Master Thesis Report (KBKM01)

Molecular Characterization of High β-Glucan Lines in Oats

(Department of Pure and Applied Biochemistry, LTH)

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Popular Science Summary –

Cereal grains are a vital part of the diets of human beings. Wheat, barley rye and oats are examples of some of the popular cereal grains being consumed across the world. Amongst these cereal grains the popularity of oats is on the rise and currently they occupy about 1 % of the world's total farmland. In comparison to the other members in the cereal grains oats have higher number of chemical substances like β-glucan, dietary fibres, and polyphenol. These are substances that helps in maintaining healthy regulation of the human body's cholesterol and sugar levels. To paint a larger picture, regular consumption of good quality of oats reduces the human body's risks to have cardiovascular and diabetic ailments. β-glucan is a compound which emphasised upon a lot while conducting this thesis and it is polymer made of Glucose. This means that β-glucan built by small glucose blocks and each of these blocks are joined by different types of linkages. CropTailor AB is a company that aims to develop healthy oat varieties by randomly changing the characteristics of one of their elite varieties called Belinda. Utilising these characteristic changes, the company has come up with three varieties lines CT-BG-53, CT-BG-21, and CT-BG-37 which have potentially higher β-glucan content in them. Through the work done in this project the various properties of these 3 new lines were found out and compared with the original line Belinda. It was found out that in comparison to Belinda these 3 lines have higher β-glucan, protein, total dietary fibre, and oil content in them. These lines also have sufficient amounts of a chemical substance called arabinoxylan which is also healthy for the body. These properties which were found out about these lines can directly be correlated to a lot of potential health benefits which is an ominous sign. Whenever oats are manufactured into products, they need to go through a heat processing step to make them fit for consumption for all and also increase their storage capacity. Through a set of experiments as a part of this project it was also confirmed that this processing step has no negative effect on the essential properties of these 3 healthy oat lines and that is an encouraging fact for all the industrialists interested in these lines. It was confirmed with the information from this project that the changes that lead to higher β-glucan in these varieties does not have a negative after effect on the content of another valuable chemical compound arabinoxylan. All in all, these 3 oat lines have the potential to turn out to be much more healthier lines than Belinda and this fact must be verified soon with some insightful clinical trials. Oats are valuable and healthy crops and the effects they can elicit are need of the hour in the current state of the world. The information from this thesis and the whole ScanOats project provides a supportive argument for the production and consumption of oats to increase.

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1. Abstract

Oats (*Avena sativa)* are part of the cereal whole grain family, and their production is on the rise for the past few years due to extensive research done on the health benefits it can provide to humans. ScanOats project in Sweden is one of the research groups across the world that has done extensive work on oats. The lines CT-BG-53, CT-BG-21, and CT-BG-37 are mutant lines harvested by Crop Tailor AB and, the type of mutation used with them is chemical mutagenesis using ethyl methyl sulphonate. The mutations were performed on the elite variety of oats called Belinda, and the aforementioned lines were screened by the company as high β-glucan (20-30%) lines. β-glucan is a hydrocolloid polysaccharide that is made up of D-glucose monosaccharides and it can effectively regulate blood sugar and cholesterol levels. The presence of high β-glucan is a commercial advantage for these mutant lines as all the health claims associated with the consumption of oats at this point are associated with the effects of β-glucan. The work conducted in this thesis has made it feasible to further characterise these mutant lines and compare them with Belinda to further elaborate on their superiority. Another important advantage for these mutant lines in comparison to Belinda is the presence of higher total dietary fibre content (20-30 %), which means that the gut will be able to absorb more advantageous dietary fibres. The mutant lines also have higher protein and lipid content in them. The total starch content of all the mutant lines is lower in comparison to Belinda and that could be related to their high dietary fibre content. The viscous behaviour of these lines is similar to that of Belinda which helps defend the fact that the health benefits these lines may provide over Belinda are primarily due to their higher nutritional content and not their physical nature. Arabinoxylan is a non-starch polysaccharide occurring in dietary fibres, and it can give rise to effects like favourable gut microbiome, antioxidant effect and, enhanced immunoregulatory responses. Most of the mutant lines have an arabinoxylan content that is close to Belinda, and it is another valuable feature for them. This arabinoxylan content in these lines also hints toward the fact that the mutation for higher production of β-glucan has not interfered with the pathway to produce arabinoxylan in them. When utilising oats industrially they go through a mandatory heat processing step and through the means of this project it has been discovered these parameters have no negative impact on the amount of the essential nutrients in these lines.

2. Introduction

Oats (*Avena sativa*) are whole grains that are predominantly found in Europe and North America and the reason for that is the crop's inclination towards cool and moist climates (Varma, *et al.*, 2016). The primary uses of oats today are as feedstocks for animals and as food formulations for humans. Production of oats is 1 % of the world's total whole grain production, and it is the sixth-largest number (Tosh & Miller, 2016). Amongst the popular whole grain oats have high amounts of β-Glucan, lipids and protein and alongside these, they have specific micronutrients and the polyphenol avenanthramide (Clemens & Klinken, 2014). Due to its nutritional composition oats can elicit a host of health benefits in humans and they can help with health conditions like cardiovascular diseases, diabetes, hypertension, obesity, etc. (Varma, *et al.*, 2016).

Most of the registered health claims related to oats have been made in connection with the consumption of β-Glucan (Ahmad, *et al.*, 2014). The nutritional composition of oats is well-rounded because overall they have considerable amounts of carbohydrates, lipids, vitamins, and proteins with good amino acid balance, and phytochemicals (Rasane, et al., 2015).

In oats, the starch present has varied physicochemical properties in comparison to other cereals and even within the different cultivars of oats (Rasane, et al., 2015). Starch is mainly located at the endosperm of the oat grain and makes up about 40-60 % of the oat grain, it has unique properties like the small size of granules, high lipid content, and well-developed granule surface (Berski, *et al.*, 2011). In the context of the rate of digestion, starch can be characterised into rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS). In oats, there is about 7 % RDS, 22 % SDS and, 25 % SDS. For human health benefits, SDS is the most important starch because it helps in moderating the glycaemic response by enabling the slow rate of starch digestion (Ovando-Martínez, *et al.*, 2013). RS is also believed to be playing an important role in the digestion mechanism and because of that it has been characterised as a functional fibre (Rasane, *et al.*, 2015). Adequate and consistent consumption of oats can therefore be considered a good source of healthy starches in the diet (Rasane, et al., 2015).

Oats are good sources of protein when compared to other cereals. About 11-15 % of oats are composed of protein. The protein composition of oats can be categorised into different classes, which are, water-soluble (albumins), alkali-soluble (globulins), alcohol-soluble (prolamins), and acid or base soluble (glutelin) (Klose, *et al.*, 2009). Almost 95 % of the proteins present in oats fall under the categories of prolamin (15 %) and globulins (80%). Albumins are a major

fraction of the total amount of metabolically active protein in oats and along with globulin is responsible for the high content of lysine in oats (Rasane, *et al.*, 2015). For people suffering from celiac disease, the protein composition of oats is advantageous in comparison to other cereals, and it is still not recommended as totally safe because the prolamin fraction in oats has a lower hydrolysis tendency (Capouchová, *et al.*, 2004). Chemical modifications are reported to have been performed on oat proteins in order to increase their utilisation as food ingredients. The properties enhanced by these modifications are the solubility and emulsification of oat protein (Mohamed, *et al.*, 2009).

Dietary fibres are those parts of plant-based foods which are resistant to the environment present in the human gut i.e., they are resistant to the enzymes that take part in the human digestion process (Anderson, *et al*., 2009). The constituents of the dietary fibres could range from polysaccharides, oligosaccharides, resistant starch, lignin, etc. (Anderson, *et al.*, 2009). In all the plant-based food products the dietary fibres can be present as a soluble and insoluble fraction (Williams, *et al.*, 2019). The dietary fibre content in oat in its unprocessed form can be up to 85 % and in its edible part, this content is about 6 – 10 % (Daou & Zhang, 2012). Some of the important health benefits provided by dietary fibres are lower blood cholesterol and regulation of blood glucose levels (Mendis & Simsek, 2014).

