characterisation of the carbohydrate metabolism by the different yeasts was achieved via HPAEC-PAD analysis of flour, doughs, and breads. *L. fermentati* FST 5.1 fermented fructans and excess fructose much more efficiently than baker's yeast and resulted in bread low in FODMAPs (below all cutoff levels known to induce symptoms). In contrast, *C. fabianii* NTCyb was unable to ferment FODMAPs in the wheat-dough-matrix. Furthermore, the yeasts' impact on the GC/MS-TOF profile of volatile aroma compounds, the sensory profile, the breads' ultrastructure, and the technological quality was examined. While *C. fabianii* NTCyb bread had poor technological and sensory attributes, the quality characteristics (volume, crumb structure, texture, sensory, aroma) of *L. fermentati* FST 5.1 bread were comparable to the baker's yeast bread. Ultimately, this study identified *Lachancea fermentati* FST 5.1 as an alternative to baker's yeast to produce low FODMAP whole wheat bread while maintaining optimal bread quality and consumer acceptance.

6.2 Introduction

Wheat-based bread (especially made from wholemeal) is an indispensable part of the Western diet, not only as a widely consumed, convenient and popular staple food but also as an important source of energy, dietary fibre, and micronutrients. However, many wheat-based products often have to be avoided by people suffering from irritable bowel syndrome (IBS), mainly due to considerably high levels of indigestible, rapidly fermentable carbohydrates, namely fructans (Biesiekierski *et al.*, 2011; Muir *et al.*, 2019).

Fructans, which are also found in other cereals such as spelt, barley or rye (Ispiryan *et al.*, 2020), are composed of ß (2 → 1) and/ or ß (2 → 6) bound linear or branched fructose chains with one single glucose residue. Different variations of linkages and the glucose moiety position define different types of fructans, including inulin- and levan- (neo)-series-type or graminan-type fructans (Livingston *et al.*, 2009; Vijn & Smeekens, 1999). The latter two are most commonly found in cereals. Fructans are considered dietary fibres as they are not digested in the small intestine, delivered to the large intestine, where they can be readily fermented by the colonic bacteria resulting in short-chain fatty acids and gas production (Roberfroid *et al.*, 2010). This mechanism is beneficial and essential for the intestinal function and microbiome of healthy individuals. However, the ingestion of fructans, with a number of other poorly absorbed dietary carbohydrates, comprised as fermentable oligo-, di-, mono-saccharides and polyols (FODMAPs), were identified to be responsible for the onset of symptoms in IBS patients (Gibson & Shepherd, 2005; Muir *et al.*, 2019). This group of carbohydrates commonly includes besides fructans, α-galactooligosaccharides (GOS), lactose, fructose in excess to glucose, and polyols, but is not limited to those. Other carbohydrates with such physiological effects may extend the list of FODMAPs (Halmos & Gibson, 2019). Dietary therapy limiting the intake of FODMAPs (the low FODMAP diet) has been shown to be effective for at least 70 % of IBS patients (Halmos *et al.*, 2014). Core grain products, including bread, are categorised as low FODMAP if the following cutoff levels are not exceeded in a serving of food (e.g., 50 g bread): 0.3 g of oligosaccharides (e.g., sum of fructans and GOS), 0.15 g of excess fructose, 0.4 g of polyols, 1 g of lactose, and a total of 0.5 g of FODMAPs excluding lactose (Varney *et al.*, 2017).

Conventional wholemeal wheat bread is commonly leavened with baker's yeast. Despite the ability of baker's yeast to ferment fructans (Nilsson *et al.*, 1987), such bread is usually found to be high in FODMAPs, as the short fermentation times of industrial bread production are often not enough to sufficiently lower FODMAP levels. In particular fructan and excess fructose levels (mainly resulting from partial fructan degradation by yeast) exceed the cutoff levels (Biesiekierski *et al.*, 2011; Ispiryan *et al.*, 2020; Varney *et al.*, 2017). At the same time, additional ingredients such as high fructose corn syrup may contribute to even higher total FODMAP levels (Varney *et al.*, 2017). Ultimately, the bread formulation, including the extraction rate of the wheat flour (i.e., refined or whole wheat flour), the yeast addition level, potential other ingredients (particularly other carbohydrate sources) and the breadmaking process (fermentation conditions) are the driving factors which are decisive whether the bread will be high or low in FODMAPs. Thus, a targeted approach is required to reduce FODMAP levels efficiently and reliably in whole wheat bread, while maintaining wheat intrinsic, slowly fermentable, and well-tolerated dietary fibres.

While the ability of baker's yeast (*Saccharomyces cerevisiae)* to ferment fructans with its invertases (encoded in SUC genes) has been known for a long time, the underlying mechanisms and the significance of fructan fermentation in the bread production process have also been studied and understood (Nilsson *et al.*, 1987; Sainz-Polo *et al.*, 2013; Struyf *et al.*, 2017a; Verspreet *et al.*, 2013). It was shown that CO_2 production during wheat dough fermentation owes not only to maltose as a primary source of fermentable carbohydrate but also to a substantial part to the utilisation of fructans (Struyf *et al.*, 2017a). Different studies in recent years researched the targeted yeast mediated fructan degradation and the inherent challenges. Ziegler *et al.* (2016) have demonstrated the importance of sufficiently long proofing times, rather than selecting a lower fructan wheat grain type, as a key element of yeast mediated FODMAP reduction strategies. Schmidt & Sciurba (2021) confirmed the significance of longer fermentation times and highlighted the difficulty to achieve sufficiently low FODMAP levels in whole wheat bread compared to bread made from refined flour. It was also recently shown that variations in the invertase activities of *Saccharomyces cerevisiae* (*S. cerevisiae*) strains from different industrial applications might allow for an effective reduction of FODMAPs in whole wheat bread (Laurent *et al.*, 2020). Struyf *et al.* (2017b & 2018) exploited the potential of an inulinase expressing *Kluyveromyces*

marxianus (*K. marxianus*) strain, which was used either in co-culture with baker's yeast or with the addition of sucrose or amylo-glucosidase, as *K. marxianus* species are unable to ferment maltose and require an alternative carbohydrate source to achieve appropriate dough rise. Due to their ability to express inulinases *K. marxianus* strains were shown to degrade wheat fructans more efficiently than the invertase mediated fructan hydrolysis by baker's yeast.

This study aimed to identify other alternative yeast strains to baker's yeast for low FODMAP baking application. Therefore, the FODMAP utilisation capability of 96 yeast isolates from different environments was screened and led to the identification of two promising non-*Saccharomyces* strains: *Lachancea fermentati* FST 5.1 (*L. fermentati* FST 5.1; originating from kombucha), and *Cyberlindnera fabianii* NTCyb (*C. fabianii* NTCyb; from a rice wine starter). Moreover, the objective of this study was to investigate the impact of the selected strains on physico-chemical whole wheat dough and bread characteristics, including the FODMAP content, the breads' ultrastructure and technological quality, and the aroma profile, compared to conventional baker's yeast.

6.3 Materials and methods

6.3.1 Materials

The ingredients for the baking trials were whole wheat flour (containing ground whole wheat and vital wheat gluten; supplier's specifications: 63.9 % carbohydrates, 2.2 % fat, 9.0 % fibre, 14 – 15 % protein, >250 s falling number) supplied by Odlums (Dublin, Ireland), salt by Glacia British Salt Limited Ltd. (Northwich, UK), sugar (Siúcra, granulated sugar) by Nordzucker (Ireland) Ltd. (Dublin, Ireland), vegetable oil by Musgrave (Cork, Ireland) and the different yeast strains. *L. fermentati* FST 5.1 (formerly KBI 1.2) was isolated from kombucha cultures as described by Bellut *et al.* (2019a & 2020). *C. fabianii* NTCyb was isolated from a dried rice wine starter and identified as described by Bellut *et al.* (2019b). *S. cerevisiae*, *L. fermentati* FST 5.1, and *C. fabianii* NTCyb are referred to as SC-BY, LF-FST 5.1, and CF-NTCyb, respectively, in this paper. All chemicals were obtained from Sigma-Aldrich (Arklow, Ireland), unless otherwise stated.

6.3.2 Yeast screening

The performance of the LF-FST 5.1 and CF-NTCyb for the production of low FODMAP whole wheat bread was compared to conventional SC-BY. These two alternative strains were selected based on the screening of 96 different yeast isolates for their potential to degrade FODMAPs in two different carbohydrate utilisation assays.

6.3.2.1 Yeast culturing and identification

The 96 yeasts were isolated from various flours, wheat sourdoughs and kombucha with several obtained from the National Collection of Yeast Cultures, Research Centre Weihenstephan for Brewing and Food Quality Technical University of Munich, Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands. *Kluyveromyces marxianus* NCYC587, that has been demonstrated as a highly potent yeast strain for low FODMAP baking (Struyf *et al.*, 2017b), and conventional *S. cerevisiae* baker's yeast were used as reference strains in the screenings. All strains were routinely cultured at 30 °C on yeast extract peptone dextrose (YPD) agar plates. Stock cultures were maintained at -80 °C in fresh YPD medium containing 40 % (v/v) glycerol. Strain typing, where necessary, was achieved by targeting the D1/D2 domain of the 26S

rRNA gene. Following DNA extraction (High Pure PCR Template Preparation Kit, according to the user manual; Roche, West Sussex, UK), the region was amplified using NL1 (50-GCATATCAATAAGCGGAGGAAAAG-30) and NL4 (50-GGTCCGTGTTTCAAGACGG-30) primers. The PCR protocol was: 95 °C/ 2 min; 30 cycles of 95 °C/ 30 s; 56 °C/15 s; 72 °C/ 60 s; 72 °C/ 5 min. Products were purified with the High Pure PCR Product Purification Kit (Roche, West Sussex, UK) and sequenced by Eurofins MWG (Ebersberg, Germany). Alignment of single-stranded sequences was performed using DNASTAR Software. Consensus sequences were analysed via Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990).

6.3.2.2 Carbohydrate utilisation assays

For assessing carbohydrate utilisation in solid or liquid assays, overnight cultures were prepared as follows. Single colonies of the respective strains, cultured on YPD agar plates, were inoculated in 5 ml YPD broth (malt extract broth was used for *L. fermentati* species). After incubation overnight at 30 °C with 250 rpm agitation, a 1 % subculture was incubated at the same conditions for 16 h. Assessment of carbohydrate utilisation was performed on Wickerham basal medium (BM) (Wickerham, 1943), supplemented individually with 0.5 % (w/v) of the following carbohydrates: sucrose, fructose, lactose, sorbitol, xylitol, maltitol, mannitol, raffinose, fructooligosaccharides (FOS) and inulin (≥ 95 % purity). Carbohydrates were obtained from Sigma-Aldrich, except FOS (from chicory inulin, enzymatically hydrolysed to DP 2 − 8) and inulin (native inulin from chicory, DP 2-60), which were purchased from Megazyme (Bray, Ireland). The unsupplemented BM, used as a base for the control growth, was composed of powdered yeast extract (4.5 g/ L, Neogen, UK), peptone (7.5 g/ L, Neogen, UK), and the pH indicator bromothymol blue (0.1 g/ L, Sigma-Aldrich, UK). For solid media, 15 g/ L of biological agar (Neogen, UK) were added.

For agar surface assays, master microtitre plates for inoculating agars were prepared by adding 200 µL aliquots of overnight cultures to a 96-well plate. Agar plates were inoculated from the master microtitre plate using a microplate replicator (Boekel Scientific ™, Philadelphia, US) and incubated at 30 °C for 72 h (Figure A-8, Appendix A-1). The development of a yellow halo around the colony indicated the individual carbohydrate fermentation (Kurtzman *et al.*, 2011). Screening assays were performed in at least two biological replicates for each carbohydrate tested.

Based on the agar screening assay results, 13 strains were selected for the broth-based microtitre assay. Yeast strains were precultured as described above and washed twice in 10 mL sterile Ringer's solution. The cell turbidity was adjusted to an equivalent of 1.0 McFarland standard (DEN-1 densitometer, Grant Instruments, UK) in Ringer's solution. Aliquots of 2 µL of standardised individual yeast strain suspensions were added to the wells of a 96-well microtitre plate (Sterilin™, Thermo Fisher Scientific, UK), containing 198 µL BM supplemented with the individual carbohydrate (0.5 % in 200 µL). Control wells were prepared for each strain, by adding 2 µL of cell suspension to 198 µL of unsupplemented BM. Plates were incubated at 30 °C for 72 h in a microplate reader (Multiskan FC Microplate Photometer, Thermo Scientific, Waltham, Ma, USA) with pulse agitation (5 by 5 s) between measurements. Readings were taken every hour against broth blanks (supplemented BM) at 595 nm. The assays were performed in four biological replicates

6.3.3 Preparation of yeast cells for dough- and bread-making

One gram of the commercial instant *S. cerevisiae* baker's yeast was suspended in 9 mL sterile Ringer's solution and cultured on a YPD agar plate. Also, LF-FST 5.1 and CF-NTCyb were cultured on YPD plates from stocks stored in 50 % glycerol at -80 °C. Plates were incubated for 48 − 72 h at 25 °C. Single yeast colonies were inoculated in 10 mL YPD broth and incubated at 25 °C for 24 h in an incubator with orbital shaker (ES-80 shaker-incubator, Grant instruments (Cambridge) Ltd., Dublin, Ireland) with 170 rpm orbital agitation. A 1 % subculture was prepared by suspending 1 mL of the preculture in 100 mL YPD broth in a 250 mL Duran Schott ® bottle, covered with sterile cotton, and incubated at same conditions. A second 1 % subculture was prepared by suspending 2 x 5 mL of the first subculture in 500 mL YPD broth in 1 L Duran Schott ® bottles, each, and incubated at the same conditions. The number of viable cells was counted after staining with Löffler's methylene blue solution (MEBAK 10.11.3.3), using a Thoma Hemocytometer (Blaubrand, Sigma-Aldrich, St. Louis, MO, U.S.A.) (MEBAK, 2011). The appropriate volume of yeast suspension was used to harvest yeast cells to achieve 9×10^7 cells/ g dough. LF-FST 5.1 was flocculating in the medium during growth. Flocs were not countable using the Hemocytometer. Thus, only cells fully suspended in the medium were counted. Yeast cells were harvested by centrifugation of the required volume of suspension (~ 200 − 1000 mL) in ~ 50 mL aliquots (5000 rpm, 10 min), washing twice by re-suspending

the pellet in 50 mL sterile tap water, centrifugation at the same conditions and finally resuspending of the combined pellet in 50 − 100 mL sterile tap water. Each inoculum for baking and rheofermentometer trials was prepared in three biological replicates.

6.3.4 Dough and bread-making

The straight dough method was applied to prepare the bread doughs, using the recipe presented in Table 6-1 to produce a total of 400 g dough. The yeast-suspension was added in the appropriate amount of water to the premixed dry ingredients and the oil, and the dough was formed by mixing for 1 min at speed 1, followed by 7 min at speed 2, using a Kenwood Chef kitchen machine equipped with a kneading hook (Kenwood Manu-facturing Co. Ltd., UK). Immediately after mixing, dough samples were either chilled at 4 °C for cell count determination according to section 6.3.5.2 (for max. 1 h until analysis) or frozen at -20 °C for chemical analyses. The dough was fermented for a total of 90 min at 30 °C and 75 % relative humidity in a humidity-controlled chamber (KOMA, SunRiser, Heinsberg-Dremmen, Germany). During fermentation, the dough was punched at 50 min and 75 min. The fermented dough was divided into four 65 g pieces, moulded, placed into greased baking tins, and proofed for 60 min at the same conditions. Three of the four proofed loaves were baked in a deck oven (MIWE Condo, Arnstein, Germany) for 16 min at 210 °C top and bottom temperature and steaming of the baking chamber, prior to loading, with 400 mL. The fourth loaf was divided and, just as the unfermented dough, either chilled at 4 °C for cell count determination or frozen at -20 °C for chemical analyses. The breads were removed from the oven, placed on a grid to cool for 1 h at room temperature before further analyses. The baking trials were performed in triplicates, using the three biological replicates of the individual yeast cell suspensions.

