

# **Laying the foundations for dough-based oat bread**

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# Laying the foundations for dough-based oat bread

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The background of the page features a light, monochromatic image of oat plants. The upper portion shows the long, slender leaves of the oat stalks, while the lower portion shows a pile of oat grain and a mound of oat flour. The overall aesthetic is clean and natural, emphasizing the agricultural origin of the subject.

# CHAPTER 1

## General Introduction

The purpose of this chapter is to introduce the readers of this thesis into the subject of oats geared towards human nutrition in the form of bread applications. This is done by reviewing the position of oat as a crop in the global context, the status of oat in the human diet and, more specific, in the gluten-free diet for people suffering from celiac disease, the state of the art of oat breeding, and the technological properties required for bread applications. Finally, this chapter presents the motivations that we had to investigate the foundations to utilize oat for bread-making applications in a dough-based system and the underlying hypothesis from which the experimental chapters contained in this thesis arose.

### *World production and uses of oats*

Less than 30% of oat production is used for human consumption, while the other 70% is used for livestock feed (Strychar, 2011). In the past, oats used to be a major source of protein for animal feed and played a role in human nutrition locally. In the Netherlands, cultivation of oat declined dramatically from 150.000 ha in 1960, to 1.500 ha in 1980. This reduction was a consequence of the introduction of mechanization in agriculture. The use of tractors displaced horse labour and, as horses were the main oat consumers in the farms, their replacement decreased oat demand for animal feed, with a subsequent decline of on-farm cultivation. Another reason that explains the reduction is that oats have been replaced by higher yielding and better valued crops such as wheat and maize.

World production of oats is estimated at 23 million tons (USDA, 2013). The crop is ranked sixth among other cereals with respect to area (Table 1). 77% of total production is concentrated in Russia, Canada, United States, the European Union (especially Sweden and Finland), and Australia. This group of countries are the main world suppliers of grain oats, seed, and oat-based foods; with Russia as the largest producer and Canada as the largest trader (Strychar, 2011). The EU produces 8.87 million tons of oats, which corresponds to 35% of world production (USDA, 2013).

The yield of oats can vary widely: while the world average yield is 2.04 tons per hectare. Ireland have reached yields of 8 tons per hectare, which is close to the yield of wheat (Table 1). This is relevant because wheat and maize are the main competitors of oats in Europe and yield is one of the relevant factors that farmers take into account to establish a crop. Another key factor that influences that decision is the market. Wheat is easier to commercialize than oats because wheat is the basis of people's diet in many countries, it is widely used to manufacture a large variety of products due to its unique viscoelastic properties conferred by gluten proteins.

**Table 1.** Average yields and maximum yields of cereal crops obtained between 2011-2012.

<b>Crop</b>	<b>Area mill. Has</b>	<b>Average yield tons/ha</b>	<b>Max. yield tons/ha</b>	<b>Country Max. Yield</b>
Wheat	225.0	3.0	8.3	UK
Rice	158.1	4.2	10.0	Egypt
Corn	157.1	5.1	9.7	US
Barley	55.8	2.8	6.8	France
Sorghum	42.2	1.5	6.2	France
Oats	12.7	2.0	8.0	Ireland
Rye	6.5	2.7	6.1	UK

Source: USDA. <http://www.fas.usda.gov/psdonline/circulars/production.pdf>



### *Oats in the human diet*

Oat is considered to be distinct among cereals because it is the highest grain protein and dietary fibre crop among cereals. Its nutritional profile includes various components like phenolic compounds with antioxidant activity, unsaturated oils, and beta-glucans that have been associated with health benefits (Andon and Anderson, 2008). Oat grains are rich in dietary fibre that is the part from the grain that is resistant to digestion in the gastro-intestinal tract. Oat fibre is composed of two fractions: a water soluble and a water insoluble fraction. Beta-glucans are the major component of the water soluble fraction. These fibres are polysaccharides with outstanding functional properties exhibiting high viscosities even at low concentrations (Butt, 2008). Beta-glucans are of particular importance in human nutrition because their solubilisation in the intestine slows down intestinal transit and, as a consequence, delays absorption of glucose and sterols preventing them from reaching the blood (Anderson and Chen, 1986). This is beneficial for human health as it reduces the amount of blood sugar and cholesterol and helps to prevent cardiovascular disease and diabetes (Andon and Anderson, 2008). For these properties, and based on observational and clinical studies, the U.S. Food and Drug Administration (FDA) granted oats and soluble fibres from oats with a health claim that allows their use for therapeutic and disease prevention purposes (FDA, 1997). The EU has also granted four health claims to oats (Regulations EC 1160/2011, and EC 432/2012). This makes oats the crop with the highest number of health claims.

The recommended beta-glucan intake is 0.75 g per serving and 3 g per day (FDA, 1997). This daily intake is estimated to lower serum cholesterol by 5-15%. It has been established that every 1% decrease in serum cholesterol would lead to a 2-3% reduction in the prevalence of cardiovascular disease, which is one of the major causes of death in Western societies (Behall and Hallfrisch, 2011). Some research has also suggested that dietary fibre may have a role in the management of obesity (Pereira and Ludwig, 2001).

Oats have a great nutritional value as it contains iron, vitamins and minerals that contribute to improve the quality of the diet (Butt et al., 2008), in addition to the high content of soluble fibres and the associated health benefits. However, despite this, oats have been under-utilized in human nutrition. The main reason for this is that oat has a lower yield than wheat, which is a major competitor crop. Oat, as well as other crops, have been gradually squeezed by higher yielding and better valued crops such as maize, soybean, and wheat. The use of oats as food has increased from 10 to 30% of total production in the last 40 years (Strychar, 2011). Their main uses in the diet are related to porridge, flakes or breakfast cereals made from crushed or rolled oats. More recently, they are also used to enrich the nutritional value of other products like bread, pasta, noodles and gluten-free products. The industrial applications of oats are limited, but

this is mainly due to a gap of knowledge about their technological properties, which is an obstacle for the food industry to capitalise the healthy image of oats in a wider variety of innovative foods.

### *Oats in the gluten-free diet*

The popularity of oats is growing in the market of gluten-free products because oats constitutes an interesting alternative to diversify and supplement the diet of people that suffer from Celiac Disease (CD). People with CD normally have nutritional deficiencies as a consequence of the disease (Thomson, 2000; Peraaho, et al. 2004).

CD is an autoimmune disease triggered by some peptide sequences present in the prolamin fraction from wheat (gliadins and glutenins), rye (secalins), and barley (hordeins). The prolamin fraction of these cereals is commonly known as gluten proteins. When people with CD eat gluten proteins, some peptides that can resist digestion by enzymes in the stomach reach the small intestine. There, an immune response mediated by T cells may occur if binding of those peptides to T cells happens. The binding depends on very specific nine amino acid sequences. These can cause an immune response that leads to a high turnover of gut cells, with a loss of the finger shaped villi and an increased thickness of the basal part leading to flattening of intestinal mucosa. This causes diverse symptoms like mal-absorption of nutrients and chronic diarrhoea, among others. The only remedy for people with CD is to stick to a long-life gluten free diet.

The disease affects about 1% of the population and has a genetic component (Fasano, et al., 2003), meaning that individuals that have a member in the family diagnosed with the disease, have a larger probability of developing the disease at some point in their lives. The disease prevalence is about 8%-15% among first-degree relatives (Lionetti et al., 2012). Consumption of gluten proteins is a risk factor in genetically predisposed individuals with a HLA-DQ2/8 genotype. Therefore, one of the strategies to prevent CD is decreasing the exposure of consumers, whether diagnosed or undiagnosed, to gluten proteins. This is a challenge because gluten is used in food industry as a “textural quality improver” due to its unique viscoelastic properties. Gluten can be found as a “hidden” ingredient in unexpected products such as sweets, sausages, canned vegetables, and even medical formulations.

The inclusion of oats in the gluten-free diet has been subject of debate since 1950. The doubt has been always present although the safety of pure oats for the majority of CD patients is supported by several clinical and follow-up studies (Thomson, 2003; Janatuinen et al., 1995; Kaukinen et al., 2013), and by the fact that oats are consumed

without any problem by 70% of people diagnosed with CD in Nordic countries (Salovaara et al., 2010). The major concern about oats is contamination with wheat, rye and barley. Hernando et al. (2008) tested commercial oat products for gluten contamination, and found that most of the samples tested were indeed contaminated. This is not surprising because wheat, rye, and barley are cultivated at a larger scale than oats and all are processed in the same mills. Because the chance of contamination of oats with gluten from other cereals is large, CD diagnosed people should not consume oats, unless strict measures are in place to prevent contamination in the whole chain, from cultivation to consumer. In Europe, oat products containing less than 20 ppm gluten are allowed to be labelled and sold as “gluten-free” (Regulation EC 41/2009).

Nevertheless, some CD patients have been shown to respond to two peptides derived from oat avenins (Arentz-Hansen et al., 2004), which are the prolamin fraction of oats. These peptides from avenins, to which immunogenicity was attributed, were different from all known T cell epitopes from wheat, rye, and barley that trigger the disease. The frequency of the response is unknown but seems to be rare. Arentz-Hansen et al. (2004) found 2 out of 9 patients that responded to oats, but the individuals for the study were selected because they presented some discomfort after eating oats.

In addition, monoclonal antibodies (mAbs) raised to detect harmful gluten sequences from wheat, rye, and barley, give signals to oat extracts and these signals vary in strength among oat varieties (Comino et al., 2011; Mujico et al., 2011). This suggested that oats might contain also some of the harmful CD sequences these cereals.

### ***Genetic background of oats***

Oat belongs to the tribe Avenae and not to the tribe Triticeae, to which wheat, rye, barley, spelt, and triticale belong. This explains why oat prolamins are mostly tolerated by CD patients, while prolamins from Triticeae lead to disease.

The cultivated oat plant is a self-pollinated allohexaploid, containing seven chromosomes that combine genetic information from three diploid genomes named AA, CC, and DD (Rajhathy and Thomas, 1974). Cultivated oat evolved later than wheat, as it probably occurred first as a weed contaminant among wheat and barley. It is believed that oat had two centers of origin: the Near East (Iran, Iraq and Turkey) and the western Mediterranean (the Iberian Peninsula, and north-western Africa). Domestication took place simultaneously and independently in different regions, especially in North-Western Europe, because oat grow best in cool, moist, and maritime climates. The earliest cereals were probably genetically uniform, but diversity increased due to accumulation of mutations and to the geographical separation. This resulted

in heterogeneous landraces, some of which have survived, while others have been lost (Valentine et al., 2012).

The genus *Avena* contains diploid, tetraploid, and hexaploid species. In contrast to wheat, wild forms of oat like *A. sterilis*, which is the most likely progenitor of cultivated oats (Li et al., 2000), exist at the hexaploid level. The common cultivated *Avena sativa* L. spread from the Near East to central-north Europe. The red oat, *A. byzantina* K. Koch, was the main cultivated species in North Africa. *A. byzantina* was more suitable for autumn sowing, while *A. sativa* became the basis of the spring-sown crop of higher latitudes. Both were introduced into United States in the sixteenth century, and there they were interbreed, so that modern cultivated *A. sativa* contains some germplasm from *A. byzantine* (Valentine, 2012). Next to the husked oats, naked oats have been originated in China and are becoming increasingly popular in Europe (Burrows, 2011).

### ***Status of oat breeding for food***

The breeding of oats has produced high yielding oat varieties, helping the crop to gain terrain in agriculture and in the market. Yield is the main objective of most breeding programs as yield is one of the major factors that affect decision making in farmers. Oat competes with other cereal crops, like maize and wheat, that have had historically higher yields and have concentrated most of the research efforts, not only related with breeding, but also with developing the food industry.

Some of the breeding strategies to obtain high yielding varieties have pointed towards lodging resistance, disease and pest resistance, and tolerance to abiotic conditions (winter and drought). Resistance to lodging has been achieved by introduction of dwarfing genes to reduce plant height. The sources of resistance against crown rust and powdery mildew derived from the hexaploid *A. sterilis*, and the diploids *A. strigosa* and *A. pilosa* (Valentine et al., 2012).

Milling yield is a very important economical factor also targeted in breeding programs (Valentine et al., 2000). Millers demand varieties with a high milling yield which is conferred by high kernel content, thin-husked grains, and hullability -how strong the husks are attached to the grains-. Grain uniformity and distribution are also important criteria for millers, as they do not want to use different settings to separate grains.

Regarding composition, breeding oats for human nutrition purposes, has aimed to produce varieties with higher beta-glucan content because of its health related benefits. The beta-glucan content in commercial oat varieties ranged between 4.5 and 5.5% (Peterson et al., 1995; and Sastamoinen et al., 2004), but through several hybridizations using a high beta-glucan base population, it was possible to increase beta-glucan

content to 7.1% (Cervantes-Martinez et al., 2007). Breeding for high beta-glucan has focussed on total content but not on composition regarding molecular weight, which has a large effect on the rheological behaviour of beta-glucans (Colleoni, et al., 2003).

Oats have not been bred for other applications different to flakes, porridges or cereal breakfast. It is accepted that oats are not suitable for pastry or bread applications because it lacks gluten, even though not much research has been done to explore new applications. Differences in oat cultivars regarding bread-making potential for gluten-free formulations based on a pancake-like system (known as batter) were reported by Huttner et al. (2011). However, it is unknown which factors are relevant for bread-making and whether high beta-glucan content, for example, will be suitable for bread applications. The same applies to fat content that is remarkably high in oats in comparison to wheat. This high fat content in oats requires an extra processing step to avoid damage of the sensory quality of the grains. While a bulk of research has been done in wheat to set the criteria to determine whether a genotype is suitable for a specific purpose, e.g., pasta, bread or pastry applications, such criteria for selection are lacking for oats.

### ***Technological properties required for bread applications***

Wheat flour is widely used for a wide variety of food applications thanks to its unique viscoelastic properties, which are conferred mainly by gluten proteins. Gluten forms a strong reversible network essential to develop a dough with the ability to retain gas during proving and baking (Lee, et al., 2003). The term gluten is used to make reference to two types of proteins: glutenins and gliadins. Both types of proteins fulfil different roles in the formation of the protein network in wheat flour; while glutenins form a network that can be stretched and recover after deformation, gliadins act as a plasticizer affecting the viscous properties of the dough. A good quality dough is therefore the result of a balance between elastic and viscous properties (Wieser, 2007).

Oats lack gluten proteins. This has been the main reason mentioned to explain the small diversity of oat products available in the market (especially limited to flakes, porridges and breakfast cereals), as these kind of products do not require the viscoelastic properties that are relevant for bread or pastry applications. Oats have been used mainly to enrich the fibre content of wheat breads after optimizing the formulation and the process to avoid compromising quality, because oat flour/meal interferes with gluten formation (Flander et al., 2007). Gluten-free bread applications based on oats have been explored using batter systems instead of dough systems, but the results obtained regarding quality (specific volume and texture) are inferior to those obtained for wheat based products (Huttner et al., 2011). Batter systems have the disadvantage

of containing a large amount of water (95 -120%), and this is a clear downside because affects shelf life and microbiologic activity of the final breads. Consequently, working with a dough system would be beneficial as any reduction on the amount of water in the system would help improving quality parameters, such as shelf life, in the final breads. Oats represent an interesting alternative to current gluten free formulations because with oats it is not necessary to add additional (expensive) thickeners on which current formulations rely on. In addition to this, oats constitute a healthy and tasty base for bread -and other - food applications.

### ***Motivation of this thesis and underlying hypothesis***

The motivation to perform this study was to generate the fundamentals to use oats for bread-making applications, with the view to obtain an oat bread as similar as possible to wheat bread. This will offer consumers a healthier alternative product to wheat bread in their daily diet. This will contribute to improve the nutritional quality of the diet, helping to reduce the risk of cardiovascular problems and diabetes due the intake of soluble fibres. This thesis focusses on the dough system for bread-making, so that processing could be done using the existing technology that bakers are familiar with, which is adapted to work with dough instead of batter. To be able to lay the foundations for oats for bread-making purposes, we needed to answer a series of questions and set experiments based on the following hypothesis:

- 1) Oats are safe for the majority of people that suffer from celiac disease because the toxic peptides from wheat, rye, and barley are absent in oats.
- 2) Reproduction of dough extensibility properties of wheat flour is possible using oat flour in combination with a network-forming agent.
- 3) The current kilning and milling processing methods applied to oat grains interfere with bread-making purposes.
- 4) Beta-glucans, that are beneficial for health, interfere with bread-making purposes.

We address the underlying hypothesis in this thesis, which is divided in two parts and five experimental chapters (Figure 1). In Part I, we clarified the status of oats in the gluten-free diet. In Part II, we developed first a dough testing system using gluten to study how compositional and processing factors could affect dough properties for bread-making purposes. Then, we studied whether whey protein particles (WPP) could be used as structuring agent, instead of gluten, to improve the technological properties of oat flour in a gluten-free setting. In the general discussion we present a summary of the most relevant findings and their implications for the use of oats for bread applications.

