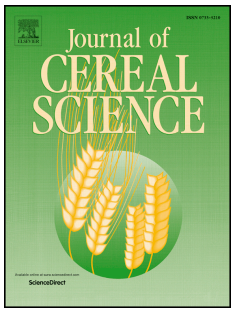


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**CRediT author statement**

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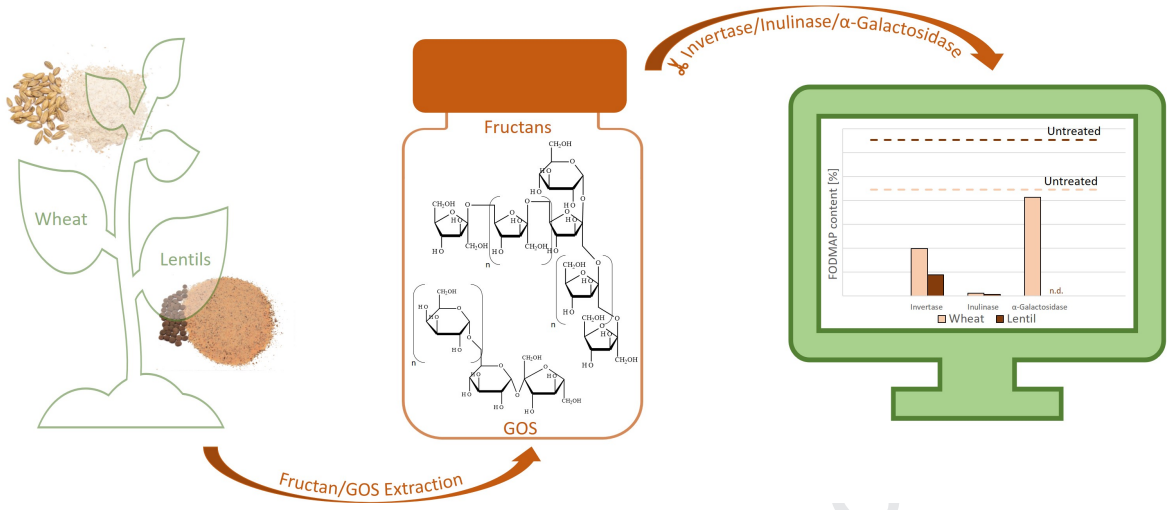

# Enzymatic Degradation of FODMAPS via application of β-fructofuranosidases and α-galactosidases- A fundamental study

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**Keywords**

FODMAPs

Enzymatic degradation

fructans

GOS

*Abbreviations:* IBS, Irritable Bowel Syndrome; FODMAP, fermentable oligo-, di, monosaccharides and polyols; HPAEC-PAD, high performance anion exchange chromatography coupled with pulsed amperometric detection; FOS, fructooligosaccharides; GOS, galactooligosaccharides; DPav, average degree of polymerization; RFO, raffinose family oligosaccharides; TOC, Total Oligosaccharide Content; %DM, percentage in dry matter; S/I ratio, ratio of relative activity towards sucrose and relative activity towards inulin

## Abstract

Cereals and pulses often contribute to the intake of Fermentable Oligo-, Di-, Monosaccharides, and Polyols (FODMAPS) due to high amounts of fructans or galactooligosaccharides (GOS). FODMAPs can trigger symptoms of Irritable Bowel Syndrome (IBS) and therefore, the development of foods and beverages with a lower FODMAP-content are favourable for IBS patients. Enzyme technology is a promising tool to reduce the FODMAP-content in foods and to maintain product quality. This fundamental study investigates the efficiency of invertase, inulinase, and α-galactosidase as potential food additives to reduce the total FODMAP content of food ingredients. Extracts of high FODMAP ingredients, such as wheat and lentil, and standard solutions of various fructans and GOS were incubated with invertase, inulinase and α-galactosidase for 1 hour and 2 hours. Contents of oligosaccharides before and after treatment and related IBS-triggering reaction products were quantified using ion chromatography. Inulinase showed a high degradation yield (over 90 % of degradation) for both GOS and fructans. For invertase only low degradation yields were measured. α-Galactosidase showed the highest efficiency in decomposing GOS (100 % of degradation) and led to non-IBS triggering degradation products. This indicates a high potential for a combined inulinase/α-galactosidase treatment for products containing both fructans and GOS.

## 1. Introduction

Irritable Bowel Syndrome (IBS) is a gastrointestinal disease that gathered attention among scientists in recent years. The high prevalence of 8 % of the population in Europe suffering from IBS and the common association between diet and the occurrence of symptoms, present a vast potential for research in the field of food science (Sperber et al., 2017). Studies revealed an improvement in IBS symptoms when patients followed a diet which includes less than 0.50 g of a total content of Fermentable Oligo-, Di-Monosaccharides and Polyols (FODMAPs) per standard serving (de Roest et al., 2013; Gibson and Shepherd, 2010). The term FODMAP describes carbohydrates which are not digestible in the human intestine and, therefore, are fermented by the gut microbiota. In addition, FODMAP are osmotically active in the human colon. These characteristics lead to, firstly, an increased water retention in the human gut resulting in a bigger luminal volume and, secondly, to the production of gases such as methane and hydrogen. Examples for these carbohydrates , which are often discussed in literature, are fructose in excess of glucose, fructans, α-galactooligosaccharides (GOS) and the polyols mannitol and sorbitol (Barrett et al., 2010).

Food products made from cereals and pulses play a major role in the FODMAP intake due to their significant contents of fructans and GOS. Wheat in particular has a major impact, since it is one of the most consumed cereals in Europe and America and it contains between 0.6-2.6 % fructans and minor amounts of GOS (Henry and Saini, 1989; Huynh et al., 2008). Lentils are commonly used for the development of gluten-free and health-promoting products due to their technological properties and nutritional benefits, such as a high protein content (Foschia et al., 2017). However, lentils contain high levels of GOS ranging from 1.8-7.5 % and, hence, contribute to the total FODMAP intake (Frias et al., 1994; Vidal-Valverde and Frias, 1992). The replacement of these raw ingredients could affect the techno-functional properties of foods, but also result in products with inferior nutritional value.