Table 6-1. Recipe for bread-making with different yeasts

Ingredient	% based on flour	w [g]
Whole wheat flour	100	227.3
Water + yeast suspension*	70	159.1
NaCl	2	4.5
Sucrose	1	2.3
Oil	3	6.8
Total	176	400

*50 − 100 mL yeast suspension prepared according to section 6.3.3; water addition based on Farinograph measurement according to section 6.3.5.1

6.3.5 Flour, dough, and bread analyses

6.3.5.1 General flour characterisation

Moisture and protein (nitrogen to protein conversion factor for whole wheat flour: 5.83) contents of the flour were determined according to AACC International Methods 44-15.02 and 46-12.01, respectively. Water absorption was determined using a Farinograph-TS equipped with an automatic water dosing system (Brabender GmbH and Co KG, Duisburg, Germany), following the AACC Method 54-21.02, to achieve a consistency of 500 Farinograph units (FU). Resistant starch, digestible starch, and total starch contents were analysed using the Resistant Starch Assay Kit K-RAPRS (version August 2019, Megazyme, Bray, Ireland). Damaged starch was determined using the Starch Damage Assay Kit K-SDAM (Megazyme). Sample extracts for the Megazyme assays were prepared in duplicates. Further duplicates of each extract-replicate were prepared for the final colour reaction steps according to assay procedure. All other analyses were performed in triplicates for each biological replicate.

6.3.5.2 Determination of cell count, pH, and total titratable acids

The yeast cell count, pH, and total titratable acids (TTA) were determined in the unfermented (t_0, after mixing) and in the fermented doughs (t_{end}, after 90 min fermentation and 60 min proofing). Each timepoint was analysed in duplicate for each of the three fermentation replicates. For the determination of the cell counts, 10 g dough (stored at 4 °C for max. 1 h until analysis after sample taking) were homogenised with 90 mL sterile Ringer's solution in sterile stomacher bags, using a Seward Stomacher ® 400 lab blender (London, UK). Samples were further diluted by serial dilution (1 mL diluted dough sample added to 9 mL sterile Ringer's solution) and plated on two YPD agar plates per dilution (in total 4 plates per fermentation replicate, per dilution). The enumeration of colony forming units per g dough (CFU/ g dough) was carried out after incubation of the plates for 48 h at 25 °C. For the determination of pH and TTA, dough samples were frozen immediately at -20 °C after sampling. Thawed samples (10 g) were homogenised with distilled water (95 mL) using a T10 basic Ultra Turrax ® (IKA Werke, Staufen, Germany). Analyses were conducted according to standard procedures (Arbeitsgemeinschaft Getreideforschung e.V., 1954).

6.3.5.3 Dough development and CO_2 production

The dough development and CO_2 production by the three different yeasts were analysed using a Rheofermentometer F3 (Chopin, Villeneuve-la-Garenne Cedex, France). The doughs (300 g) were prepared according to section 6.3.4, placed into the fermentation chamber, a weight constraint of 1.5 kg was placed on top, and the fermentation was monitored over a period of 180 min at 30 °C. Fermentation trials were performed in triplicate, using three biological replicates for the inoculum, prepared according to section 6.3.3.

6.3.5.4 Bread compositional analyses

Protein, damaged starch, resistant starch, digestible starch, and total starch contents of the freeze-dried bread samples were analysed as described in section 6.3.5.1. The dry matter of the freeze-dried samples was determined according to AACC 44-15.02. The mass before and after freeze-drying for 3 days (3 × 6 − 10 g aliquots of bread sample per fermentation replicate) was recorded and used for calculations of FODMAPs on fresh weight basis, as described in the following section. The sum of the remaining moisture in the freeze-dried products and the mass difference from the freeze-drying was calculated as moisture of the breads.

6.3.6 Quantification of acids and carbohydrates

The whole wheat flour was used for analysis as supplied. Dough and bread samples were freeze-dried and ground to a fine powder using a QUIAGEN Tissue Lyser II (Hilden, Germany).

6.3.6.1 Quantification of FODMAPs and other carbohydrates

Extraction and quantification of mono-, di, oligosaccharides and polyols were carried out as previously described by Ispiryan *et al.* (2019; 2020), without any modifications. Analyses were performed on a high-performance anion-exchange chromatography system coupled with pulsed amperometric detection (HPAEC-PAD). Fructans were quantified after enzymatic hydrolysis to the monomers glucose and fructose, while all other carbohydrates were quantified using authentic references standards as previously described (Ispiryan *et al.*, 2019; 2020). Therefore, xylitol, sorbitol, mannitol, rhamnose, galactose, glucose, fructose, sucrose, melibiose, maltose, raffinose,

maltotriose, verbascose, kestose, nystose and kestopentaose were used for external standard calibrations in the ranges 0.05 – 1 mg/ L and 1 – 20 mg/ L.

Extractions were carried out in duplicate, for each of the three fermentation replicates. Results comparing carbohydrate changes in the flour, the unfermented dough and the final bread are presented in g analyte per 100 g of sample on a dry weight basis (g/ 100 g DM). The dry matter (DM) of the flour and the freeze-dried samples was determined as described in section 6.3.5. Finally, compliance of FODMAP levels with low FODMAP criteria (cutoff levels for each FODMAP to trigger IBS symptoms) according to Varney *et al.* (2017) was assessed for the breads fermented with the different yeast. A typical serving for bread was estimated as 50 g (Edwards, 2017). Therefore, FODMAP levels are additionally expressed on a fresh weight basis (g/ 100 g 'as is'). The mass difference from the freeze-drying was used for calculations on fresh weight basis, as described in section 6.3.5.4.

6.3.6.2 Quantification of organic acids

Extraction and quantification of organic acids was performed according to the method described by Hoehnel *et al.* (2020), with slight modifications. To 2 g ± 0.5 mg of ground freeze-dried bread sample 15 mL of 80 % ethanol (v/v) at 55 ± 5 °C were added, and the mixture was subjected to the first extraction step using a sonicator. After centrifugation and separation of the supernatant, a second extraction of the pellet, at the same conditions, was carried out. The supernatants were combined and the ethanol in the extracts was evaporated using a vacuum centrifuge. The residue was dissolved in 10 mL ultrapure water containing 50 mg/ L sodium azide. After filtration through 0.2 µm syringe driven filters, extracts were analysed on a Dionex Ultimate 3000 system (Thermo Fisher Scientific, Waltham, MA, USA) and ultraviolet light/ diode array detection (UV/DAD, quantification at 210 nm; Thermo Fisher Scientific). Separation of the organic acids was achieved with a Hi-Plex H column (7.7 × 300 mm; Agilent Technologies, Santa Clara, CA, USA) at 60 °C and isocratic elution with 5 mM sulfuric acid. Reference standards of lactic acid, succinic acid and acetic acid were used for an external standard calibration in the range of 0.03 – 6 g/ L. Extractions were carried out in duplicate, for each of the three fermentation replicates.

6.3.7 Bread quality analyses

6.3.7.1 Technological quality characteristics

To assess quality characteristics of the breads prepared with the different yeast cultures, several bread quality analyses were performed. The analyses were conducted after 1 h cooling of the baked loaves. Three loaves or two slices (20 mm) from the centre of each of the three loaves were analysed for each of the three fermentation replicates (in total at least 9 − 18 measurements were taken for each bread type, per analysis). Bake loss was determined gravimetrically by weighing the dough after moulding and the final loaf after baking. Specific volume was measured (rotation speed 1 rps, 2 mm vertical steps) with a Volscan profiler (Stable Micro Systems, Godalming, UK). Crumb structure (slice area, number of cells, area of cells, cell diameter) was analysed using a C-Cell Imaging System (Calibre Control International Ltd. Warrington, UK). Crumb hardness was determined using a TA-XT2i Texture analyser (Stable Micro Systems Godalming, UK), equipped with a 25 kg load cell and 20 mm cylindrical probe. The following measurement parameters have been applied: slice-compression to 40 % of its height, 5 mm/ s test speed, 10 mm/ s post-test speed, 5 s waiting time between compressions, 0.05 N trigger force. Lightness (L*) and redness (a*) of crust (5 measurements per loaf) and crumb (3 measurements per slice) were analysed with a Colorimeter CR-400 (Konica, Minolta, Tokyo, Japan), using the CIE L*a*b* colour space. Water activity of the crumb was measured using a water activity meter (HygroLab, Rotronic, Bassersdorf, Switzerland).

6.3.7.2 Sensory analysis

Sensory analysis of the breads prepared with the three different yeast strains was performed by aromaLAB GmbH (Tentamus Company, Martinsried, Germany). The bread samples were orthonasally analysed by a trained panel (n = 6). The sensory profile was established in accordance with DIN EN ISO 13299:2016-09. Prior to the sensory analysis 9 aroma characteristics (‘malty’, ‘roasty’, ‘fatty’, ‘butter’, ‘fruity’, ‘sour’, ‘honey’, ‘earthy’, ‘dusty/ plastic/ oatflakes’, ‘potato’) were fixed. Samples of the bread crumb were filled into sensory glasses and ranked on a scale from 0 (indiscernible) to 3 (strongly discernible).

6.3.7.3 Aroma profile analysis

The volatile aroma compounds of the crumbs were screened using gas chromatography with time-of-flight mass spectrometry (GC/MS-TOF) by aromaLAB GmbH (Tentamus Company, Martinsried, Germany). Therefore, 50 g of crumb were extracted with 150 mL of diethyl ether and 50 mL of water. The volatile compounds were isolated via SAFE distillation (solvent assisted flavour evaporation). The extract was dried over sodium sulphate and concentrated to 100 µL using a vigreux column. Screening of the aroma compounds was achieved on a GC (Agilent 7890B GC system) coupled with TOF (Pegasus BT, LECO). One microlitre of sample was injected on a DB-FFAP column (30 m × 0.25 mm inner diameter, 0.25 µm film thickness, Phenomenex) at 240 °C, in split mode (1:10). The following temperature program was applied: 6 °C/ 1 min to 35 °C/ 5 min to 240 °C/ 5 min. The MS was operated in electron ionisation (EI, 70 eV) mode in a scan range of m/z 35-350 at 10 spectra/ s.

6.3.8 Scanning electron microscopy

Samples of bread crumb were freeze-dried and cross-sectioned using a scalpel. Scanning electron microscopy (SEM) was performed as described by Ispiryan *et al.* (2021). The Specimens, coated with gold/palladium (Au:Pd = 80:20), were examined on a JSM-5510 scanning electron microscope.

6.3.9 Statistical analysis

Growth curve analysis of the broth microtitre assay was performed using "Growthcurver" (Sprouffske & Wagner, 2016) and "DescTool" (Signorell, A., et mult. al., 2021) packages in Rstudio, version 1.4.1106 (RStudio Inc, Boston, MA, USA; R Core Team, r-project), to fit the growth curve data to a standard form of the logistic equation and to calculate the maximum optical densities (OD_{max}). Dunnett's multiple comparison test assessed significant differences between the OD_{max} of an individual strain in the carbohydrate supplemented BM and substrate-free BM (control). Results were categorised as good growth "+++" ($p < 0.001$), moderate growth "++" ($0.001 \leq p < 0.01$), limited growth "+" ($0.01 \leq p < 0.05$), and no significant difference "-" ($p > 0.05$) to the control, thus no fermentation of the individual carbohydrate in broth. Statistical analyses of the baking trials were performed with SPSS Statistic 26 (IBM Corp., Armonk, NY, USA). One-way ANOVA followed by Tukey's post hoc test was applied to show significant differences between the data from the breads

prepared with the different strains, between the unfermented and fermented doughs, and between the flour, the unfermented doughs, and the final breads ($p < 0.05$).

6.4 Results

6.4.1 Yeast screening and selection of potent strains

Ninety-six yeast strains were isolated from various environmental sources as detailed in Table A-5 (Appendix A-1). These included *Saccharomyces cerevisiae*, *Saccharomyces cerevisiae/ paradoxus*, *Schizosaccharomyces pombe*, *Zygosaccharomyces kombuchaensis*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Zygotorulaspora florentina*, *Brettanomyces anomalus*, *Brettanomyces bruxellensis*, *Cyberlindnera fabianii*, *Cyberlindnera misumaiensis*, *Cyberlindnera mrakii*, *Lachancea fermentati*, *Cyberlindnera subsufficiens*, *Cyberlindnera (Pichia) jadinii*, *Debaromyces hansenii*, *Pichia kudriavzevii*, *Pichia membranifaciens*, *Pichia scaptomyzae* and other unidentified yeast isolates. Their carbohydrate fermentation characteristics were determined on Wickerham basal solid medium (Wickerham, 1943) supplemented individually with sucrose, fructose, lactose, mannitol, raffinose, FOS or inulin at 0.5 % (w/v). Potential for sugar (FODMAP) utilisation was assessed by a discolouration zone and an increase in colony size. The majority showed good FODMAP utilisation characteristics on agar surface as summarised in Figure 6-1.

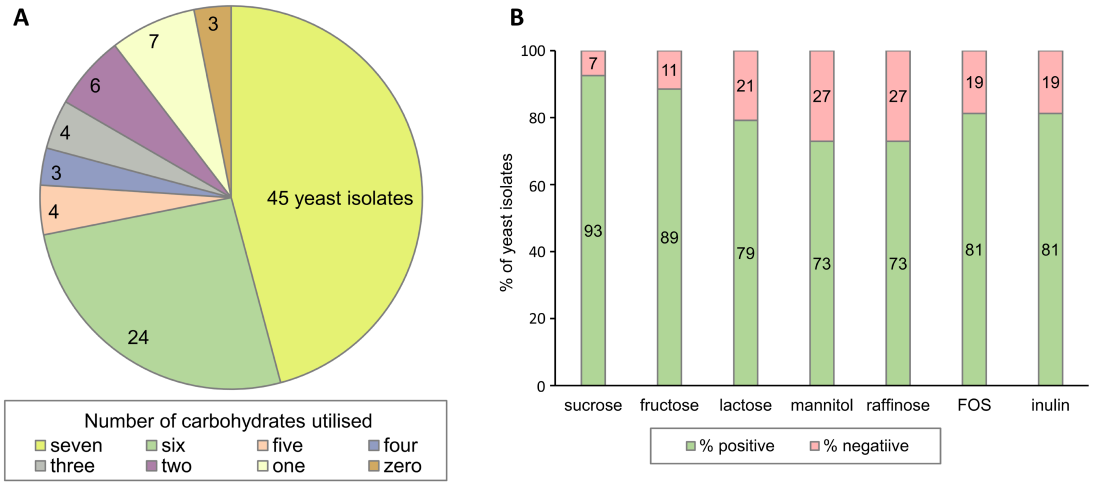

Figure 6-1. Results of carbohydrate fermentation analysis of 96 yeast isolates as determined on Wickerham basal solid medium (Wickerham, 1943) supplemented with either sucrose, fructose, lactose, mannitol, raffinose, FOS, inulin at 0.5 % (w/v). A: Isolates fermenting all seven carbohydrates were most numerous at 45, with 24 isolates fermenting six carbohydrates. The remaining isolates showed more limited carbohydrate utilisation capacity. B: Percentage of yeast strains established as positive and negative for individual carbohydrate fermentation

Isolates fermenting all seven carbohydrates were most numerous at 45, with 24 isolates fermenting six carbohydrates. The remaining isolates showed more limited carbohydrate utilisation capacity. Sucrose and fructose were utilised by the majority of strains (93 % and 89 % respectively), followed by inulin and FOS (81 % for both). Lactose was utilised by 79 % of tested strains, whereas both mannitol and raffinose utilisation was observed for 73 % of the strains tested in a qualitative agar FODMAP utilisation assay. Selection of the strains for further analysis was based on the utilisation of the most abundant FODMAP carbohydrates, typically found in a fermenting wheat-dough matrix (fructans and fructose) established via agar assay. The yellow discolouration as well as the growth capacity in artificial medium (as an indication of high adaptability of the strain – data not shown) were taken into consideration. Of the 96 strains evaluated, thirteen judged to have the highest potential for FODMAP fermentation were further screened in microtitre assays in Wickerham basal broth medium supplemented individually with the same carbohydrates. Of the thirteen selected strains, *S. cerevisiae* NCYC77 did not have the ability to ferment mannitol, yet showed relatively high fermentation of FOS, similar to *L. fermentati* FST5.1 which did not ferment lactose. *C. subsufficiens* CBS5763 and *C. subsufficiens* C6.1 failed to grow on lactose, mannitol and raffinose supplemented basal medium, yet grew relatively well on fructans and fructose. The other strains selected for microtitre assay in Wickerham basal broth medium grew on all seven carbohydrates, and strong utilisation of fructose and fructans was observed. Only *K. marxianus* strains showed significant inulin fermentation. The results of the microtitre assay are summarised in Table 6-2, which also includes the conventional baker's yeast *S. cerevisiae* and *K marxianus* NCYC587, a strain previously recognised for FODMAP breakdown in whole wheat bread (Struyf *et al.*, 2017b). It was observed that while there was reasonably good correlation between results on agar surface and growth in liquid medium, there were some cases when behaviours on solid medium did not carry through to good growth in liquid. Of the 13 strains, *L. fermentati* FST 5.1 and *C. fabianii* NTCyb were selected as the most promising alternatives to conventionally used *S. cerevisiae* baker's yeast for baking trials.