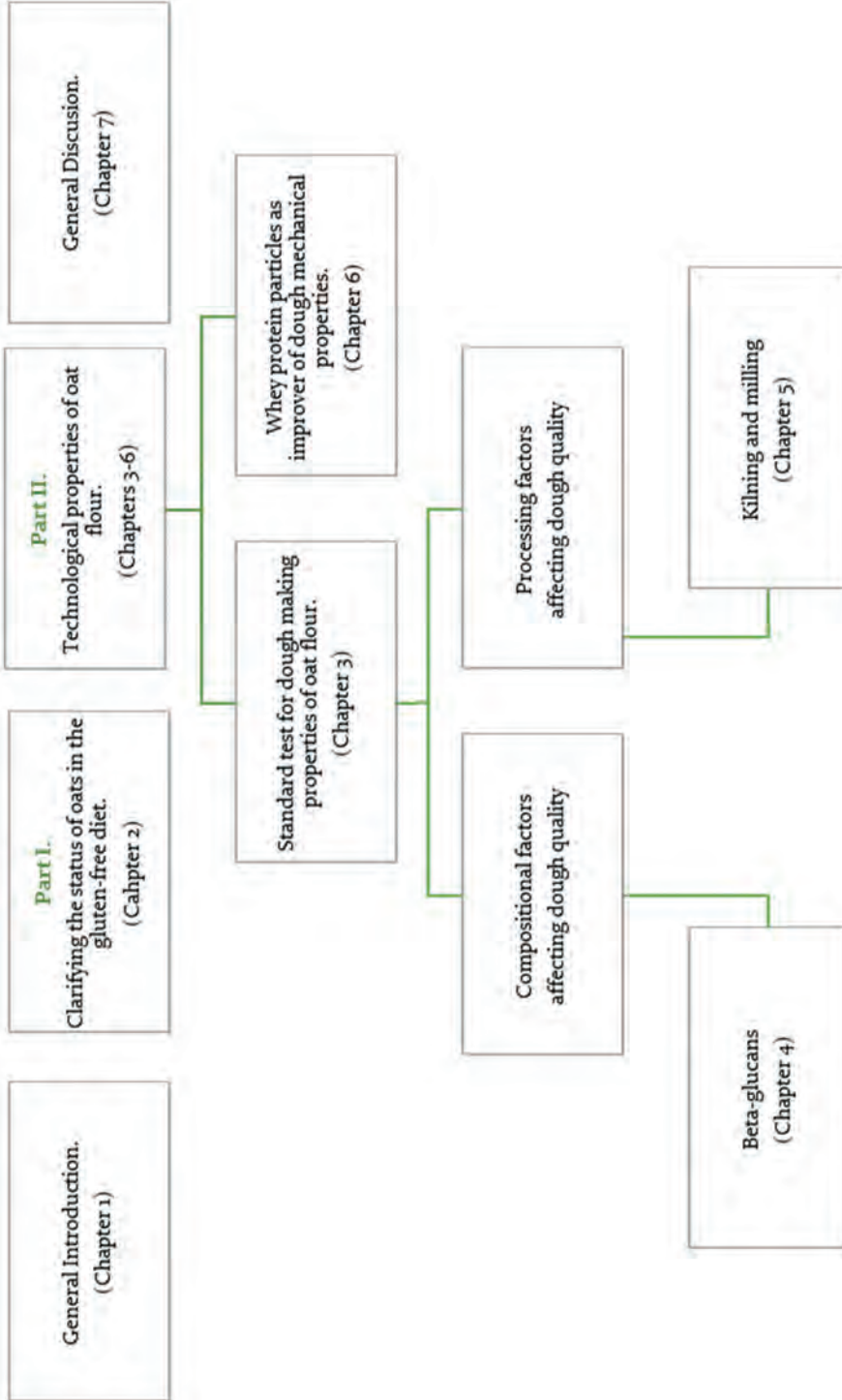


Figure 1. Schematic overview of the thesis.



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# **Part I**

**Clarifying the safety of oats  
for people with celiac disease**



## CHAPTER 2

### **Avenin diversity analysis of the genus *Avena* (oat). Relevance for people with celiac disease**

#### **ABSTRACT**

Oat is widely consumed by people with celiac disease (CD). Its safety has been disputed because two peptides from oat avenins can be recognized as T cell epitopes by some CD patients. Differential signals of gluten-specific monoclonal antibodies and in-vitro T cells to oat varieties have suggested the existence of differences in immunogenicity. We aimed to clarify the nature of such responses by cloning avenin genes from 13 *Avena* species. A single oat plant contained up to 10 different avenin genes. Based on sequence homology, the 78 different avenin proteins clustered in four groups of which two contained the two avenin CD epitopes. All *Avena* species examined harbored avenins of these two groups, and as a consequence all contained avenins with the two avenin-specific epitopes, which makes it very unlikely to find oat cultivars that are devoid of these sequences. The established gluten epitopes from wheat, rye, and barley were not present in oat avenins; some variants with two and three amino acid substitutions occurred, but they were predicted to not resist proteolysis in the gastro-intestinal tract. Perfect recognition sites of antibodies R5 and G12 were also not present in avenins. Thus, monoclonal antibody signals to oat are probably due to cross-reactive or promiscuous recognition of avenin peptides, and such signals should not be interpreted as differences in immunogenicity of oat varieties for CD patients.

Keywords: Gluten-free, celiac disease, epitope, R5 antibody, G12 antibody.

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## INTRODUCTION

Celiac disease (CD) is an intolerance to gluten proteins (prolamins) from wheat, rye and barley that leads to a chronic inflammation of the small intestine and affects about 1% of Western population. The only remedy for CD patients is a gluten-free diet. There is an increasing demand for gluten-free products to diversify this diet and oat is considered an interesting alternative because it contains healthy compounds (essential amino acids, unsaturated fatty acids beta-glucans, polyphenols) that can supplement the diet of patients and help to prevent diabetes and vascular diseases (Andon and Anderson, 2008).

It has generally been accepted that CD patients can consume oats without detrimental inflammation of the small intestine (Thompson, 2003). A systematic study with 52 patients revealed that most of them, whether in remission or newly diagnosed, can add moderate amounts (about 50 g/day) of oats to their gluten-free diet without any harmful effects (Janatuinen et al., 1995). Another study described how children with CD tolerated oats in their gluten-free diet for a period of two years in which they were regularly monitored (Koskinen et al., 2009). Pulido et al. (2009) analyzed the studies published between 1995 and 2009, and concluded that the majority of adults and children with CD can tolerate moderate amounts (from 20 g/day for children to 70 g/day for adults) of pure oats. Probably the largest problem with oats for CD patients is the fact that most commercial oat products are contaminated with wheat (Gelinass et al., 2008). Contamination can start in the field when wheat or barley plants grow among oat plants and are not removed systematically, but contamination can also occur later on in the food production chain, during storage or processing. Consequently, a guaranteed gluten-free oat production chain is an essential requirement for CD-safe oat production. Such a chain exist in several Nordic countries (Sweden, Finland) where 70% of patients eat oats regularly as a healthy component of their gluten-free diet (Salovaara et al., 2010) and it was recently established in The Netherlands. Oat products containing less than 20 ppm gluten are allowed to be labeled and sold as “gluten-free” in Europe, because of its general safety and its contribution to a healthy diet.

Medical research has produced an extensive list of epitopes intrinsically present in wheat, rye and barley that are recognized by T cells from CD patients (Sollid et al., 2012). In wheat, HLA-DQ2/8 T cell epitopes occur in various gluten protein families:  $\alpha$ - and  $\gamma$ -gliadins, LMW- and HMW-glutenins. Presence and number of T cell epitopes per gluten molecule differ among members of these multigene storage protein families (van Herpen et al., 2006) and among individual cultivars (Spaenij-Dekking et al., 2005), due to naturally occurring amino acid substitutions in the epitopes (Mitea et al., 2010). Oat contains only one family of prolamins called avenins, which makes up 10-15% of the total

seed protein content, in contrast to wheat, in which prolamins represent 80% of the total seed protein content. Four cases of oat avenin-sensitive CD patients have been described and the response has been attributed to three specific avenin-derived peptides that are different from all known T cell epitopes from wheat, rye and barley (Arentz-Hansen et al., 2004; Lundin et al., 2003; Vader et al., 2003). Recently a new nomenclature for CD epitopes was released based on unified criteria that the peptides should accomplish in order to be considered CD epitopes (Sollid et al., 2012). This list only includes two avenin-specific epitopes present in oats that can induce T cells.

Recently, a new discussion emerged based on responses of monoclonal antibodies (mAbs) and T cell clones to oat extracts *in vitro*. Silano et al. (2007) found that avenins from four oat varieties activated peripheral T cell clones from ten CD children on a gluten-containing diet. Comino et al. (2011) found that protein extracts from two of three groups of commercial oat varieties induced G12 mAb binding, and especially variety OM719 was able to induce proliferation and IFN-gamma production of peripheral blood mononuclear T cells from patients who were on a gluten-containing diet. The reactions were not due to contamination of the oats used. Mujico et al. (2011) observed differences in the binding of mAbs raised against a wheat LMW-glutenin derived peptide to oat cultivars. A possible relation was recently suggested between oat consumption and the persistence of duodenal intraepithelial lymphocytosis in CD patients on a gluten-free diet (Tuire et al., 2012).

These results raised the question whether toxicity in oats may have been overlooked, which would have implications on whether promotion of oat consumption by CD patients is justified. Alternative explanations of these results include cross-reactivity of mAbs with other peptides that are not necessarily immunogenic for CD patients and promiscuity of T cells that have been induced by wheat gluten and that cross-react with variants of the canonical T cell epitopes (Anderson et al., 2000; Vader, Stepniak et al., 2003). Unfortunately, only few avenin sequences are known (Chesnut et al., 1989), and diversity within a single oat variety has not been studied, so it is unknown whether peptides in avenins could cause such responses.

The objective of this study was to determine the frequency of avenin-specific T cell epitopes, and whether T cell epitopes known from wheat, rye or barley are present in avenins. We also aimed to determine variation in copy number and sequence similarity among genes, and to estimate diversity between varieties and species. To achieve these objectives, we cloned and sequenced avenin genes present in the commercial hexaploid oat cultivar 'Gigant' and in accessions of 12 diploid and tetraploid *Avena* species, representing various A and C genomes within the genus *Avena* (Loskutov, 2008). We also analyzed an EST library from another hexaploid oat cultivar, 'Dancer'.

## MATERIALS AND METHODS

### *Plant material*

Genomic DNA of twelve diploid and tetraploid *Avena* species representing the major genomes in *Avena* was obtained from the Vavilov Institute of General Genetics, Moscow, Russia (Table 1). We also included the hexaploid *Avena sativa* cultivar ‘Gigant’ from which genomic DNA (gDNA) was extracted using the DNeasy Plant Mini kit (Qiagen, Venlo, The Netherlands).

Grains in two different developmental stages (watery and early milk stage) were collected from the hexaploid *Avena sativa* ( $2n=6x=42$ ) cv. ‘Gigant’ in an agricultural production field located in Munnekezijk, Groningen, The Netherlands. The hulls were removed to classify the grains according to developmental stage. The naked grains were frozen in liquid nitrogen and stored at -80 C until processing. Messenger RNA was extracted using TRIzol and the RNeasy Plant Mini kit (Qiagen, Venlo, The Netherlands), and cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands).

### *Primer design*

The genes for storage proteins in cereals are often present in multiple copies per genome, which may be highly similar to each other. To obtain avenins from oat we implemented a strategy used to reliably obtain gamma-gliadin sequences from wheat despite the high similarity between various gene family members, only accepting a sequence if it was obtained in two independent PCR reactions (Salentijn et al., 2012). Primers were designed on conserved regions at the 5’ and 3’ ends of the coding sequence of 5 avenin genes described by Chesnut et al. (1989) and on avenin ESTs from ‘Dancer’ available in EMBL/GenBank, which assembled into 11 contigs of > 99% identity (Table 2). Forward primers were designed on a part of the avenin signal peptide MKTFLI. Reverse primers were designed on the 3’ end of the avenin genes, the stop codon TAA being present in the centre or at the 3’ end of the reverse primer. The primers were synthesised by Biolegio, Nijmegen, The Netherlands. In total, 7 different combinations of the following forward (F) and reverse (R) primers were used:

F<sub>2</sub> 5’-ATGAAGAACTTCCTCATC-3’

F<sub>4</sub> 5’-ATGAGGACCTTCCTCATC-3’

F<sub>d1</sub> 5’-ATGAARACMTTTCYCATC-3’

F<sub>d2</sub> 5’-ATGAAGAMCTTYCTCATC-3’

R<sub>1</sub> 5’-AGTGTTCCTTAGAAGCCAC-3’



R2 5'-AGTCTTCTCAGAAGCCAC-3'

R3 5'-AATCTTCTTAGCAACCAC-3'

R6 5'-CCCTTATAGTCTTCTCAG-3'

Rd2 5'-CYCTTRTAGTSTTCTYAG-3'

### ***Cloning and sequencing of avenin genes from gDNA and cDNA***

The set of seven primer pairs was used to amplify avenins from cDNA of cv. Gigant and gDNA of diploid and tetraploid *Avena* species (Table 1). Each reaction contained 50 ng gDNA or 2 ml cDNA, 75 mM Tris-HCl pH 9.0, 20 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.01 % (w/v) Tween-20, 2.5 mM  $\text{MgCl}_2$ , 250 mM of each dNTP, 200 nM of specific primer 01200 nM of degenerate primer, and 1 U Goldstar Taq (Eurogentec, Liege, Belgium). The PCR reactions were performed in a Dyad Thermal Cycler PTC220 (Bio-Rad) starting with an initial denaturation step for 5 minutes at 94°C followed by 1 cycle at 94°C for 1 minute, 56°C for 1 minute and 72 °C for 2 minutes. The 56°C annealing temperature was subsequently reduced by 1°C for the next 9 cycles, and continued at 47 °C for a remaining 26 cycles. A final extension followed at 72°C for 10 minutes.

The PCR products, containing a mixture of gene sequences, were ligated into the pGEM®-T Easy Vector (Promega Benelux, Leiden, The Netherlands) and used for transformation of *E. coli* XL1-blue supercompetent cells (Stratagene Europe, Amsterdam, The Netherlands). The white colonies were picked and grown overnight at 37°C in LB-freezing medium into 96-well flat bottom plates. Each colony PCR contained 1 µl of overnight culture, 75 mM Tris-HCl pH 9.0, 20 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.01 % (w/v) Tween-20, 1.5 mM  $\text{MgCl}_2$ , 100 mM of each dNTP, 250 nM of both M13 forward and reverse primer and 0.5 U Goldstar Taq (Eurogentec). PCR was performed in a PTC200 (MJ Research, Bio-Rad) with an initial denaturation step at 94 °C for 5 minutes followed by 35 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 2 minutes and a final extension step of 72 °C for 10 minutes. The colony PCR products were sequenced with M13 forward and reverse primers using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences Benelux, Roosendaal, The Netherlands) on an ABI3710 automated sequencer (Applied Biosystems). On average 16 clones per product were sequenced.

### ***Analysis of sequences***

The traces were checked manually and subsequently assembled using Lasergene 8.0 (DNASTar, Madison, WI). The mismatches within each contig were checked manually on the forward and reverse traces. Only sequences containing SNPs that were supported

by two or more clones from at least two different PCR events were considered as genuine. Sequences obtained from cDNA and gDNA of cv. Gigant were assembled into 7 and 10 contigs of >99% identical sequences, respectively. Sequences obtained from diploid and tetraploid *Avena* species assembled into 41 contigs of >99% identical sequences; some contigs contained identical avenin genes from different *Avena* species. The sequences reported in this paper have been deposited in EMBL under Accession numbers FN706441-FN706450, HE576937-HE576976, and HE801178-HE801213.

An EST library containing 231 sequences of cv. ‘Dancer’ found in NCBI on May 2009 was analyzed also. The sequences with accession numbers [NCBI: G0584576.1, G0583621.1 and G0584748.1] were removed from the data set because they did not have the expected size and did not share the common features with the other sequences, e.g. the characteristic presence of eight cysteine residues in the translated amino acid sequence. All sequences were translated into amino acid sequences and used to perform a Bootstrap Neighbor-Joining test (1000 replicates) using MEGA 4.

### ***Screening for CD toxic epitopes***

All translated avenin protein sequences were screened for the presence of celiac-immunogenic or -toxic peptides (epitopes) described up to date in the literature (Sollid et al., 2012) as well as all possible variants of these epitopes in oats with 1, 2 or 3 amino acid substitutions. In silico digestion of avenin proteins was done using PeptideCutter (<http://expasy.org/>) taking into account the specificities of trypsin, pepsin (pH 1.3, and pH>2), and chymotrypsin (high and low specificity).