Enzyme technology provides the advantage of specific FODMAP degradation and therefore promises less interference with the nutritional and technological properties of products made from cereals and pulses. Invertase – which is commonly used in the food industry- is a β-fructofuranosidase enabling

the hydrolysis of β-(2-1)-linkages in sucrose and short-chain fructans up to an average degree of polymerization (DPav) of 5 (Schorr-Galindo et al., 2000; Struyf et al., 2017). Inulinase is a β-fructofuranosidase, which hydrolyses the glycosidic bonds in fructans with a higher DPav, such as inulin (Goosen et al., 2008; Ricca et al., 2009). The mechanism for the hydrolysis via β-fructofuranosidases is depicted in figure 1 (Koshland and Stein, 1954; Rye and Withers, 2000). Invertase and inulinase which are both β-fructanosidases, are able to cleave the β-(2-1)-linkage between fructose and glucose of the sucrose moiety in GOS, such as raffinose, stachyose and verbascose (Teixeira et al., 2012). Therefore, those two enzymes were investigated regarding the simultaneous hydrolysis of both fructans and GOS. However, no action on α-(1-6)-linkages between galactose residues is reported. Even though inulinase is available as a food grade enzyme, it is not as frequently used in the food industry as invertase.

α-Galactosidase catalyses specifically the cleaving of glycosidic-linkages between α-(1-6)-galactose-residues, as depicted in figure 1 (Ademark et al., 2001; Frias et al., 2003; Fujimoto et al., 2003; Rye and Withers, 2000). Tuck et al. (2018) investigated the effect of this enzyme as a supplement for IBS patients and showed that the intake decreased the frequency of symptoms.

This study investigates the efficiency of purified invertase, inulinase and galactosidase in degrading FODMAPs in wheat and lentil extracts as well as pure fructans and GOS. The products after enzyme treatment provide information about the performance and limitations of the degradation process. This study can contribute to the development of an enzyme-based treatment in food systems as a technological approach to reduce FODMAP content.

## 2. Materials and Methods

### 2.1 Materials

As raw ingredients wholemeal wheat flour (Odlum, Dublin, Ireland) and lentils of the variety Itaccia (Agroservice S.p.A., Severino Marche, Italy) were used. Lentils were milled to a particle size of 250 µm using a disc mill (Buehler Universal, Uzwil, Switzerland) before use.

Furthermore, the degradation of purified fructooligosaccharides (FOS) with a degree of polymerisation (DP) of 2-8 and inulin (DP ~ 30) from two different suppliers, Megazyme (Bray, Ireland) and Beneo-Orafti (Tienen, Belgium), represented high fructan ingredients. Both stachyose tetrahydrate and raffinose pentahydrate were purchased from Sigma-Aldrich (Neuss, Germany) and verbascose was supplied by Megazyme (Bray, Ireland).

Invertase (E-INVP; EC 3.2.1.26), Inulinase (E-FRMXP; mix of exo-inulinase (EC 3.2.1.80) and endo-inulinase (EC 3.2.1.7); ratio of 20:1) and alpha-Galactosidase (E-AGLANP; EC 3.2.1.22) were all obtained from Megazyme (Bray, Ireland).

Sodium acetate (anhydrous; ≥ 99.9%) was supplied by Thermo Scientific™ (Dionex™ AAA-Direct Reagents; Dublin, Ireland) and glacial acetic acid was provided by Fisher Chemical (J.T. Baker™; Loughborough, UK).The extra pure, 50 % (w/w) sodium hydroxide (NaOH) solution was supplied by Thermo Fischer Scientific (ACROS Organics, Dublin, Ireland). All components of the HPAEC-PAD and the ThermoScientific DionexIC Pure Water purification system were purchased from Thermo Scientific Dionex (Sunnyvale, USA).

### 2.2 Methods

#### *2.2.1 Moisture*

The moisture content of the wholemeal wheat flour and milled lentils was measured using the official AACC method 44-15.02.

*2.2.2 Extraction and preparation of standard solutions*

The extraction of FODMAPS in wholemeal wheat flour, milled lentils, FOS and inulin was conducted as described by Ispiryan, et al. (2019). Extractions were executed using a sodium azide solution (50 ppm) at 80 °C and homogenisation of the sample suspension using a BANDELIN Sonoplus HD 3100 homogenizer (Berlin, Germany).Fructan stock solutions were prepared reaching final concentrations of 70 ppm, while GOS stock solutions (raffinose pentahydrate, stachyose tetrahydrate and verbascose) yielded a concentration of 80 ppm. These concentrations were chosen based on the highest levels of fructans and GOS in wheat/lentil extracts.

*2.2.3 Enzyme preparation.*

All enzyme solutions were prepared by dissolving the freeze-dried powder in sodium acetate buffer (100 mM; pH 4.5) which was prepared according to the official AOAC method 997.08. The concentration of each enzyme stock solution was 300 U/mL.

*2.2.4 Incubation*

For the incubation 1 mL of each extract or standard solution was used. Initial concentrations of standards were adjusted to the amount of fructans or GOS extracted from wheat and lentil. Enzymes were applied singly and an enzyme to substrate ratio of 1 U per 0.001 mg FODMAP was applied. All incubations were carried out in a water bath at 40° C for 1 h and 2 h respectively. The reaction was stopped by deactivating the enzymes on a block heater at 100 °C for 10 minutes.

The degradation of the oligosaccharides was determined by the difference of untreated sample (sample blank) including sodium azide (50 ppm) instead of enzyme solution and treated sample.