Table 6-2. Carbohydrate utilisation by selected yeasts over 72 h at 30 °C in broth.

Yeast	Carbohydrate utilisation in broth [a]						
	Sucrose	Fructose	Lactose	Mannitol	Raffinose	FOS	Inulin
K. marxianus NCYC587	+++	+++	++	-	-	+++	+++
K. marxianus NCYC179	+++	+++	+++	+++	+++	+++	+++
K. marxianus NCYC744	+++	+++	+++	+++	+++	+++	+++
K. marxianus NCYC970	+++	+++	+++	-	+++	+++	-
K. marxianus NCYC1425	+++	+++	+++	-	+++	+++	+++
***S. cerevisiae* (baker's yeast)**	**+++**	**+++**	**+**	**-**	**+++**	**+++**	**-**
S. cerevisiae NCYC77	+++	++	-	-	-	+++	-
L. fermentati CBS707	+++	+++	-	-	-	+++	-
L. fermentati KBI12.1	+++	+++	-	-	-	+++	-
***L. fermentati* FST5.1**	**+++**	**+++**	**-**	**-**	**+++**	**+++**	**-**
C. subsufficiens CBS5763	-	++	-	-	-	+++	-
C. subsufficiens C6.1	+++	+++	-	-	-	+++	-
***C. fabianii* NTCyb**	**+++**	**+++**	**-**	**-**	**+**	**+++**	**-**

[a] Results are categorised based on significant differences in OD_{max} in supplemented medium and substrate-free basal medium: good growth "+++" ($p < 0.001$), moderate growth "++" ($0.001 \leq p < 0.01$), limited growth "+" ($0.01 \leq p < 0.05$), and no significant difference "-" ($p > 0.05$). Yeast strains highlighted with bold font were used for baking trials.

6.4.2 Flour characteristics

Whole wheat flour was used for dough preparation and bread-making. The flour characteristics are summarised in Table 6-3. The moisture content of the flour was 14.55 ± 0.05 %, and the optimal water absorption for dough preparation was 70 %. Furthermore, the flour contained 15.00 ± 0.09 % protein on dry matter basis (DM), 64.63 ± 0.31 % DM starch (2.60 ± 0.00 % DM resistant and 7.24 ± 0.05 % DM damaged starch), 1.84 ± 0.01 % DM fructans, 0.81 ± 0.00 % DM sucrose, 0.22 ± 0.00 % DM raffinose, and glucose, fructose, maltose and maltotriose levels ≤ 0.06 % DM (Figure 6-2).

Table 6-3. Flour characteristics and bread composition

Variable	Flour	SC-BY	LF-FST 5.1	CF-NTCyb
Moisture [%]	14.55 ± 0.05 [a]	41.74 ± 0.58 [b]	40.67 ± 0.24 [c]	41.90 ± 0.36 [b]
Water absorption* [%]	69.9 ± 0.3	-	-	-
Total protein [% DM]	15.00 ± 0.09 [a]	13.99 ± 0.15 [b]	14.35 ± 0.24 [c]	13.95 ± 0.15 [b]
Damaged starch [% DM]	7.24 ± 0.05	-	-	-
Resistant starch [% DM]	2.60 ± 0.00 [a]	0.83 ± 0.01 [b]	0.80 ± 0.03 [b]	0.82 ± 0.02 [b]
Digestible starch [% DM]	62.03 ± 0.32 [a]	58.39 ± 1.13 [b]	58.68 ± 0.69 [b]	58.39 ± 0.65 [b]
Total starch [% DM]	63.63 ± 0.31 [a]	59.22 ± 1.13 [b]	59.47 ± 0.71 [b]	59.21 ± 0.66 [b]

* Determined with Farinograph to reach 500 FU. Means ± standard deviation with different superscript letters in the same row are significantly different ($p < 0.05$) SC-BY: *Saccharomyces cerevisiae* BY; LF-FST 5.1: *Lachancea fermentati* FST 5.1; CF-NTCyb: *Cyberlindnera fabianii* NTCyb

6.4.3 Fermentation performance of different yeast strains

The fermentation performance of the yeast strains *Cyberlindera fabianii* NTCyb (CF-NTCyb) and *Lachancea fermentati* FST 5.1 (LF-FST 5.1) was compared to conventional *Saccharomyces cerevisiae* baker's yeast (SC-BY). The three different doughs and breads were prepared using the same recipe and procedure, and the respective yeast suspensions were prepared identically. Thus, any differences observed during the proofing and in the breads' composition and quality characteristics can be attributed to the different yeasts' fermentation performance.

6.4.3.1 Gas production during fermentation

The CO_2 production capacity of the different yeast strains was measured over a fermentation period of 180 min (Table 6-4). LF-FST 5.1 resulted in higher CO_2 (V_{tot}) production than SC-BY, with ~ 500 mL more CO_2 produced, and required a shorter time to achieve the maximal dough height (Time Hm). CF-NTCyb, in contrast, produced only very low CO_2 levels of ~ 80 mL, resulting in no detectable dough rise.

The LF-FST 5.1 dough had a comparably high gas holding capacity (retention coefficient) to the SC-BY dough, with 97 % of the CO_2 produced retained in the dough. Similarly, the maximal dough height reached over the fermentation period (Hm) did not significantly differ for SC-BY and LF-FST 5.1, but a tendency for a slightly higher dough hight could be seen for LF-FST 5.1. Both results, the gas holding capacity and the maximal dough height, indicate similar strength and rheological properties for the SC-BY and the LF-FST 5.1 doughs.

Table 6-4. Fermentation characteristics and gas production of different yeast strains.

Variable *	SC-BY	LF-FST 5.1	CF-NTCyb
Cell count [× 10^7 CFU/ g]			
t_0	4.66 ± 0.82 $^{a, A}$	17.21 ± 5.94 $^{b, A}$	5.92 ± 0.07 $^{a, A}$
t_{end}	4.77 ± 0.59 $^{a, A}$	20.88 ± 3.92 $^{b, A}$	7.32 ± 1.03 $^{a, A}$
pH			
t_0	6.25 ± 0.01 $^{a, A}$	6.20 ± 0.02 $^{b, A}$	6.28 ± 0.01 $^{a, A}$
t_{end}	6.02 ± 0.03 $^{a, B}$	5.99 ± 0.02 $^{a, B}$	6.15 ± 0.08 $^{b, B}$
bread	6.04 ± 0.01 $^{a, B}$	5.98 ± 0.03 $^{b, B}$	6.24 ± 0.01 $^{c, A, B}$
TTA [mL 0.1M NaOH]			
t_0	3.55 ± 0.10 $^{a, A}$	4.13 ± 0.26 $^{b, A}$	3.46 ± 0.17 $^{a, A}$
t_{end}	4.90 ± 0.10 $^{a, b, B}$	5.17 ± 0.23 $^{b, B}$	4.48 ± 0.26 $^{a, B}$
bread	3.83 ± 0.03 $^{a, C}$	4.25 ± 0.15 $^{b, A}$	3.47 ± 0.06 $^{c, A}$
Rheofermentometer			
Maximal dough height (Hm) [mm]	34.1 ± 1.6 ^{a}	36.8 ± 2.2 ^{a}	0.0 ± 0.0 ^{b}
Time Hm [min]	156 ± 5 ^{a}	96 ± 3 ^{b}	180 ± 0 ^{c}
Total CO_2 volume (V_{tot}) [mL]	910 ± 50 ^{a}	1452 ± 65 ^{b}	77 ± 6 ^{c}
Volume CO_2 retained (V_{ret}) [mL]	902 ± 47 ^{a}	1411 ± 82 ^{b}	74 ± 7 ^{c}
Retention coefficient [%]	99.1 ± 0.3 ^{a}	97.2 ± 1.4 $^{a, b}$	95.7 ± 1.7 ^{b}

* t_0 = samples taken after mixing, t_{end} = samples taken after 150 min of fermentation and proofing. Means ± standard deviation with different superscript lower-case letters in the same row and capital letters in the same column are significantly different ($p < 0.05$).

6.4.3.2 Fermentation characteristics: cell count, pH, TTA, organic acids

The cell count, pH, total titratable acids (TTA; Table 6-4) and levels of organic acids were determined in the different doughs and breads, representing fermentation characteristics for the different yeast strains used in this study.

The yeast suspensions were harvested and used for dough preparation after cell count determination with the Hemocytometer to achieve an inoculation level of 9×10^7 cells/ g dough. The cell count of the different yeast in the inoculated t_0 doughs resulted in 4.7×10^7 CFU/ g dough and 5.9×10^7 CFU/ g dough, for SC-BY and CF-NTCyb, respectively, and 1.7×10^8 CFU/ g dough for LF-FST 5.1. The numbers of viable cells in the respective doughs did not change over the fermentation and proofing period.

For all three yeast strains a drop in pH, paralleled by a higher TTA value was observed comparing the t_0 doughs to the fermented doughs (Table 6-4). No change in pH

occurred between the fermented doughs and the breads, while a decrease of the TTA value was determined. The pH and TTA values of the SC-BY and the LF-FST 5.1 fermented doughs did not differ. In contrast, the pH of the CF-NTCyb dough was slightly higher and the TTA correspondingly slightly lower. The main organic acid detected in all breads was acetic acid (0.11 − 0.15 % DM). Only traces of succinic acid and lactic acid were detected in all three breads.

6.4.3.3 Impact of different yeasts on carbohydrate profile

Carbohydrate levels were determined in the "unfermented" doughs (t_0 doughs, after mixing) and the final breads and compared to the initially present carbohydrate composition of the flour. The impact of the fermentation by the different yeast strains on mono-, di-, tri-, oligosaccharides and polyols is shown in Figure 6-2 on a dry matter basis.

The t_0 doughs prepared with the suspensions of the different yeast strains contained similar levels of fructans. The initially present fructans from the wholemeal flour (1.84 % DM) were slightly lowered in the t_0 doughs (1.57 − 1.60 % DM) but did not differ for the different yeasts. After fermentation and proofing of the bread fermented with the SC-BY, a degradation of the fructans to 1.3 % DM was observed. As opposed to SC-BY's low fructan degradation, a higher degree of degradation was observed in the bread fermented with LF-FST 5.1, resulting in 0.6 % DM fructans in the final bread. Interestingly, the bread fermented with CF-NTCyb did not result in any further fructan degradation, despite its ability to utilise FOS in the agar and microtitre assays.

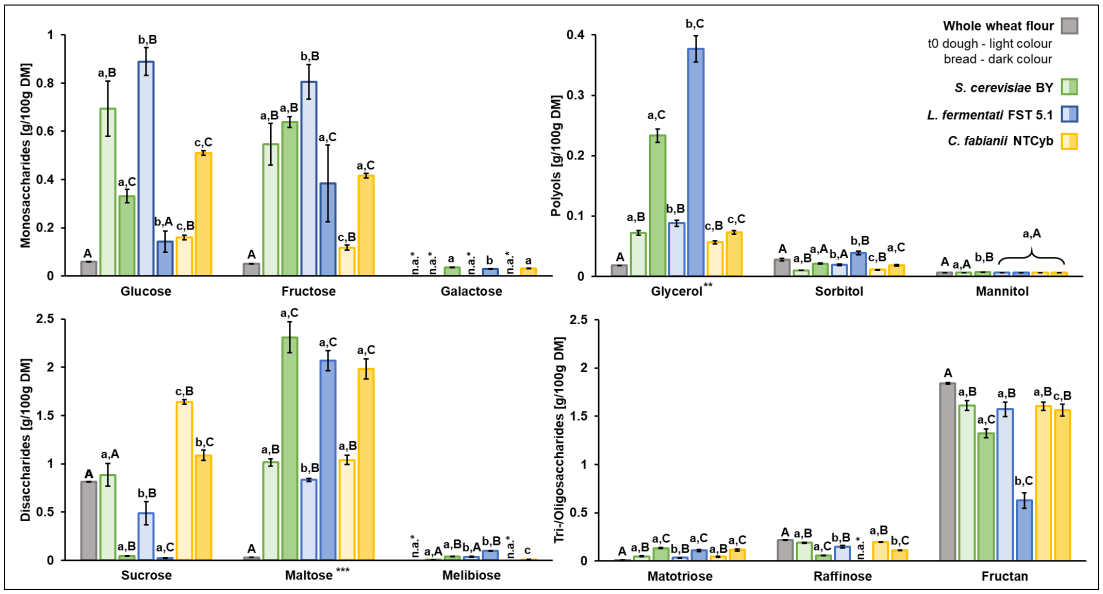

Figure 6-2. Carbohydrate profile of wholewheat flour (first, grey column in each chart, respectively), unfermented doughs after mixing (t_0 dough; light colour shade for each yeast), and breads fermented with different yeasts (darker colour shade for each yeast). * n.d., not detected or levels below 0.005 g/100 g DM. Different capital letters above each column represent significant difference between flour, t_0 dough and bread for the respective yeast for each carbohydrate, while different lower-case letters indicate significant difference between levels for the different yeast fermentations for each carbohydrate ($p < 0.05$)

Like fructans, raffinose contents were slightly lower in the t_0 doughs (SC-BY: 0.19 % DM, LF-FST 5.1: 0.15 % DM, CF-NTCyb: 0.20 % DM) compared to the flour (0.22 % DM). Finally, no raffinose was detected in the LF-FST 5.1 fermented bread, while residual amounts of 0.06 % DM and 0.11 % DM were determined in SC-BY and CF-NTCyb fermented breads, respectively. In reversed order to the raffinose degradation, the highest melibiose levels were found in the LF-FST 5.1 fermented bread (0.10 % DM), followed by the SC-BY bread (0.04 % DM) and the CF-NTCyb bread (0.01 % DM). Very low levels of galactose (<0.03 % DM) were determined in all breads.