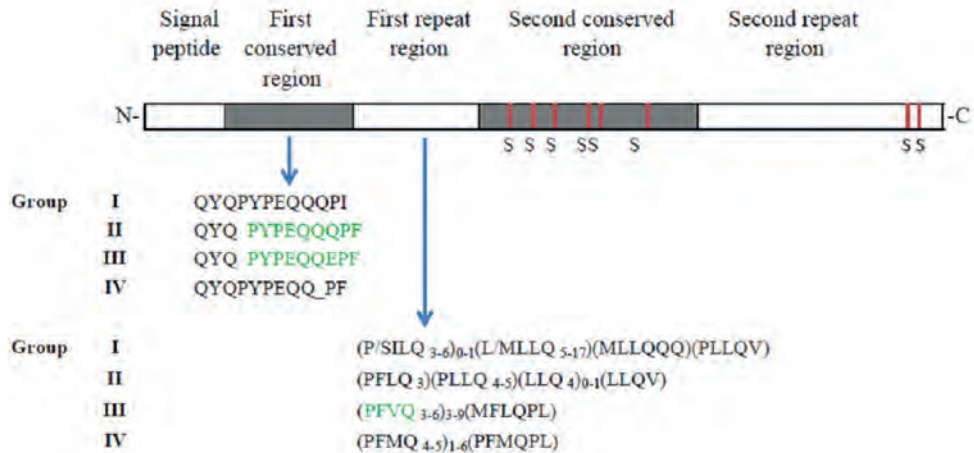
### ***SDS-PAGE***

Oat avenins were extracted from 100 mg of commercial oat flour by addition of 0.5 ml of 50% (v/v) aqueous iso-propanol with continuous mixing (MS1 Minishaker, IKA Works, Inc.) at 1000 rpm for 30 min at room temperature, followed by centrifugation at 10,000 rpm for 10 min at room temperature. The residue was re-extracted once with 0.5 ml 50% (v/v) aqueous iso-propanol. The two obtained supernatants were combined and can be considered to be the complete avenin protein extract, as shown by van den Broeck et al. (2011). The avenin protein content was quantified using the Bio-rad Protein Assay (Bio-Rad), based on the Bradford dye-binding procedure, according to manufacturer’s instruction with BSA as a standard. Avenins were separated on SDS-PAGE gels (11%) using a Hoefer SE 260 mighty small II system (GE Healthcare) followed by staining with PageBlue (Fermentas, Thermo Scientific, St. Leon-Rot, Germany).

## RESULTS

### *The avenins of oat*

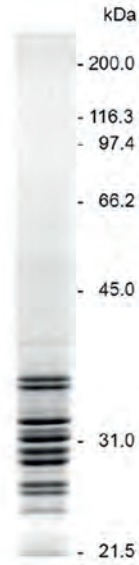
The general structure of avenin proteins is depicted schematically in Figure 1. Similar to other prolamins they have a high proline and glutamine content, low content of lysine, and they are insoluble in water. In total 717 avenin sequences were obtained from gDNA of 13 diploid, tetraploid, and hexaploid *Avena* species containing the three major genomes (A, D, C) that occur within the genus. These sequences assembled in 83 contigs of >99% identical sequences, conservatively representing 78 different genes and 5 pseudogenes (with internal stopcodons). Some contigs were composed of sequences from different species. The number of genes obtained from diploid and tetraploid accessions ranged from five to seven in diploid species, and from four to eight in tetraploid species. The number of genes obtained from gDNA of the hexaploid *A. sativa* cultivar ‘Gigant’ was 10 (Table 1).



**Figure 1.** Schematic structure of avenin proteins with typical amino acid motifs indicated. Gray areas correspond to highly conserved domains. Vertical red lines represent cysteines (S) conserved throughout avenin proteins. The location of the avenin epitopes according to current (Sollid et al., 2012) and old nomenclature (Vader et al., 2003) are indicated in green within the motifs. After Shewry et al. (1995).

Gene expression was studied in kernels of ‘Gigant’ in early stage of development. From cDNA we cloned and sequenced 120 PCR products (from two independent reactions) that assembled into only 7 contigs of >99% identical sequences (606 to 810 bp in length), indicating that 7 of the 10 different avenin genes obtained from gDNA were

expressed during early development. It is likely that during later developmental stages of 'Gigant' a few more genes will be expressed since SDS-PAGE analysis of proteins from mature seeds of 'Gigant' showed 12 clear protein bands (Figure 2). The EST library of cultivar 'Dancer' assembled into 11 contigs (S<sub>1</sub>).



**Figure 2.** SDS-PAGE (11%) analysis of an avenin extract from *A. sativa* cultivar Gigant followed by PageBlue staining. The apparent weight on the gel is somewhat higher than the molecular weight of avenins of 'Gigant' (21-31 kDa) as predicted based on the sequences.

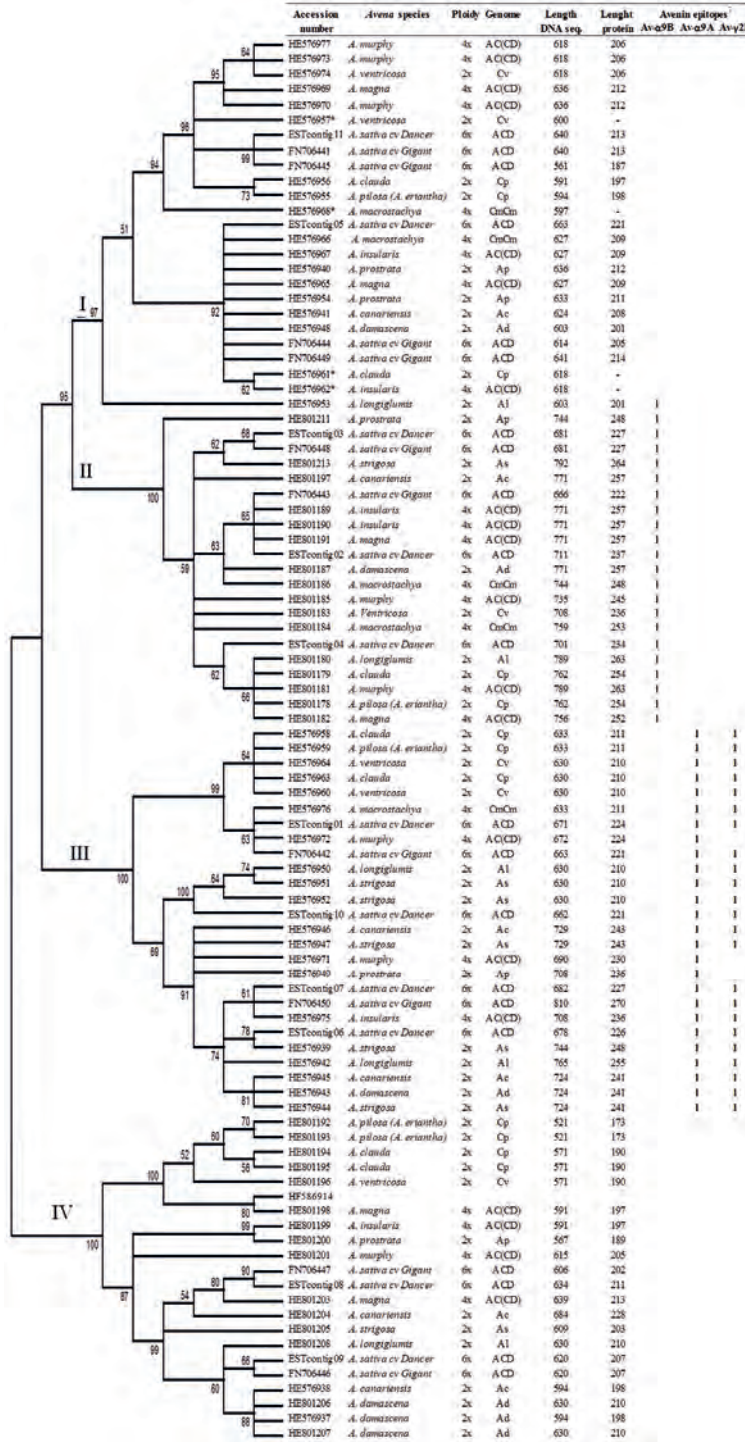
Diploid species (*A. ventricosa* and *A. clauda*) and tetraploid species (*A. macrostachya*, *A. murphy* and *A. insularis*) contained one sequence with internal stop codons (Table 1). According to an unrooted neighbor joining analysis, avenin genes clustered into four groups (I-IV; Figure 3), with *Avena* species representative of all genomes in each group. From this we deduce that, even if we not have cloned all members of the gene family from each accession, we did cover the diversity present in the gene family across the genus. The N-terminal sequences exhibited a very high homology (S<sub>2</sub>), which is in accordance with the results presented by Pernollet et al. (1987). The length of the predicted proteins ranged from 187 to 270 AA. Differences in length were mainly due to presence of repetitive motifs within non-conserved regions (between positions 34-89 and 161-301 after the signal peptide). The predicted molecular weight of the avenins of 'Gigant' is 21-31 kDa, which is low in comparison to prolamins from wheat, rye and barley whose molecular weights range from 25-100 kDa (Tatham et al., 2000).

**Table 1.** Summary of avenin genes obtained from genomic DNA of diploid, tetraploid, and hexaploid *Avena* species.

<i>Avena</i> species	Ploidy	Sample <sup>1</sup>	Cat#	Genome <sup>2</sup>	# of avenin genes	# of pseudogenes	# of genes with avenin epitopes
<i>A. canariensis</i>	2x	121	k292	Ac	6	0	3
<i>A. clauda</i>	2x	72	CN19217	Cp	6	1	3
<i>A. ventricosa</i>	2x	53	CN21992	Cv	5	1	3
<i>A. longiglumis</i>	2x	534	k87	Al	5	0	4
<i>A. strigosa</i>	2x	127	k5196	As	7	0	6
<i>A. pilosa</i>	2x	148i	CN73755	Cp	5	0	2
<i>A. prostrata</i>	2x	86	2055	Ap	5	0	2
<i>A. damascena</i>	2x	57	CN19457	Ad	6	0	2
<i>A. macrostachya</i>	4x	142	-	CmCm	4	1	3
<i>A. murphy</i>	4x	542	2088	AC <sup>2</sup>	8	1	4
<i>A. magna</i>	4x	123	k1852	AC <sup>2</sup>	6	0	2
<i>A. insularis</i>	4x	138	-	AC <sup>2</sup>	5	1	3
<i>A. sativa</i> cv Gigant	6x	-	-	ACD	10	0	4

<sup>1</sup> ID of the gDNA of diploid and tetraploid *Avena* species in the Vavilov Institute of General Genetics, Moscow, Russia.

<sup>2</sup> Genome AC(CD) of tetraploid species is represented by AC in this article.



**Figure 3.** Unrooted Neighbour Joining (NJ) tree of avenin genes from diploid, tetraploid and hexaploid *Avena* species. The avenin genes of *Avena* species with different ploidy levels formed four groups of sequences that are indicated as I-IV. Bootstrap values based on 1000 replicates are indicated (as %). Sequences containing avenin epitopes Av-α9B and Av-α9A (Sollid et al., 2012) and Av-γ2B (Vader et al., 2003) are also indicated on the corresponding column for each epitope. The epitopes from wheat, rye and barley are absent in avenin sequences of the species analyzed.



### ***Presence of celiac disease epitopes in avenins***

To estimate the T-cell stimulatory capacity of oat avenins, all predicted avenin proteins were screened for the presence of known CD T cell epitopes (Sollid et al., 2012) and variants thereof, as some substitutions may not abolish T cell activation (Mitea, et al., 2010). Next, the potential protease resistance of the deduced peptides was analyzed. Finally, the occurrence of recognition sites for the R5 and the G12 antibodies was verified.

The avenin-specific T cell epitopes PYPEQQQPF (Av- $\alpha$ 9B) and PYPEQQEPF (Av- $\alpha$ 9A) were present and occurred only once per protein. They were located in the first conserved region of avenin proteins: Av- $\alpha$ 9B was present in all genes of group II, whereas Av- $\alpha$ 9A occurred in all genes of group III. The motif QQP $\nu$ VQQQPFVQQ, which used to be considered as an epitope (Av- $\gamma$ 9B) but according to the new nomenclature it does not fulfill all criteria (Sollid, Qiao et al., 2012), was located immediately downstream of Av- $\alpha$ 9A in the first repetitive region in most of the sequences of group III (Figure 1). Avenins of groups I and IV did not contain the canonical avenin-specific T cell epitopes but variants thereof: PYPEQQPF $\underline{M}$  in proteins of group IV, and PYPEQQQPI or PYPEQQQSI in proteins of group I. As a consequence, Av- $\alpha$ 9B and Av- $\alpha$ 9A were found in avenins of the A/D and the C genomes, and in each of the 13 *Avena* species studied. Four of 10 avenin genes of hexaploid cultivar ‘Gigant’ contained at least one of the canonical avenin-specific T cell epitopes. In ‘Dancer’ 7 of 11 transcripts encoded one of these T cell epitopes (3 contained Av- $\alpha$ 9B and 4 contained Av- $\alpha$ 9A).

None of the gluten T cell epitopes from wheat, rye and barley were detected in avenins, but some variants with one, two and three amino acid substitutions were found (Table 2). The two variants with one amino acid substitution corresponded to the already described avenin T cell epitopes. Interestingly, the variants with two and three substitutions were predicted to be proteolysed in the gastrointestinal tract when we performed the analysis using the known specificities of the three enzymes, trypsin, chymotrypsin, and pepsin. In this analysis also the two described avenin epitopes did not completely resist proteolysis as the phenylalanine was removed, leaving QPYPEQQQP and QPYPEQQEP peptides. If pepsin is omitted from the analysis, some variants are predicted to survive digestion (Table 2).

We further screened our set of avenin proteins for the 5-mer and 6-mer amino acid sequences that are recognized by the R5 antibody and the G12 antibody, respectively, which are used to detect gluten in an ELISA test. The R5 antibody recognizes QQPFP, LQPFP, LQPFP, QLPYP, and, to a lesser extent, QLPTF, QQSFP, QQTFF, PQPFP, QQPYP, and PQPFP (Valdes et al., 2003), with an overall recognition motif of Q(P)-Q(P,L)-

Q(L)-PF/YP (Osman et al., 2001). The G<sub>12</sub> antibody recognizes the sequences QPQLPY, QPQQPY and QPQLPF (Moron et al., 2008). All those antibody recognition sequences were absent in avenin proteins. Variants of these recognition sites were present: with one amino acid difference for the R<sub>5</sub> recognition site (Table 3), and with at least two amino acid differences for the G<sub>12</sub> recognition site (Table 3). Two out of four R<sub>5</sub>-related variants and four out of six G<sub>12</sub>-related variants are present in sequences that contain also the avenin-specific epitopes, so it might be possible that they relate to the number of those epitopes but this relation should be proved. All those variants are predicted to be degraded by pepsin, trypsin and chymotrypsin.

**Table 2.** T cell epitope variants present in oat avenins that are predicted to resist trypsin and chymotrypsin digestion. T cell epitopes from wheat, rye and barley were not present in oat avenins, but variants with one, two, and three amino acid differences (in red and underlined) were present in avenin sequences. Of the 89 proteins of Figure 3 only the eight variants listed here resisted in silico trypsin and chymotrypsin proteolysis. None of them remained completely intact if pepsin was also added.

T cell epitopes Deamidated	Epitope variants present in oat avenins that are predicted to be resistant to trypsin and chymotrypsin Digestion								
DQ2.5-glia-γ2 IQPEQPAQL (from wheat)	<u>N</u>	Q	P	Q	Q	<u>Q</u>	A	Q	<u>F</u>
	I	Q	P	Q	Q	<u>L</u>	<u>P</u>	Q	<u>Y</u>
DQ2.5-glut-L2 FSQQQESPF (from wheat)	<u>V</u>	<u>Q</u>	Q	Q	Q	Q	<u>Q</u>	P	F
DQ2.5-hor-1 PFPEQPEPF (from barley)	P	<u>Y</u>	P	<u>E</u>	<u>Q</u>	Q	Q	P	F
DQ2.5-sec-1 PFPEQPEPF (from rye)	P	<u>Y</u>	P	<u>E</u>	<u>Q</u>	Q	Q	P	F
DQ2.5-ave-1a PYPEQEEPF (from oat)	P	Y	P	E	Q	Q	<u>Q</u>	P	<u>I</u>
DQ2.5-ave-1b PYPEQEPPF (from oat)	P	Y	P	E	Q	Q	Q	<u>S</u>	<u>I</u>
	P	Y	P	E	Q	Q	Q	<u>Q</u>	<u>L</u>



**Table 3.** Variants of the recognition sites of R5 and G12 antibodies with one and two amino acid substitutions. No perfect recognition sites were present in oat avenins, but some variants with one or two amino acid substitutions (in red and underlined) were found.