*2.2.5 Quantification of FODMAPs and degradation products*

Sugar alcohols, mono-, di- and oligosaccharides were quantified using high performance anion exchange chromatography coupled with Pulsed amperometric detection (HPAEC-PAD). The system used for analysis was a Dionex ICS-5000$^{+}$ equipped with a SP Single Pump (analytical gradient

pump), an AS-AP Autosampler, a 10 µL injection loop (full-loop was used for injection) and an ED Electrochemical Detector cell coupled with a gold working electrode and a PdH reference electrode. The eluents used for separation were purified water (solution A), 225 mM NaOH (solution B), and 500 mM NaOAC (solution C). A $N_2$ atmosphere was used for the storage of the eluents and this atmosphere was created by using a Peak Scientific (Inchinnan, UK) Corona Air Compressor and a Corona Nitrogen generator (pressure of 4.5 – 5 bar). A Thermo Scientific Dionex CarboPac PA 200 analytical column (3x250 nm) was used for the quantification of the mono-,di-, and oligosaccharides, while a Thermo Scientific Dionex CarboPac PA1 analytical column (2x250 nm) was used for the quantification of mono-, disaccharides and polyols. The applied methodis described by Ispiryan et al. (2019). Mixtures of reference standards were used in ranges from 0.05 – 1 mg/L and from 1 – 20 mg/L. This corresponds to molar concentrations which range from 0.28/0.15 -5.6/2.9 mmol/L and from 5.6/2.9 – 111.0/58.5 mmol/L depending on the quantification of either mono- or disaccharides. In order to distinguish between raffinose and stachyose, degradation products, such as melibiose and manninotriose, were determined, after the treatment with invertase or inulinase. The quantification of fructans was carried out as described by Ispiryan et al. (2019). Samples were treated with two different enzyme mixtures (enzyme mixture A and B). Enzyme mixture A contains α-galactosidase (E-AGLANP)/amyloglucosidase(E-AMGPD)/sodiumacetate-buffer (pH 4.5) in a ratio of 1:1:1 and mixture B consists of α-galactosidase (E-AGLANP)/amyloglucosidase(E-AMGPD)/inulinase (E-FRMXPD) in a ratio of 1:1:1. The amounts of glucose and fructose were measured after the treatment with both enzyme mixtures. The differences between the amounts of fructose and glucose detected after treatment with mixture A and B were used for the quantification of fructans. Fructan contents were calculated as the sum of the detected amounts of glucose and fructose.

The quantification of the degradation products manninotriose (originating from stachyose) and manninotetraose (originating from verbascose) required a special procedure, since pure standards for HPAEC-PAD are not available. Therefore, firstly, a total hydrolysis of different standard concentrations of stachyose and verbascose by inulinase was conducted. Concentrations ranged from 0.05 – 1 mg/L and from 1 – 20 mg/L. This corresponds with ranges of molar concentrations from

0.10/0.08 – 2.0/1.5 mmol/L and from 2.0/1.5 – 39.6/30.0 mmol/L depending on the quantification of either manninotriose or manninotetraose.

For total hydrolysis 30 U of Inulinase were added to different concentrations of stachyose and verbascose and incubated at 60 °C for 30 minutes, followed by the deactivation of inulinase by heat treatment at 100 °C for 10 minutes.

Afterwards, enzyme treated solutions were directly measured using the HPAEC-PAD and the concentrations of manninotriose and manninotetraose were calculated based on the molecular weight, considering degradation of 1 mole stachyose or verbascose would result in 1 mole of manninotriose or manninotetraose, respectively.

### 2.3 Statistical Analysis

One-way ANOVA (Tukey HSD; $p < 0.05$) and correlation analysis (Pearson; $p < 0.05$) were performed using SPSS. Furthermore, the determination of the average, standard deviation and confidential interval was conducted using Microsoft Excel 2016.

## 3. Results

### 3.1 Change in total oligosaccharide content

The total oligosaccharide content (TOC) was defined as the sum of the detected amount of fructans and the total amount of GOS (sum of raffinose, stachyose, and verbascose). This value can be used as an indicator for the limitations of the investigated enzymes, since it reveals the enzyme-efficiency towards both groups of oligosaccharides. Outcomes of the TOC measured before and after incubation of wheat and lentil extracts are presented in figure 2.

For untreated wheat extracts, the presence of fructans in a higher ratio than GOS (in the form of raffinose) was discovered. In untreated lentil extracts, only GOS could be determined. The application of all three enzymes lowered the TOC in both substrates (Fig. 2).

A decline of the TOC over 90% in the lentil extracts was observed for all three enzymes (Fig. 2). In wheat extracts, only inulinase could accomplish a similar rate of degradation. Invertase gradually decreased the TOC in both substrates and yielded the lowest degradation rate among the three enzymes. Treatment of lentil extracts with α-galactosidase resulted in a complete decrease, while treated wheat extract showed a degradation rate of 8.46% after 1 and 2 hours of incubation.

### 3.2 Enzyme treatment of extracts from wholemeal wheat flour and fructan standards

#### *3.2.1 Changes in fructan concentration*

The content of fructans before and after incubation was analysed to investigate their degradation by invertase and inulinase. Extracts of two fructan standards which varied in the DPav were used to evaluate the influence of the chain-length of the oligosaccharides. The values of these experiments are illustrated in Table 1.

Application of both enzymes led to a decrease in fructan concentration in wheat extracts, as well as in solutions of FOS and inulin. However, the degree of degradation and the decrease of the fructan concentration over time varied. For all substances, a partial deterioration after 1 hour was observed. Incubation of FOS with invertase for 1 h led to the detection of approximately 5 % of the initial concentration. In both wheat and inulin, a partial degradation of less than 50 % could be determined, while the treatment of FOS by invertase resulted in a complete decomposition after 2 hours. In general, FODMAP degradation via invertase was more efficient in the first hour (50% degradation rate) compared to the second hour (11.25% degradation rate). For the trials with inulin, no significant differences between the two-time points were detected. Inulinase showed for all substrates the efficiency to decompose over 90 % of the determined fructans during the first hour and resulted in concentrations under the detection limit after 2 hours.

#### *3.2.2 Reaction products of fructan hydrolysis*

Reaction products resulting from fructan degradation by inulinase or invertase, such as fructose and glucose, were analysed to obtain a clearer picture of the reaction. Both enzyme treatments led to a

detected increase in excess fructose in all extracts (Table 1). Differences were seen in the quantity of excess fructose depending on the used enzyme and substrate. Inulinase resulted in a higher amount of excess fructose in wheat extract and inulin compared to invertase. Treatment with invertase and inulinase resulted after 2 hours in amounts of excess fructose of 0.552 %DM and 0.935 %DM in wholemeal wheat flour.

### 3.3 Enzyme treatment of extracts from milled Lentils and GOS standards

*3.3.1 Changes in GOS concentration*

Three types of GOS were determined in the substrates analysed: Raffinose, stachyose, and verbascose. The GOS levels before and after enzymatic treatment are shown in Table 2.

Untreated lentil extract contained all three types of GOS. In untreated wheat extract only raffinose was determined, which showed the lowest concentration (6.01 ± 0.50 mg/L) of one type of GOS in the untreated flour samples.