Fructose levels in all three t_0 doughs increased quickly during the dough-mixing, alongside a glucose increase. This can be primarily attributed to hydrolysis of sucrose. The calculated level of flour intrinsic and added sucrose is 1.9 % DM, in contrast to 0.49 − 1.64 % DM determined in the t_0 doughs. While similar levels of glucose (0.69 % DM and 0.89 % DM) and fructose (0.55 % DM and 0.80 % DM) were present in the t_0 doughs of SC-BY and LF-FST 5.1, respectively, only 0.16 % DM glucose and 0.12 % DM fructose were found in the CF-NTCyb t_0 dough. After fermentation and proofing, however, the fructose content in the final SC-BY and CF-NTCyb fermented

breads further increased to 0.64 % DM and 0.42 % DM, respectively. In the LF-FST 5.1 bread, on the contrary, the fructose content decreased significantly, paralleled by declining glucose levels. Furthermore, the highest levels of glycerol were found in LF-FST 5.1 fermented breads (Figure 6-2).

Maltose levels increased quickly in the t_0 doughs (0.83 − 1.04 % DM) compared to the flour (0.03 % DM). The final maltose concentrations in the three breads did not differ (~2 % DM). Lastly, only trace levels of the sugar alcohols sorbitol and mannitol were detected in the flour, doughs, and breads (<<0.05 % DM).

6.4.4 FODMAP contents and composition of different breads

Based on the amount of each group of FODMAP in 100 g of fresh bread, the compliance of the breads with the low FODMAP criteria, according to Varney *et al.* (2017), was assessed. The FODMAP contents in the three different breads are presented in Table 6-5.

The amounts for excess fructose and polyols were below the cutoff levels (0.15 g and 0.4 g per 50 g fresh bread, respectively) for all bread types. However, the SC-BY and the CF-NTCyb fermented breads exceeded the cutoff level for oligosaccharides in grain products (Σ fructans, raffinose and melibiose > 0.3 g/ 50 g fresh bread). Only the bread fermented with LF-FST 5.1 met the low FODMAP criteria for all individual groups of FODMAPs and could thus be categorised as a functional low FODMAP product.

Finally, in contrast to the different FODMAP contents, the nutritional composition of the breads prepared with the three yeast strains was very similar with ~ 14 % DM protein, ~ 0.8 % DM resistant starch and ~ 59 % DM digestible starch (Table 6-3).

Table 6-5. FODMAP contents of breads referred to low FODMAP cutoff levels

Yeast	FODMAP contents ± standard deviation [g/ 100 g 'as is'] [a]						Serve [g] [e]	Meets low FODMAP criteria [f]
	Mono-/Disaccharides [b]		Polyols		Oligosaccharides			
	Glucose	Fructose	EF [c]	∑ Polyols (sor, man)	∑ GOS (raf, mel [d])	Total fructan		
S. cerevisiae BY	0.20 ± 0.02 [A]	0.38 ± 0.01 [A]	0.18 [A]	0.02 ± 0.00 [A]	0.06 ± 0.00 [A]	0.78 ± 0.03 [A]		No
L. fermentati FST 5.1	0.09 ± 0.03 [B]	0.23 ± 0.09 [A]	0.14 [A]	0.03 ± 0.00 [B]	0.06 ± 0.00 [A]	0.38 ± 0.05 [B]	50	Yes
C. fabianii NTCyb	0.30 ± 0.01 [C]	0.25 ± 0.01 [A]	-	0.01 ± 0.00 [C]	0.07 ± 0.00 [B]	0.92 ± 0.04 [C]		No

[a] extractions carried out in duplicates for each of the three fermentation replicates, and measured via HPAEC-PAD, results referred to fresh weight ('as is). [b] no lactose detected in any of the breads. [c] EF, excess fructose = glucose – fructose. [d] melibiose produced from fermentation of raffinose by all three yeasts, accounted as FODMAP. [e] one serving of bread estimated as 50 g (Edwards, 2017). [f] cutoff levels per serve for each FODMAP (Varney *et al.*, 2017): 0.3 g oligosaccharides, 0.4 g polyols, 0.15 g excess fructose, 1 g lactose, total FODMAPs excluding lactose 0.5 g. Means ± standard deviation with different superscript capital letters in the column are significantly different ($p < 0.05$)

6.4.5 Bread quality characteristics

The breads fermented with the different yeast cultures and the SEM images depicting the microstructure of the respective crumbs are shown in Figure 6-3. Visual evaluation of the LF-FST 5.1 fermented bread revealed no distinct differences in loaf size and shape, colour or crumb structure, apart from marginally larger appearing slices, and slightly more browning of the crust, compared to the SC-BY fermented bread. The CF-NTCyb fermented bread, in contrast, resulted in a markedly smaller and flatter loaf, no visible browning of the crust and a much denser crumb structure. No differences were seen in the different crumbs' ultrastructure.

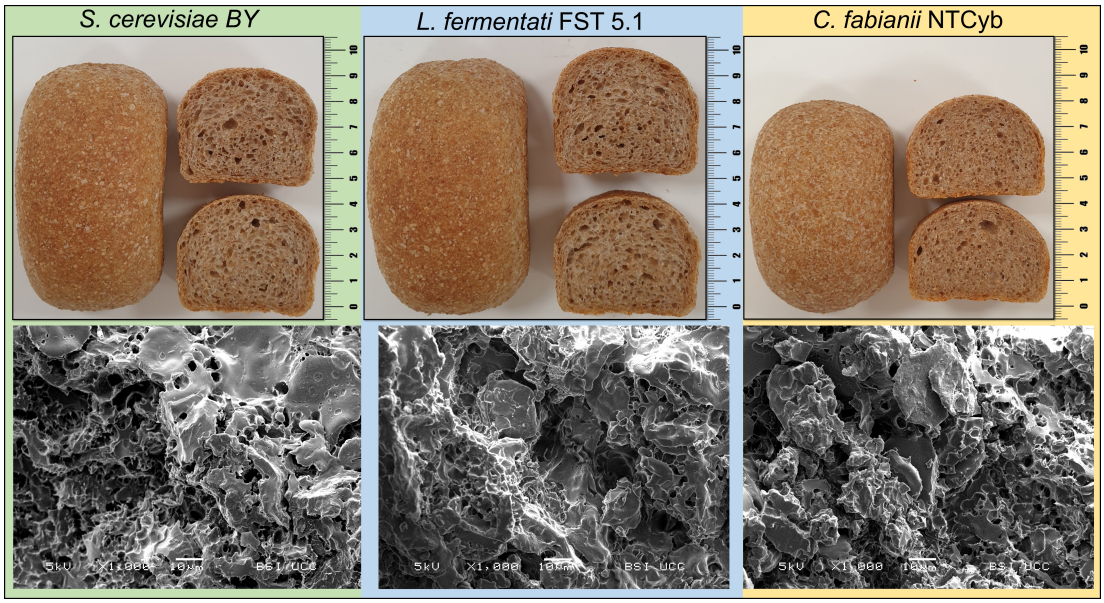

Figure 6-3. Photographs and SEM micrographs (magnification: ×1000; scale: 0.5 cm ≙ 10 µm) of bread fermented with *S. cerevisiae* BY (images on left), *L. fermentati* FST 5.1 (images in middle), *C. fabianii* NTCyb (images on right).

The results of the technological bread quality characteristics (Table 6-6) support the visual evaluation. There was no significant difference in the specific volumes of the LF-FST 5.1 and the SC-BY fermented breads. Yet, a slight trend towards higher volumes than for the SC-BY fermented bread was seen (2.09 ± 0.06 mL/ g vs. 2.27 ± 0.13 mL/ g). This is in line with the parameters describing the crumb structure: the slice area (1727 – 1782 mm^2), the number of cells (1286 – 1369), the area of cells (49.3 – 49.6 %) and the cell diameter (1.6 mm^2). Also, the similar crumb texture, again with a slight trend towards a softer crumb for the LF-FST 5.1 bread fully agrees with the other findings (8.14 ± 0.62 N vs. 7.77 ± 0.78 N). The CF-NTCyb bread analyses, in contrast, revealed despite a similar number of cells (1361), smaller cell diameters

(1.3 mm^2), a smaller area of the cells (47.1 %), a smaller slice area (1428 mm^2), a lower specific volume (1.45 mL/ g) and correspondingly a much higher hardness (18.24 N). In contrast to the similar results of the above-described quality characteristics for the LF-FST 5.1 and SC-BY fermented breads, the fermentation of the latter resulted in lower redness (a*: 16.5 vs 15.0) and higher lightness (L*: 61.7 vs 53.6) of the crust colour. The CF-NTCyb fermented bread, in turn, had the lowest crust redness and highest lightness (a*: 10.3, L*: 66.6).

Table 6-6. Technological bread quality characteristics

Variable	*S. cerevisiae* BY	*L. fermentati* FST 5.1	*C. fabianii* NTCyb
Specific volume [mL/ g]	2.09 ± 0.06 [a]	2.27 ± 0.13 [a]	1.45 ± 0.02 [b]
Bake loss [%]	13.97 ± 0.52 [a]	13.81 ± 0.45 [a]	12.05 ± 0.43 [b]
Water activity	0.967 ± 0.004 [a]	0.965 ± 0.003 [a]	0.971 ± 0.003 [a]
Hardness [N]	8.14 ± 0.62 [a]	7.77 ± 0.78 [a]	18.24 ± 1.39 [b]
Chewiness	6.27 ± 0.53 [a]	6.21 ± 0.66 [a]	12.18 ± 0.73 [b]
Colour crust			
Lightness (L*)	61.7 ± 1.5 [a]	53.6 ± 1.5 [b]	66.6 ± 1.1 [c]
Redness (a*)	15.0 ± 1.0 [a]	16.5 ± 0.9 [b]	10.3 ± 0.6 [c]
Colour crumb			
Lightness (L*)	58.6 ± 2.1 [a]	51.8 ± 1.9 [b]	60.2 ± 1.9 [a]
Redness (a*)	7.2 ± 0.6 [a]	9.0 ± 0.5 [b]	8.3 ± 0.4 [c]
Slice area [mm^2]	1727 ± 49 [a]	1782 ± 69 [a]	1428 ± 40 [b]
Number of cells	1286 ± 72 [a]	1369 ± 75 [a]	1361 ± 64 [a]
Area of cells [%]	49.6 ± 0.6 [a]	49.3 ± 0.5 [a]	47.1 ± 0.5 [b]
Cell diameter [mm^2]	1.6 ± 0.1 [a]	1.6 ± 0.1 [a]	1.3 ± 0.1 [b]

Means ± standard deviation with different superscript letters in same row are significantly different ($p < 0.05$)

6.4.6 Aroma profile: flavour compounds and sensory characteristics

Sensory analysis was performed to determine the impact of the different fermentations on the aroma profile of the breads. Additionally, GC-MS screening provided information on the most dominant aroma compounds in the different breads. The relative areas of the volatile compounds in the crumbs from the GC-MS screening, with the corresponding organoleptic properties as reviewed by Pico *et al.* (2015), are shown in the bar chart of Figure 6-4.

For the sensory analysis nine aroma characteristics (‘malty’, ‘roasty’, ‘fatty’, ‘butter’, ‘fruity’, ‘sour’, ‘honey’, ‘earthy’, ‘dusty/ plastic/ oatflakes’, ‘potato’) were established

following DIN EN ISO 13299:2016-09. The analyses of the bread samples by the trained panel revealed very similar intensities in perception of the odour impressions ‘malty’, ‘butter’, ‘fruity’, ‘sour’, ‘honey’, and ‘potato’ in all three breads (Figure 6-4, sensory chart). A more subtle ‘roasty’ odour note was determined in the CF-NTCyb fermented bread. The aroma characteristics ‘fatty’ and ‘dusty/ plastic/ oatflakes’ were perceived as most distinctive; they were more dominant in the CF-NTCyb fermented bread, were perceived to a lesser extent in the SC-BY fermented bread, and only faint intensities were determined in the LF-FST 5.1 fermented bread. The latter was perceived as slightly more earthy than the other two breads.

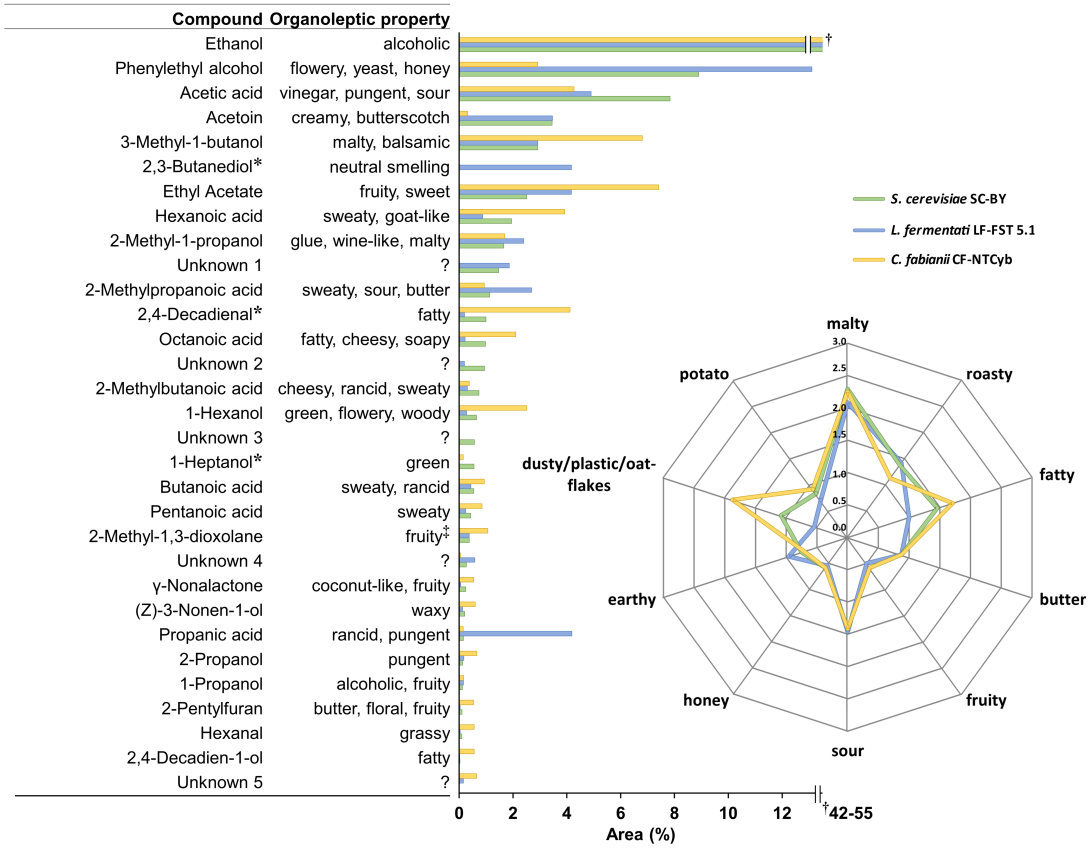

Figure 6-4. GC-MS aroma profile with typical organoleptic properties for each aroma compound according to Pico *et al.* (2015) and sensory chart for breads produced with different yeast. *S. cerevisiae* (SC-BY; green bars/ lines), *L. fermentati* (LF-FST 5.1; blue bars/ lines), *C. fabianii* (CF-NTCyb; yellow bars/ lines). Aroma compounds of different fermentations with respective relative areas > 0.5 %, presented in descending order of compounds in SC-BY fermented bread. * Low probability of compound identification. † Break in area axis: ethanol in all types of bread had relative areas of 42 – 55 %. ‡ No data available, predicted organoleptic property based on general perception of aldehydes.