One of the most abundant and crucial dietary fibre in oats is β-glucan, and it is present in the soluble fraction of the dietary fibre (Stevenson & Inglett, 2009). The β-glucan is a hydrocolloid polysaccharide that is made up of D-glucose monosaccharides and these monomers are linked with either β (1,4) or β (1,3) glycosidic linkages. (1,3) (1,4) β-D-glucan is the primary component of the oat soluble fibre (Stevenson & Inglett, 2009). In oats, most of the β-glucan present is in the endosperm cell walls. In the endosperm cell wall, the highest concentration of β-glucan is found in the thick sub-aleurone layer, and a small concentration is present in the aleurone layer (Wang & Ellis, 2014)

Oat β-glucan is linear in structure, consisting of D glucose monomers and they are all held together with the help of β-(1 → 3) and β-(1 → 4) linkages. It can provide health benefits like low levels of total and more essentially low-density lipoprotein cholesterol and controlling blood sugar levels (Wang & Ellis, 2014). The physical properties of the β-glucan in the different cereal fibres vary depending on the tetrasaccharide/trisaccharide ratio in them and in oats this ratio is around 2:1 (Ghotra, *et al.*, 2007). The visualisation of this structure is presented in figure 1.

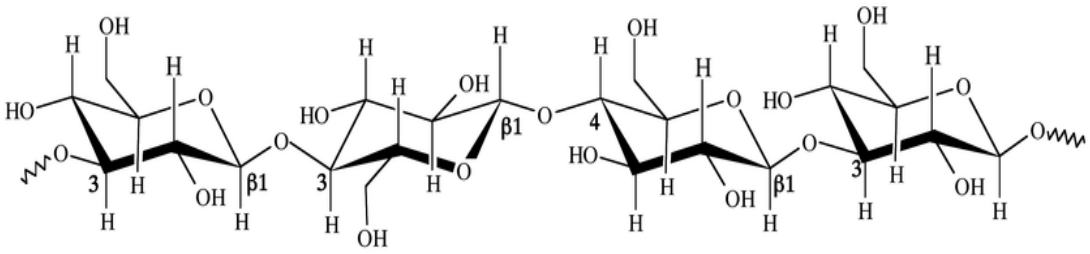

Figure 1 Structure of β-D Glucan (Pillai, et al., 2005)

The significance of oat β-glucans is high because at this moment most of the health claims registered with the consumption of oats under the European Union Regulation are associated with the health benefits of consuming β-glucan. Following are the claims registered –

1. Oat beta-glucan has been shown to lower/reduce blood cholesterol. High cholesterol is a risk factor in the development of coronary heart diseases. (Commission Regulation (EU) No 1160/2011).
2. Consumption of beta-glucan from oats as part of a meal contributes to the reduction of the blood glucose rise after that meal. (Commission Regulation (EU) No 432/2012.
3. Oat grain fibre contributes to an increase in faecal bulk. (Commission Regulation (EU) No 432/2012).

Molecular weight (MW) and the viscosity of β-glucan play a crucial role in determining the kind of health effect it will elicit. β-glucan with higher MW and viscosity is known to be responsible for hyperglycaemic and hypercholesteraemic effects (Du, *et al.,* 2019). Bile acid-binding capacity of low MW oat β-glucan is more than high MW oat β-glucan. (Kim & White, 2010). Solubility is another important criterion for β-glucan's functional activities like stability, emulsifying property, drug delivery, and membrane-forming properties. β-glucan in lower MW ranges exhibits better water solubility in comparison to higher MW ranges (Kim & White, 2011). The viscosity of any polysaccharide solution is dependent on the MW of the polysaccharide, its concentration, and its coagulation ability. After entering the gastrointestinal tract β-glucan increases the viscosity there and that in turn leads to a delay in the absorption of carbohydrates by the intestine, this viscosity increase is directly proportional to the MW of oat β-glucan (Du, *et al.*, 2019). Therefore, the consumption of varied MW ranges of oat β-glucan could lead to synergistic health benefits for the human body.

Arabinoxylans are the primary non-starch polysaccharide occurring in dietary fibres and the health benefits associated with its consumption have only been discovered in the last few years

(Mendis & Simsek, 2014). These health benefits include good digestive health, controlled blood sugar levels, favourable gut microbiome, antioxidant effect and, enhanced immunoregulatory responses (Mendis & Simsek, 2014). Arabinoxylan (AX) is localized in the cell wall of starchy endosperm (aleurone) in the bran tissue as well as in the husk. Although the structure of AX can differ slightly depending on the source, the main structure consists of a β-1.4-D-xylopyranosyl backbone with ᵅ-L-arabinofuranosyl residues substituted at positions 2 and/or 3 (Chen, et al., 2009).

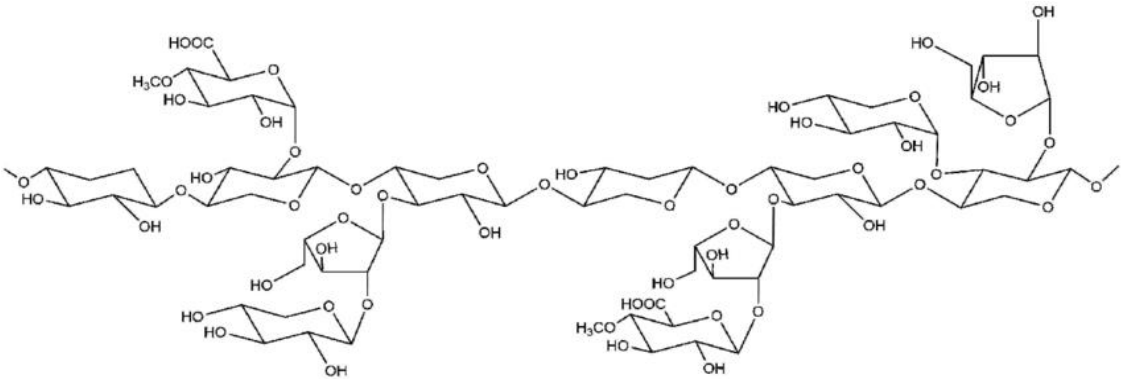

Figure 2 Structure of Arabinoxylan (Li, et al., 2020)

In comparison to the other popular cereal grains, the overall lipid content of oats is higher i.e., about 5-9 % percent of the total oat grain. These lipids present in oats are present in the endosperm along with that they are substantial sources of energy and unsaturated fatty acids (Rasane, *et al.,* 2015). Oat grains also contain native lipases which can act under low moisture levels and this fact combined with high amounts of lipids in the oats result in problems like rancidity and short storage life of the product. (Lehtinen, et al., 2003). The stability of the lipid content in oats is high as well because they retain the same value for almost a year at room temperature and this results in further processing problems like excessive browning of toasted products and poor flavour (Keying, *et al.,* 2009).

Whenever any whole grain is thought of to be used as a food product there is a variety of expectations that the consumers have concerning for properties like flavour, shelf life, texture, and formulation of the product. To meet these demands, just like other cereals oats have to undergo food processing steps as well. The difference in the form of treatment has varying effects on the nutritional and physical quality of pats and that in turn will have implications on the health benefits elicited by oats (Zhang, *et al.,* 2021). Oat kernel in its unprocessed form is non-digestible for the body and therefore it must go through food processing techniques in order to make the nutrition present in them absorbable for the body. The most important steps in processing oats is dehulling, milling, and heat processing and amongst all the industries

dealing with the manufacturing of oat-based products, these are the most popular processing steps. Heat processing is a vital step in the processing of oats as it primarily helps in the inactivation of the enzymes that catalyse the process of the oats getting rancid. So, heat treatment is an industrial activity that is vital to improving the shelf life of oat products that are launched in the market (Decker, *et al.*, 2014). A fact that must be kept in mind regarding this processing step is that the heat involved here may have an adverse effect on the essential fibres present in oats.

Crop Tailor AB is a biotechnology company from Sweden which develops mutagenized oat lines using random mutations with the overall aim being to screen out nutritionally superior lines from these mutagenized lines. The high β-glucan and arabinoxylan lines concerned with this project have been screened out when the mutation was chemically induced using ethyl methyl sulphonate (EMS) on the elite variety 'Belinda' (Lantmännen, n.d.) The EMS mutation technique used here is an example of an unguided approach of inducing mutations and it serves as a tool for unearthing novel characteristics which could ultimately lead to discovering new lines. EMS is an ethylating agent which converts the GC pair in the DNA to AT by inducing ethylation of guanidine. Additional advantages of this mutation technique are that it is inexpensive induces point mutations at a high rate and, is easy to implement (Gillmor & Lukowitz, 2020).