The application of invertase, inulinase, and α-galactosidase resulted in a lower concentration of each type of raffinose family oligosaccharides (RFO) in all substrates. However, differences in degradation yield occurred depending on the enzymes and the substrate.

Invertase showed the lowest impact on the degradation of GOS present in the lentil extracts, as well as in the solutions of stachyose and verbascose. On the contrary, the treatment of raffinose standard solution and wheat extract with invertase resulted in a total degradation of GOS after 1 hour. The degradation rate of stachyose and/or verbascose in the standard solutions (stachyose: 90.1 % / verbascose: 75.6 %) was less efficient compared to lentil extract (stachyose: 100 % /verbascose: 100 %)

Inulinase caused a higher yield of degradation compared to invertase and resulted in complete degradation in most substrates after 2 hours. The comparison of degradation of verbascose in lentil extracts and in standard solutions resulted in a similar trend as observed for the invertase treatment.

α-galactosidase was the only enzyme that hydrolysed 100 % of all types of GOS in extracts of wheat and lentil in the first hour of the experiments and caused to complete decomposition in all standard solutions after 2 hours.

*3.3.2 Reaction products of GOS hydrolysis*

Besides the differences in the degradation, the three enzymes showed variances in the products of the hydrolysis. The two β-fructofuranosidases invertase and inulinase resulted in the production of melibiose, manninotriose, and manninotetraose, depending on the type of GOS cleaved (Table 3). Invertase also led to a gradual increase of manninotriose and manninotetraose in experiments with lentil extracts, stachyose, and verbascose standard solutions. Inulinase caused a continuous production of manninotriose in trials with lentil extracts and of manninotetraose for treatments of verbascose standard solutions. Inulinase treatment of wheat/lentil extracts and standard solutions of stachyose and verbascose resulted in higher quantities of melibiose, manninotriose or manninotetraose compared to the invertase trials. The use of invertase and inulinase led to total concentrations of melibiose, manninotriose, and manninotetraose of 2.37 %DM and 2.68 %DM, respectively. The increase of fructose and excess fructose was discovered in all substrates (Data not shown). Contents of excess fructose of 0.33 %DM and 0.75 %DM were measured in lentil flour after treatment with invertase and inulinase for 2 hours.

Experiments conducted with α-galactosidase resulted in the production of sucrose and α-galactose in all substrates. Variances occurred in the quantity of these reaction products for the various substrates (Table 4). The enzyme treatment of lentil extracts resulted in the highest increase of sucrose and galactose, while wheat extracts showed the lowest production rate of sucrose and galactose after incubation.

## 4. Discussion

Fructans are widely reported as the main component involved in the FODMAP intake via cereal based products (Huynh, et al., 2008). Thus, the determined ratio between fructans and GOS in wheat can be considered as an example for ingredients with a high content of fructans and low amounts of GOS. In

the extracts of lentils, α-galactooligosaccharides are detected as the only type of FODMAPs. Therefore, lentils can be used as an example of ingredients high in GOS. In order to investigate the influence of various substrates on the performance of FODMAP-degrading enzymes, the comparison between complex ingredients (wheat and lentil) and pure fructan-/GOS-standards is vital.

Treatments with invertase and inulinase were used for the determination of fructan degradation, since α-galactosidase is reported to specifically hydrolyse the glycosidic bond between α-galactose residues (Ademark et al., 2001). Discrepancies in the rate of degradation caused by invertase or inulinase occurred due to differences in branching and average degree of polymerization (DPav) of the investigated fructans.

The fact that invertase caused the lowest rate of degradation in inulin trials can be explained by the long chain-length of inulin, which the supplier specified as 30. On the contrary, fructans extracted from wheat and from FOS standard showed a relatively short chain-length (DPav of 5; DPav of 2-8 respectively). It was reported that invertase has a higher affinity towards sucrose and fructans with a DPav up to 5 (Schorr-Galindo et al., 2000). This affinity is also a reason for a decrease of the fructan concentration occurring during the first hour of inulin treatment. Furthermore, the presence of branched graminan-type fructans in wheat compared to fructan standards resulted in a lower degradation yield (Struyf et al., 2017; Verspreet et al., 2015).

Interestingly, the degradation of fructans in wheat extract showed a slower decline in the second hour. This is putatively due to the cleavage of the easier accessible linear fructans in the first hour, followed by a slower hydrolysis of the branched fructans in the second hour. (Nilsson et al., 1987; Struyf et al., 2017).

Inulinase possesses a high affinity towards fructooligosaccharides with a higher DPav and is also able to degrade branched fructans, leading to the higher degradation yield observed in these trials. The different affinity regarding the DPav is usually expressed by ratio of relative activity towards sucrose and relative activity towards inulin (S/I ratio), which was measured for these enzymes. Inulinase typically shows low S/I ratios (between 1.5 and 20) (Vandamme and Derycke, 1983), while invertase possesses higher S/I ratios (up to 4.000) (Nilsson et al., 1987). Similar trends and differences for fructan degradation via these two enzymes were observed in a study conducted by Struyf, et al.

(Struyf et al., 2017). The degradation of fructans is an advantage regarding the FODMAP content, however, degradation of fructans can as well result in a lowered dietary fibre content, since fructans share certain characteristics - such as indigestibility and fermentability - with dietary fibres.

Both invertase and inulinase were able to decrease the amount of all three GOS-species found in lentil extracts and the investigated standards. This effect was caused by the hydrolysis of the β-(2-1)-bond between glucose and fructose in the sucrose residue of the investigated types of GOS and not by an activity towards α-(1-6)-linkages between galactose residues. (Teixeira et al., 2012). Treatment of raffinose, stachyose, and verbascose revealed that the DPav of GOS affects invertase similar to the DPav of fructans. This dependency is caused by the overall affinity of invertase towards smaller oligosaccharides (Sainz-Polo et al., 2013). Both inulinase and α-galactosidase, on the other hand, have a higher affinity towards substrates with a higher DPav (de Vries et al., 1999; Ricca et al., 2009). Despite a similar mechanism of degradation for invertase and inulinase differences in the efficiency of hydrolysis were found. A similar discrepancy was found between invertase and α-galactosidase. Both inulinase and α-galactosidase have a higher affinity towards substrates with a higher DPav (de Vries et al., 1999; Ricca et al., 2009).On the contrary astudy conducted by Arand et al. (2002) showed a higher affinity of inulinase for raffinose and stachyose compared to invertase. However, no notable differences in the affinity for these two substrates were found (Arand, et al., 2002). The efficiency of invertase depends on the concentration of verbascose, since the yield of degradation detected for verbascose as a standard (initial concentration of 78.98 ± 2.53 mg/L) was lower than for verbascose in lentil extracts (initial concentration of 40.64 ± 3.40).