Different alcohols (phenylethyl alcohol, 3-methyl-1-butanol, 2-methyl-1-propanol, 1-hexanol, 1-heptanol, (Z)-3-nonenol, 2-, and 1-propanol, 2,3-butanediol, 2,4-decadien-1-ol), aldehydes (2,4-decadienal, hexanal, 2-methyl-1,3-dioxalane), one ketone (acetoin), one ester (ethyl acetate), acids (acetic acid, hexanoic acid, 2-methyl-

propanoic acid, octanoic acid, 2-methybutanoic acid, pentanoic acid, butanoic acid, propanoic acid) and heterocyclic compounds (γ-nonalactone, 2-pentylfuran) and 5 unknown compounds characterised the aroma profiles of the SC-BY, LF-FST 5.1 and CF-NTCyb fermented bread to somewhat different extents (Figure 6-4). While 2,4-decadienal, octanoic acid, 2,4-decadienol, 1-hexanol, (Z)-3-nonenol and hexanal (with grassy, waxy, and fatty organoleptic properties) had a much higher impact on the CF-NTCyb profile, the same compounds had the least impact on the LF-FST 5.1 fermented bread's aroma profile. Furthermore, phenylethyl alcohol, 2,3 butanediol, 2-methyl-1-propanol, 2-methylpropanoic acid, and propanoic acid were more prominent in the LF-FST 5.1 profile.

6.5 Discussion

Out of 96 yeast isolates from different environments screened in carbohydrate utilisation tests, two alternative strains to the conventionally used *Saccharomyces cerevisiae* baker's yeast (SC-BY), namely *Lachancea fermentati* FST 5.1 (LF-FST 5.1) and *Cyberlindnera fabianii* NTCyb (CF-NTCyb), were the most promising strains for low FODMAP bread production.

Sucrose, raffinose, and inulin utilisation by LF-FST 5.1 and CF-NTCyb in microtitre broth assays were in accordance with other studies, although growth on inulin has been reported as "variable" for *L. fermentati* species (Bellut *et al.*, 2019a; 2020; Lachance & Kurtzman, 2011; Porter *et al.*, 2019). No literature is available on the ability of LF-FST 5.1 and CF-NTCyb to utilise shorter chain FOS, and the underlying mechanisms. In contrast, the ability of baker's yeast to ferment fructans, such as those found in wheat, has been extensively studied (Laurent *et al.*, 2020; Nilsson *et al.*, 1987; Struyf *et al.*, 2017b; Verspreet *et al.*, 2013). *S. cerevisiae* species degrade wheat fructans using cell-wall associated invertases, which have a higher affinity towards shorter fructan chains (average degree of polymerisation, DP, ~5). Other yeast strains known to utilise fructans, including long-chain inulin, belonging to *Kluyveromyces marxianus* species, can hydrolyse inulin more efficiently due to the expression of inulinases, which have a much higher affinity towards inulin molecules than invertases (Atzler *et al.*, 2020; Kurtzman *et al.*, 2011; Struyf *et al.*, 2017b). However, *K. marxianus* species is known to be unable to metabolise maltose, which is essential to achieve an appropriate dough rise during bread-dough fermentation (Struyf *et al.*, 2017b). In

contrast both non-Saccharomyces strains selected for the baking trials have been reported to utilise maltose (Bellut *et al.*, 2019b; Bellut *et al.*, 2020).

In the microtitre broth assay, both non-*Saccharomyces* strains and the baker's yeast showed comparable ability to ferment sucrose, fructose, and FOS, the most critical carbohydrates contributing to the final FODMAP content of a yeast-fermented wheat bread. However, the carbohydrate utilisation tests were performed in media with a single carbohydrate source and under aerobic conditions. Hence, different behaviour of the yeasts in a multi-nutrient environment, namely the complex wheat-matrix, and anaerobic conditions in the fermenting dough, was expected. To the best of our knowledge, both alternative strains have not been applied in bread-making before. The application of the two species has been recently investigated for low-alcohol beer production (Bellut *et al.*, 2019b; Bellut *et al.*, 2020). The nomenclature of both species has changed several times. *Lachancea* genus includes species previously characterised as *Zygosaccharomyces*, *Kluyveromyces* and *Saccharomyces* (Kurtzman, 2003). *Cyberlindnera* genus comprises species previously known as *Saccharomyces*, *Zygosaccharomyces*, *Hansenula*, *Pichia*, *Candida* and *Lindnera* (Minter, 2009).

6.5.1 Fermentation performance of selected yeast strains in whole wheat dough and bread

In the production of whole wheat dough and bread the two selected strains displayed a highly variable fermentation performance, compared to baker's yeast.

Firstly, the consumption of carbohydrates by yeast during the bread-making is essential for the leavening of the bread and is ultimately decisive for the bread's FODMAP content. During bread dough fermentation, yeast ferments sugar to produce ethanol and CO_2, under anaerobic conditions, while glycerol is produced as a secondary metabolite (Kulp & Lorenz, 2003). Therefore, free glucose and fructose as well as the invertase mediated release of these monosaccharides from raffinose, sucrose, and fructans serve as primary source of fermentable sugars for baker's yeast in the first hour of fermentation (Struyf *et al.*, 2017a). Hence, the results of the gas production by the different yeast strains are directly related to their carbohydrate fermentation pattern. Highest gas levels were measured in the LF-FST 5.1 fermented dough, while minimal CO_2 levels were detected in the CF-NTCyb dough. Accordingly, LF-FST 5.1 consumed fermentable sugars more efficiently than SC-BY

and CF-NTCyb during fermentation and proofing of the bread-making process. LF-FST 5.1 degraded more fructans, raffinose and sucrose, resulting in the release of higher glucose and fructose levels which could be metabolised over the fermentation period. The underlying mechanism of fructan, sucrose and raffinose degradation by *L. fermentati* species, however, remains to be elucidated. A possible explanation might be the expression of invertases with a higher substrate specificity than baker's yeast invertases, rather than inulinases, given that inulin was not utilised in the carbohydrate utilisation assay in broth. However, further studies are required to support such hypothesis. Besides the above-discussed sugars, also glucose, released from maltose by the yeasts' maltase, is a major fermentable carbohydrate during the bread-making process (Struyf *et al.*, 2017a). A possible explanation for the CF-NTCyb's unexpected low fermentation rate in the dough might be a pronounced high sensitivity towards glucose repression, and hence, repression of the SUC2 (invertase) and MAL (maltase) genes (Barnett & Entian, 2005). This could be induced on the one hand by the yeasts' growth in YPD, on the other hand by the increased glucose levels in the dough after the fast initial sucrose degradation in the t_0 dough (Verspreet *et al.*, 2013).

Furthermore, the fermentation metabolites acetic acid and succinic acid are the main organic acids typically found in yeast-fermented dough (Struyf *et al.*, 2018). In this study, acetic acid was the main organic acid detected in the different breads. Despite studies reporting succinic acid is the main pH-influencing acid in *S. cerevisiae* fermented dough (Jayaram *et al.*, 2014; Struyf *et al.*, 2018), no free succinic acid was found in any of the breads. Apart from the possibility that no or very little succinic acid was produced during the fermentations under the given conditions (e.g., low activity of enzymes from the oxidative pathway of the tricarboxylic acid cycle (Rezaei *et al.*, 2015)), coelution of the acid associated to other matrix-components or partial depletion during the baking process are also likely. The first and latter reasons would contradict the pH defining character of succinic acid, as there was no difference in pH between the fermented doughs and breads, but it would explain the slightly lower TTA values in the respective breads, compared to the fermented doughs. Moreover, levels of lactic acid were determined in the breads, as *L. fermentati* species, recently applied for low alcohol beer production, are known to have the unique ability to produce considerable amounts of lactic acid (Bellut *et al.*, 2020). However, only traces of lactic acid were found in the breads. The fermentation time during bread-making (150 min)

is much shorter than the beer-fermentation time (several days) and not sufficient for the yeast to produce quantifiable amounts of lactic acid.

6.5.2 FODMAP contents in the breads

The FODMAP contents of the breads fermented with the different yeast were most significantly defined by (a) the extent of fructan degradation and (b) the amount of fructose present in the bread in excess to glucose. The excess fructose value in turn was defined by (a) the amount of fructose released from sucrose, raffinose and fructans and glucose released from sucrose and starch, which were (b) left unconsumed by the yeast in the final breads. LF-FST 5.1 degraded fructans, raffinose and sucrose more effectively than SC-BY, and hence, allowed for a more efficient consumptions of the released monosaccharides over the fermentation period. This resulted in lower fructan and excess fructose levels. Furthermore, invertase and inulinase mediated degradation of raffinose results in the formation of melibiose and fructose (Atzler *et al.*, 2020). None of the yeast species used in this study were able to utilise melibiose (Kurtzman *et al.*, 2011). As indigestible sugar such as GOS (Adamberg *et al.*, 2018), melibiose accounted towards the total FODMAP content. Finally, while it is very common for sourdough fermentation to results in polyol production, yeast-leavened bread does not accumulate sugar alcohols (Loponen & Gänzle, 2018). Even though some yeasts are known to be xylitol-producers (Sahin *et al.*, 2019), neither of the yeast species investigated in this study has been reported to produce sugar alcohols during fermentation.

Ultimately, only the FODMAP contents of the LF-FST 5.1 fermented bread, in a 50 g serving of fresh bread, were below all individual cutoff levels for oligosaccharides, excess fructose, and polyols, as well as a total of 0.5 g FODMAPs. In contrast the SC-BY and CF-NTCyb fermented bread exceeded the individual and total FODMAP cutoff levels (Table 6-5).

6.5.3 Bread quality

The results of the technological bread quality characteristics largely agree with the findings of the individual fermentation performance of the different yeast strains. Except for the colour, there was no significant difference in the technological bread quality characteristics of the SC-BY and the LF-FST 5.1 fermented breads. While the analysis of the CF-NTCyb breads revealed, as expected from the poor fermentation performance, a significant reduction in bread quality parameters. Concerning one of

the most important quality characteristics, the specific volume, no significant difference was measured between the SC-BY and the LF-FST 5.1 fermented breads. This may seem contradictory to the higher capacity for gas production seen during the rheofermentometer measurements of the LF-FST 5.1 fermenting dough. The CO_2-production was measured over a fermentation period of 180 min in the resting dough. In contrast, the bread-making process involved several mechanical dough modification steps within only 150 min fermentation and proofing. Indeed, the undifferentiated bread volumes can be attributed to 30 min shorter fermentation time and partial loss of CO_2 during the punching, dividing, and moulding steps of the bread-making process. The inferior quality characteristics of the CF-NTCyb fermented bread are a direct consequence of the low fermentation rate and low levels of gas retained in the dough of the CF-NTCyb, compared to the SC-BY and LF-FST 5.1 fermentations. Finally, a weaker crust browning of the CF-NTCyb and the SC-BY fermented breads was determined, compared to the LF-FST 5.1 fermented bread. This can be, supposedly, attributed to a limited educt-availability to form Maillard products. The Maillard reaction of reducing sugars and primary amines during the baking process mainly contributes to the browning of the crust (Sluimer, 2005). High levels of reducing sugars were present in the final SC-BY and CF-NTCyb fermented breads (Figure 6-2); thus, the lack of browning was likely to be caused due to limited availability of amines.

6.5.4 Aroma profile

Besides the technological quality characteristics of bread, the aroma is of particular interest, especially in the application of unexplored non-conventional yeast species. Several volatile compounds dominated the aroma profiles of the respective breads and thus, contributed to odour impressions perceived in the sensory analysis.

The different volatile aroma compounds that characterised the aroma profile of the SC-BY fermented bread are in line with frequently reported aroma compounds in wheat bread (Pico *et al.*, 2015). 2-Methyl-1,3-dioxalane has not been previously reported. However, a similar compound (2,4,5- trimethyl-1,3-dioxolane) was found in bread crust by Folkes & Gramshaw (1977).

The slightly more ‘earthy’ note in the LF-FST 5.1 fermented bread, perceived by the sensory panel, could not be explained by volatile compounds, typically responsible for

an earthy aroma. No pyridines, pyrazines or octanol were found in considerably high levels to contribute to the aroma perception of the LF-FST 5.1 fermented bread (Paraskevopoulou *et al.*, 2012; Pico *et al.*, 2015).

The weaker 'roasty' note from the CF-NTCyb fermented bread determined in the sensory analysis could also not be explained by volatile compounds but is in line with the findings of the weak crust browning. Maillard and caramelisation products, mainly formed in the bread crust, contribute substantially to the bread aroma (Pico *et al.*, 2015). The crumb was analysed by the sensory panel, yet an odorant transfer between crust and crumb has been reported (Onishi *et al.*, 2011).

Furthermore, the sensory analysis resulted in a weaker perception of the odour note 'fatty' from the LF-FST 5.1 fermented bread, than from the other two samples. Indeed, the relative areas of aroma compounds determined, responsible for a fatty olfactory reception of bread, including 2,4-decadienal, octanoic acid and 2,4-decadienol had a lesser impact on the overall aroma profile of the LF-FST 5.1 fermented bread, compared to the profile of the CF-NTCyb and SC-BY fermented breads. In combination with 1-hexanol, (Z)-3-nonenol, and hexanal, the same compounds might have contributed to a higher intensity in the perception of a 'dusty/ plastic/ oatflakes' note in the CF-NTCyb fermented bread. The higher propanonic acid and 2-methylpropanonic acid production in the LF-FST 5.1 fermentation (relative as well as absolute areas, latter not shown) might be related to *L. fermentati* strains' unique ability to produce higher levels of lactic acid, which in turn can be an intermediate compound in the formation pathway of those volatile acids (Bellut *et al.*, 2020; Pico *et al.*, 2015). It was shown that volatile compounds, such as those dominant in the LF-FST 5.1 fermented profile (phenylethyl alcohol, 2-methyl-1-propanol, 2-methylpropanoic acid), are typically also found in higher levels in sourdough bread, compared to yeast-leavened bread (Hansen & Hansen, 1996; Pico *et al.*, 2015). However, no distinct 'sour' note (also no off-flavour) was perceived from the LF-FST 5.1, compared to the baker's yeast fermented bread. This could be explained if the concentrations of the respective compounds were below or very close to the odour thresholds. Yet, the absolute quantities of the aroma compounds were not determined in this study. At last, an explicitly fruity aroma due to higher levels of esters, as it was seen for *L. fermentati* applications in beer, was not determined (Bellut *et al.*, 2020). Overall, the aroma profile of the LF-FST 5.1 fermented bread was very similar to the

baker's yeast fermented bread, indicating a good consumer acceptance of the bread, not only regarding its technological quality characteristics but also its aroma.

6.5.5 Conclusion

This study explored the potential application of two non-conventional yeast strains, isolated from kombucha and a rice wine starter, as an alternative to baker's yeast, to produce low FODMAP whole wheat bread. It demonstrated the great potential of *L. fermentati* FST 5.1, which has never been applied in baking, to the best of our knowledge. Fermentation with the *L. fermentati* FST 5.1 resulted in significantly lower fructan levels in the final bread compared to baker's yeast fermented bread, without the accumulation of high levels of excess fructose or any other FODMAP. The bread's FODMAP levels were below all cutoff levels, which categorises it as a low FODMAP product. Concerning the second alternative strain, *C. fabianii* NTCyb, it was shown that, despite the strain's potential ability to ferment fructans and other FODMAPs (based on single-sugar-medium utilisation tests), the application in a wheat-dough-matrix resulted in a very low fermentation rate. The carbohydrates in the dough-formulation were barely utilised, leading to high FODMAP levels, low CO_2 production and an overall poor bread quality, which indicates *C. fabianii* NTCyb may not be suitable for baking applications. Ultimately, this study has shown comparable quality characteristics of the *L. fermentati* FST 5.1 and the baker's yeast fermented breads, with a slight tendency towards a higher bread volume and the production of sourdough-like aroma compounds. The availability and application of an optimised preparation of the *L. fermentati* FST 5.1 strain as baking ingredient may even outperform technological and sensory bread quality characteristics of conventional baker's yeast fermented bread and should be further investigated.