Antibody	Reported antibody recognition sites	Variant in oat avenins with one (R5) or two (G12) amino acid substitution						% sequences containing the variant	linked to avenin epitopes	
R5	QQQFP	Q	Q	P	F	<u>L</u>		23.4	+	
		Q	Q	P	F	<u>V</u>		27.6	+	
		Q	Q	P	F	<u>M</u>		23.4	-	
	QQQYP	<u>Y</u>	Q	P	Y	P		100	-	
G12	QPQLPY	Q	P	Q	L	<u>Q</u>	<u>Q</u>	73.4	+	
	QPQQPY	Q	P	Q	Q	<u>Q</u>	<u>A</u>	40.4	+	
		Q	Q	Q	Q	P	F		48.9	+
		Q	P	Q	Q	<u>L</u>	<u>P</u>		14.9	-
		Q	P	Q	Q	<u>L</u>	<u>S</u>		6.4	-
	QPQLPF	Q	P	Q	L	<u>Q</u>	<u>L</u>	8.5	+	

## DISCUSSION

This work presents, for the first time, a thorough evaluation of the sequence diversity of avenin genes across the entire genus *Avena* (oat). We have studied the presence of celiac disease DQ2/8 T cell epitope sequences of wheat, barley and rye (according to current international agreement (Sollid et al., 2012)), the occurrence of the two avenin-specific CD epitopes (Arentz-Hansen et al., 2004), and the existence of recognition sequences of several mAbs that are applied in commercially available gluten detection kits. The results enable to discuss deeply the safety of oats for patients suffering from celiac disease and the limitations of *in vitro* immunogenicity data in heterologous plant material.

### *Avenin gene number*

According to the estimations in oat made by Chesnut et al. (1989) based on Southern hybridization, and in line with large numbers of the homologous gliadin genes in wheat (van Herpen et al., 2006), at least 25 avenin genes (and pseudogenes) were expected to be present in oat. However, we detected only 10-11 avenin genes in the hexaploid cultivars ‘Gigant’ and ‘Dancer’. It is possible that some cultivars contain loci with more genes through additional gene duplications. Alternatively, we may have underestimated

the number of genes as we conservatively combined all sequences with 99% or higher similarity into one gene contig, thus possibly lumping highly similar genes. On the other hand, indirect evidence that the gene family is not very large comes from the fact that we did not find any pseudogenes in hexaploid *A. sativa* cultivars, and only very few in the sequences from other *Avena* species (overall 5 of 78 distinct avenin genes, or 6%). Pseudogenes may be the hallmark of an expanded gene family of storage proteins (in wheat, 87% of the  $\alpha$ -gliadins were pseudogenes (van Herpen et al., 2006)). It is not likely that our primers systematically would have missed particular groups because EST sequences clustered in the same groups.

A low number of avenin genes compared to gluten genes in wheat is consistent with the difference in the prolamins fraction of the storage protein composition (10-15% of the total seed protein content in oats, compared to about 80% in wheat (Tatham et al., 2000)). The low number of proteins is supported by the similar number of bands on an SDS-PAGE gel. Van den Broeck et al (2011) extracted avenins from a commercial oat flour and distinguished 12 protein bands (SDS-PAGE) and found that each band corresponded to a single avenin protein on a 2D-gel.

### ***Avenin gene family expansion***

A neighbor-joining analysis showed that the avenin gene sequences of the entire *Avena* genus representing the diploid, tetraploid and hexaploid species and genomes (A/D and C) (Loskutov, 2008) obtained in this paper, clustered in four groups (Figure 3). The genes in the four groups differed from each other by sequence differences in the two conserved regions, and by differences in repeat motifs and the number of repeat units in the two repetitive regions (S<sub>2</sub>). The codon-based test for selection (Z-test) showed evidence for purifying selection for genes of each of the four groups. Each group consists of avenins from the A and C genomes of the diploid species, and of tetraploid and hexaploid *Avena* species. This indicates that the most recent common ancestor (MRCA) of the *Avena* genus most likely contained one or more representatives of each of the four avenin groups before the A and C genomes diverged. In the Groups III and IV, two genome-specific subgroups appear to exist: one subgroup with sequences from genome A and the other one with sequences from genome C. Repeat motifs within the first and the second repetitive regions of group IV contained methionine while the others did not. Chesnut et al. (1989) regarded this group as a separate protein subfamily. Absence of genome-specific subgroups in group II could be due to a lack of initial divergence (in agreement with the low diversity within this group) and/or incomplete lineage sorting. Presence of two A-genome subgroups in group III, each of them containing sequences of *A. strigosa* and *A. longiglumis*, may indicate a specific

duplication of sequences of this group in the MRCA of at least some of the A genome diploid species. The allohexaploid species *A. sativa* contains avenins from each of the four groups and subgroups except the C-genomic subgroup of group IV, indicating that the allohexaploid has maintained all groups brought in by the three genomes.

### ***Phylogenetic origin***

There are some structural differences between avenins and other prolamins (especially those from wheat, barley and rye) regarding polymorphism, molecular weight and number and location of repetitive domains and cysteine residues (Shewry et al., 1995; Tatham et al., 2000). Alignment of the second conserved region of the avenins against EMBL/GenBank sequences (not shown) indicated that the highest degree of homology are gamma-type S-rich prolamins, such as the gamma-secalins in barley and the gamma-hordeins in rye that all contain eight cysteine residues at similar positions. Similarly to secalins, none of the avenin sequences contained tryptophan. We confirmed the presence of aspartic acid in position 7 and its replacement by asparagine as shown by Pernollet et al. (1987), or, in some avenins, by tyrosine.

Thus, genus-wide cloning and sequencing of the avenins clearly showed the limited range of diversity of the avenin gene family. With this approach, for the first time, a complete picture of the avenins in oats has been obtained, as a basis to establish the safety of oat for CD patients.

### ***The safety of oats for people with celiac disease***

The safety and the diet-improving effect of oats for the majority of celiac patients is currently well-recognized (Pulido et al., 2009). It is fair to say that a large intervention study (although not intended as such) regarding the safety of oats is currently ongoing in the form of daily pragmatic practice of oat consumption in Scandinavia and The Netherlands, where Celiac patient societies stimulate oat-based food consumption from established gluten-free oat production chains, and where patients have a positive attitude on the gluten-free oat products. Seventy percent of CD patients in Nordic countries and an increasing number of patients in The Netherlands eat oats regularly with full contentment (Salovaara et al., 2010). In addition, the advantages of oat consumption by diabetic individuals, as CD and diabetes regularly happen to coincide, are welcomed (personally communicated by the board of the Dutch Diabetic Society).

Toxicity problems of oat products, in general, are related to contamination with gluten, and, in some rare cases, to the presence of the two avenin-specific sequences harboring the epitopes Av- $\alpha$ 9A (PYPEQQEPF) and Av- $\alpha$ 9B (PYPEQQQPF) that can induce an

immune response in few celiac patients (Arentz-Hansen et al., 2004; Lundin et al., 2003; Vader et al., 2003). Although the number of oat-sensitive patients appears to be very low, this aspect attained much attention and definitely caused reluctance in oat consumption by the celiac population. Peptides containing these two avenin epitopes turned out to be common in oat (Figure 3).

### ***Antibody and T cell tests***

Based on our extensive set of deduced avenin proteins we were, for the first time, able to determine that the 17 internationally accepted CD epitopes from wheat, rye and barley that are recognized by CD4+ T cells (Sollid et al., 2012), as well as the p31-43 epitope that has been implicated in the innate immune response, are all absent in oat avenins. Variants of these epitopes do occur in oats, but they differ at two or three of the nine amino acid residues. None of them is predicted to survive digestion by pepsin, trypsin and chymotrypsin. Thus, they are unlikely to be clinically relevant. The two avenin-specific epitopes described by Arentz-Hanzen et al. (2004), to which few CD patients specifically react, were found in two of the four groups of avenins that occur in each of the genomes. Hence, these epitopes are probably present in all *Avena* species. Based on in silico proteolysis we predict that they also represent the most abundant protease-stable peptides. So, the question is what could cause the differential reaction of monoclonal antibodies and peripheral T cells in vitro to oat varieties. None of the recognition sites of the R5 and the G12 antibody tests are present in avenins. Only variant sequences with one (in case of R5) or two (in case of G12) amino acid residue difference were detected, some of which were linked to the avenin-specific epitopes (Table 3). Thus, the immunogenic responses that are described to oat extracts of some varieties (Comino et al., 2011; Mujico et al., 2011) are most likely the result of cross-reactivity with sequences present in avenins with one (R5) or two (G12) amino acid difference and this should be considered to be beyond the identified clinically toxic sequence profile (Sollid et al., 2012). Hence, the relation between the antibody signal and a T cell response as established for gliadins in wheat and prolamins in rye and barley should not simply be extrapolated to oat.

### ***Limitations of test systems***

Thus, test systems that do not directly detect the intact epitopes are limited in their applicability, as the correlation between the antibody signal and the presence of intact epitopes as established for gliadins in wheat and prolamins in rye and barley, should not be extrapolated beyond these species (Sollid et al., 2012). This would appear obvious, but antibodies developed to detect gluten in wheat were used on the proven

gluten-safe crops like the dicot Quinoa (Zevallos et al., 2012), which is not even a cereal. Without confirmation by other methods such data are misleading to the overall patient population. Here, the scientific world should take its responsibility, and should be reluctant in sending out signals that might cause commotion among the patient society before relevance and extent of possible harm are reliably demonstrated.

## CONCLUSION

The genus-wide genomics analysis of the avenin gene family with regard to celiac disease (CD) has shown that none of the internationally agreed epitopes from gluten of wheat, barley and rye are present in oat avenins. The presence of the two described avenin-epitopes was confirmed within a conserved avenin region in all *Avena* species examined, making it unlikely to find oat varieties that lack these sequences. Therefore, if responses of T cells of CD patients to oat extracts occur, they are most likely towards these two peptides. The monoclonal antibody signals (R5 and G12) are probably due to cross-reactive or promiscuous recognition of avenin peptides, and unless the relation with the two avenin-epitopes is proven, such signals should not be interpreted as differences in immunogenicity of oat varieties for CD patients.

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## **Part II**

### **Technological properties of oat flour**



# CHAPTER 3

## Development of a standard test for dough-making properties of oat cultivars

### ABSTRACT

Bread is consumed all over the world. However, so far, production of large volume bread is only possible with wheat. Alternatives, such as oats, are less suitable but this is partly due to the lack of knowledge about their functionality for other purposes than porridge, which is their most common use. Existing standard tests for the dough making characteristics of wheat flour are not suitable for oat flour, hampering research to optimize oats for bread-making purposes. We therefore set out to develop a test to evaluate oat in relation to mixing and dough making properties using wheat as a model. It was possible to reproduce the profile of various qualities of wheat flour using mixtures of oat flour and gluten in different proportions. Our standard test was based on a dough system composed of 87.2% oat flour and 12.8% gluten and it presented similar properties to a wheat flour with regard to resistance to extension. This dough system was sensitive and reliable (coefficient of variation lower than 10%) for detecting differences among oat cultivars. It can be used to screen oat varieties and individual oat components in relation to relevant properties for bread-making purposes.

Keywords: oat flour, oat bread, gluten-free bread

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## INTRODUCTION

Bread is an important staple especially in European countries with wheat (*Triticum aestivum*) bread being most popular among consumers. There is an increasing demand for new products for different target groups that should meet different quality criteria related to texture, taste, nutrition, and health. Oat is an interesting alternative for people with celiac disease (Pulido et al., 2009; Londono et al., 2013), or people who for example like to benefit from the health-related compounds present in oat (Butt et al., 2008).

The inclusion of oat in the daily diet is encouraged because it contains components that have been associated to health benefits, notably beta-glucans, that help to decrease cholesterol and glucose in the blood (Butt et al., 2008; Jenkins et al., 2002). High blood cholesterol is a major risk factor for coronary artery disease which is one of the main causes of death in Western countries (Butt et al., 2008). Beta-glucans are considered as a functional component for prevention of cardiovascular diseases and of type II diabetes (Jenkins et al., 2002).

People that suffer from celiac disease, a chronic disorder caused by ingestion of gluten proteins that affects about 1% of the Western population, should stick to a long-life gluten-free diet. But gluten free breads –and, for that matter, gluten free products in general–, have an inferior quality compared to those made of wheat flour (Hager et al., 2012). In a comparison of different gluten-free formulations loaves made of wholegrain oat flour presented similar specific volume to loaves made of wholegrain wheat flour, the loaf specific volumes were 2.40 for oat bread and 2.62 ml/g for wheat bread (Hager et al., 2012). However, despite their similarity, these volumes are considered of inferior quality in comparison to standard white wheat bread, which have specific volumes between 3.5 to 4 mL/g (Belitz et al., 2004).

Gluten-free bread making is normally based on low viscosity systems known as batter systems. These account for a water addition ranging between 95 and 120% w/w (Hager et al., 2012; Huttner et al., 2009). This approach has been used to test bread-making performance of commercial oat flours and oat cultivars, and differences have been reported using the same formulation for all of them: 100% oat flour, 120% water, 1.75% salt, 1% sugar, and 2% dried yeast (Hüttner et al., 2010; Hüttner et al., 2011). The largest differences among oat cultivars regarding bread-making were observed in crumb texture while no significant differences in loaf specific volume occurred (about 1.5 ml/g). Additionally, various technologies have been tested to improve quality of oat bread by treating the batters with high pressure (Huttner et al., 2009), adding enzymes (Renzetti and Arendt, 2009; Renzetti et al., 2009), hydrocolloids (Huttner et al., 2010), or bacteria (Moore et al., 2007).

The baking quality of wheat is mainly determined by gluten content and its composition (Bushuk 1998). Gluten proteins confer the unique viscoelastic properties to wheat dough which are essential for gas retention. Of all gluten proteins the high molecular weight (HMW) glutenins contribute most to the elastic properties of wheat dough and to the loaf volume (Bushuk 1998). The lower quality of oat bread compared to wheat bread has been mainly attributed to the absence of gluten proteins in oat. Normally, when people use the term 'oat bread', they refer to composite breads made of mixtures of oat meal and wheat flour in various proportions. So far, the maximum amount of oat meal that has been used in a composite oat/wheat bread without compromising texture is 51% together with an adjustment of the formulation and the baking process (Flander et al., 2007).

From a practical point of view, it would be more convenient for bakers to use a dough system instead of a batter system to make oat bread because batters are sticky and difficult to handle, but also to avoid the use of thickening agents on which batter systems rely because they are costly. There is however a gap of knowledge concerning the relevant functional aspects of oat flour and the technology required for making oat bread. There are no standard parameters to test oat flours using dough systems that can fulfil the same functions as the parameters that exist for wheat flour. The Farinograph and extensibility parameters to test bread quality of wheat flours are well defined, but cannot be used as such for oat flour. Therefore, we aimed to develop a standardised dough system to test the intrinsic technological properties of oat cultivars and to be able to study the functionality of different oat components on the bread-making properties. Our approach was based on a replacement of a fraction of oat flour with vital gluten to determine if it was possible to reconstruct, partly or completely, typical wheat-like properties, using the maximum amount possible of oat flour as basis. The standard system proposed does not reflect the final oat bread aimed for with respect to optimal quality, but only forms a first step towards good quality oat bread. The standard system simply serves an analytical purpose in order to fill the knowledge gap regarding the effects of components of oat.

## **MATERIALS AND METHODS**

### ***Flours***

For the experiments we used commercial oat meal (De Vlijt, Wageningen, the Netherlands), wheat flour –which we will refer to as wheat flour “C”-, and its gluten fraction (provided by Cargill, the Netherlands), and commercial wheat Patent flour

(C1000, the Netherlands). As the texture of oat meal was visibly coarser than the texture of wheat flours, the size of the particles in 50 g of oat meal was characterized using a series of sieves of 0.500, 0.300, 0.250, 0.180, 0.150, and 0.071 mm opening. We decided to use only the fraction that passed through the 0.250 mm sieve to eliminate possible detrimental effects of large particles on the gluten network (Noort et al., 2010). This fine fraction was packed in plastic bags, sealed and stored in the freezer until use. This fine fraction (<0.250 mm) is what we refer to as oat flour in this study.

Moisture content of oat meal, oat flour, and wheat flour was calculated using the AACC method 44-15A. Nitrogen was determined by combustion (AACC approved method 46-30) using a NA 210 nitrogen and protein analyser (ThermoQuest, Ronado, Italy) to calculate the protein content, using a factor of 6.25. Total starch was quantified using the AACC method 76-13, and  $\beta$ -glucan content by the AACC method 32-23.

Once the standard test was developed using the sieved fraction of commercial oat meal, grains of 10 oat cultivars (DLO, the Netherlands) were put in a 0.5 m<sup>3</sup> steel container to undergo the kilning process. First, a grain layer of three cm was placed in the container and steamed for three minutes at 100 °C. Then, the grains were let cool down for 30 minutes and placed in a drying oven at 85 °C overnight. The grains were milled at 8000 rpm (Hosokawa Alpine D-86199, Augsburg), and sieved in the same way described for oat meal to remove the bran particles. The fraction that passed through the <0.250 mm sieve was used to compare their extensibility properties to test the sensitivity of the standard dough system to detect differences. The composition of the oat varieties is presented as supporting information (S1).