Aim of the project –

- To functionally characterise the high β-glucan lines on the parameters of β-glucan content, arabinoxylan content, protein content, total dietary fibre content, total starch content, viscosity, and total oil content.
- To verify if the industrial heat processing parameters have any negative impact on the essential components present in the high β-glucan line of oats.
- To inspect if the effect of mutation for high β-glucan production and the effect of mutation for high arabinoxylan production are dependent on each other or are they 2 independent effects.

3. Materials and Methods

3.1. Sample collection

The seeds from the oat lines LT20-Belinda FLM (Belinda), CT-BG-53, CT-BG-21 and CT-BG-37, were collected from Crop Tailor. The three lines and the reference line have been picked on the basis that they were all harvested in the same year. This was done to equalise as many abiotic factors as possible since they were all grown in the same field and endured the same climatic conditions.

Here Belinda was the reference line, and the other three lines were previously screened for high β glucan. Apart from these for several experiments, lines with high arabinoxylan content were also collected from Crop Tailor.

3.2. Milling

The samples were manually dehulled and were milled using the Pulverisette machine. An appropriate number of kernels were dehulled and milled based on the weight of the sample needed for every analysis. The frequency of the machine was set at 50 s^{-1} and the seeds were milled twice for 2 minutes each.

3.3. Heat treatment

The heat treatment of the samples was done in an oven which heat-treats with the utilisation of moisture. The parameters of this were set such that they would mimic the industrial heat-treatment process. The exact values of the same cannot be disclosed due to the confidentiality clause. The oven used for the heat treatment is displayed in Figure 3.

Figure 3 Rational Self-cooking centre® Steam oven

3.4. Characterisation using Grainsense

Grainsense analysing makes the use of small equipment that functions on the principle of near-infrared (NIR) technology. This helps in giving the real-time values of protein, carbohydrate, moisture, and oil in the oat kernels. First, measurement is taken with the empty equipment, and this is taken as the reference measurement (Grainsense, n.d.). Then a handful of oat kernels of each sample is placed by opening the lid of the equipment. To standardise all the results for this project all the measurements were done after dehulling the oat kernels.

3.5. β-Glucan Assay

The β-glucan assay was performed using the kit procured from Megazyme (K-BGLU) and, the principle of this method is based on degrading the β-glucan polysaccharides into glucose monosaccharides by using the enzymes Lichenase and β-glucosidase. The β-glucan amount is backtracked from the amount of glucose present and that is done by measuring the OD at 510 nm. The flow chart of how the β-Glucan polysaccharide is broken down into monosaccharides and the enzymes involved at each stage is depicted in Figure 4.

The enzymes and the reagents used for this assay are the same as the ones provided by the kit. However, the protocol used for this experiment is not the same as the protocol present in the kit and it has been modified by Crop Tailor AB to create a standardized method for more efficient estimation of the β-Glucan amount in all the different oat cultivars. The exact modification made for this purpose cannot be revealed in this report as it is the confidential

property of the company. In case the protocol needs to be accessed the author for this report or the company Crop Tailor AB must be contacted.

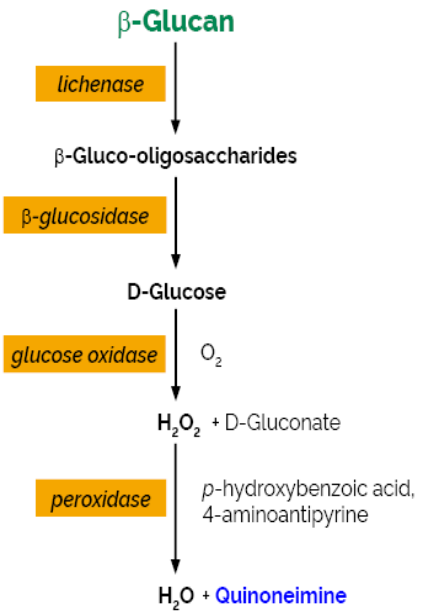

Figure 4 Functionality of the β-glucan assay kit (Megazyme Product code - K-BGLU)

3.6. Arabinoxylan Quantification (Chromatographic Assay)

3.6.1. **High-Pressure Anion Exchange Chromatography (HPAEC)**

HPAEC was performed for the oat lines LT20-Belinda FLM (Belinda), CT-BG-53, CT-BG-21, CT-BG-37 to estimate the concentration of arabinoxylan present in the milled flour. The standard for this is a mixture of pure mono sugars Arabinose, Galactose, Glucose, Mannose and Xylose was. PA-20 column was used for the mono sugar analysis. The A, B, and C eluents used were Milli Q water, NaOH 2mM and, NaOH 200mM respectively. The separation of the sugars was carried out with a mixture of 62.5% of eluent A and 37.5% of eluent B with a sample flow rate of 0.5 ml/min.

3.6.2. **Sample preparation for HPAEC –**

3.6.2.1.Solubilization of fibres (polysaccharides) -

Approximately 25 mg of the milled flour from each line was transferred to autoclavable test tubes and hydrolysed in 250 µl 72% Sulphuric acid that was incubated for 30 minutes at 200 Hz in a shaker incubator at 30ºC

3.6.2.2.Hydrolyzation of Carbohydrates

6.8 ml of Milli Q water was added to the tubes with the hydrolysed samples and the tubes were autoclaved for 1 hour at 121ºC to break the polysaccharides to monosaccharides. 2 ml of the samples were then transferred to 2 ml tubes and centrifuged at 4500 rpm for 10 minutes.

3.6.2.3.Neutralization of samples -

1 ml of the supernatant from the centrifuged tubes was transferred to 15 ml tubes and the samples were neutralized to approximately pH 7 by adding 0.1 M barium hydroxide in a stepwise manner. The pH was controlled using a pH indicator and the dilution volume was noted down. The pH should be less than 8 as a higher pH will destabilize and break down the saccharides.

3.6.2.4.Filtration of samples -

The samples were filtered twice using a 0.2µm syringe filter and collected in HPLC tubes.

3.7. Protein Estimation

Approximately, 25 mg of milled flour from each line was used for the protein analysis by the Dumas method. Dumas method is the protocol that quantifies the amount of nitrogen in chemical substances, and it was first described by Jean-Baptiste Dumas. The principle here is that when a nitrogenous compound such as protein is heated with cupric oxide in the excess of CO_2 it yields the production of free nitrogen. The free nitrogen is directly proportional to the amount of protein in the sample. The conversion factor (Jones Factor) used here was 6.25. This was performed at the Department of Food Technology, Lund University.

3.8. Total Dietary Fibre Extraction

The Megazyme-kit (K-TDFR) was used to extract the soluble and insoluble dietary fibre from the milled samples. In order to degrade the starch and proteins in the samples, the kit contained the enzymes Amylose, Protease, and Amyloglycosidase. The extracted fibre was split into two parts- the insoluble and the soluble fraction. The fibre portions were collected using glass crucibles and, after vacuum filtration, dried overnight in a hot air oven at 100º C. The weights of the extracted soluble and insoluble dietary fibre were estimated by subtracting the tare weight of the crucible from the weight of the crucible with fibre. The online Megazyme calculator (Mega-calc) was used to estimate the amount of insoluble, soluble, and the total dietary fibre in the oat samples.

3.8.1. **Sample preparation**

The untreated seeds and heat-treated seeds from the oat lines LT20-Belinda FLM (Belinda), CT-BG-53 and CT-BG-21, were dehulled and milled and approximately 2 g of the milled flour was taken for the TDF estimation.

3.8.2. **Insoluble dietary fibre estimation**

3.8.2.1. 2g of the milled flour was taken in a conical flask, and it was dissolved in MES-TRIS Buffer with pH 8.2

3.8.2.2. 100µl of α-amylase was added to this mixture and was incubated for 30 minutes at 98-100 ºC. Then 200µl of protease was added and incubated for 30 minutes at 60ºC. The pH was adjusted to 4.2-4.8 before adding amyloglycosidase. Once the pH is adjusted, 400µl of amyloglycosidase was added and was incubated for 30 minutes at 60ºC.

3.8.2.3. After the incubation with the enzymes, the sample is filtered using a vacuum filter into a fritted crucible. The liquid fraction is saved for the extraction of SDF. The crucible is then dried in a hot air oven at 100ºC overnight.

3.8.2.4. The crucible with the lowest amount of fibre is ashed in an incinerator at 525ºC for 5 h while the other crucible is scraped out to collect the IDF and the collected IDF is stored in 2 ml tubes.

3.8.2.5. The amount of IDF, protein weight, and the ash weight are noted and the %w/w IDF is calculated by using the online Megazyme calculator.