The specific cleaving of the bond between α-galactose residues in GOS via α-galactosidase is the reason for the complete deterioration caused by this enzyme. The decomposition of RFOs in lentil flours by endogenous and commercial α-galactosidase isolated from *Aspergillus niger* were already discussed in literature, and similar results were obtained (Frias et al., 2003).

The change in the TOC correlates with the degradation of fructans and GOS caused by the investigated enzymes. Invertase and inulinase are able to degrade both fructans and GOS, which explains the degree of decomposition observed in both substrates. The low rate of degradation

detected for α-galactosidase treatments of wheat extracts depended on the specificity of this enzyme and the ratio between GOS and fructans detected.

It was shown that α-galactosidase was most efficient in lentils and that inulinase possesses the ability to sufficiently decrease the TOC in ingredients high in both fructans and GOS. These results suggest that both inulinase and α-galactosidase can sufficiently lower the amount of FODMAPs in ingredients with a high amount of GOS. For ingredients with a higher ratio of fructans only inulinase led to promising results, since invertase and α-galactosidase resulted in a TOC in wheat extracts that could lead to FODMAP levels above the cut-off values.

The enzyme treatment resulted in a degradation of FOS or GOS but also caused the production of various types of monosaccharides and oligosaccharides, which could also be considered as FODMAPs. Therefore, the advantages and disadvantages of these reaction products need to be discussed to fully evaluate the suitability of invertase, inulinase and α-galactosidase. The degradation of both fructans and GOS via invertase and inulinase produced excess fructose due to the higher ratio of fructose to glucose in fructans and no release of glucose from GOS. Differences in the amounts of excess fructose produced by invertase and inulinase can be linked to the varying efficiency of fructose-release caused by the degradation of fructans and GOS. The synthesis of fructose and excess fructose as a result of fructan degradation was reported before (Struyf et al., 2017). This study also showed that yeast fermentation during food production leads to a decrease in fructose content and excess fructose. This effect is especially relevant in food products which include a step of fermentation, such as bread making. The accumulation of fructose in excess of glucose caused by enzyme treatment needs to be taken into account in non-fermented food products. The outcomes of this study highlight this disadvantage, since amounts of excess fructose exceeded the cut-off value for a low FODMAP diet using the ingredient as a main component in food.The production of melibiose, manninotriose and manninotetraose by β-fructofuranosidases, such as invertase and inulinase, occurred due to their action towards fructose linkages (Teixeira et al., 2012). A correlation between the degradation efficiency and the reaction products was determined. The production of melibiose, manninotriose, and manninotetraose have to be critically discussed, due to the remaining bond between the α-galactose residues which cannot be cleaved by the enzymes of the human

366 gastrointestinal tract. Hence, these compounds need further investigations, since they possess
367 characteristics of FODMAPs and might also cause IBS symptoms. One of these characteristics is the
368 rapid fermentability by the human gut microbiota. An example for this is the fermentation of
369 melibiose and manninotriose by *Bifidobacteria*, which are a common part of the human gut
370 microbiota (Rada et al., 2002; Schell et al., 2002). The complete transformation of GOS to these
371 compounds can result in concentrations which could still trigger IBS.
372 Incubation with α-galactosidase resulted in the production of galactose and sucrose in all substrates
373 containing GOS. Differences in the quantity of α-galactose and sucrose detected in the different
374 standard solutions are caused by the increasing number of α-galactose residues, which also changes
375 the ratio between sucrose and galactose. For treatments of verbascose for 1 hour with α-galactosidase,
376 the presence of raffinose/stachyose as an intermediate product was observed (Data not shown), which
377 is caused by incomplete cleavage of α-galactose linkages. Further release of α-galactose led to the
378 decomposition of raffinose/stachyose during the second hour. This outcome indicates that treatment of
379 ingredients high in verbascose or GOS with a DPav above 5 for a shorter amount of time would only
380 result in shorter GOS and not in a lower GOS concentration. Galactose and sucrose can be digested
381 and absorbed in the human gut and are therefore not active as FODMAPs. Clinical trials with
382 fermented peas showed that the degradation of GOS due to α-galactosidase improved bloating (Jha
383 and Verma, 1980).

384 These outcomes highlight the advantages of α-galactosidase. However, the production of sucrose and
385 galactose could lead to disadvantages for the product quality, such as higher sweetness or darker
386 colour due to increasing amounts of Maillard-products. Higher amounts of fructose released during
387 fructan degradation caused by invertase and inulinase could also result in an increase of sweetness and
388 a higher possibility of the formation of Maillard-reactions.

389 The production of D-chiro-inositol in lentil extracts (Data not shown) results from the αdegradation of
390 the galactosyl-cyclitol Ciceritol [α-D-galactopyranosyl-(1-6)-α-D-galactopyranosyl(1-2)-4-O-methyl-
391 quiro-inositol] which contains galactose residues and is reported to occur in pulses (Quemener and
392 Brillouet, 1983). The decomposition of ciceritol, which could be fermented by the gut microbiota and

393 can also be assumed to remain in ingredients treated with β-fructofuranosidases, indicates another
394 advantage of α-galactosidase.

395 **5. Conclusion**

396 The results of this study suggest that the addition of the three investigated enzymes during food
397 production can lead to a reduction of the FODMAP content. However, their application was observed
398 to be suitable under certain conditions. The findings indicate that two main factors are restricting the
399 use of the enzymes. One limiting factor for the use of the enzymes is the ratio between fructans and
400 GOS. Results showed that the efficiency especially of invertase and α-galactosidase depended on the
401 type of oligosaccharides. Inulinase was observed to be able to hydrolyse both fructans and GOS and
402 also shows the advantage of degrading fructans in a time independent manner and therefore could
403 enable shorter processing times in industrial use. The other limiting factor is the accumulation of
404 reaction products after treatment with invertase or inulinase, which can be potentially considered as
405 FODMAPs. However, in-vitro and in-vivo studies are needed to verify this hypothesis.