### ***Making a dough system***

We prepared dough using pure oat flour and mixtures of oat flour and vital gluten in different proportions in a total weight of 10g (14% moisture). In total seven flours were used to make dough: pure oat flour and mixtures in which a fraction of oat flour was replaced with 0.08, 0.16, 0.32, 0.64, 1.28, and 2.56 g of gluten. The wheat flour “C” that was used as source to extract the gluten was used as control for its functionality in the mixtures; dough made of commercial wheat Patent flour was included just for comparison. 2% (mixture basis) NaCl was added to all flours to prepare a dough according to the method 54-21 (AACC, 1995).

A Micro-Farinograph (Brabender instruments, Mod.-No. 8 110) was used to determine the amount of water that each flour required to get a consistency of 500 BU, which is the standard consistency to test quality of wheat flour, and to establish the mixing time required to reach the peak consistency (dough development time). First, we determined the water absorption that each of the flours required. Then, we proceeded to prepare

the dough using the Micro-Farinograph. The dough was allowed to relax in a plastic container within an incubator for 20 minutes at 30 °C and a constant relative humidity of 85%. After relaxation the dough was homogenized by hand and pressed between two oiled grooved forms to make dough strips. These strips were let to relax again for 40 minutes within the grooved bases in a plastic container at 24 °C and constant relative humidity. Subsequently, its maximum extensibility (mm) and resistance to extension (g) were measured using a Texture Analyser fitted with the SMS/Kieffer Extensibility Rig (Stable Micro systems). The standard settings for wheat flour were used according to instructions of manufacturer:

Mode: Measure force in tension

Option: Return to start

Pre-test speed: 2.0 mm/s

Test speed: 3.3 mm/s

Post-test speed: 10.0 mm/s

Distance: 120 mm

Trigger force: 5g

Data acquisition rate: 200pps

### ***Standardisation of consistency and mixing time***

Initially we selected a mixture of oat flour and gluten that presented intermediate values of maximum extensibility, resistance to extension, and strain hardening in comparison to wheat flour at a consistency of 500 BU, used as standard consistency to test wheat flours. Low variation between measurements was also a criterion for the selection. Subsequently, that system was used to prepare dough at different consistencies (500, 700, and 900 BU) to see if there was an effect of consistency on the extensibility parameters. Finally, the effect of mixing time on extensibility parameters of a selected dough system was tested after mixing for 2, 3, 4, and 5 minutes.

### ***Strain hardening***

Strain hardening defines the level of elastic or plastic behaviour of a material. We calculated the strain hardening of the dough by fitting the extensibility data obtained with the Texture Analyser (Stable Micro systems) to the stress-strain curve to the formula described by Dunnewind et al. (2003), between the measured strain-range of 20-95%. The sample volume extended was assumed as constant and banding distance

was neglected. The formula used to calculate strain hardening was:

$$\sigma = k \cdot e^{\epsilon-n}$$

Stress ( $\sigma$ ) and Henky-strain ( $\epsilon$ ) were determined at fracture. The coefficient  $n$  is the strain hardening index. Regression coefficient ( $R^2$ ) was calculated for each sample.

## RESULTS

### *Characterization of flours*

Commercial oat meal had a coarser texture than wheat flour; only 31.4% (weight basis) passed through the 0.250 mm sieve (Table 1). This fraction is referred to as 'oat flour' in this article and was used to prepare the flour mixtures to perform the experiments. The composition of oat meal (and its respective fractions), wheat flour and vital gluten are given in Table 1. Complete oat meal and its sieved 'oat flour' fraction differed considerably: oat meal had 30% more protein, 78% more beta-glucan and 14% less starch than its sieved 'oat flour' fraction. The fat content was the same. The medium fraction (0.250 – 0.500 mm) had the highest content of fat.

### *Oat dough system at 500 BU*

The farinograms of pure oat flour and of the mixtures made by replacing 0.08, 0.16, and 0.32 g of oat flour with gluten (in 10 g, 14% moisture), showed an initial short peak consistency that declined rapidly and a narrow fluctuation that looks like a line. This peak consistency is related to hydration but dough development does not occur (Figure 1a). In contrast, significant improvements of dough strength, observed as a longer peak time and as a wider amplitude of the band in the farinograms, were obtained when replacing 0.64, 1.28, and 2.56 g oat flour with gluten; the dough development of oat flour improved gradually with the increase of gluten at the mentioned amounts (Fig 1b, c, d). The water absorption of flour mixtures increased proportionally to gluten content, from 5.77 ml in the mixture containing 0.08 g gluten to 7.9 ml in the mixture containing 2.56 g gluten.

The wheat flour 'C', used to extract the gluten used in the mixtures, absorbed 5.46 ml water/10 g flour and the commercial wheat Patent flour absorbed 6.7 ml/10 g flour to reach a consistency of 500 BU. Farinogram consistency of these two wheat flours became stable around 500 BU, while consistency of oat flour mixtures, independently of their gluten content, dropped off to 300 BU (Figure 1).

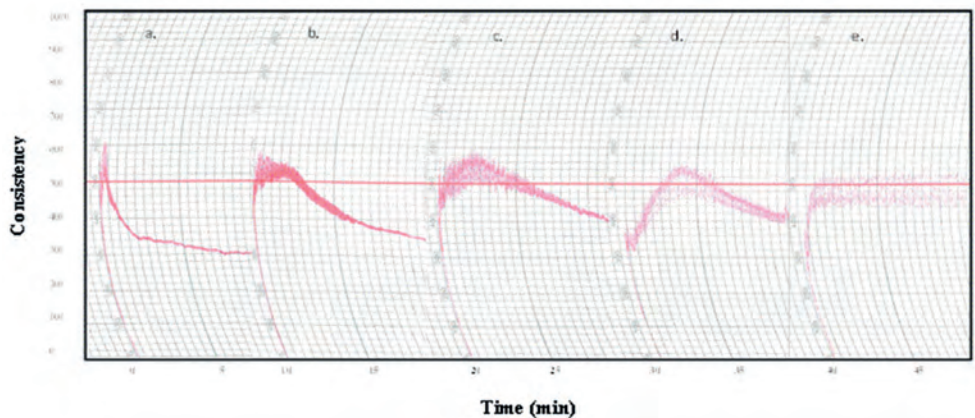


**Table 1.** Composition of oat meal and its respective fractions, wheat flours and vital gluten (as is), used to develop a standard oat dough system.

Material	Proportion of oat meal (weight) %	Moisture Mass %	Composition (mg/100 mg)				
			Starch	Protein	B-glucan	Fat	%
<b>Oat meal:</b>	100	6.98	61.75	12.50	5.54	11.05	97.82
Fraction >0.500 mm	44.41	11.46	44.68	16.98	8.35	11.90	93.37
Fraction 0.250 - 0.500 mm	15.16	12.78	51.92	22.20	0.40	13.91	101.22
Fraction <0.250 mm <sup>1</sup>	31.36	16.18	72.12	8.70	1.20	11.30	109.50
Not recovered	9.07	-	-	-	-	-	-
<b>Wheat flour 'C'</b>	-	14.9	70.29	10.4	0.2	4.82	100.61
<b>Wheat Patent Flour<sup>2</sup></b>	-	11.2	70.3 (70.9)	10.6 (11)	0.2	4.72 (1.0)	97.02
<b>Vital gluten</b>	-	2.50	4.88	80	-	10.5	97.88

<sup>1</sup> Fraction used to perform the experiments and what we refer to as 'oat flour'.

<sup>2</sup> Composition of commercial wheat Patent flour as listed on the packaging is shown between brackets.



**Figure 1.** Farinograms of pure oat flour and of mixtures of oat flour and vital gluten: Pure oat flour (a), mixtures containing gluten: 0.64 g (b), 1.28 g (c), 2.56 g (d) and wheat flour 'C' (e).

### *Extensibility properties of oat dough at 500 BU*

Maximum resistance to extension (g) and maximum extensibility (mm) of the dough at 500 BU were exponentially proportional to the gluten content in the flour mixtures. There was no difference in extensibility properties between pure oat flour and oat flour mixtures containing 0.08, 0.16, and 0.32 g gluten/10 g, the strain hardening values of these dough were below one (Table 2). In contrast, clear improvements were observed in flour mixtures containing 0.64, 1.28, and 2.56 g gluten/10 g, which is consistent with the improvement on dough development observed in the Farinograph test at the same levels of gluten. At these levels of gluten the dough had a strain hardening of 1.29, 1.22, and 1.4, respectively (Table 2).

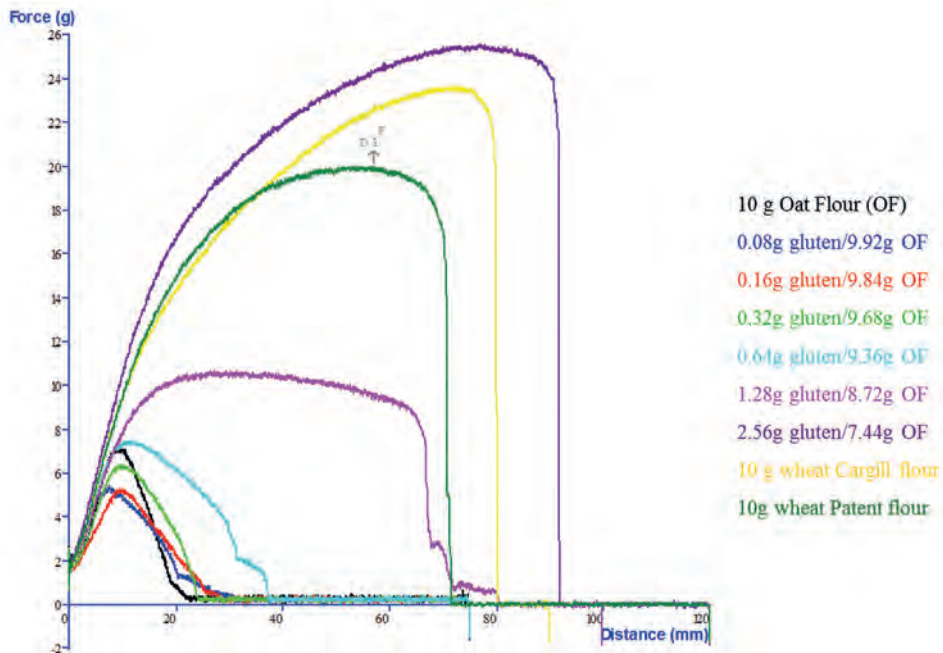
Values for maximum resistance to extension and maximum distance of the wheat flour 'C' and of commercial wheat Patent flour fell between the values obtained for oat flour mixtures containing 1.28 and 2.56 g gluten / 10 g (Figure 2). The values of strain hardening for wheat flour 'C' and wheat Patent flour were 1.39 and 1.35, respectively. These results suggest that reconstruction of technological properties of wheat flour using oat flour as basis and gluten is feasible. Because our purpose was to develop a sensitive test system, it should not contain too much gluten since this could mask any differences that may exist among oat cultivars. We therefore selected the system composed of 8.72 g oat flour and 1.28 g gluten, the coefficients of variation for the extensibility test were 6.7 and 9.6% for maximum resistance to extension and distance, respectively. This system had a similar behaviour to a wheat flour suitable to be used in pastry applications (Figure 2).

**Table 2.** Water absorption (flour basis) and strain hardening of oat flour mixtures containing different levels of gluten.

Gluten content (g)	Water %	Strain hardening value	R <sup>2</sup>
0	57.7	0.73	0.89
0.08	57.7	0.37	0.93
0.16	58.5	0.23	0.74
0.32	60.0	0.76	0.99
0.64	61.8	1.29	0.99
1.28	66.4	1.22	0.99
2.56	78.7	1.40	0.99
Wheat flour 'C'	54.6	1.39	0.99
Wheat Patent flour	67.0	1.35	0.99

### *Effect of consistency on extensibility properties*

The selected system, composed of 8.72 g oat flour and 1.28 g gluten, was used to prepare dough in the Farinograph at different consistencies by adjusting the level of water. Consistency of the dough system was very sensitive to water, peak consistency increased from 500 to 1000 BU with a decrease of 1.2 ml water/10 g flour mixture. Maximum resistance to extension of the selected dough system increased with consistency while extensibility decreased (Figure 3). This dough system at a consistency between 900 and 1000 BU had similar extensibility properties to wheat Patent flour at 500 BU; therefore, and because the maximum consistency of the Farinograph is 1000 BU, a consistency of 900 BU was chosen as standard for the selected oat dough system. The strain hardening of the dough at 500 BU was 1.22, at 700 BU was 1.17 and at 900 BU was 1.20.



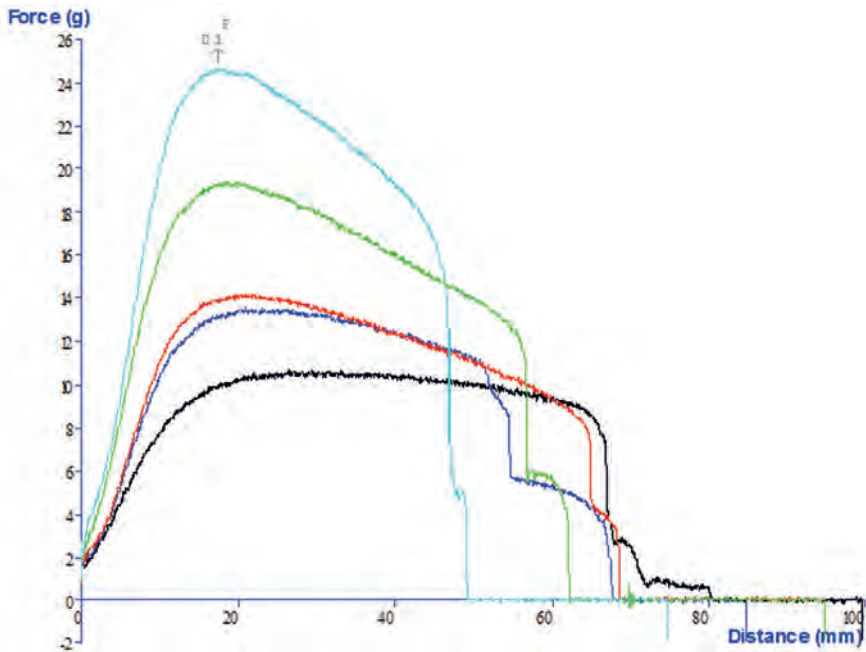
**Figure 2.** Resistance to extension (force) and extensibility (distance) of oat flour with a replaced fraction with vital gluten.

### *Effect of mixing time on extensibility properties*

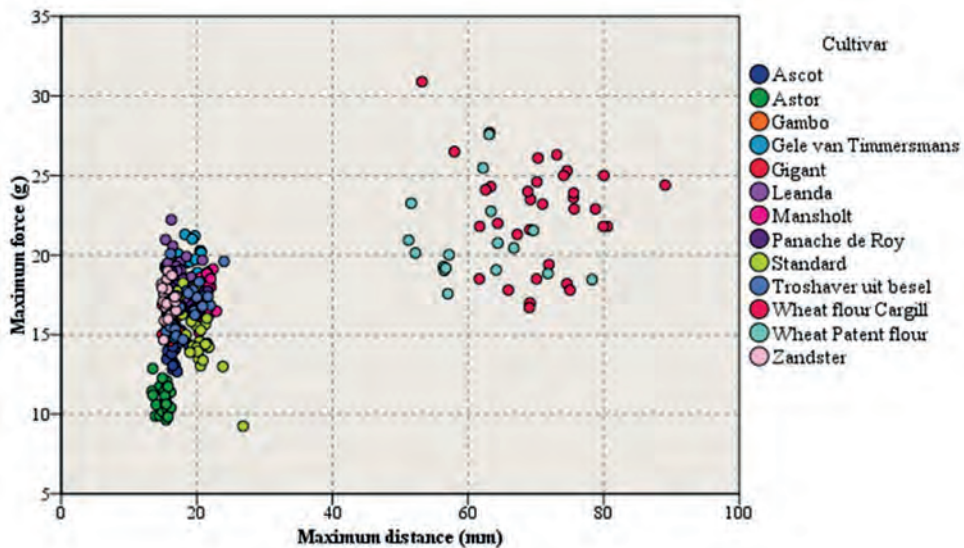
To conclude with the standardisation, extensibility properties of the selected dough system, at a consistency of 900 BU, were tested after mixing for 2, 3, 4, and 5 minutes. The dough system kept its extensibility properties after mixing for 2 or 3 minutes, but a decline was observed with longer mixing times (results not shown). As the peak time might vary between cultivars, we decided to use a criterion of peak time plus one minute.