3.8.3. **Soluble dietary fibre estimation**

3.8.3.1. The liquid fractions from the IDF filtration are precipitated using 4 times the volume of ethanol and are filtered similarly as that of IDF. The crucibles are dried overnight at 100ºC, and the lowest weighing crucible is ashed at 525ºC for 5 h while the other crucible is scraped out and the SDF is collected and stored in 2ml tubes.

3.8.3.2. The amount of SDF, protein weight and ash weight are noted, and the %w/w SDF is calculated by using online Megazyme calculator

3.8.4. **Total dietary fibre calculation**

The total dietary fibre is obtained by adding the %w/w IDF and %w/w SDF

3.8.5. **Estimation of Ash content**

The collection of insoluble and soluble dietary fibre was carried out in duplicates and the crucible with the lowest amount of the fibre was analysed for the ash content by incinerating it in the muffle furnace for 5 hours at 525 ºC. The ash weight was estimated by subtracting the tare weight of the crucible from the ash weight of the crucible.

3.8.6. **Estimation of protein content**

Estimation of protein in the insoluble and soluble fractions was done by the Dumas estimation and the steps followed were exactly similar to section 3.7. The only difference here is that instead of milled flour the samples would be
extracted insoluble and soluble fractions.

3.8.7. **Estimation of arabinoxylan**

Estimation of arabinoxylan in the insoluble and soluble fractions was done by conducting the arabinoxylan assay and steps followed were exactly similar as in section 3.6. The only difference here is that instead of milled flour the samples would be extracted insoluble and soluble fractions.

3.8.8. **Estimation of β Glucan**

Estimation of β Glucan in the insoluble and soluble fractions was done by conducting the β Glucan assay and steps followed were exactly similar as section 3.5. The only difference here is that instead of milled flour the samples would be extracted insoluble and soluble fractions.

3.9. Total oil content –

The total oil content in each of the oat lines was analysed by extracting the oil from the oat kernels using the solvent extraction method. The principle of this method is that the solvent made of a mixture of alcohols percolates via the oat flour particles and releases the oil and when the solvent evaporates only the oil is left.

3.9.1. 5-10 seeds and dehulled and milled for each sample. Their weight is measured and noted down as W_s.

3.9.2. 1.2 ml of the solvent (3 parts heptane and 2 parts Isopropanol) is added to each sample.

3.9.3. The oat flour and solvent are mixed thoroughly using the Precellys mixer. The parameter of mixing was 5000 rpm for 5 * 90 seconds with 10 seconds intervals.

3.9.4. The samples were now centrifuged at 9000 rpm for 5 minutes.

3.9.5. New tubes for each sample were now taken and the weight of each of them was noted down as W_t.

3.9.6. All the supernatant in each tube after the centrifugation step was transferred to the new tubes. The detail that was carefully followed was that no flour particle was transferred as it could have an exaggerating effect on the oil percentage.

3.9.7. Now leave these tubes opened in an air hood for 2 days during which the solvent will completely evaporate. All that will be left in each tube will be the oil content, note the weight of each tube as W_{to}

3.9.8. Calculate oil percentage using *Equation 1*.

$$Oil\ \% = ((Wto - Wt) * 100)/Ws \text{ (Equation 1)}$$

3.10. Viscosity comparison with reference Line –

The viscosity comparison was made between the reference line Belinda and the three mutagenized lines by plotting a viscosity versus shear rate graph by using a rheometer shown in figure 5. The formulation that was used to analyse the viscosity behaviour of each sample was a solution of oat flour and water.

Figure 5 Rheometer from Kinexus along with Geometry used for this Experiment

3.10.1. Dehull and mill enough seeds from each sample to make up to 1 g of flour.

3.10.2. Add the oat flour to 20 ml of MilliQ water and mix properly with use magnetic stirrers. Ensure that there are no clumps of the flour forming.

3.10.3. Boil each solution in a water bath with constant stirring for 10 minutes.

3.10.4. Set up the rheometer by initialising it.

3.10.5. Take a zero-gap measurement in order to calibrate the instrument.

3.10.6. Run the program that measures the viscous behaviour of each sample under 2 set points of shear stresses at 25 ºC. The program is set up such that it reads 5 points between each interval.

3.10.7. Compare the trends of the viscosity vs shear rate graphs of each sample to estimate which sample is more viscous.

3.11. Total Starch Content –

3.11.1. Dehull and mill enough kernels to have 25 mg oat flour for each sample

3.11.2. Add 1.25 mL of EtOH (80 % v/v) to each sample.

3.11.3. Incubate each tube at 80-85 ºC for 5 minutes. Mix using a vortex and dispense another 1.25 mL of EtOH (80 % v/v) in the tube.

3.11.4. Centrifuge each tube for 10 minutes at 3000 rpm and discard the supernatant afterward.

3.11.5. Resuspend the pellets with 2.5 mL of EtOH (80 % v/v). Centrifuge again for 10 minutes at 3000 rpm and discard the supernatant afterward.

3.11.6. Add a magnetic stirrer bar and 0.5 ml of 2M KOH to each tube and resuspend pellets to dissolve the resistant starch. Stir for 20 minutes in an ice bath.

3.11.7. Add 2 mL of 1.2 M sodium acetate buffer to each tube.

3.11.8. Quickly add 25 µL thermostable α-amylase and follow it up with 25 µL of amyloglycosidase.

3.11.9. Incubate each tube at 50 ºC water bath for 30 minutes coupled with intermittent mixing.

3.11.10. Quantitatively transfer the tube content to a 50 ml falcon tube and adjust to 25 ml with distilled water and mix well.

3.11.11. Centrifuge a 1 mL aliquot from each tube at 3000 rpm for 10 minutes.

3.11.12. Dispense 25 µL from each tube to a newly prepared Eppendorf tube.

3.11.13. Prepare the D-Glucose control by adding 25 µL of the D-glucose standard solution

3.11.14. Prepare reagent blank by adding 25 µL of water.

3.11.15. Add 0.75 mL of GOPOD reagent to each sample tube along with the control and the blank.

3.11.16. Incubate the tubes at 50 ºC for 20 minutes

3.11.17. Measure the OD_{510} for each of the samples.

3.11.18. Calculate the amount of starch with the Megazyme Starch calculation template (K-TSTA).

4. Results and Discussion

4.1. Grainsense Characterisation

Table 1 consists of the Grainsense characterization done for all the lines which were dealt with during this project. The Grainsense equipment has estimated different parameters such as the moisture, carbohydrate, protein, and oil content for each of these lines.

Based on the results obtained by the Grainsense equipment in Table 1, it is noticeable that the high β-glucan lines and high arabinoxylan lines have more protein content in them in comparison to Belinda. The difference is more significant in the high β glucan lines, and this could add another attractive feature for these lines as the products manufactured by using them will potentially contain high β glucan and protein content.

The moisture content for the high arabinoxylan lines is significantly lower than that of Belinda and that is an interesting observation. There could be multiple factors that contribute to this observation. One of these reasons could be the presence of higher arabinoxylan and the insoluble nature of the arabinoxylan fibre.

Overall, the measurements made by the Grainsense are not accurate, but they help point out the correct trend and this feature could be useful when the need is for rapid measurements.

Table 1 The protein, carbohydrate, Moisture and Oil content of the High β-glucan lines, High Arabinoxylan lines (LT 17 CT0005 M7, LT 17 CT0076 M7, LT 17 CT0107 M7 and LT 17 CT0320 M7) and the reference line Belinda

Sample ID	Protein (% DW)	Carbohydrate (% DW)	Moisture (% DW)	Oil (% DW)
LT20-Belinda FLM	11.69	83.31	13.2	4.99
CT-BG-53	15.33	79.28	11.17	5.39
CT-BG-21	15.11	79.7	11.38	5.19
CT-BG-37	15.41	79.26	11.22	5.33
LT 17 CT0005 M7	11.70	83.55	8.25	4.75
LT 17 CT0076 M7	12.08	83.59	8.04	4.33
LT 17 CT0107 M7	12.18	83.92	8.22	3.90
LT 17 CT0320 M7	12.99	82.81	7.93	4.19

4.2. Characterisation of High β-glucan Lines

4.2.1. β-glucan, Arabinoxylan and Protein Content

Table 2 represents the β-glucan, Arabinoxylan, and protein content of the three lines CT-BG-53, CT-BG-21, and CT-BG-37 along with the reference line Belinda. All of these lines were harvested in the year 2020.