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Chapter 7
General discussion

7.1 General discussion

The concept of the low FODMAP diet is a rather new therapeutic dietary approach for the management of functional gastrointestinal disorders; it was first described only ~ 15 years ago (Gibson & Shepherd, 2005). Consequently, research on the development of functional foods with a lowered FODMAP content is still in its infancy. In this matter, as whole grain cereals (i.e., wheat, rye, barley) and pulses, and products made from those, represent a major source of FODMAPs (fructans and GOS, respectively), the choice of healthy and nutritious products with a low FODMAP content is strongly limited. The global market on functional products with a low FODMAP claim is currently dominated by gluten-free products, which also coincide to have low FODMAP contents (***Chapter 2***). This is because commonly used ingredients of gluten-free products (e.g., flours and starches from rice, maize, or potato) also have very low FODMAP contents. As opposed to wheat or rye, and pulses, those do not accumulate fructans or GOS (***Chapter 4***). Ultimately, less than 10 % of commercially available products with a low FODMAP claim are not gluten-free but utilise currently known FODMAP reduction strategies to some extent. However, the wheat protein gluten is an essential component for the production of bakery products and pasta; it does not have to be eliminated from the product formulations, such as it is necessary for individuals suffering from coeliac disease or other gluten sensitivities/ allergies. In addition, gluten-free products often have inferior sensory and nutritional characteristics compared to conventional products (Miranda *et al.*, 2014; Pellegrini & Agostoni, 2015). Aiming to overcome this, research over the past few years has already demonstrated the potential of different, more targeted approaches to produce appealing and nutritionally beneficial functional low FODMAP products (reviewed in ***Chapter 2***). Hence, with the apparent lack of such products on the global market, a growth of the market segment is forecast, especially for bakery products (Mellentin, 2020).

First and foremost, in the research for functional products, the accurate quantification of FODMAPs poses a challenge (***Chapter 3***). Especially the appropriate choice of an analytical technique for the quantification of fructans deserves careful consideration. The analysis of fructans can be divided into two main approaches: a direct quantification (each signal in a chromatogram from a fructan molecule is evaluated) and an indirect quantification (fructans are hydrolysed and the resulting monomers are

quantified to calculate the total fructan content) (Stöber *et al.*, 2004). The applicability of the direct approach for the analysis of fructans as part of the FODMAP quantification in food products is restricted by different factors. Firstly, the availability of authentic reference standards for quantifying different fructan molecules is strongly limited. And although alternative approaches, such as the separation and purification of the compounds of interest from fructan rich matrices (using preparative chromatographic techniques) (Pitsch *et al.*, 2021), or the application of molar calibration curves with an available standard, corrected by the respective molecular weights (Ziegler *et al.*, 2016), are technically possible, they are not reasonable. An adequate application of such techniques in highly complex and diverse food matrices (e.g., containing different types of fructans in different DPs) is extremely laborious. Hence, the most commonly used approach is the indirect analysis of fructans after hydrolysis, either enzymatic (AOAC 997.08, 2003) or using a mild acid treatment (Verspreet *et al.*, 2012). An important yet often neglected factor using this indirect approach is the consideration of fructose and glucose monomers released from other compounds than those of interest. In this matter, fructan hydrolysing enzymes (inulinases/ fructanases) used to hydrolyse fructans prior to quantifying the monomers, also release fructose from GOS (raffinose, stachyose, verbascose). This inevitably leads to the overestimation of the total fructan content and might provide an explanation for contradictory reports of fructan contents in pulses (McCleary *et al.*, 2019). While pulses do accumulate high amounts of GOS, they are not known as fructan accumulating seeds (***Chapter 4*** and ***5***). Nonetheless some studies reported considerable amounts of fructans in pulses, supposedly as a result of an analytical error (Biesiekierski *et al.*, 2011; Dodevska *et al.*, 2013). Two commercial enzyme assay kits from Megazyme (K-FRUC and K-FRUCHK) serve as convenient photometric quantification techniques, which do not require advanced chromatographic equipment. Nevertheless, it is crucial to choose the respective kit according to its application and to amend both assays (as recommended in the assays' protocol) with an additional α-galactosidase treatment step, if GOS are expected in the food matrix (always the case in cereal- and pulse-based products). The K-FRUCHK assay kit has been specifically developed to overcome the underestimation of partially hydrolysed fructans (have a reducing end as opposed to native fructans with non-reducing ends; final fructose residues are reduced with free non-fructan sugars during the sodium borohydride reduction step in the K-FRUC assay); yet its application for the fructan quantification

in cereal-based matrices is limited. The assay's accuracy is strongly impaired and results in high blank absorbances if relatively low fructan and high sucrose, fructose, glucose, and maltose levels are present in the sample extracts, again leading to false fructan results. Hence, the K-FRUC assay that eliminates absorbances from non-fructan sugar with the borohydride reduction step is better suited for the analysis of cereal-based matrices. Lastly, the appropriate choice of an extraction medium for fructans is essential to fully solubilise the higher DP fructan molecules. While ethanol and water at room temperature do not solubilise the higher DP fructans, hot water (80 °C) results in a complete extraction (Haskå *et al.*, 2008). The analytical method for the quantification of fructans and all other FODMAPs, proposed as part of this thesis (***Chapter 3***), is based on the direct quantification of polyols, mono-, disaccharides and GOS with reference standards via HPAEC-PAD and the calculation of the fructan content from enzymatically released fructose and glucose (also quantified via HPAEC-PAD). Interfering saccharides in the fructan-analysis are taken into consideration with the inclusion of amyloglucosidase and α-galactosidase in two separate enzymatic treatments, where only one hydrolyses fructans; the calculation of the fructose and glucose contents is based on the difference of both treatments. The specificity, accuracy and reproducibility of the method has been validated and the method served as analytical tool for FODMAP quantifications throughout the work undertaken for this thesis.

As already indicated, cereals and pulses accumulate two main classes of FODMAPs: the oligosaccharides fructans and GOS, respectively (***Chapter 4***). In agreement with existing literature, the work of this thesis confirmed that wheat (any variety of modern or ancient wheat), barley and rye grains contain considerably high fructan levels. In contrast, other gluten-free cereal grains, including oat, millet, rice, and maize do not accumulate relevant amounts of fructans (Verspreet *et al.*, 2015; Ziegler *et al.*, 2016). Reports of higher fructan levels in oat- or maize-based products, without any other source of fructans, could supposedly be attributed to the analytical errors described above. Furthermore, also other ingredients typically used in gluten-free products, including vegetables and pseudo cereals, such as potato, quinoa, and buckwheat, do not contain fructans or GOS. Buckwheat, however, accumulates another class of indigestible oligosaccharides (0.2 – 3 % fagopyritols found in different milling fraction) with similar structural and biochemical properties to GOS (Horbowicz *et al.*,

1998; Steadman *et al.*, 2000); those are suspected to also act as FODMAPs in IBS patients. Fagopyritol concentrations in buckwheat are lower than GOS in pulses. Nonetheless, they may be sufficient to trigger IBS symptoms (cutoff level for oligosaccharides is 0.3 g per serving). However, the potential of buckwheat to induce symptoms in IBS patients and the consequent necessity of an inclusion of fagopyritols into routinely analysed FODMAPs remain to be elucidated.

Although barely utilised by food industry to date, to lower FODMAP levels in cereals and pulses and produce low FODMAP products, different targeted approaches are available. On the one hand, products can be based on the use of ingredients where FODMAPs have been physically removed. For instance, formulations where wheat flour is replaced with its isolates starch and gluten (contain only low levels of fructans), fortified with IBS-friendly fibres, could serve as a simple, yet efficient and reliable approach in the low FODMAP product development, such as recently demonstrated for bread (Atzler *et al.*, 2021). Also, the isolation of protein ingredients from pulses can, depending on the technology applied (e.g., membrane filtration is very effective), remove the majority of GOS from pulses and provide thereby low FODMAP pulse-based ingredients (Joehnke *et al.*, 2021; Vogelsang-O'Dwyer *et al.*, 2020). Those can be easily used for example for protein fortification of baked products and pasta or dairy alternative products, while partly or fully replacing high FOMDAP ingredients in the formulations (Hoehnel *et al.*, 2020b; 2020a; Vogelsang-O'Dwyer *et al.*, 2021). However, although the fortification of products with pulse protein has been of research and industry interest especially in recent years, this approach has received very little attention in the context of FODMAPs.

On the other hand, FODMAPs can be biotechnologically reduced during food processing, mediated by seed endogenous enzymes (***Chapter 5***), added enzymes or microbial enzymes during a fermentation process (***Chapter 6***). Using biotechnological FODMAP reduction techniques, the targeted carbohydrates can be degraded while preserving beneficial, slowly fermentable whole-grain intrinsic dietary fibres (Laatikainen *et al.*, 2016; Loponen & Gänzle, 2018). An important factor of bio-technological FODMAP degradation is that the degradation of the oligosaccharides, especially fructans, should always be linked to a strategy for reducing the resulting monomers to avoid an accumulation of other FODMAPs.

The indigestible linkages in GOS are cleaved by α-galactosidases. While this can be

achieved via enzyme addition or fermentation with selected microorganisms (Nyyssölä *et al.*, 2020; 2021), seed endogenous α-galactosidases can also be activated during the seeds' germination and lead to a significant GOS reduction in pulses. This effect has been known for a long time and proven its potential to reduce GOS levels in a variety of pulses (Martínez-Villaluenga *et al.*, 2008; Reddy & Salunkhe, 1980). In this framework and although not commonly applied for pulses, malting (***Chapter 5***) can serve as an effective tool to produce stable and storable pulses with significantly lower GOS levels. The malting process is a limited germination process under controlled conditions, terminated by exposure of the germinated seeds to a heating/ drying step; throughout the malting process, GOS in lentils and chickpeas were shown to be reduced by 80 − 90 %. Also, fagopyritols in buckwheat were degraded during the germination process due to elevated activities of α-galactosidases (Jia *et al.*, 2015). In contrast to the GOS degrading effect of malting in pulses, fructan levels in cereals can be further elevated as a result of the germination process. Although biochemical changes of fructans during the germination of cereals have received much less attention, the work of this thesis, in agreement with other studies, demonstrated a clear trend of fructan synthesis during the germination of cereals, followed by a partial decline during the kilning process (Cozzolino *et al.*, 2016; Harris & MacWilliam, 1954; Krahl *et al.*, 2008; MacWilliam *et al.*, 1956). This led to a slight increase of fructans in barley and wheat malt and 0.8 % DM *de novo* synthesised fructans in oat malt, and even 1.4 % DM in the germinated grains before kilning (raw oat contains only traces of fructans). Consequently, malted cereals should either be avoided or subjected to further biochemical treatment, such as fermentation, for low FODMAP applications. However, in contrast to the results of this thesis and other studies reporting the fructan developments in malted cereals, one recent study reported very low fructan levels in sprouted barley and rye grains (Tuck *et al.*, 2018). Conventional sprouting of grains for direct consumption aims the seeds' germination under less controlled conditions than malting and does not involve a drying/ heating step. The fresh germinated seeds, soaked with water, are prepared for direct consumption or immediate further processing. The results of that study are reported based on the seeds' fresh weight. Hence, the low fructan contents can be at least partly attributed to a 'dilution' effect (germinated seeds contain ~ 50 % water, compared to ~10 % water in dry raw seeds). Nonetheless, a potential shift of dominating fructan-anabolic enzymes (observed during malting) towards catabolic enzymes with alteration of the

germination conditions, deserves more scientific attention.

Finally, specific strains of yeast and lactic acid bacteria (LAB) produce α-galactosidases, inulinases (fructanases) or invertases. The most commonly used leavening agent in baked products, baker's yeast (*Saccharomyces cerevisiae*, also one of the most commonly used microorganisms in food production) expresses invertases. Baker's yeast has been shown to be capable of degrading the majority of wheat fructans during the fermentation process of bread-making (40 – 90% degraded) (Gélinas *et al.*, 2016; Struyf *et al.*, 2017; Ziegler *et al.*, 2016). However, with very short industrial fermentation times and an affinity of invertases mainly towards shorter chain fructans (up to DP 5, degradation of longer chains is slower) (Nilsson *et al.*, 1987; Struyf *et al.*, 2017), conventional yeast-leavened products, especially if whole wheat based, are usually high in FODMAPs. If the yeast's fructan degradation capacity is slow, insufficient fermentation times can additionally lead to an accumulation of excess fructose as the yeast does not have enough time to metabolise the released fructose monomers (Ziegler *et al.*, 2016). Hence, recent studies focussed on the investigation of alternative, more potent fermentation cultures. For instance, the yeast strain *Kluyveromyces marxianus* NCYC587 or the LAB strain *Lactobacillus crispatus* DSM 29598 were identified as very potent FODMAP degraders, used either in a baker's yeast co-culture leavened bread or in a sourdough bread with baker's yeast addition, respectively. Within this thesis (***Chapter 6***) the FODMAP degradation capabilities of various yeast isolates from different environments were first screened in carbohydrate utilisation tests. This led to the identification of two promising strains: *Lachancea fermentati* FST 5.1 (from a kombucha culture) and *Cyberlindnera fabianii* NTCyb (from a rice wine starter), subsequently used for baking application. While the latter displayed a very low fermentation rate during whole wheat dough fermentation, resulting in high FODMAP bread, *L. fermentati* FST 5.1 outperformed baker's yeast with a much more efficient fructan degradation and concomitant metabolism of released fructose. The resulting bread had not only overall low FODMAP contents but also optimal quality characteristics (volume, texture, sensory) comparable to conventional baker's yeast leavened bread. Although the involved metabolic enzyme(s) in the fructan degradation mechanisms remain to be elucidated, it could be hypothesised that invertases with a higher wheat fructan substrate specificity might be rather involved than inulinases (*L. fermentati* FST 5.1 was unable to utilise inulin in the preceding screening assays). Further studies could focus on an optimised

preparation of the yeast growth (e.g., minimise effect of glucose repression and stimulate invertase expression with modified growth media, Verspreet *et al.*, 2013). This could further enhance the strain's performance, potentially allowing a shorter fermentation time, in the interest of industrial baking (2.5 h applied in this thesis).

Furthermore, alongside research efforts leading to the identification of different powerful FODMAP degrading enzyme preparations, yeast and LAB strains, the synergistic effects of the respective approaches have received very little attention so far. Few recent studies have demonstrated the potential of combining selected potent LAB strains with fructan degrading enzymes and/ or baker's yeast (Acín Albiac *et al.*, 2020; Li *et al.*, 2020). Investigations of the synergistic effects of different approaches in a larger variety of products and product formulations, will contribute to a further expanded repertoire of reliable tools for the production of appealing and nutritionally beneficial low FODMAP products. Nevertheless, it is crucial to bear in mind that, regardless of the approach used, a complete removal of FODMAPs is not desired, but only a reduction to tolerable levels; especially fructans are important prebiotics, that stimulate the growth of beneficial gut bacteria (Muir *et al.*, 2019). Finally, collaboration of biomedical and food scientists should target the investigation of other carbohydrates that may act as FODMAPs, while identifying their dietary sources and relevance to extend the list of routinely analysed FODMAPs. Ultimately, the work presented in this thesis contributes practical and in-depth fundamental knowledge to the novel concept of low FODMAP food production on the way of providing an improved quality of life with a better food choice for IBS patients.