### *Extensibility properties of ten oat cultivars*

Extensibility properties of ten oat cultivars were evaluated using the standard dough system developed: 1.28 g gluten plus 8.72 g oat flour, 900 BU consistency, mixing at peak time plus one minute. The test was sensitive to detect differences among oat cultivars; the largest difference in maximum resistance to extension was 74% and occurred between cultivars Astor and Gele (Figure 4). The largest difference detected in extensibility at maximum resistance was 40% between cultivars Astor and Mansholt (Figure 4). The average coefficient of variation was 6%. There was no correlation between the two parameters for the group of oat varieties tested, which means that breeding to optimise both may be possible. The results proved that our standard test based on a dough system can be used to detect differences among oat cultivars with respect to technological parameters relevant for dough based bread-making.



**Figure 3.** Maximum force and distance of a selected dough system composed of 8.72g oat flour and 1.28g gluten at different consistencies.



**Figure 4.** Differences among ten oat cultivars using a standard dough system. The standard conditions were: 1.28 g gluten/8.72 g oat flour; 900 BU consistency, mixing time was peak time plus one.

## DISCUSSION

In this study we demonstrated that it is possible to reconstruct the technological properties of wheat flour by using a mixture of oat flour and gluten powder (80% protein), and that reproduction of various wheat flour quality profiles depends on the amount of gluten present in the mixture.

The reproduction of the extensibility parameters of wheat flour used as a source of gluten at 500 BU consistency -standard for wheat flour-, was achieved with a mixture that contained 25.6% gluten powder (2.56 g gluten powder / 7.44 g oat flour), which is very high in comparison with the gluten content of its counterpart wheat flour (10.4% protein, from which about 80% is expected to be gluten (Hamer 2003)). Reproduction of the extensibility properties of another wheat flour profile -wheat Patent flour-, was possible by decreasing the amount of gluten in the mixture by half, 12.8% (1.28 g gluten powder / 8.72 g oat flour), and by increasing dough consistency from 500 to 900 BU. Consistency was very sensitive to water as a small decrease of water already caused a large increase in consistency and a considerable improvement of resistance to extension of the dough, without affecting to a large extend its maximum distance. From this we conclude that the standard Farinograph consistency of 500 BU, used to prepare wheat dough, should not be extrapolated to prepare oat dough.

What people normally call 'oat bread' is in fact a composite bread made of a mixture of oat meal and wheat flour. Mixing oat and wheat flour is done mainly to improve the fiber content and nutritional value of wheat bread with beta-glucans from oats, but some detrimental effects of bran particles, which are also rich in beta-glucans (Table 1), on bread structure have been reported. So far, 51% is the maximum proportion of whole oat meal used in composite oat/wheat bread in a dough system without compromising textural characteristics (Flander et al., 2007). However our approach was different and it is important to highlight that our standard dough system was based on a sieved fraction of oat meal (<0.250 mm). It closely resembled the texture of normal wheat flour, which means that detrimental effects of bran particles were removed (Gan et al., 1992; Noort et al., 2010), but also a large proportion of the beta-glucans (Table 1). Oat flour of similar texture should be used to replicate the results obtained. The fact that we were able to reproduce extensibility properties of wheat flour using oat flour as a base instead of oat meal is novel. We were not able to do this using oat meal (data not shown) suggesting that removal of bran fractions can be a way to improve the textural quality of oat bread. On the other hand, the nutritional quality of oats is also based on the presence of high amounts of beta-glucans. So, any process should try to improve texture while maintaining the highest possible content of beta-glucans.

The standard test presented in this paper was specifically designed with the application of oats in a dough system in mind. So far, differences in bread-making performance of commercial oat flours and of oat cultivars have been reported based on a batter system, and under the same formulation for all cultivars and flours (Hüttner et al., 2010; Hüttner et al., 2011). This approach of using the same amount of water for different flours, implicitly assumes that flours do not differ in their water binding capacity. However, it is known that oat cultivars can strongly differ in the content of water binders: starch, damaged starch, proteins and beta-glucans. Consequently, comparing oat cultivars using the same amount of water for all of them might mask differences in their bread-making potential related to their composition, which is not only affected by the cultivar used to make the flour, but also by the milling process (Gray et al., 2000). Our standard system is developed to make the comparison under the same conditions of Farinograph consistency (all cultivars were compared at 900 BU), just like it is done for wheat flour (at 500 BU).

Our standard test was sensitive to detect differences among oat cultivars. There can be several explanations for these differences, such as differences in beta-glucan content, protein content and protein composition. However, the main purpose of this paper is to present the standard method and demonstrate its sensitivity in a relevant way.

## **CONCLUSION**

There is a considerable lack of understanding of the effects of differences (both quantitative and qualitative) in oat components in relation to bread making. Oat is either used as an addition to wheat-based doughs or in an oat-based batter system. We have produced dough using a mixture of oat flour and vital gluten that had similar extensibility characteristics to a pure wheat dough. This dough forms a sensitive test system to detect differences among oat cultivars and can be used to study the contribution of various oat components (avenins, beta-glucans and oil) to the quality of oat bread. Our method will help fill the knowledge gap regarding the effects of components of oat, and forms a first step towards good quality oat bread.



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# CHAPTER 4

## Understanding the role of beta-glucans in oat-based dough systems

### ABSTRACT

Beta-glucans are one of the components that make oats special among other cereals. Because of this, the health-related value of oats is increasingly recognized. However, so far oats cannot easily be applied in bread-like products without losses in product quality. Here we have studied how the content and viscosity of beta-glucans affect the technological properties of oat dough in both a gluten-free and a gluten-containing system. In both systems, increasing the beta-glucan concentration resulted in an increase of dough stiffness and in a reduction of dough extensibility. Beta-glucans negatively impacted the elastic properties that additional wheat gluten conferred to oat dough. This effect was smaller for medium-viscosity beta-glucans than for high-viscosity beta-glucans. Interestingly, dough made with low beta-glucan flour (< 2 %) had increased gas retention capacity. Overall, the impact of beta-glucans on the properties of oat dough systems was governed by concentration and viscosity, with or without additional wheat gluten. Our findings point out that beta-glucans are a key component that determines the rheology of oat-based dough systems and, with that, the technological functionality of oat in dough systems.

Keywords: Oat flour, oat bread, gluten-free bread.

Submitted to Journal of Cereal Science: Londono, D.M., Smulders, M.J., Visser, R.G., Gilissen, L.J., Hamer, R.J., 2013.

## INTRODUCTION

Oat constitutes a healthy basis for food products. Oat has gained relevance in human nutrition because it is one of the few cereals with a high content of soluble fiber -namely beta-glucans-, and is a good source of proteins, vitamins, and minerals (Butt et al., 2008). Oat consumption enriches the nutritional quality of the diet, especially of those that suffer from celiac disease (Butt et al., 2008).

Celiac disease is a chronic inflammatory disorder of the small intestine produced by ingestion of gluten proteins, and one of the symptoms is the malabsorption of nutrients. In addition to the malabsorption, people suffering from celiac disease do not realise the required intake of nutrients and fibers because gluten-free (GF) bakery products are commonly based on starches that have a lower nutritional quality than their cereal counterparts that include gluten (Thomson, 2000)

Beta-glucans are polysaccharides composed of  $\beta$ -(1-4)-linked glucose units separated every two to three units by a single  $\beta$ (1-3)-linked glucose (Bell, et al., 1999). Beta-glucans are a very interesting oat component because they help to reduce cholesterol and post prandial glucose (PPG) levels in blood. A high cholesterol level, and high PPG levels are considered important risk factors for coronary heart disease and diabetes type II respectively (Behall et a., 2004; Mathews, 2011; Othman et al. 2011)-. The positive physiological effect of beta-glucans has been attributed to the increased viscosity beta-glucans confer in the intestine, which slows down the intestinal transit and the absorption of sterols and glucose (Anderson and Chen, 1986). Because of that, the American Food and Drug Administration and the European Commission granted oat beta-glucans with a health claim. The recommended intake to help prevent cardiovascular diseases is 3 g/day (FDA, 1997).

Beta-glucans are used as a functional food ingredient (Brennan and Clearly, 2005), but can also be used to enhance viscoelastic properties of GF bread formulations (Lazaridou and Biliaderis, 2007). Rheological studies of beta-glucans extracted from different oat varieties showed a positive correlation between total beta-glucan content and water viscosity (Colleoni, et al., 2003). A correlation with molecular weight was also reported as -at the same beta-glucan concentration- oat lines containing the highest molecular weight beta-glucans presented the highest viscosity (Colleoni, et al., 2003).

Oats are used mainly as flakes, porridges and as a component of breakfast cereals. Applications that require more processing, such as bread-making, have been explored, but knowledge on the technological properties of oat flour/meal is scarce. This limits product innovations like the development of breads that comply with consumer standards regarding volume, structure and texture.

The potential of oats for GF bread applications has been studied in formulations based on batter systems (Huttner et al., 2010). Recently Londono et al. (2013) developed a standard dough system using gluten in the formulation, resulting in a dough system with similar rheological characteristics as wheat dough with respect to its extensibility properties. Differences among oat varieties regarding bread-making potential have been reported for batter as well as dough systems (Londono et al., 2013; Huttner et al., 2011). In this paper, we aim to understand how beta-glucan content and viscosity affect the extensibility properties of an oat dough. For this we studied the effects both in a gluten-free and in a gluten-containing system. The results from this comparative study will help to better manage the technological properties of oat flour, and to determine whether oat varieties with high beta-glucan content, which are desired for health purposes, are compatible with bread making applications.

## **MATERIALS AND METHODS**

### **Materials**

The materials used were commercial oat meal (De Vlijt, Wageningen, the Netherlands), vital gluten (provided by Cargill, the Netherlands), and oat beta-glucans of high and medium-viscosity (Megazyme, lots 86608c and 31205a, respectively). The purity of the beta-glucan extracts was more than 94%, and the average molecular weights were 361 Kd for the high-viscosity and 272 Kd for the medium-viscosity samples.

### ***Flour mixtures***

The oat meal was sieved using a series of sieves of 0.500, 0.300, 0.250 mesh. Sieving was done to eliminate bran particles, which can have detrimental effects on dough rheology (Noort, et al., 2010). The sieved fine fraction of oat meal (<0.250 mm diameter) is what we refer to as 'oat flour' in this study. This flour contained 16% moisture, 8.7% protein, 1.2% beta-glucans, and 72% starch. Since the major proportion (~80%) of the beta-glucans was present in the bran, This fraction was substantially lower in beta-glucans. This was considered a positive as this allowed us to study a wider range of beta-glucan concentrations.

We performed two types of experiments to study the effect of beta-glucans on dough properties: the first type was based on a standard dough system containing 12.8% gluten, developed by Londono et al. (2013). This system behaves similar to a weak wheat flour regarding extensibility properties. In the other type of experiment, gluten was excluded from the system. We prepared flour mixtures using oat flour (1.2% beta-glucan) and

we added beta-glucans of high and medium-viscosity, to test both the effect of beta-glucan content and the effect of its viscosity properties on dough extensibility. In the gluten-containing system, the beta-glucan concentration was increased to 2 and 5%, by adding 0.8 and 3.8% of beta-glucans, respectively. In the gluten-free system, the concentration was increased to 5% only.

In order to further demonstrate the effect of beta-glucans, we designed an experiment in which beta-glucans were enzymatically degraded. For this purpose, Endo- and exo-glucanase (Megazyme, Lots 91102a and 91101, respectively) were used as follows. Prior to the experiment, a glucanase solution was prepared by dissolving 60  $\mu$ L endo- and 60  $\mu$ L exo-glucanase in 20 mL distilled water. This solution was used to treat the dough mixture containing 5% beta-glucans of high-viscosity. An overview of the experimental conditions is presented in Table 1.

**Table 1.** Overview of experiments. The total content of beta-glucans corresponds to the sum of the amount of beta-glucans added and the beta-glucans present in the oat flour (1.2%).

System	Flour type	Oat Meal	Oat Flour	Addition beta-glucan HV <sup>1</sup>	Addition beta-glucan MV <sup>2</sup>	Total beta-glucan concentration	Gluten	Total <sup>3</sup>
		%	%	%	%	%	%	%
<b>With gluten</b>	Oat meal	87.2	-	-	-	5.5	12.8	100
	Oat flour (control)	-	87.2	-	-	1.2	12.8	100
	Oat flour + 0.8% HV	-	86.4	0.8	-	2.0	12.8	100
	Oat flour + 0.8% MV	-	86.4	-	0.8	2.0	12.8	100
	Oat flour + 3.8% HV	-	83.4	3.8	-	5.0	12.8	100
	Oat flour + 3.8% MV	-	83.4	-	3.8	5.0	12.8	100
<b>Gluten-free</b>	Oat meal	100	-	-	-	5.5	-	100
	Oat flour (control)	-	100	-	-	1.2	-	100
	Oat flour + 3.8% HV	-	96.2	3.8	-	5.0	-	100
	Oat flour + 3.8% MV	-	96.2	-	3.8	5.0	-	100

<sup>1</sup>HV: Refers to high-viscosity beta-glucans.

<sup>2</sup>MV: Refers to medium-viscosity beta-glucans.

<sup>3</sup>NaCl (2%) was added to all mixtures (Method 54-21, AACC).

### ***Dough preparation and extensibility test***

Dough was prepared using a Micro-Farinograph (Brabender instruments, Mod.-No. 8110, Germany), using a 10 g mixer head. Water was added to prepare a dough system containing gluten with a consistency of  $850 \pm 20$  Brabender units (BU), while the gluten-free system was prepared to give a consistency of  $650 \pm 20$  BU. The reason for the difference in consistency is that flours containing gluten and extra beta-glucans were very sticky and difficult to handle at consistencies  $< 850$  BU. The dough was mixed until the consistency reached the peak level in the gluten-containing system (from two to five minutes), or until the consistency became stable in the gluten-free system (from 2.5 to 10 minutes). The mixing time depended on the experimental conditions because the process of hydration to reach the desired consistency was slower for flours with high beta-glucan content and also for the commercial oat meal. Each system, with and without gluten, was compared to its respective control at the same consistency.

After mixing, the dough was allowed to relax in a sealed plastic container within an incubator for 20 minutes at 30 °C and 85% relative humidity. Then, the complete dough was moulded into a ball by hand (Figure 1D) and pressed between two oiled lubricated and grooved Teflon blocks to make dough strips. The strips were allowed to relax again for 40 minutes within the mould in a plastic container at 24 °C and 80% relative humidity. Subsequently, the dough strip was placed in its holder and maximum resistance to extension (g) and extensibility at maximum resistance (mm) and total extensibility (mm) of each strip were measured using a Texture Analyser fitted with the SMS/Kieffer Extensibility Rig (Stable Micro systems). The standard settings for wheat flour dough were used according to instructions of manufacturer. The test was performed in triplicate and about 7-9 measurements were taken per dough sample. The resulting graphs represent the average of 21-27 measurements.

### ***Gas retention test***

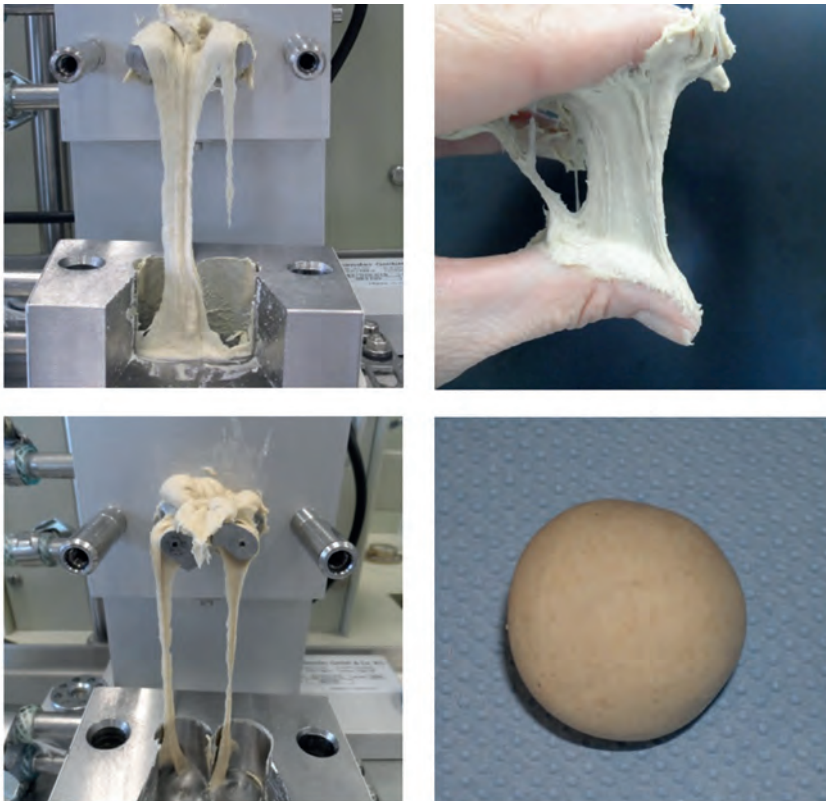
To evaluate the gas holding capacity of the dough, 1.8% of dried active bakery yeast (Bruggeman Instant, Belgium, Lot 50109T2) and 1.0% D-glucose (Sigma) were added to the flour mixtures. Then the dough was prepared in the Farinograph using the conditions described above and placed in the system described by Peighambardoust et al. (2010) to measure the gas production of a piece of dough (5 g). The volume of the dough was measured immediately after mixing and again after 20 mL of gas production. A calibrated cylinder filled with 50 mL of n-hexane (Sigma) was used to measure the volume displaced by the dough. Five samples per dough mixture were tested and results were averaged to calculate specific volume increase of the dough between mixing and after the production of 20 mL gas.