β-glucan, Arabinoxylan, and protein are a few of the most important elements of oats. Through the data represented in Table 2, it is confirmed that the lines CT-BG-53, CT-BG-21, and CT-BG-37 are lines with higher β-glucan content. The intriguing feature about these lines is that they have higher protein content in comparison to Belinda as well. This difference however was less significant after the analysing by the protein analyser than what was observed using the Grainsense equipment (Table 1). The presence of higher amounts of β-glucan can correspond to the fact that the people who consume products from these lines regularly would have better regulations of cholesterol and blood sugar levels (Wang & Ellis, 2014). The higher amounts of protein can position the products from these lines as a valuable contributor toward the total amount of protein that should be consumed regularly. The potential for the consumption of these lines by the patients with celiac disease must be further assessed by analysing the total content of prolamins present in these lines (Capouchová, *et al.*, 2004).

The arabinoxylan content in these lines is slightly lower than Belinda, the differences however are not very significant which suggests that these values are close to each other. The presence of arabinoxylan in substantial amounts by these lines hints that these lines may possess a prebiotic potential and may go on to build a favourable gut microbiome.

Table 2 β-glucan, Arabinoxylan and Protein contents of the three high β-glucan lines along with the reference line Belinda. Each value is present along with the Standard deviation between the replicates

Sample ID	β Glucan Concentration (% dw) ± SD (n = 2)	Arabinoxylan Concentration (% dw) ± SD (n = 2)	Protein (% dw) ± SD (n = 2)
LT20-Belinda FLM	4.71 ± 0.24	4.18 ± 0.15	15.32 ± 0.32
CT-BG-53	6.47 ± 0.05	3.68 ± 0.34	16.60 ± 0.07
CT-BG-21	5.55 ± 1.00	2.95 ± 0.04	16.56 ± 0.06
CT-BG-37	6.68 ± 0.05	3.74 ± 0.03	16.67 ± 0.14

4.2.2. Total Lipid Content

Table 3 consists of the values of the total lipid present in the lines CT-BG-53, CT-BG-21, and CT-BG-37 along with the reference line Belinda.

With the support of the data present in Table 3, it is evident that the high β-glucan lines have a slightly higher amount of lipids present in them in comparison to Belinda. The percentage of the difference between the sample lines and the reference line is not very high and therefore it can be affirmed that the total lipid present in these lines are close to each other. Upon comparing these values with the values obtained by Grainsense equipment in Table 1 it is again evident that the NIR technology underestimates the lipid content to a certain extent and as the lipid content goes higher the discrepancy between these values will increase.

The presence of higher amounts of lipids does not essentially point towards proven health benefits. However, in recent times oat polar lipids have been studied to have potential nutraceutical properties by modulating acute and second meal postprandial metabolic responses (Hossain, *et al.,* 2021). So, if further characterised it will be possible to find out the amount of polar lipids in the total lipid content. If the content of polar lipids is substantial, then it can combine with the previously found high β-glucan to potentially create a synergistic effect in the regulation of glycaemic index.

Table 3 Lipid contents of the three high β-glucan lines along with the reference line Belinda. Each value is present along with the Standard deviation between the replicates

Sample ID	Total Lipid Content (% w/w) ± SD (n = 2)
LT20-Belinda FLM	6.84 ± 0.15
CT-BG-53	7.13 ± 0.11
CT-BG-21	7.25 ± 0.01
CT-BG-37	7.39 ± 0.13

4.2.3. Total Extracted Starch Content

Table 4 contains the values of the total starch extracted from the lines CT-BG-53, CT-BG-21, and CT-BG-37 along with the reference line Belinda. As seen in the data available in the table, the starch content in the lines with high β-glucan has lower amounts of starch present in them when compared to Belinda. A plausible explanation for this discrepancy is the fact that in oat grains both starch and β-glucan are located at the endosperm (Berski, *et al.,* 2011) (Wang & Ellis, 2014). If carefully noticed the major differentiating factor between the lines CT-BG-53, CT-BG-21, and CT-BG-37 and Belinda is the presence of higher β-glaucan amounts and because of their common location it could be that the β-glucan molecules have occupied more of the endosperm in these lines and that is why the starch produced in them is lower.

The effects with respect to health created by effective oat starches and oat β-glucans are more or less similar with possibly different mechanisms. Based on these facts it can be affirmed that the presence of a comparatively lower amount of starch in high β-glucan lines is not a major shortcoming for them.

Table 4 Total Starch contents of the three high β-glucan lines along with the reference line Belinda. Each value is present along with the Standard deviation between the replicates

Sample ID	Total Starch (% w/w) ± SD (n = 2)
LT20-Belinda FLM	47.02 ± 1.29
CT-BG-53	39.95 ± 3.89
CT-BG-21	43.72 ± 5.93
CT-BG-37	44.05 ± 2.70

4.2.4. Comparing the Viscous Behaviour with Belinda

The comparison of the viscous behaviour of the lines with Belinda was possible by plotting the viscosity of all the samples against certain shear rates over a period of specific shear stresses. The rheometer works in a way where it has to stabilise for 10 seconds and then it goes on to recognise a particular point for the viscosity and the shear rate. This is the reason why when carefully observing the graphs in Figure 6, it can be observed that the data points of all the lines are different. Therefore, the trend of the individual graphs must be analysed in order to successfully compare the viscous behaviour of the high β-glucan lines with Belinda.

As visible from the graphs in Figure 6 there is no significant difference between the viscous nature of the high β-glucan lines and Belinda. The viscosity of a sample which has the potential to regulate the glycaemic index is an important factor because if the viscosity is high for a sample, it enables a delay in the absorption of carbohydrates in the intestines (Du, *et al.*, 2019). Therefore, when the specific effect of each line is observed in humans through some form of a clinical study, the fact that the regulations are taking place primarily due to the difference in viscosity could be ruled out.

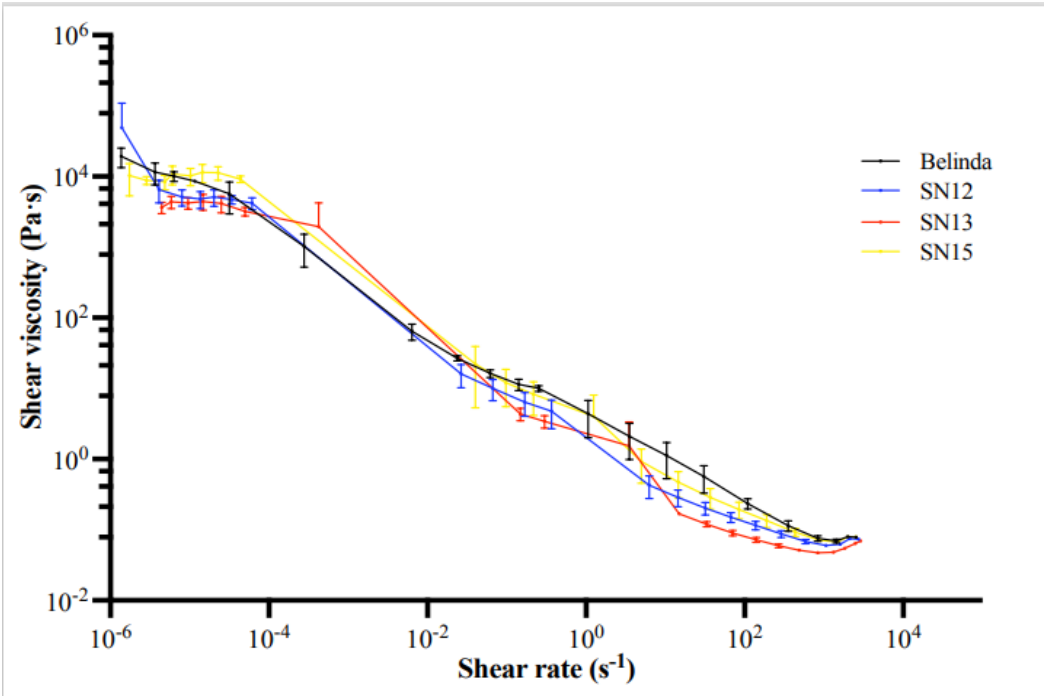

Figure 6 Shear rate vs Viscosity (where SN12 - CT-BG-53; SN13 - CT-BG-21; SN15 - CT-BG-37)

4.2.5. Total Dietary Fibre Content

Table 5 consists of the dietary fibre content in the mutant lines CT-BG-53, CT-BG-21, and the reference line Belinda. The table consists of the insoluble dietary fibre, soluble dietary fibre, and their sum as the total dietary fibre content of each line. Based on the data presented in the table it can be concluded that the mutant lines which have a higher β-glucan content are also the lines with a higher dietary fibre content in them. One of the obvious reasons for this is that β-glucan itself is a dietary fibre and that is why the total dietary fibre content in them is higher content is higher in them. This fact is supported by the higher soluble fraction in both the mutant lines. As previously known dietary fibre fraction of a plant-based food item consists of a wide range of substances like polysaccharides, oligosaccharides, resistant starch, lignin etc. (Anderson, *et al.*, 2009), the reason for a higher amount of dietary fibre cannot be limited to just higher β-glucan.