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Appendix

Appendix

A-1 Supplementary figures and tables

Table A-1. Composition of mobile phases for chromatographic separation on CarboPac PA200 (1) and CarboPac PA1 (2)

(1) Gradient method on CarboPac PA200 [a]				
time [min]	A [b] [%]	B [c] [%]	C [d] [%]	
0	70	27.5	2.5	
30	42.5	27.5	30	
50	26.5	27.5	46	separation of analytes
70	15.3	27.5	57.2	
75	0	27.5	72.5	
80	0	0	100	column clean-up
85	0	0	100	
86	0	100	0	regeneration gold surface
101	0	100	0	
102	70	27.5	2.5	re-equilibration
117	70	27.5	2.5	
(2) Isocratic method on CarboPac PA1 [a]				
time [min]	A [b] [%]	B [c] [%]	C [d] [%]	
0	91.9	8.1	0	separation of analytes
25	91.9	8.1	0	
26	0	0	100	column clean-up
31	0	0	100	
32	0	100	0	regeneration of gold surface
47	0	100	0	
48	91.9	8.1	0	re-equilibration
60	91.9	8.1	0	

[a] flow rate 0.25 mL/ min, column temperature 25 °C.
[b] ultrapure water.
[c] 225 mM NaOH.
[d] 500 mM NaOAc.

Figure A-1. Impact of extraction temperature and MeOH addition on α-amylase activity in wheat.
(1) Extraction according to K-CERA procedure with room-temperature buffer, (2) Extraction with buffer heated to 80 °C, (3) Extraction with a mixture of MeOH and buffer, (4) Extraction with a mixture of MeOH and buffer heated to 80 °C. Error bars represent standard deviations of triplicates.

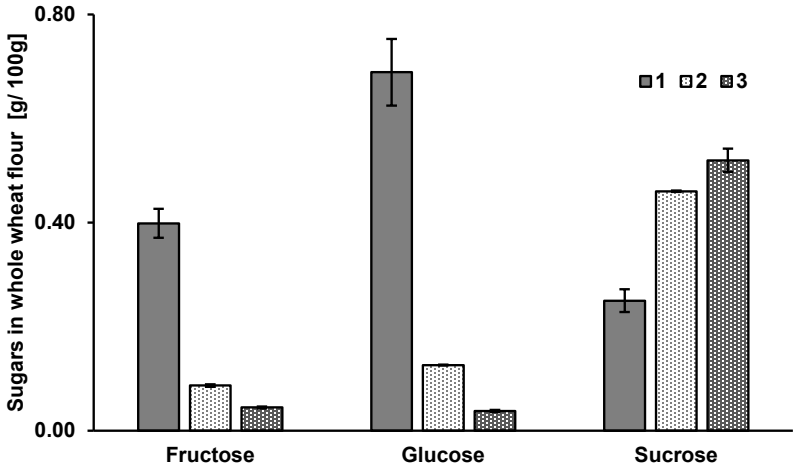

Figure A-2. Impact of extraction temperature and MeOH addition on glucose, fructose, and sucrose determination in whole wheat flour (HPAEC-PAD analysis).
(1) Extraction at room temperature without MeOH addition, (2) Extraction at room temperature with MeOH addition, (3) Extraction with H2O heated to 80 °C with MeOH addition. Error bars represent standard deviations of triplicates.

Table A-2. Retention times of carbohydrates for CarboPac PA200 and CarboPac PA1

Compound	Rt [min] CarboPac PA200 [a]	Rt [min] CarboPac PA1 [a]
Xylitol	3.6	**2.4**
Sorbitol	3.7	**3.1**
Mannitol	3.9	**3.7**
Maltitol	4.7	**10.0**
Rhamnose	**4.5**	**9.5**
Galactose	**5.1**	**15.2**
Glucose	**5.1**	15.9
Fructose	**5.6**	21.6
Melibiose	5.6	**27.5**
Sucrose	**6.3**	24.4
Manninotriose	5.8 [b]	34.6 [b]
Manninotetraose	6.1 [b]	53.0 [b,c]
Lactose	**6.4**	39.5 [c]
Raffinose	**7.8**	52.5 [c]
Stachyose	**7.8**	61.9 [c]
Verbascose	**8.7**	-
Kestose	**8.8**	-
Nystose	**13.0**	-
Kestopentaose	**16.3**	-

[a] Only peaks with retention times printed in bold have been used for quantitative analyses.
[b] Identification based on enzymatic hydrolysis; reference standards were not available.
[c] Extended instrument method for CarboPac PA1 (not shown) used for qualitative identification.

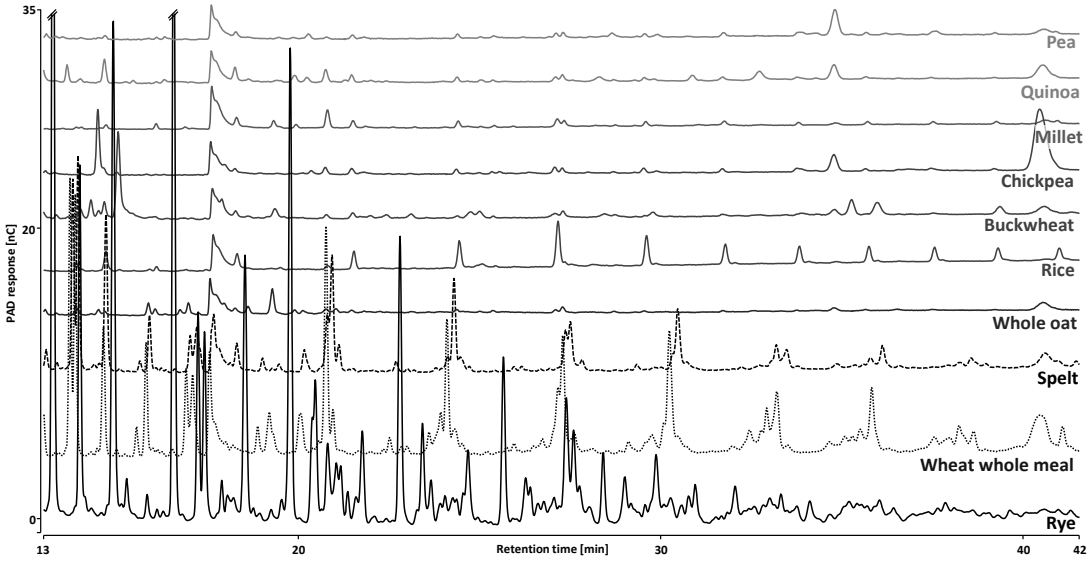

Figure A-3. HPAEC-PAD CarboPac PA200 profile in higher-DP area from different flour extracts showing the diversity and complexity of composition of different material

Table A-3. Cereal-product ingredients screened for the FODMAP profile

Type of flour	Supplier	Country of origin [a]
Group I		
Whole meal	Odlums	Ireland
Bakers' flour	Unifood	UK
Biscuit flour	Odlums	Ireland
Semolina	Buchanan	Northern Ireland
Vital gluten	Roquette	France
Wheat starch	Roquette	France
Wheat bran	Boecker	Germany
Spelt	Rettenmeier Muehle	Germany
Rye	Thylmann	Germany
Barley (whole grains)	SOC (var. Beatrix)	France
Group II		
Whole oat flour	Flahavan's	Ireland
Oat bran	ABS food	Italy
Quinoa	Ziegler Organic	Germany
Millet	Ziegler Organic	Germany
Buckwheat flour	Trouw	Netherlands
Brown rice	Doves farm	UK
Potato starch	Emsland group	Germany
Corn starch	Cargill	US
Group III		
Lentil (whole seeds)	Societa' Agricola Zannini D. e C.	Italy
Chickpea flour	Doves Farm	UK
Soy (whole seeds)	Tesco	Ireland
Green pea (whole seeds)	Quay-Coop	Ireland
Yellow pea (whole seeds)	Quay-Coop	Ireland
Fababean (proteinrich flour)	Fraunhofer IVV (research)	Denmark
Group IV [b]		
Fababean protein (85 %)	Fraunhofer IVV (research)	Denmark
Carob protein (≥ 48 %)	DuPont Danisco	UK
Pea protein (≥ 83 %)	Roquette	France
Lupin protein (94 %)	Fraunhofer IVV (research)	Denmark
Lupin protein (> 38 %)	Lup'Ingredients Protilup	France
Group V		
Quinoa sprouts		
Pea sprouts		
Lupin sprouts	Keimkraft	Germany
Buckwheat sprouts		
Lentil sprouts		

[a] The country of origin is referred to the product, not the raw material the company used to produce the ingredient. [b] The number in brackets indicates protein content of the ingredient.

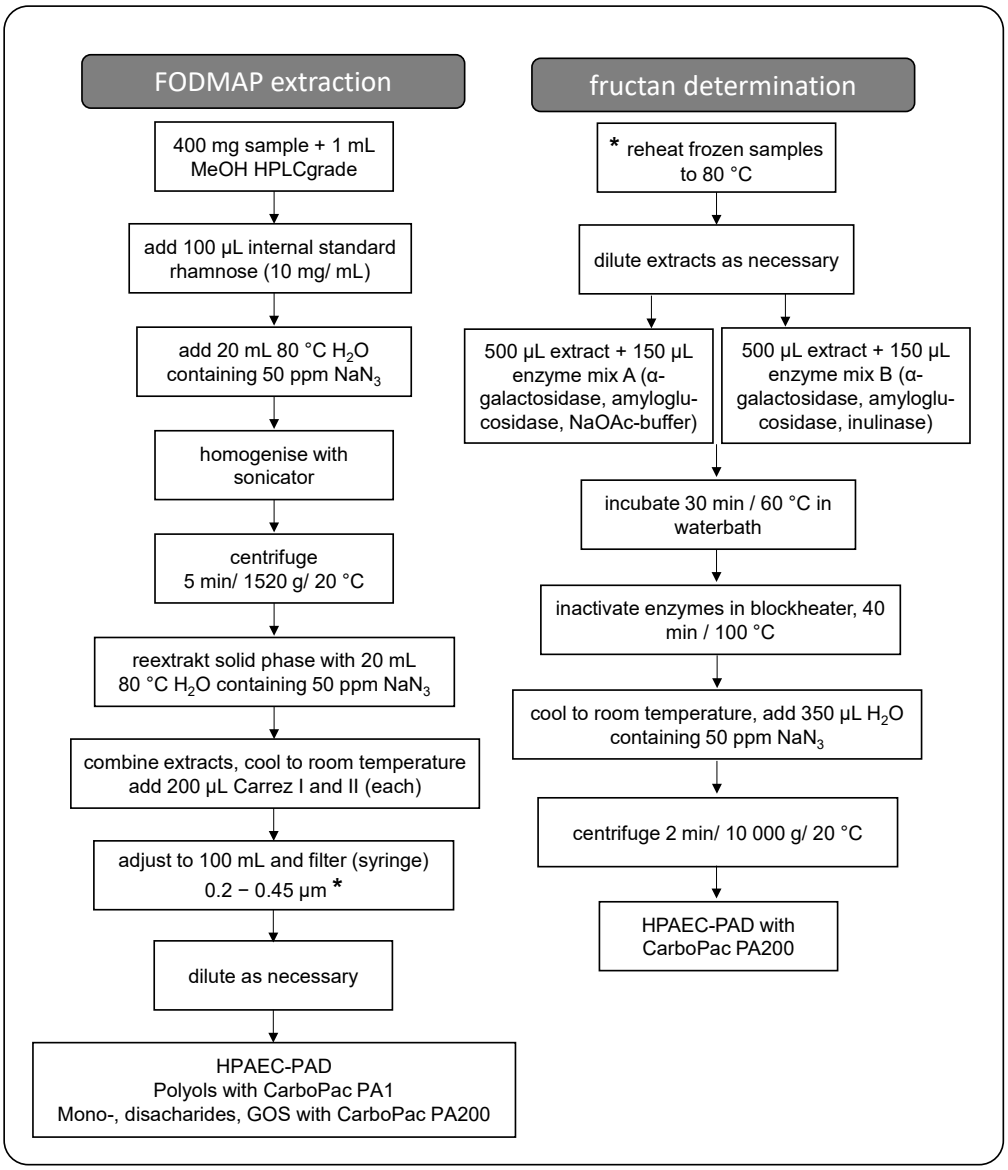

Figure A-4. Flow-chart of method for FODMAP determination via HPAEC-PAD according to Ispiryan *et al.* (2019)

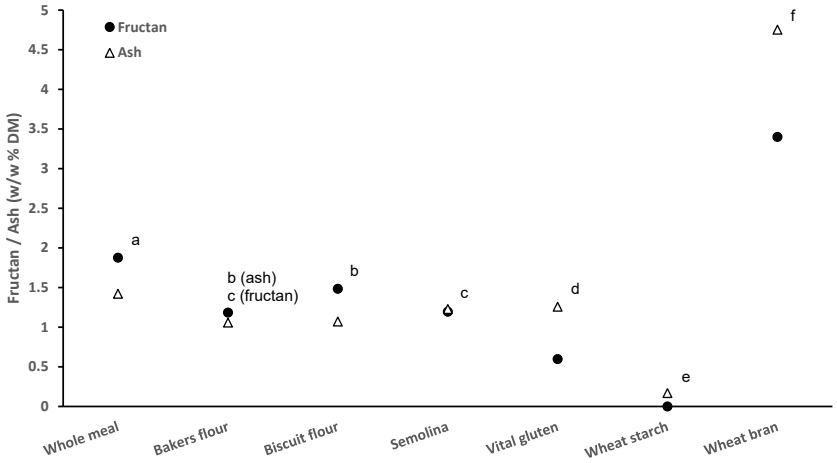

Figure A-5. Ash content and fructan content in different wheat-based ingredients. The small letters indicate significant difference ($p < 0.05$) of the fructan and ash contents between the ingredients

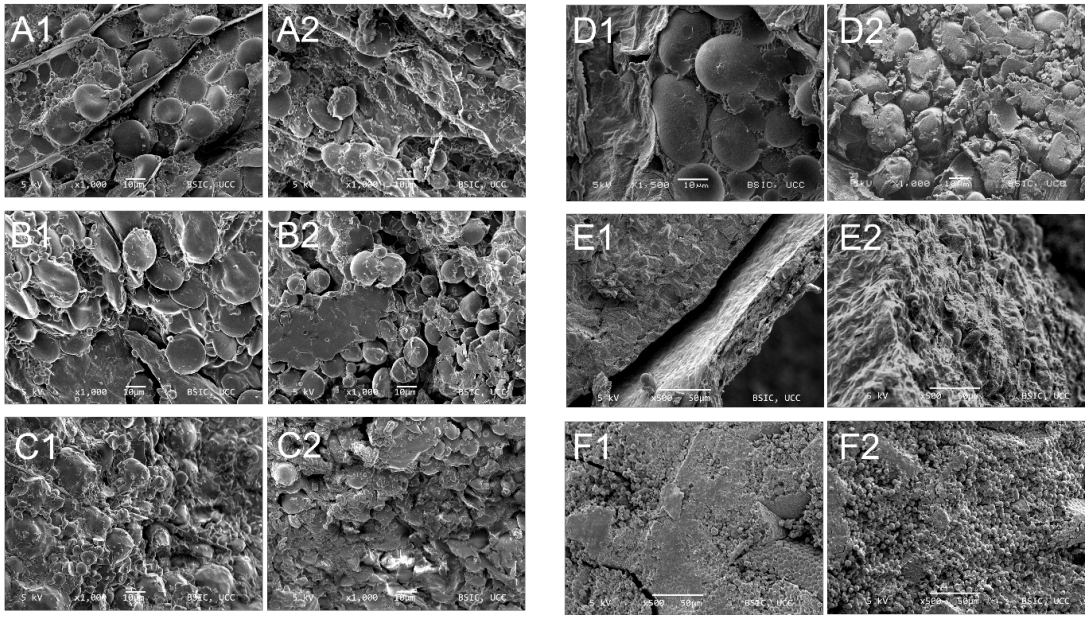

Figure A-6. SEM micrographs of raw and malted A: barley, B: wheat, C: oat, D: lentil, E: chickpea, F: buckwheat. A1-F1: raw seeds, A2-F2: malted seeds