## RESULTS

### *Effect of beta-glucans in a gluten-containing oat-based dough system*

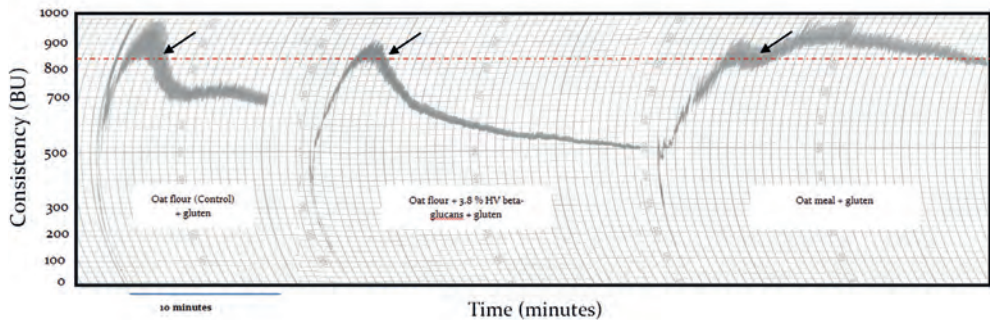
The effect of high and medium-viscosity beta-glucans in a gluten-containing oat flour dough system was determined at concentrations of 1.2, 2 and 5% beta-glucan (Table 1). These concentrations were obtained by addition of 0.8 and 3.8% beta-glucans to the oat flour described, which already contained 1.2% beta-glucans and was used as control. The dough was prepared at  $850 \pm 20$  BU consistency because at lower consistencies the system was very sticky and became impossible to handle when extra beta-glucans were added (Figure 1).



**Figure 1.** Dough appearance of flour mixture containing 5% high viscosity beta-glucans and gluten. Water was added to reach a consistency of 650 BU (upper row) and 850 BU (bottom row). It was impossible to manipulate the dough at 650 BU in the presence of gluten, and even at 850 BU the system was very sensitive to over-mixing.



Flours containing 0.8% of added beta-glucans (bringing the total concentration to 2%) did not present significant differences with respect to the control regarding maximum resistance to extension (force). Apparently, a viscosity increasing effect was prevented by adding more water during dough mixing (required to reach the same consistency). However, the extensibility at maximum resistance (mm) and total extensibility (mm) were reduced (Figure. 2). In contrast, in flours that received 3.8% beta-glucans extra (bringing the total concentration to 5%) the resistance to extension increased from 12 to 20 N in the case of high-viscosity beta-glucans, and from 12 to 16 N in the flour containing medium-viscosity beta-glucans (Figure 2). In this case, adding water to achieve the standard consistency was not adequate to prevent an increase in stiffness. Extensibility at maximum resistance and total extensibility were also significantly reduced (Figure 2). There was no significant difference between oat meal and the flour containing 3.8% of added high-viscosity beta-glucans in the gluten-containing system (Figure 2). Oat meal and flours with higher beta-glucan content required more water and a longer time to reach the  $850 \pm 20$  BU consistency (Figure 3). High and medium-viscosity beta-glucans had the same water binding capacity. All results are summarised in Table 2.



**Figure 3.** Farinograms of three gluten-containing dough systems: oat flour, oat flour plus 3.8% high-viscosity beta-glucans, and oat meal. The dotted line indicates the consistency at which the dough were prepared. The arrows indicate the moment when mixing was stopped. All the dough were very sensitive to over-mixing.

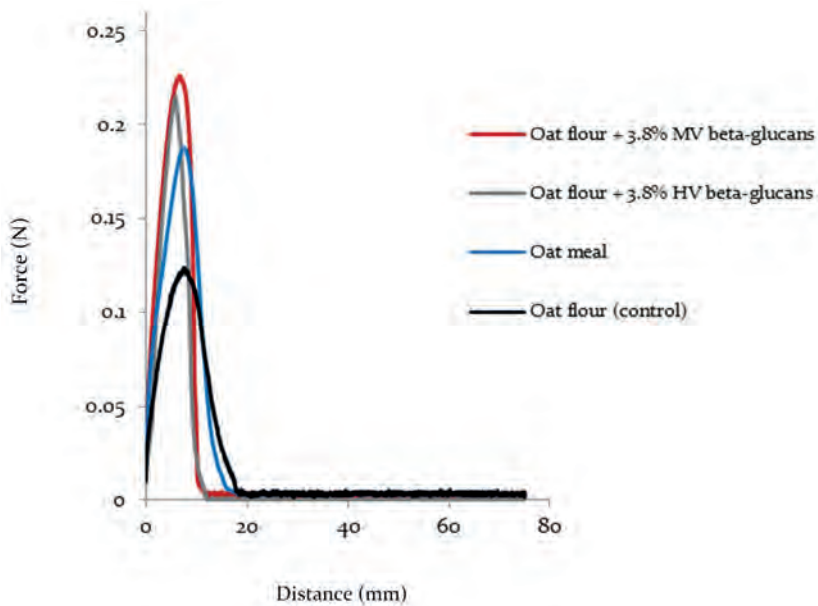
Table 2. Summary of results of extensibility and gas retention capacity tests.

System	Flour type	Farinograph Consistency <sup>1</sup> (BU)	Water added (mL)	Resistance to extension (N)	Mixing time (Min)	Extensibility at max. resistance (mm)	Extensibility total (mm)	Specific volume <sup>2</sup> (mL/g)	Specific vol. gain <sup>3</sup> %
<b>With gluten</b>	Oat flour (control)	850	6.2	0.12 ± 0.08	2.0	21.0 ± 1.6	61.0 ± 7.4	1.29 ± 0.07	51 ± 0.09
	Oat flour + 0.8% MV	850	6.6	0.12 ± 0.05	2.0	18.2 ± 1.3	56.7 ± 6.2	-	-
	Oat flour + 0.8% HV	850	6.6	0.10 ± 0.06	2.0	17.1 ± 0.8	55.9 ± 4.8	-	-
	Oat flour + 3.8% MV	850	7.8	0.16 ± 0.07	2.0	16.0 ± 0.8	38.2 ± 2.2	-	-
	Oat flour + 3.8% HV	850	7.8	0.20 ± 0.01	3.0	13.5 ± 1.0	26.1 ± 2.6	-	-
	Oat meal	850	6.7	0.20 ± 0.01	5.0	10.9 ± 1.1	26.0 ± 3.4	1.31 ± 0.10	48 ± 0.10
<b>Gluten-free</b>	Oat flour (control)	650	4.4	0.12 ± 0.09	2.5	7.9 ± 0.5	15.0 ± 2.4	1.26 ± 0.8	45 ± 0.14
	Oat flour + 3.8% MV	650	5.4	0.22 ± 0.13	4.0	6.3 ± 0.6	12.4 ± 1.2	-	-
	Oat flour + 3.8% HV	650	5.4	0.22 ± 0.14	10.0	5.8 ± 0.6	11.2 ± 1.5	-	-
	Oat flour + 3.8% HV (treated with Glucanase)	650	5.5	0.13 ± 0.2	6.0	6.0 ± 0.9	11.0 ± 1.3	-	-
	Oat meal	650	5.7	0.18 ± 0.18	10.0	7.5 ± 0.9	12.2 ± 1.0	1.19 ± 0.02	42 ± 0.09

<sup>1</sup> Consistency ± 20 BU.<sup>2</sup> Measured at 20 mL total gas production, average of 5 samples.<sup>3</sup>  $\text{Specific vol. gain} = \frac{\text{specific vol. after proofing} - \text{specific vol. after mixing}}{\text{specific vol. after mixing}}$

### *Effect of beta-glucans in a gluten-free oat-based dough system*

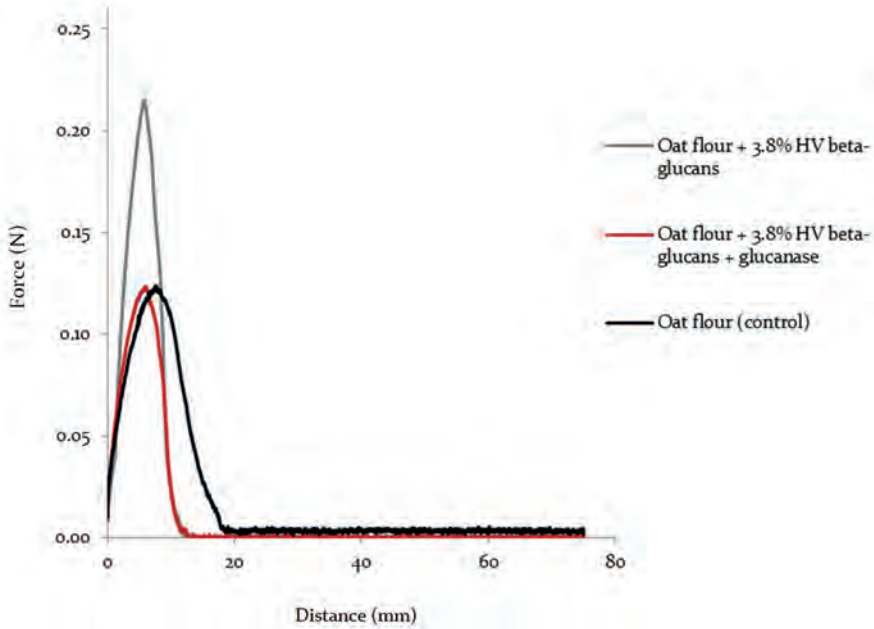
In the absence of gluten, addition of 3.8% beta-glucans to oat flour caused an increase of 73% in dough maximum resistance to extension (force) and a reduction of ~20% of extensibility at maximum resistance and total extensibility (mm); there was no significant difference between flours containing high or medium-viscosity beta-glucans and oat meal (Figure 4).



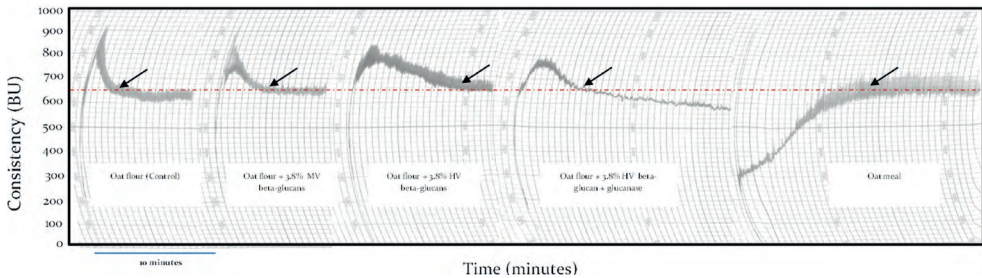
**Figure 4.** Effect of medium- and high-viscosity beta-glucans on extensibility properties of a gluten-free oat-based system. Measurements were performed at  $650 \pm 20$  BU consistency.

Glucanase treatment of the dough containing an extra 3.8% high-viscosity beta-glucans was effective to reverse the viscosity effect of added beta-glucans. The dough had the same maximum resistance to extension (force) as the control, but showed lower total extensibility (mm) (Figure 5).

In the Farinograph the control dough had a peak time of two minutes. Addition of beta-glucans increased the peak time (Figure 6). The flour containing high-viscosity beta-glucans and oat meal needed about 10 minutes to reach stability consistency (Figure 6). The effect of the enzymes was also observed in the farinogram as a reduction of the width of the oscillations and a reduction in the time needed to reach the stability consistency (Figure 6). Flours with higher beta-glucan content did require more water to reach the desired peak consistency of 900 BU.



**Figure 5.** Effect of glucanase treatment on extensibility of an oat-based gluten-free system with added beta-glucans. The effect on beta-glucans was reversed after treating the oat flour with an additional 3.8% beta-glucans with endo- and exo-glucanase (Megazyme). The treatment resulted in a loss of the extensibility observed in the control.



**Figure 6.** Farinogram behaviour of gluten-free dough systems with different beta-glucan content and viscosities. The dotted line indicates the consistency at which the dough were prepared. The arrows indicate the moment when mixing was stopped. HV = high-viscosity.

***Effect of beta-glucans on gas retention capacity***

The effect of beta-glucans on gas retention capacity was tested by comparing the specific volumes of oat flour and oat meal in the gluten-containing and the gluten-free system. There was no significant difference among the specific volumes, which ranged

between 1.19 and 1.31 mL/g. However, a small but significant difference between the specific volume gain of the dough after proofing was observed. In both systems the dough made of oat flour gained 3% more specific volume than the oat meal dough (Table 2).

The specific volume gain in the gluten-containing system was 6% larger than in the gluten-free system for both oat flour and oat meal (Table 2). However, this result cannot be only attributed to the presence of gluten, because the dough consistency was also different. The appearance of the dough after proofing was also different in both systems: oat flour dough did not expand uniformly but more to the sides, and presented pores on the surface, while oat meal dough had a firm and rounded shape.

## DISCUSSION

Applications of oats in bread-like products are restricted due to problems in dough handling and a loss of product quality (Flander et al., 2007). Here we have determined the effects of beta-glucan content and viscosity/molecular weight on the extensibility properties of an oat dough. To confirm the functionality of beta-glucans we either added high- or medium-viscosity beta-glucans, or used a glucanase to remove (part of) such effect.

In order to create a point of reference, we decided to perform our experiments at the same consistency. This required us to adjust the amount of water added and the mixing time, as flours with higher beta-glucan content needed more water to reach the same consistency than flours with low beta-glucan content. This behaviour is comparable with the observed by Wang et al. (2002) in gluten-containing flours that received additions of water extractable pentosans. Surprisingly, the high-viscosity beta-glucans used in this study required the same amount of water as the medium-viscosity type to reach the desired Farinograph consistency. We expected high viscosity beta-glucans to absorb more water as the solubility and viscosity of polysaccharides are controlled partly by molecular weight (Wood, 2011), but as we added the beta-glucans on a weight/weight basis, the molecular weight of the beta-glucans apparently does not affect the total water binding capacity per unit of mass. It may affect the time required for hydration, as high molecular weight beta-glucans required more time to fully absorb the same amount of water (Table 1). In contrast, in the gluten-free as well as the gluten-containing system the effect of beta-glucans on dough extensibility properties was related to both concentration and molecular weight (viscosity). This result is in accordance with the results reported by Skendi et al. (2009), who studied by creep-

recovery measurements the effect of barley beta-glucan extracts on dough rheology of a poor and good bread-making quality wheat flour. They found that the rheological behaviour of flours enriched with beta-glucans depended on concentration and on molecular weight, but also on the type of flour used; beta-glucan-enriched wheat flours presented an increased stiffness and resistance to deformation (Skendi et al., 2009). Increasing the concentration of beta-glucans in oat flour increased dough resistance to extension (stiffness) and reduced dough extensibility, with high-viscosity beta-glucans having a larger impact. However, the associated reduction of dough extensibility not necessarily would result in a lower volume but in a coarser dough and crumb structure (Kloek, et al., 2001). If dough extensibility is a measure of dough elasticity, an increase would mean a higher stability of small gas cells that are mixed in and act as growth nuclei. The more nuclei, the better (finer) the crumb structure (Kloek, et al., 2001).

Bran particles can also affect dough properties (Noort et al., 2010). Previous studies indicated that when wheat flour is mixed with oat flour to enrich the fiber content of wheat bread, this resulted in loaves with a reduced specific volume (Flander et al., 2007; Noort et al., 2010; Popa, et al., 2012; Tiwari, et al., 2013), indicating that the gluten network was disrupted. This disruption was attributed to the bran particles present in oat meal and to its high content of beta-glucans. We compared commercially available oat meal and sieved oat flour. The concentration of beta-glucans was significantly higher in the oat meal (5% versus 1.2%), indicating that the bran fraction of the meal contains a higher concentration of beta-glucans. Dough of the sieved oat flour had a higher extensibility than oat meal. When beta-glucans were added to the flour up to 5%, the extensibility decreased to the level of oat meal. This means that the beta-glucan content is sufficient for this effect, as we observed no additional effect of bran particles on extensibility. So the increase in dough stiffness, the loss of extensibility and the lower specific volume gain observed in oat meal dough can be attributed to its high content of beta-glucans (5%). Similarly, Skendi et al. (2009) observed that beta-glucans from barley can affect wheat dough rheology already at very low concentrations: additions of 1.0% (w/w) produced a hampering effect on the rheology of good quality wheat flour, while an improving effect was observed in weak wheat flour. In our case, increasing the concentration of beta-glucans in oat flour from 1.2% to 2% did not interfere with the formation of the gluten network in the gluten-containing system.