The higher dietary fibre content is a healthy and economically attractive feature for the mutant lines because of its ability to regulate blood cholesterol and sugar levels (Anderson, *et al.*, 2009). Along with these features, studies have also shown that dietary fibres can enhance the satiety (Savastano, *et al.*, 2014) by employing mechanisms like increasing stomach distention to delay its emptying (Kristensen & Jensen, 2011) or fermenting the gut microbiota which

promotes the production of short-chain fatty acids (Bouhnik, *et al.*, 2004). This effect of the dietary fibre is helpful for people who are suffering from health conditions like obesity as regular consumption of a sufficient quantity of dietary fibres could help them control their weight along with maintaining their nutrition intake (Bouhnik, et al., 2004). Therefore, the presence of higher dietary fibre content positions these mutant lines as a healthier product in comparison to Belinda.

Table 5 Total Dietary Fibre contents of the 2 high β-glucan lines along with the reference line Belinda. Each value is present along with the Standard deviation between the replicates

Lines	Insoluble Dietary Fibre (% w/w) ± SD (n = 2)	Soluble Dietary Fibre (% w/w) ± SD (n = 2)	Total Dietary Fibre (% w/w) ± SD (n = 2)
LT20-Belinda FLM	6.80 ± 1.11	3.40 ± 0.28	10.19 ± 0.82
CT-BG-53	9.71 ± 0.71	5.79 ± 1.30	15.50 ± 2.01
CT-BG-21	9.18 ± 1.12	4.10 ± 1.45	13.28 ± 0.33

4.3. Effect of Heat Treatment

4.3.1. Effect on β-glucan and Arabinoxylan

Table 6 represents the effect of heat treatment on the β-glucan and arabinoxylan content after the heat treatment protocol. β-glucan and arabinoxylan are two of the most important and broadly studied dietary fibres present in oats and that is why it is important to understand what kind of effect the heat treatment parameters have on their content. The comparison of the β-glucan and arabinoxylan content before (Table 2) and after (Table 6) heat treatment gives rise to the graphical representations in Figures 7 and 8.

Table 6 β-glucan and Arabinoxylan contents of the three high β-glucan lines along with the reference line Belinda after heat treating them. Each value is present along with the Standard deviation between the replicates

Sample ID	β Glucan Concentration (% DW) ± SD (n = 2)	Arabinoxylan Concentration (% DW) ± SD (n = 2)
LT20-Belinda FLM	4.64 ± 0.15	3.25 ± 0.15

CT-BG-53	6.25 ± 0.07	3.74 ± 0.86
CT-BG-21	6.44 ± 0.25	3.75 ± 0.20
CT-BG-37	6.17 ± 0.13	3.57 ± 0.94

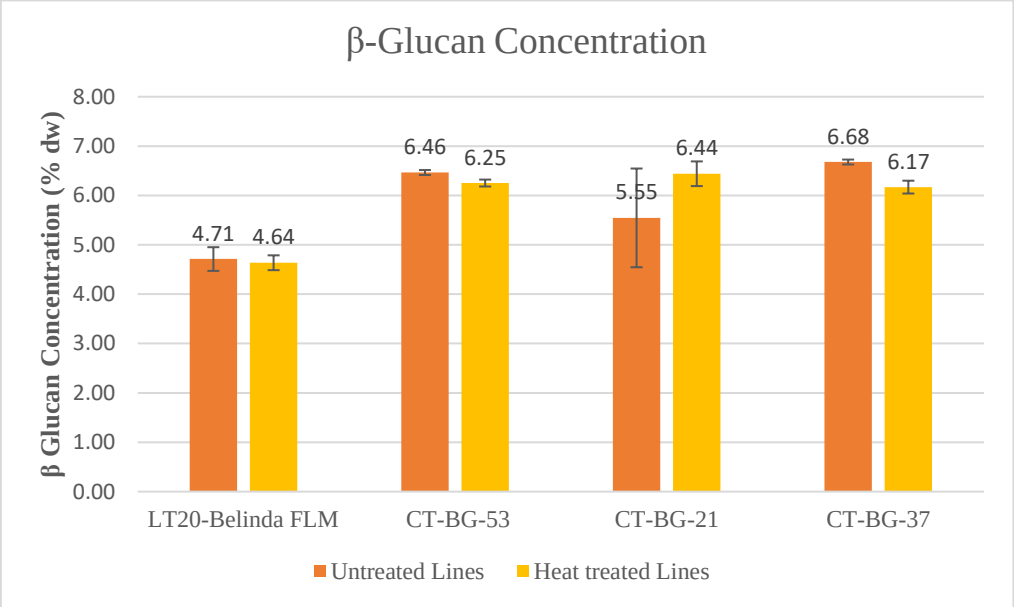

Figure 7 Graph comparing the β-glucan content before and after heat treatment

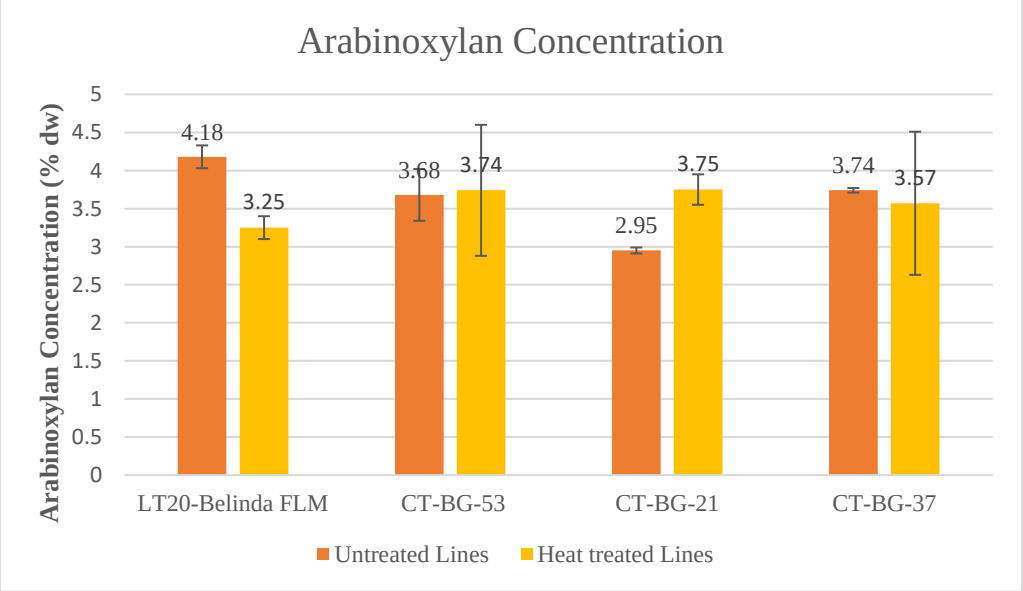

Figure 8 Graph comparing the arabinoxylan content before and after heat treatment

As seen in the aforementioned figures the heat treatment does not lead to a major decrease in the content of these essential fibres. Since these lines are high β-glucan lines in comparison to Belinda, β-glucan would be positioned as their major attractive feature from a commercial

standpoint. With regards to this fact, an encouraging pattern for these lines is that the β-glucan content in them is still higher in them as compared to Belinda after the heat treatment protocol. With regards to the arabinoxylan content, it is important to notice that there is no significant decrease observed. In some instances, for both the dietary fibres, it is noticed that there is concentration observed in their values after the heat treatment. There could be multiple explanations for this observation. Some of the most likely ones amongst these are that either due to the heat there has been a reduction of other elements in the oat kernel and that is why the percentage of an individual element has propped up or it could be because there is steam involved in the protocol, the solubility of the fibres has been enhanced and it is now possible to extract them more efficiently (Stevenson & Inglett, 2009).