406 In summary, this study shows the conditions, such as the oligosaccharides present in raw ingredients
407 or the reaction products, under which enzyme application can lead to beneficial effects. Inulinase
408 showed the highest potential for use in ingredients with a high fructan content, while application of α-
409 galactosidase seems the most suitable in ingredients with a high GOS amount. Combination of
410 inulinase and α-galactosidase for ingredients containing both types of oligosaccharides represents a
411 theoretically promising approach. Invertase was identified as the enzyme with the lowest suitability
412 for use in food production.

413

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# Tables

**Table 1:** Fructan concentration [mg/L] and excess fructose [mg/L] of extracts of wheat, FOS and inulin before and after treatment with invertase or inulinase

| | **Fructan concentration [mg/L]** | | | | | **Excess Fructose [mg/L]** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *No treatment* | *Invertase* | | *Inulinase* | |
| | *No treatment* | *Invertase* | | *Inulinase* | | | | | | |
| | *0 h* | *1 h* | *2 h* | *1 h* | *2 h* | *0 h* | *1 h* | *2 h* | *1 h* | *2 h* |
| **Wheat extract** | 69.2 ± 4.56$^{d}$ | 34.57 ± 2.32$^{c}$ | 24.56 ± 3.52$^{b}$ | 2.1 ± 0.22$^{a}$ | n.d. | n.d. | 2.46 ± 0.32$^{a}$ | 3.85 ± 0.19$^{b}$ | 3.20 ± 0.44$^{a}$ | 6.52 ± 0.74$^{b}$ |
| **FOS extract** | 65.26 ± 5.73$^{b}$ | 4.86 ± 0.62$^{a}$ | n.d. | n.d. | n.d. | 3.62 ± 0.05$^{a}$ | 11.66 ± 0.41$^{b}$ | 11.95 ± 0.21$^{b}$ | 10.12 ± 0.13$^{c}$ | 11.63 ± 0.18$^{b}$ |
| **Inulin extract** | 69.09 ± 2.02$^{a}$ | 49.36 ± 0.94$^{b}$ | 48.73 ± 3.98$^{b}$ | n.d. | n.d. | 0.23 ± 0.01$^{a}$ | 1.21 ± 0.09$^{b}$ | 1.72 ± 0.03$^{b}$ | 12.46 ± 0.52$^{c}$ | 13.56 ± 0.32$^{c}$ |

All values are given as average ± confidence interval
All values in one row for the same enzyme marked with a different superscript are significantly different ($p < 0.05$)
*n.d.- not detectable due to no significant differences in values of fructose and sucrose in the enzyme assays of A and B used for the fructan determination

509 **Table 2:** Concentration of galactooligosaccharides [mg/L] in extracts from Lentil, Raffinose, Stachyose and Verbascose before and after treatment with invertase, inulinase or α-galactosidase

| | | **Wheat Extract** | **Lentil Extract** | | **Raffinose [mg/L]** | **Stachyose [mg/L]** | **Verbascose [mg/L]** |
|---|---|---|---|---|---|---|---|
| | | *Raffinose/Stachyose [mg/L]* | *Raffinose/Stachyose [mg/L]* | *Verbascose [mg/L]* | | | |
| **Untreated Sample** | *0 h* | 6.01 ± 0.50$^{a}$ | 72.58 ± 1.23$^{d}$ | 40.64 ± 3.40$^{d}$ | 70.49 ± 0.60$^{a}$ | 82.76 ± 5.12$^{d}$ | 78.98 ± 2.73$^{f}$ |
| **Invertase** | *1 h* | n.d. | 5.34 ± 0.34$^{c}$ | 10.63 ± 1.08$^{c}$ | n.d. | 22.26 ± 3.14$^{c}$ | 31.48 ± 0.51$^{e}$ |
| | *2 h* | n.d. | 0.53 ± 0.04$^{b}$ | 3.90 ± 0.12$^{b}$ | n.d. | 7.55 ± 0.06$^{b}$ | 18.81 ± 0.50$^{d}$ |
| **Inulinase** | *1 h* | n.d. | 0.39 ± 0.01$^{a}$ | 0.93 ± 0.01$^{a}$ | n.d. | n.d. | 5.44 ± 0.23$^{c}$ |
| | *2 h* | n.d. | n.d. | n.d. | n.d. | n.d. | 2.58 ± 0.25$^{a}$ |
| **α-Galactosidase** | *1 h* | n.d. | n.d. | n.d. | n.d. | 1.19 ± 0.10$^{a}$ | 4.29 ± 0.50$^{b}$ |
| | *2 h* | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

510 All values are given as average ± confidence interval
511 All values in one column marked with a different superscript are significantly different ($p < 0.05$), values for each enzyme treatment are compared to the initial value
512 *n.d.- not detectable or contents below a value of 0.05 mg/L
513
514

**Table 3:** Molar Concentration [mmol/L] of reaction products (Melibiose, Manninotriose, Manninotetraose) of GOS hydrolysis via invertase or inulinase