Table A-4. Fructans in oat malting determined via HPAEC-PAD and K-FRUC assay

	Fructan [g/100g DM] ± standard deviation	
	K-FRUC [a]	HPAEC-PAD [b]
raw oat	n.d.	n.d.
steeped	n.d.	n.d.
1. s.o.g.[c]	n.d.	n.d.
2. s.o.g.	0.39 ± 0.02	0.44 ± 0.06
3. s.o.g.	0.61 ± 0.02	0.63 ± 0.02
4. s.o.g.	0.73 ± 0.07	0.82 ± 0.04
green malt	1.37 ± 0.04	1.44 ± 0.00
oat malt	0.74 ± 0.03	0.77 ± 0.01

[a] n.d., not detected in means of levels below LOD of the K-FRUC assay. [b] n.d., not detected in means of no significant difference in sucrose values and fructose values determined from difference of assay A and B in fructan determination, or levels below 0.1 g/ 100 g DM. [c] s.o.g., stage of germination

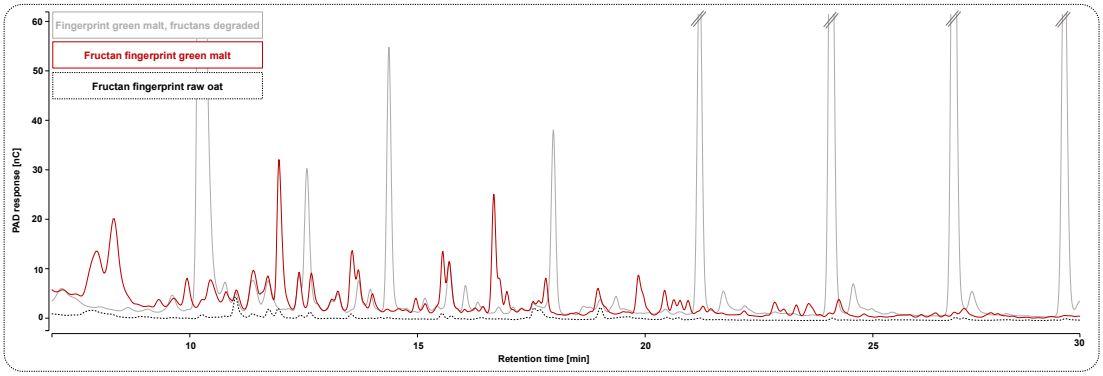

Figure A-7. HPAEC-PAD profiles of extracts of oat grain and green malt incubated with different enzyme preparations as elucidation for fructan peak assignment. Fructan fingerprint profiles represent peaks which could be assigned to fructans after incubation of the extracts with amyloglucosidase and α-galactosidase to hydrolyse malto-oligosaccharides and GOS. Fingerprint of the green malt incubated with inulinase (grey line) was reduced by fructan-peaks (and sucrose, GOS)

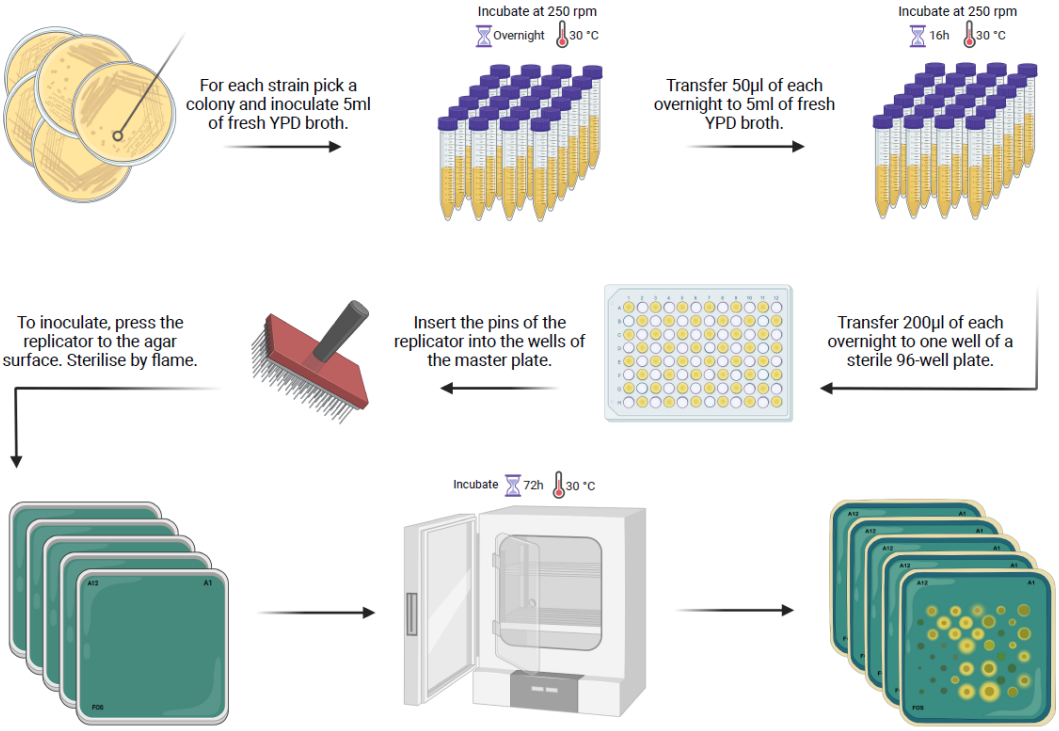

Figure A-8. Flowchart explaining the agar carbohydrate utilisation assay. Graphic created with BioRender.com

Table A-5. Collection of 96 yeast isolates. Untyped isolates are designated by isolate number

Strain	Isolated from	Strain	Isolated from
Brettanomyces anomalus KBI 15.1	Kombucha	CP1 7.3.1A	Chickpea flour
Brettanomyces anomalus KBI 31.1	Kombucha	CP1 7.4.1A	Chickpea flour
Brettanomyces anomalus KBI 6.1	Kombucha	CP2 9.4.1A	Chickpea flour
Brettanomyces anomalus KBI 6.5	Kombucha	CP3 7.4.1A	Chickpea flour
Brettanomyces anomalus UCC 7.4	Kombucha	CP3 7.4.1B	Chickpea flour
Brettanomyces bruxellensis KBI 10.2	Kombucha	CP3 7.4.1C	Chickpea flour
Brettanomyces bruxellensis KBI 25.4	Kombucha	GP1 5.3.1A	Green pea flour
Brettanomyces bruxellensis KBI 25.6	Kombucha	GP2 4.3.1A	Green pea flour
Brettanomyces bruxellensis UCC 11.2	Kombucha	GP2 4.3.2A	Green pea flour
***Cyberlindnera fabianii* NTCyb**	**Rice wine starter**	GP3 4.3.1A	Green pea flour
Cyberlindnera misumaiensis 837 A	Brewery cellar	GP3 4.3.2A	Green pea flour
Cyberlindnera misumaiensis 837 B	Brewery cellar	GP3 7.1.8A	Green pea flour
Cyberlindnera mrakii CBS 1707 ^{T}	Soil	M1 4.3.1A	Millet flour
Cyberlindnera subsufficiens CBS5763 ^{T}	Soil	M1 5.3.1A	Millet flour
Cyberlindnera subsufficiens C 6.1	Coconut	M1 5.3.2A	Millet flour
Cyberlindnera (Pichia) jadinii L1	Fruit Naranjilla	M3 5.3.1A	Millet flour
Debaromyces hansenii NCYC 9	Cheese	M3 5.3.2A	Millet flour
Kluyveromyces marxianus NCYC 1425	Yoghurt	MZ 4.3.1A	Maize flour
Kluyveromyces marxianus NCYC 179	Cheese	MZ 5.3.1A	Maize flour
Kluyveromyces marxianus NCYC 587	Souring figs	MZ 5.3.2A	Maize flour
Kluyveromyces marxianus NCYC 744	Yoghurt	MZ 5.3.3A	Maize flour
Kluyveromyces marxianus NCYC 970	Yoghurt	SP1 3.3.1A	Spelt flour
Lachancea fermentati CBS 707 ^{T}	Vegetation	SP3 3.3.1A	Spelt flour
***Lachancea fermentati* FST 5.1**	**Kombucha**	TF1 5.3.1A	Teff flour
Lachancea fermentati KBI 12.1	Kombucha	TF1 5.3.1B	Teff flour
Lachancea fermentati KBI 3.2	Kombucha	TF1 5.4.1A	Teff flour
Lachancea fermentati KBI 5.3	Kombucha	TF1 5.4.1B	Teff flour
Pichia kudriavzevii UCC 7.3	Kombucha	TF2 5.3.1A	Teff flour
Pichia membranifaciens MTU074	Sourdough	TF2 5.3.1B	Teff flour
Pichia scaptomyzae KBI 26.1	Kombucha	TF2 5.4.1A	Teff flour
Saccharomyces cerevisiae	**Baker's yeast**	TF2 5.4.1B	Teff flour
Saccharomyces cerevisiae NCYC 77	Sourdough	TF3 5.3.1A	Teff flour
Saccharomyces cerevisiae/paradoxus KBI 30.2	Kombucha	TF3 5.3.1B	Teff flour
Schizosaccharomyces pombe UCC 4.2	Kombucha	TP1 7.3.1A	Tapioca flour
Zygosaccharomyces kombuchaensis UCC 3.2	Kombucha	TP1 7.3.1B	Tapioca flour
Zygotorulaspora florentina MTU064	Sourdough	TP1 7.4.1A	Tapioca flour
MTU01B	Sourdough	TP1 7.4.1B	Tapioca flour
MTU01D	Sourdough	TP2 7.3.1A	Tapioca flour
MTU01E	Sourdough	TP2 7.3.1B	Tapioca flour
MTU01F	Sourdough	TP2 7.4.1A	Tapioca flour
UCC 9.1	Kombucha	TP2 7.4.1B	Tapioca flour
B1 5.3.1A	Buckwheat flour	TP3 5.3.1A	Tapioca flour
B1 5.3.1B	Buckwheat flour	TP3 5.3.2A	Tapioca flour
B2 5.3.1A	Buckwheat flour	TP3 5.4.1A	Tapioca flour
B2 5.3.2A	Buckwheat flour	TP3 5.4.2A	Tapioca flour
B2 7.1.3A	Buckwheat flour	YP1 3.3.1A	Yellow pea
B2 7.1.5A	Buckwheat flour	YP3 3.3.1A	Yellow pea
BZ 7.1.4A	Buckwheat flour	YP3 3.3.1B	Yellow pea

Yeast isolates typed in bold were used for baking trials.

A-2 List of publications and presentations and awards

First author publications

Ispiryan, L., Heitmann, M., Hoehnel, A., Zannini, E., & Arendt, E. K. (**2019**). Optimization and Validation of an HPAEC-PAD Method for the Quantification of FODMAPs in Cereals and Cereal-Based Products. *Journal of agricultural and food chemistry*, *67 (15)*, *4384–4392*, doi: 10.1021/acs.jafc.9b00382.

Ispiryan, L., Zannini, E., & Arendt, E. K. (**2020**). Characterization of the FODMAP-profile in cereal-product ingredients. *Journal of Cereal Science*, *92*, *102916*, doi: 10.1016/j.jcs.2020.102916.

Ispiryan, L., Kuktaite, R., Zannini, E., & Arendt, E. K. (**2021**). Fundamental study on changes in the FODMAP profile of cereals, pseudo-cereals, and pulses during the malting process. *Food chemistry*, *343*, *128549, 128549*, doi: 10.1016/j.foodchem. 2020.128549.

Ispiryan, L., Borowska, M., Sahin, A. W., Zannini, E., Coffey, A., & Arendt, E. K. (**2021**). *Lachancea fermentati* FST 5.1: an alternative to baker's yeast to produce low FODMAP whole wheat bread. *Food & function,* doi: 10.1039/D1FO01983J.

Ispiryan, L., Zannini, E., & Arendt, E. K. (**2021**). FODMAP modulation as a dietary therapy for IBS: Scientific and market perspective. *Comprehensive review in Food Science and Food Safety (under peer review)*. Date of submission 26/08/2021

Other publications

Atzler, J. J., Ispiryan, L., Gallagher, E., Sahin, A. W., Zannini, E., & Arendt, E. K. (**2020**). Enzymatic degradation of FODMAPS via application of β-fructofuranosidases and α-galactosidases- A fundamental study. *Journal of Cereal Science*, *95*, *102993*, doi: 10.1016/j.jcs.2020.102993.

Vogelsang-O'Dwyer, M., Bez, J., Petersen, I. L., Joehnke, M. S., Detzel, A., Busch, M., Krueger, M., Ispiryan, L., O'Mahony, J. A., Arendt, E. K., & Zannini, E. (**2020**). Techno-Functional, Nutritional and Environmental Performance of Protein Isolates from Blue Lupin and White Lupin. *Foods*, *9 (2)*, doi: 10.3390/foods902023

Joehnke, M. S., Jeske, S., Ispiryan, L., Zannini, E., Arendt, E. K., Bez, J., Sørensen, J. C., & Petersen, I. L. (**2021**). Nutritional and anti-nutritional properties of lentil (*Lens culinaris*) protein isolates prepared by pilot-scale processing. *Food Chemistry: X*, *9*, *100112*, doi: 10.1016/j.fochx.2020.100112.

Oral presentations

Ispiryan, L., Axel C., Heitmann, M., Zannini, E., Arendt, E.K. (**2018**). Oligosaccharides in food: Sample preparation and troubleshooting. *Ion Chromatography User Meeting*, Dublin, Ireland, May 2018

Ispiryan, L., Axel C., Heitmann, M., Rozalia, K., Zannini, E., Arendt, E.K. (**2018**). Biotechnological approach to reduce FODMAPs. *7th International Sourdough Symposium*, Cork, Ireland, June 2018

Ispiryan, L., Axel C., Heitmann, M., Zannini, E., Arendt, E.K. (**2018**). FODMAP levels of cereal product ingredients & Impact of malting on the FODMAP profile. *AACC International Annual Meeting Cereals & Grains 18*, London, England, October 2018

Ispiryan, L., Zannini, E., Arendt, E.K. (**2019**). Approach to address the putative non coeliac gluten sensitivity trigger - FODMAPs. *5th International Symposium on Gluten-Free Cereal Products and Beverages*, Leuven, Belgium, June 2019

Ispiryan, L., Zannini, E., Arendt, E.K. (**2020**). A HPAEC-PAD method for the analysis of FODMAPs in cereals and cereal-based products. *Virtual global IC symposium*, Cork (virtual), Ireland, October 2020

Ispiryan, L., Zannini, E., Arendt, E.K. (**2021**). New Technologies to Remove FODMAPs from Cereal-Based Ingredients and Pulses to Improve Product Development for People Suffering from IBS. *Spring workshop 2021 Health Grain Forum*, Cork (virtual), Ireland, April 2021

Poster presentations

Ispiryan, L., Zannini, E., Arendt, E.K. (**2017**). Low FODMAP food strategies to reduce irritable bowel syndrome – The TALENTFOOD project. *Monash Prato Gastrodiet 2017 Conference*, Prato, Italy, November 2017

Awards

Best presentation award for: Ispiryan, L., Axel C., Heitmann, M., Rozalia, K., Zannini, E., Arendt, E.K. (**2018**). Biotechnological approach to reduce FODMAPs. *7th International Sourdough Symposium*, Cork, Ireland, June 2018

Publication of the year 2019 in Food and Nutritional Sciences award from College of Science, Engineering and Food Science (UCC) for: Ispiryan, L., Heitmann, M., Hoehnel, A., Zannini, E., & Arendt, E. K. (**2019**). Optimization and Validation of an HPAEC-PAD Method for the Quantification of FODMAPs in Cereals and Cereal-Based Products. *Journal of agricultural and food chemistry*, *67 (15)*, *4384–4392*, doi: 10.1021/acs.jafc.9b00382.