4.3.2. Effect on the Protein content

Table 7 represents the protein values in the lines CT-BG-53, CT-BG-21, and CT-BG-37 along with reference line Belinda after the heat treatment protocol. Protein as discussed before is an important component for maintaining a balanced diet and these high β-glucan lines have higher protein content in them in their unprocessed form in comparison to Belinda. Therefore, it will be an even more attractive feature for these lines if the protein content in them is not affected harshly due to the heat processing. Protein is the kind of substance that is susceptible to denaturation due to heat and that is why the graphical representation presented in Figure 9 paints a very interesting picture. Figure 9 contains the graphical comparison of the protein in the aforementioned lines before and after the heat treatment protocol. What is observed in that is the fact that there is no adverse effect on the protein content after the heat treatment. If carefully observed the content in almost all the lines, the difference between the values observed is not very significant.

Table 7 Protein contents of the three high β-glucan lines along with the reference line Belinda after heat treating them. Each value is present along with the Standard deviation between the replicates

Sample ID	Protein (% dw) ± SD (n = 2)
LT20-Belinda FLM	15.63 ± 0.16
CT-BG-53	16.92 ± 0.15
CT-BG-21	15.29 ± 0.16
CT-BG-37	16.27 ± 0.33

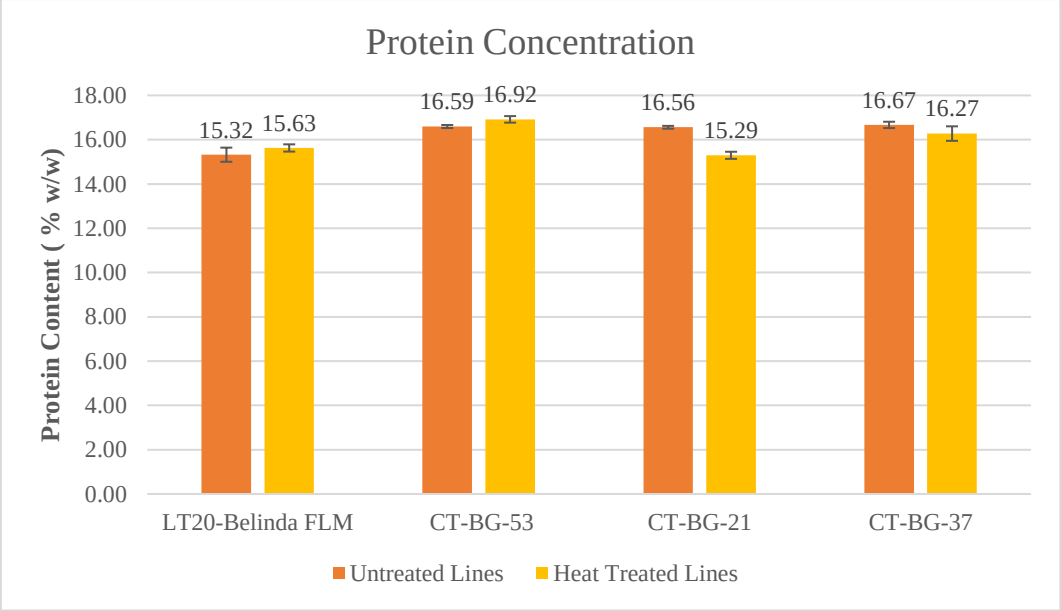

Figure 9 Protein Content in High β-glucan lines before and after heat treatment

The expected behaviour in this case owing to the nature of proteins would be to observe a decrease in the content of the protein but that is not what can be observed here. A major reason for this could be the method of analysis involved here. Since the measurement is based on the Dumas principle and there the protein content is quantified based on the nitrogen amount detected in the sample. So, after heat treatment, it could be a case where the protein might have denatured but the amount of nitrogen remains the same in the sample and so when that value is multiplied by the protein factor the value obtained is very close to the original value. Therefore, it is a plausible scenario where the protein content after the heat treatment might have been overestimated by the denaturation method. A more accurate method to check the actual state of protein content would be using a qualitative method like SDS-PAGE.

4.3.3. Effect on Total Dietary Fibre Content

Table 8 contains the total dietary fibre content in the lines CT-BG-53, and CT-BG-21, along with reference line Belinda after the heat treatment protocol. Figure 10 provides the graphical description of the comparison between the total dietary fibre content in the lines before and after heat treatment.

A consistent pattern noticed for all the lines is that the dietary fibre content seems to be concentrated post the heat treatment. One of the possible explanations for this could be improved in the extractability of the fibres due to enhanced solubility provided by the steam involved in the treatment and this fact is supported by the significant increase in the soluble fraction of each line. However, it could also be possible that this value is being overestimated

as there could be an amalgamation of starch due to the heat, but the possibility of this is rather low as there is no dry heating here.

Table 8 Total Dietary Fibre contents of the three high β-glucan lines along with the reference line Belinda after heat treating them. Each value is present along with the Standard deviation between the replicates

Lines	Insoluble Dietary Fibre (% w/w) ± SD (n = 2)	Soluble Dietary Fibre (% w/w) ± SD (n = 2)	Total Dietary Fibre (% w/w) ± SD (n = 2)
LT20-Belinda FLM	7.02 ± 0.69	3.97 ± 0.28	10.99 ± 0.33
CT-BG-53	12.00 ± 1.55	6.69 ± 1.51	18.69 ± 1.34
CT-BG-21	10.83 ± 0.48	6.60 ± 1.75	17.43 ± 1.39

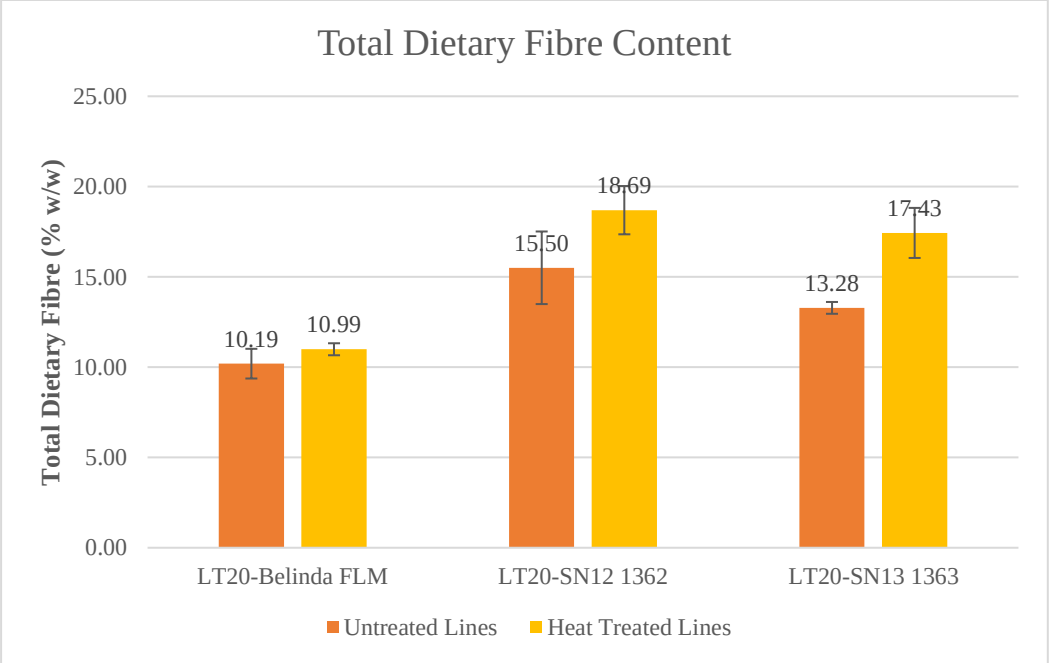

Figure 10 Total Dietary Fibre Content before and after Heat Treatment

4.4. Inspecting the Correlation of the Mutations

Both the high β-glucan and high arabinoxylan concerned with this project are oat lines developed through random mutagenesis on the reference line Belinda. The occurrence of random mutagenesis means that numerous biosynthetic pathways in oats that produce β-glucan, arabinoxylan, starch, or other macromolecules will get affected in different ways. Henceforth,

it is important to note that if the enhancement of the production of one essential fibre has any ill effect on the production of the other essential fibre.

For this project, this relationship was analysed between β-glucan and arabinoxylan through a biochemical approach. The analysis of the correlation mentioned above for the high β-glucan lines was done by finding out their arabinoxylan content in order to compare it with that of Belinda and analyse the difference. It was done the other way around for the high arabinoxylan, where their β-glucan content was compared with that of Belinda. Utilising this approach has made it feasible to compare the content of the secondary fibre in the mutant lines and the standard line, in order to see if the pathway for the high production of the primary fibre has interfered with its production.