| | | Invertase | | | Inulinase | | |
|---|---|---|---|---|---|---|---|
| | | *Melibiose [mmol/L]* | *Mannino-Triose [mmol/L]* | *Mannino-Tetraose [mmol/L]* | *Melibiose [mmol/L]* | *Mannino-Triose [mmol/L]* | *Mannino-Tetraose [mmol/L]* |
| **Wheat extract** | *1h* | $10.12 \pm 0.01^{a}$ | n.d. | n.d. | $13.39 \pm 0.98^{b}$ | n.d. | n.d. |
| | *2 h* | $11.98 \pm 0.02^{a}$ | n.d. | n.d. | $13.36 \pm 0.38^{b}$ | n.d. | n.d. |
| **Lentil extract** | *1 h* | $11.48 \pm 0.40^{a}$ | $85.67 \pm 1.19^{b}$ | $41.76 \pm 1.26^{a}$ | $12.40 \pm 0.28^{a}$ | $93.59 \pm 2.09^{a}$ | $62.11 \pm 0.90^{a}$ |
| | *2 h* | $12.71. \pm 1.30^{a}$ | $87.92 \pm 1.02^{b}$ | $52.99 \pm 0.94^{b}$ | $13.15 \pm 0.33^{b}$ | $98.27 \pm 1.82^{b}$ | $60.86 \pm 0.98^{a}$ |
| **Raffinose** | *1 h* | $129.26 \pm 2.38^{c}$ | n.d. | n.d. | $126.05 \pm 2.12^{a}$ | n.d. | n.d. |
| | *2 h* | $135.82 \pm 2.23^{d}$ | n.d. | n.d. | $128.08 \pm 1.93^{a}$ | n.d. | n.d. |
| **Stachyose** | *1 h* | n.d. | $79.96 \pm 2.81^{a}$ | n.d. | n.d. | $95.28 \pm 0.81^{a}$ | n.d. |
| | *2 h* | n.d. | $91.25 \pm 0.61^{c}$ | n.d. | n.d. | $100.80 \pm 2.00^{a}$ | n.d. |
| **Verbascose** | *1 h* | n.d. | n.d. | $58.46 \pm 2.26^{c}$ | n.d. | n.d. | $120.52 \pm 2.55^{a}$ |
| | *2 h* | n.d. | n.d. | $93.33 \pm 3.72^{d}$ | n.d. | n.d. | $129.98 \pm 1.10^{b}$ |

All values are given as average and with confidence interval
All values in one column are significantly different ($p < 0.05$)
*n.d.- not detectable or contents below a value of 0.1 mmol/L

520 **Table 4:** Molar concentration [mmol/L] of sucrose and galactose measured in extracts from wheat, lentils and standard solutions of raffinose, stachyose, verbascose before and after treatment
521 with α-galactosidase

| | **Sucrose [mmol/L]** | | | **Galactose [mmol/L]** | | |
|---|---|---|---|---|---|---|
| | *No treatment* | *1 h* | *2h* | *No treatment* | *1 h* | *2 h* |
| **Wheat extract** | 93.60 ± 12.51$^a$ | 104.09 ± 1.66$^b$ | 104.74 ± 1.29$^b$ | n.d. | 12.89 ± 0.71$^a$ | 15.58 ± 0.98$^b$ |
| **Lentil extract** | 209.12 ± 4.62$^a$ | 424.33 ± 6.05$^b$ | 431.77 ± 9.16$^b$ | 5.42 ± 0.75$^a$ | 373.55 ± 8.45$^b$ | 377.61 ± 6.36$^b$ |
| **Raffinose** | n.d. | 134.47 ± 2.60$^a$ | 145.65 ± 1.44$^b$ | n.d. | 133.07 ± 0.67$^a$ | 147.08 ± 1.86$^b$ |
| **Stachyose** | n.d. | 111.19 ± 0.96$^a$ | 111.06 ± 1.82$^a$ | n.d. | 204.68 ± 1.31$^a$ | 220.49 ± 0.57$^b$ |
| **Verbascose** | n.d. | 78.03 ± 1.05$^a$ | 88.71 ± 3.90$^b$ | n.d. | 247.71 ± 3.09$^a$ | 290.77 ± 5.31$^b$ |

522 All values are given as average and with confidence interval
523 All values in one row marked with a different superscript are significantly different ($p < 0.05$)
524 *n.d.- not detectable or contents below a value of 0.3/0.1 mmol/L corresponding to the quantification of either galactose/sucrose

525 **Figure Captions**

526

527 **Fig. 1:** Modified Koshland mechanism of hydrolysis of GOS via α-galactosidase (A) and hydrolysis
528 of fructans via invertase/inulinase (B); R1= further galactose residues or terminal sucrose residue;
529 R2= further fructose residues or terminal glucose residue

530

531 **Fig. 2:** Total Oligosaccharide content [%] of extracts from wholemeal wheat flour (A) and lentils (B)
532 before and after treatment with invertase, inulinase and α-galactosidase; FOS- determined amount of
533 fructans; GOS- determined amount of α-galactooligosaccharides; all values marked with the same
534 minor letter are not significantly different; n.d. – not detectable amounts or amounts beneath 0.01%

535

536 **Figures**

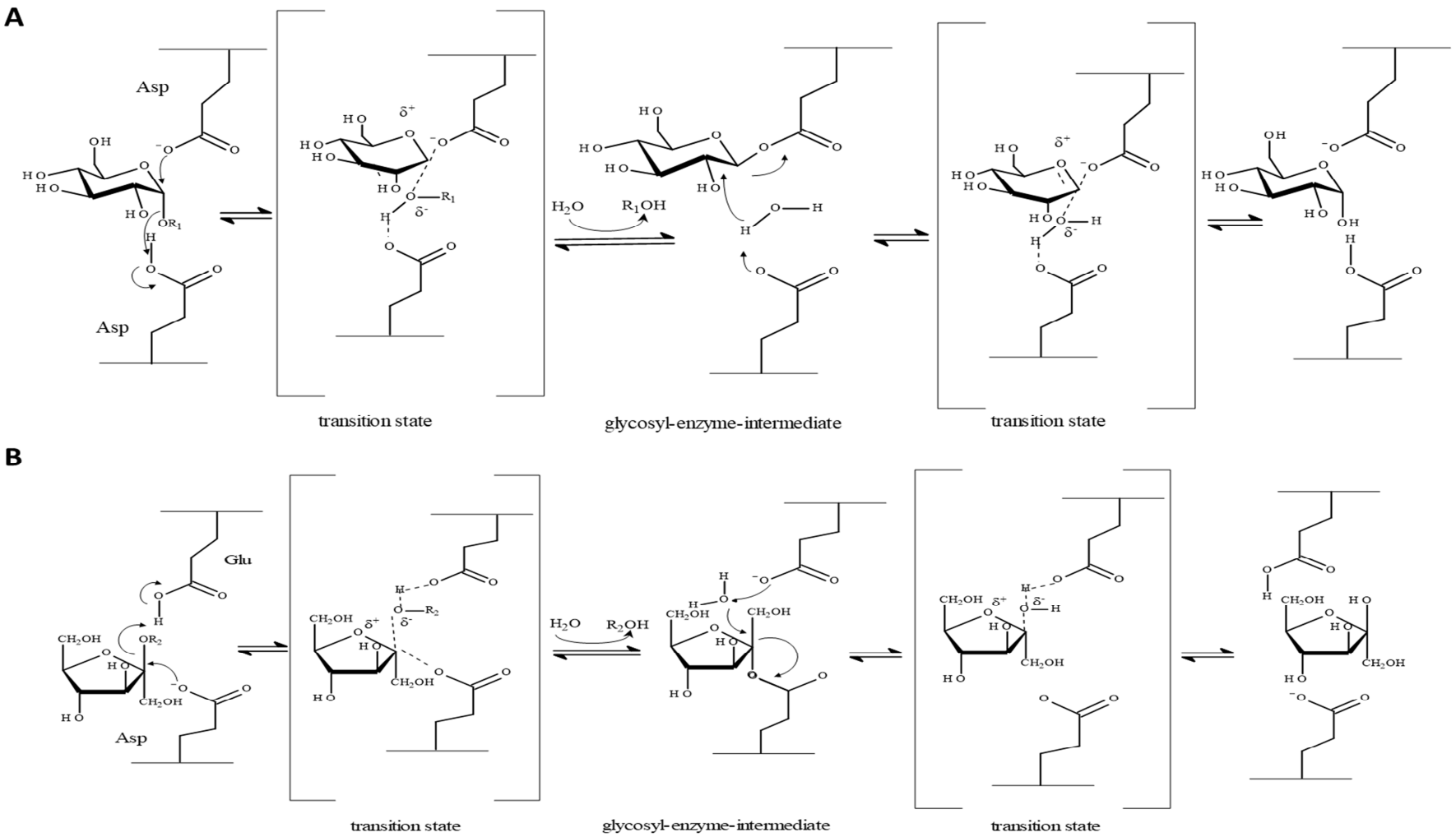


**Fig. 1:** Modified Koshland mechanism of hydrolysis of GOS via α-galactosidase (A) and hydrolysis of fructans via invertase/inulinase (B); R1= further galactose residues or terminal sucrose residue; R2= further fructose residues or terminal glucose residue

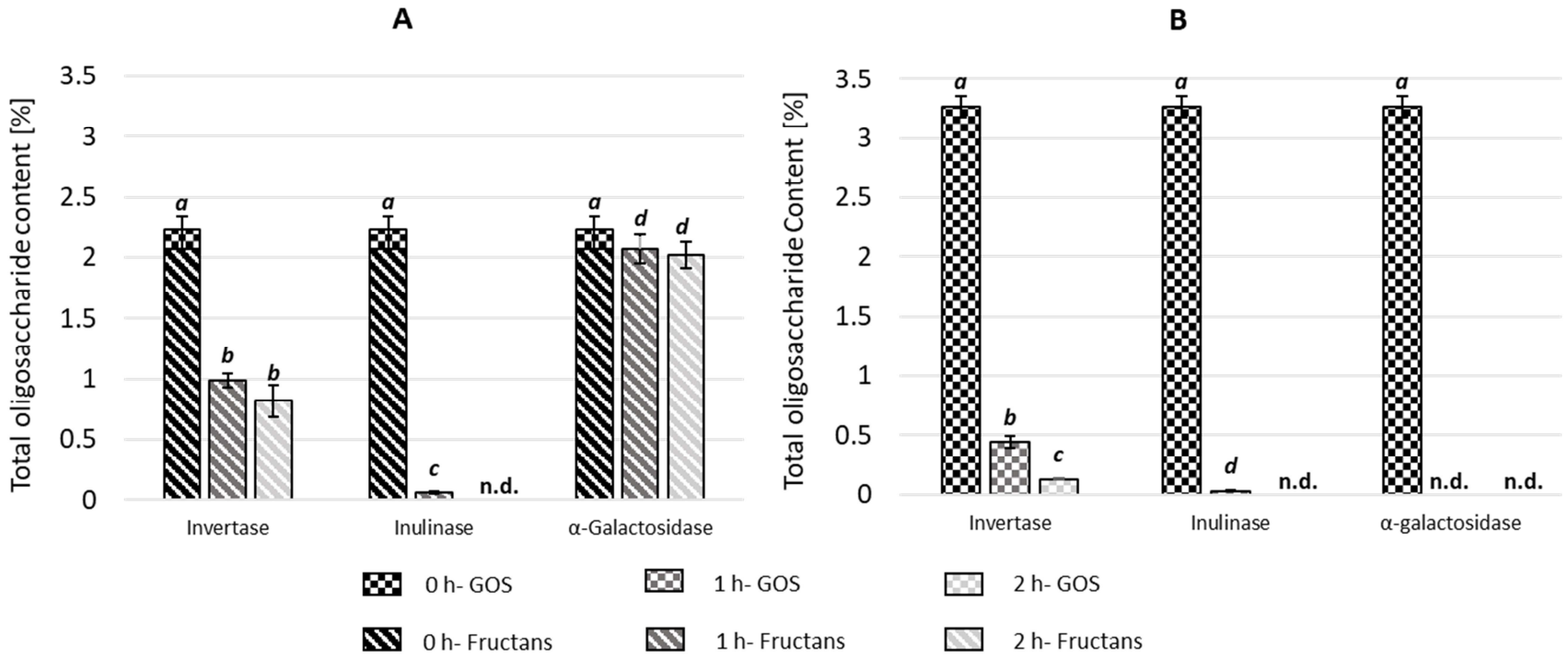


**Fig. 2:** Total Oligosaccharide content [%] of extracts from wholemeal wheat flour (A) and lentils (B) before and after treatment with invertase, inulinase and α-galactosidase; FOS- determined amount of fructans; GOS- determined amount of α-galactooligosaccharides; all values marked with the same minor letter are not significantly different; n.d. – not detectable amounts or amounts beneath 0.01%

# Enzymatic Degradation of FODMAPS via application of β-fructofuranosidases and α-galactosidases- A fundamental study

## *Highlights:*

- Efficiency of specific enzyme depends on pre-dominant type of oligosaccharide
- Inulinase shows high degradation of both fructans and α-galactooligosaccharides
- Reaction products of invertase and inulinase could possibly act like FODMAPs

# Enzymatic Degradation of FODMAPS via application of β-fructofuranosidases and α-galactosidases- A fundamental study

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## Conflicts of interest

The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.