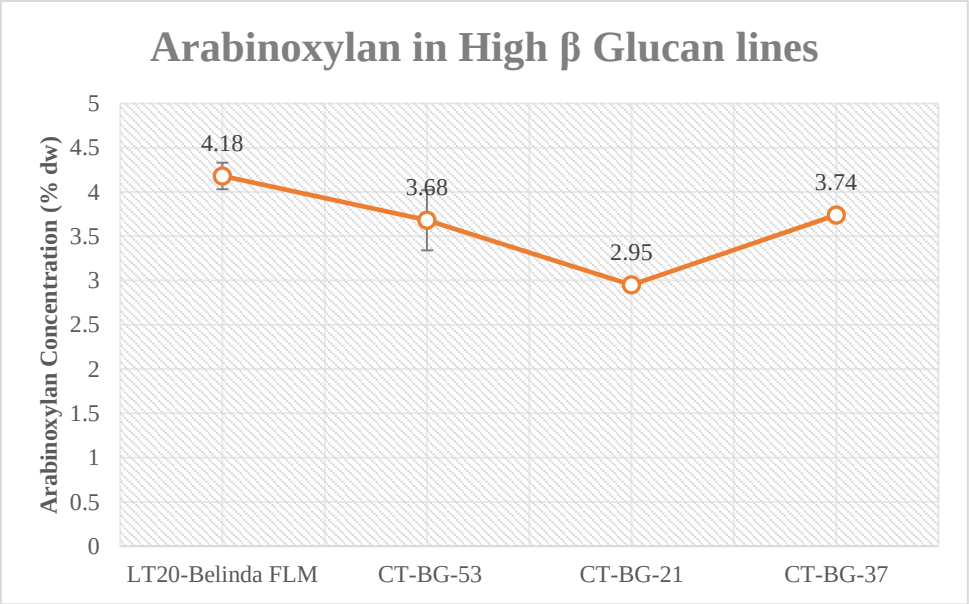

Figure 11 Arabinoxylan content in High β-glucan Lines

Figure 11 consists of the graphical presentation of the arabinoxylan content in the high β-glucan oat lines. Since the lines have certain mutations which have increased the production of β-glucan, arabinoxylan is being considered as the secondary fibre in these lines. The scatter graph in Figure 11 allows the monitoring of the difference in the arabinoxylan content between the mutant lines and the Belinda. Observing the graph, it is visible that 2 of these lines do not have a significant difference in comparison to Belinda, therefore it can be hypothesised that the mutation of the production of high β-glucan amounts has no effect on the production of arabinoxylan in these lines. Since the nature of the mutagenesis process used here is unguided and random, this hypothesis cannot be generalized for all oats, and it should be limited to these lines.

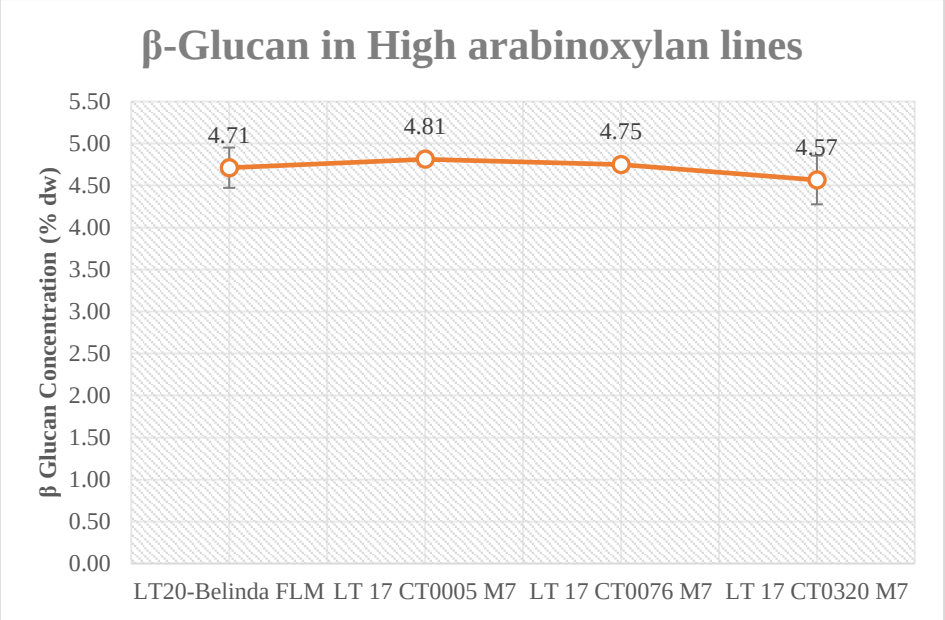

Figure 12 β-glucan content in High Arabinoxylan Lines

Figure 12 consists of the graphical presentation of the β-glucan content in the high arabinoxylan oat lines. Due to the similar reasoning as before, β-glucan is being considered as the secondary fibre for these lines. The scatter graph in Figure 12 allows the monitoring of the difference in the β-glucan content between the mutant lines and the Belinda. It is visible in the graph that all of these lines do not have a significant difference in comparison to Belinda. Therefore, it can be hypothesised that the mutation of the production of high arabinoxylan amounts has no effect on the production of β-glucan in these lines. Due to a similar mutagenesis process being followed for these lines as well this observation must also be considered valid for these specific lines only

The analysis of the 2 Figures above provides the conclusion that for these lines the mutation for the high production of one dietary fibre is and independent mutation and it has no effect on the pathway for the production of the other fibre.

5. Conclusion

This project has successfully characterized the high β-glucan mutant lines CT-BG-53, CT-BG-21, and CT-BG-37 on multiple parameters and based on those these lines show genuine health benefitting ability. The components present in higher amounts in these lines when compared to Belinda (reference line) are β-glucan, protein, total dietary fibre, and lipids. Based on the various literature works referred to in this report the abundance of these components can provide a lot of clinically proven benefits to the human body, some of which are lower blood cholesterol, regulation of blood glucose level, anti-cancerogenic effects, favourable gut microbiome, antioxidant effect, and enhanced immunoregulatory responses. If considering the trends of the global lifestyle, there is a need for natural products that have an abundance of such useful macronutrients and these mutant lines along with their related families have shown the potential to complement these needs. The next step in the functional characterisation of these lines is to first check if these superior properties stay constant over multiple generations and if that is the case then somehow plug these lines in a basic clinical study where the blood glucose regulation ability of these lines could be compared with other products. Something also worth paying attention to is the yield of these lines in the field. A high yield could enable any company to further invest in the potential of these lines and make food products out of it. If the yield of these lines is low for any reason, then research must be into trying to increase their yield, or else industrial application of these lines would not be commercially viable.

The major attractive feature of these lines is their high β-glucan content and due to the physical nature of this hydrocolloid polysaccharide it is found in higher amounts in the soluble fraction of the dietary fibre, and this would make it easier for the manufacturers to extract more β-glucan out of these lines. If the mutant lines have clinically proven health benefits, then these lines could qualify for being used as raw material for industrial manufacturing of oat-based food products. Since every valuable strain is screened out in order to have potential industrial use, they will go through the basic processing steps. Heat processing is one of the important processing steps and based on the results of this project it is proven that this step does not have a negative impact on the essential nutrients in oats.

After combining the aforementioned mutant strains with high arabinoxylan lines, the interconnection between the 2 mutations was also analysed. Based on the biochemical assays it has presented an observation that the mutation for higher production of β-glucan does not effect on the arabinoxylan production pathways in that line. This observation in a vice versa scenario also turned out to be true. If this result holds out to be true for a pool of different oat

strains, it could provide a useful general observation that the mutation for high production of one essential fibre won't interfere with the pathway of production of any other fibre. Therefore, enhancement in one aspect of the oat strain is not going to necessarily deprive it of other helpful nutrients.

All in all, oats are useful and advantageous cereal whole grains. They occupy 1 % of the world's total viable farmland and with the recent studies increasing this percentage should also be on the rise. In comparison to other popular cereal whole grains, there are added benefits attached to the consumption of oats. Sweden has made significant headway in the research done on oats and that has contributed to this crop's increasing popularity throughout this reason. The environment at this point is in severe need of sustainability and there is serious potential in oat waste streams to be utilised as some sort of product. The mutations brought about by EMS mutagenesis are random and unguided and that is where the problem of stable features comes in. Stabilising the oat characteristics over multiple generations is challenged by various biotic and abiotic stresses and there is a lot of work being put in to counter this problem.

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