The effect of beta-glucans on extensibility properties of gluten might be comparable to the effect of water extractable pentosans, which are also polysaccharides that can interfere with the formation of the gluten network (Wang et al., 2004a). To explain this phenomenon three mechanisms have been proposed: disturbed viscosity, depletion of molecular attraction between gluten molecules, and modification of Van der Waals forces between particles (Wang et al., 2004a; 2004b). As beta-glucans have a great affinity to bind water (Ahmad et al., 2010), competition with gluten and other water-binding

components can occur in the system, and this would interfere with the development of the gluten network (Wang et al., 2002). This may explain why we were not able to obtain a dough at a consistency of  $650 \pm 20$  BU in the gluten-free system.

The treatment with glucanase completely reversed the stiffness of the dough induced by addition of 3.8% high-viscosity beta-glucans. Interestingly, the glucanase-treated flour had a lower extensibility than the control. This can be explained by the intrinsic beta-glucans of the oat flour used as a control - that were also degraded by the enzyme -, being responsible for conferring the extensibility that oat flour accounted for. This supports the results of Lazaridou et al. (2007), who reported beta-glucans as improvers of the viscoelastic properties of gluten-free flours when added in concentrations from 1 to 2% w/w. Higher concentrations had a negative impact and decreased loaf specific volume (Pastuszka et al., 2012). Our findings thus confirm that beta-glucans are relevant in determining the rheology of oat dough.

Low beta-glucan flour produced a dough with better extensibility properties and gas retention capacity. It is therefore necessary to find the optimum balance between content and composition to obtain the best bread quality, and this might be done by selecting oat varieties with low beta-glucan content of low viscosity and molecular weight, and by a correct adjustment of the water in the formulation. However, reducing beta-glucan content and viscosity may compromise the health benefits. According to literature, the magnitude of the physiological effect that leads to a lowering of blood glucose level in humans is dependent on beta-glucan viscosity: the higher the viscosity, the larger the glucose reduction (Mälkki, 2004; Wood, 2007). The relation between the viscosity type of beta-glucans and cholesterol reduction has not yet been demonstrated (Wood, 2007). So, the optimum balance between health benefits and technological quality of the end product with regard to beta-glucan concentration still has to be determined. In addition, it is important to consider that a high internal water content may also be negative for shelf life properties, which are another major drawback of GF bread.

## CONCLUSIONS

The behavior of beta-glucans in dough systems is governed by two factors: concentration and molecular weight (viscosity), if the appropriate amount of water is added. Low beta-glucan oat flour has better extensibility properties than oat meal dough or oat flour dough enriched with beta-glucans. Overall, our findings indicate that beta-glucans are a key component determining rheology of oat-based dough systems. At least up to 2% beta-glucans appears to be positive for baking quality. Whether it is possible to make a good dough of oat containing significantly more beta-glucans remains to be determined.



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# CHAPTER 5

## Effect of kilning and milling on the dough-making properties of oat flour

### ABSTRACT

Oats are mostly used for porridges, flakes, and cereal breakfasts. The current oat kilning and milling methods are suited for these purposes. Bread-making applications have been explored, but the bread quality results are far from optimal. Here we studied whether kilning and milling methods may impact dough rheology. We tested oat grains treated with infrared (IR) and steam kilning for their Farinograph and dough extensibility behavior. We also assessed if particle size distribution and bran content could impact these dough properties. IR kilning had a very negative effect on the dough-making properties of oat grains, resulting in a very stiff and short dough, while steam-kilned dough showed similar behavior as observed without kilning. Oat meal resulted also in a stiff and short dough, and re-milling did not change this pattern. In contrast, removing all the bran from the oat meal improved dough-making properties. Dough rheology was negatively impacted by the bran, large and medium size bran being more harmful than fine bran. This is attributed to their high content of beta-glucans. In conclusion, current kilning and milling methods are not suitable for bread-making purposes and these treatments must be optimized. Whole grain oat meal is not a proper material for bread applications in the absence of fractionation.

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## INTRODUCTION

The most common uses of oats are related to porridges, flakes and meal, and the current technology is adapted to fit these uses. Kilning and milling are two important steps of oat processing that can influence the quality of the grains, affecting their potential use for new applications, e.g., bread-making. Oats contain high lipid and beta-glucan contents in comparison to other cereals, and these compounds represent a challenge during storage and food processing because they can have adverse effects on the sensory properties of grains and of end products. Oxidation of lipids can cause a rancid taste (Lehtinen and Kaukovirta-Norja, 2011), while beta-glucans affect food texture and mouth feeling due to their high viscosity. Therefore, special attention should be given to storage and processing of oats in comparison to other cereals.

Lipid oxidation is carried out by lipase enzymes. The lipase activity in oats is exceptionally high (O'Connor et al., 1992), which, in combination with the high lipid content, makes the stability of oat lipid a major challenge for the oat-processing industry. Lipolysis produces a rancid taste, and it generally begins when the grains are milled, although it also may start after the dehulling (Peltonen-Sainio et al., 2004). The lipase activity is localized mainly in the bran fraction, thus pearling of the outer grain layers is an alternative to preserve the sensory quality of the starchy endosperm flour (Hu et al., 2009), although the endosperm also presents some activity that needs to be neutralized. The inactivation of lipases is known as kilning. In the kilning process the dehulled grains undergo a heat treatment in which the heat must reach the outer bran layers of the grains, where enzymes are most active, and the inner endosperm layers (Lehtinen et al., 2003). This heat treatment can be performed dry or with steam, the latter being the most popular. Recently some millers have adopted the infrared (IR) technology for dry kilning because it allows to treat larger amounts of grains in a shorter time. Both methods are applied indistinctly of the food application for which the grains are intended.

Milling takes place after kilning of the grains. The purpose of grain milling is to produce flour or meal. The most common oat material used for baking applications is whole grain oat meal, which includes the bran fraction and, consequently, the beta-glucans that are mainly present in the bran. Beta-glucans are contained mainly in the bran fraction of the grains and it has been shown that their viscosity may impact negatively dough rheology when present at high concentrations (ca. 5%, Chapter 4). It is known that the texture of the resulting material (flour or meal) is important for bread applications, but also the composition (Chapter 4). Fractionation is an alternative to deal with the milling product of oats, as it would make possible to fractionate the material into fine

oat flour and bran. The fine oat flour would be suitable for bread applications because of its texture but also because of its low beta-glucan content, while the bran fraction could be used as a source of beta-glucans to enrich food products.

Wheat is the most used cereal for bread applications. Wheat does not need kilning treatment for enzyme stabilization, only drying to lower the moisture level and allow long-term storage, but milling and fractionation are key processing factors that influence wheat baking quality. In wheat it is known that heating can destroy gluten-forming properties (Schofield et al., 1983). For oats, it is unknown whether the current kilning and milling methods may impact negatively the dough making properties of the grains. Here we studied whether the kilning methods currently used (steam and IR kilning) as well as milling and fractionation can affect the dough extensibility properties of oat grains. The results provide information for future standardization specifically for bread applications.

## **MATERIALS AND METHODS**

### ***Materials***

We tested the effect of steam and IR kilning methods and of particle size distribution on dough extensibility properties of oat flour. For the kilning experiments we used oat grains from cultivar Gigant provided by a commercial miller (De Halm, The Netherlands). For the fractionation experiments we used commercial oat meal (De Vlijt, The Netherlands). Vital gluten (Cargill, The Netherlands) and salt (Merck) were used to prepare the dough according to Londono et al. (Chapter 3). The composition (as is) of oat meal was 12.5% protein (AACC Method 46-30), 5.54% beta-glucan (AACC Method 32-23), 62% starch (AACC Method 76-13), and 7% moisture.

### ***Kilning treatment***

The oat grains were treated following two procedures that are normally used to inactivate lipase activity: steam kilning and infrared (IR) kilning. Untreated grains were used as control. We did not test the affectivity of these methods to inactivate the enzyme because both are used often and known to be effective to prevent lipid oxidation.

The steam kilning treatment was performed in a 0.5 m<sup>3</sup> steel container as described by Londono et al. (Chapter 3). First, a grain layer of three cm (2 kg) was placed in the container and steamed for three minutes at 100 °C. Then, the grains were placed in a

drying oven at 80 °C overnight for tempering.

The INR kilning was performed following the protocol used by the miller that provided the grains (De Halm, The Netherlands) in a custom made IR device. First, the grains were placed in a Vibronet (Nebraska, USA) at 17-19% moist for one hour. Then, the grains were placed on a belt to heated by eleven IR high pressure burners (HOAF, The Netherlands) for about 15 seconds. Finally, the grains were stored in a tempering bunker for 15-20 minutes.

### ***Flours***

Flour was prepared from the grains of cultivar Gigant by milling at 8000 rpm using a laboratory pin mill (Hosokawa Alpine D-86199, Augsburg). After that, the resulting material was fractionated using a series of sieves of 0.500, 0.300, and 0.250 mm mesh. Only the fraction that passed through the <0.250 mm sieve was used to perform the experiments using the standard method developed by Londono et al. (2014).

### ***Particle size distribution***

We used two approaches to study the effect of particle size distribution on dough properties of oat flour. In the first approach we re-milled commercial oat whole meal using a laboratory mill (Hosokawa Alpine D-86199, Augsburg) at maximum speed (16.000), to reduce the particle size of the material. The particle size distribution was characterized by sieving the materials using a series of sieves of 0.50, 0.30, and 0.25 mm mesh. The particle size distribution of oat meal and re-milled oat meal is shown in Table 1.

**Table 1.** Particle size distribution of oat meal and re-milled oat meal.

Sieved fraction	Oat meal <sup>1</sup>	Re-milled oat meal <sup>2</sup>
	%	%
>0.500 mm	45	27
0.250 – 0.500 mm	15	15
<0.250	32	52
Total recovered	92	93

<sup>1</sup> The particle size of the commercial product.

<sup>2</sup> Re-milled at 16.000 rpm.

In the second approach we fractionated the oat meal into bran and fine flour using a series of sieves (0.50, 0.30, and 0.25 mm mesh). The particle size of the fine flour was <0.25 mm. This was used as the oat flour base for the experiments. Then, the bran fraction was re-milled at 16,000 rpm and fractionated again using the same series of sieves into three fractions. These fractions were used to prepare mixtures based on 60% fine flour and 40% of each of the re-milled bran fractions. Fine flour was used as control. The contents of protein (AACC Method 46-30) and beta-glucan (AACC Method 32-23) of the re-milled bran fractions were quantified. The composition of the bran fractions used in the mixtures is shown in Table 2.

**Table 2.** Protein and beta-glucan contents of the re-milled bran fractions used in the flour mixtures.

Fraction size	Protein (%)	Beta-glucans (%)
Large (>0.50 mm)	16.9	8.4
Medium (0.30-0.50 mm)	12.2	8.2
Fine (<0.25 mm)	10.2	1.6

### ***Dough preparation and extensibility test***

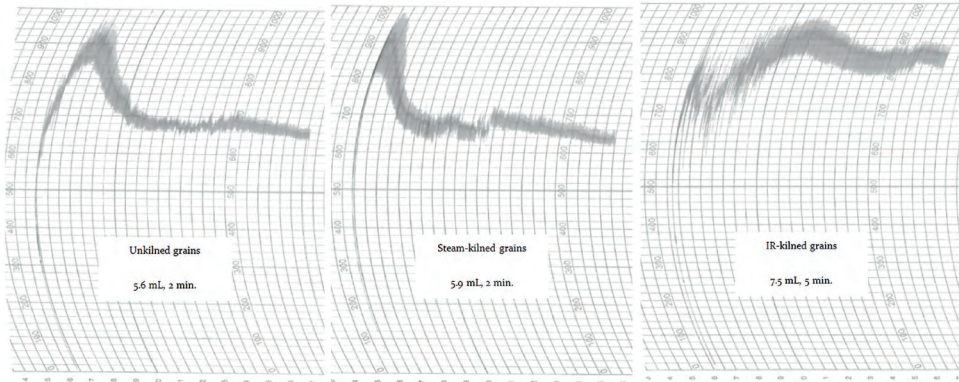
The dough for the extensibility test was prepared as described by Londono et al. (2013). Dough was prepared using a Micro-Farinograph (Brabender instruments, Mod.-No. 8110, Germany) and a 10 g mixer head. Water was added to prepare a dough system containing gluten (12.8%) at a peak consistency of  $850 \pm 20$  Brabender units (BU). The mixing time depended on the experimental conditions because the process of hydration to reach the desired consistency was slower in some treatments than in others. After mixing the dough was allowed to relax in a sealed plastic container within an incubator for 20 minutes at 30 °C and 85% relative humidity. Then, the complete dough was moulded into a ball by hand and pressed between two oiled lubricated and grooved Teflon blocks to make dough strips. The strips were allowed to relax again for 40 minutes within the mould in a plastic container at 24 °C and 80% relative humidity. Subsequently, the dough strip was placed in its holder and maximum resistance to extension (g) and extensibility at maximum resistance (mm) and total extensibility (mm) of each strip were measured using a Texture Analyser fitted with the SMS/Kieffer Extensibility Rig (Stable Micro systems). The standard settings for wheat flour dough were used according to instructions of manufacturer. The test was performed in triplicate and about 7-9 measurements were taken per dough sample. The resulting graphs represent the average of 21-27 measurements.



## RESULTS

### *Effect of kilning on dough extensibility*

Steam kilning and IR kilning, commonly used to inactivate the lipase activity in oat grains, were compared to check if heating had an effect on the dough extensibility properties of oat flour using the standard dough system developed by Londono et al. (2014). Flour from untreated grains was used as a control. Kilning treatments affected the extensibility properties of oat flour. With steam kilning a similar Farinograph behavior was obtained as with untreated grains, although the kilning increased the amount of water needed to reach the peak consistency by 5%. In contrast, IR kilning completely changed the Farinograph behavior of the dough: water absorption was increased by 34% and the time required to reach peak was increased from 2 to 5 min (Figure 1).



**Figure 1.** Farinograms of oat flour made of untreated grains (left), steam-kilned grains (middle), and IR-kilned grains (right).

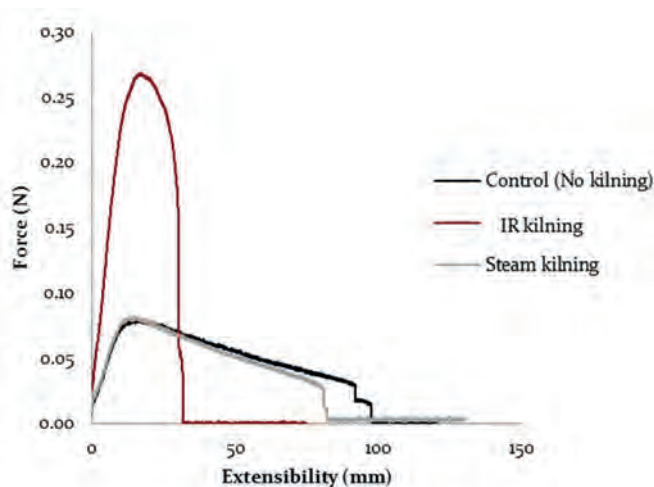
Using the micro-extension rig the dough samples prepared in the Farinograph (at peak time) were tested. Again, the non-kilned and steam kilned dough showed similar behavior. The IR kilned oat gave a very stiff and short dough (Figure 2).

### *Effect of particle size distribution on dough extensibility*

We tested the effect of particle distribution on dough extensibility to determine whether composition was more relevant for dough rheology than particle size. This effect was assessed in two ways. First, we compared the Farinograph and extensibility behavior of normal oat whole meal, as bought in a shop, with the same oat whole meal but re-milled at 16.000 rpm. Second, we prepared mixes of the 0,25 mesh flour fraction with



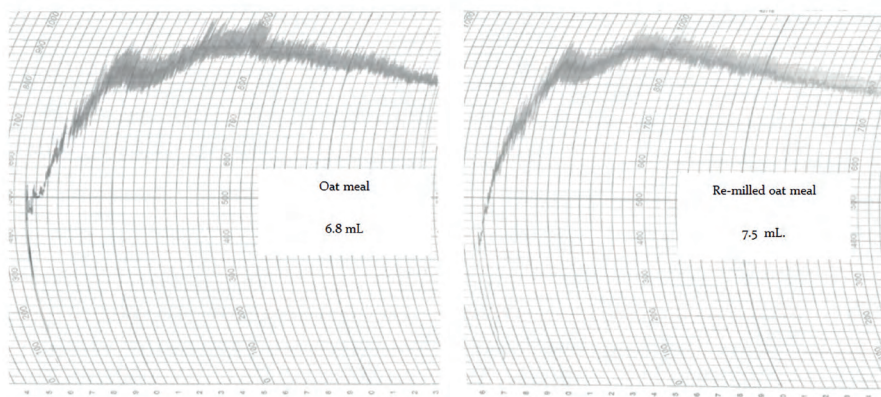
re-milled bran varying in particle size (<0.25, 0.30-0.50, and >0.50 mm) in proportions 60% fine flour and 40% re-milled bran. Fine flour without addition of bran was used as a control. We used the standard dough system developed by Londono et al. (2014).



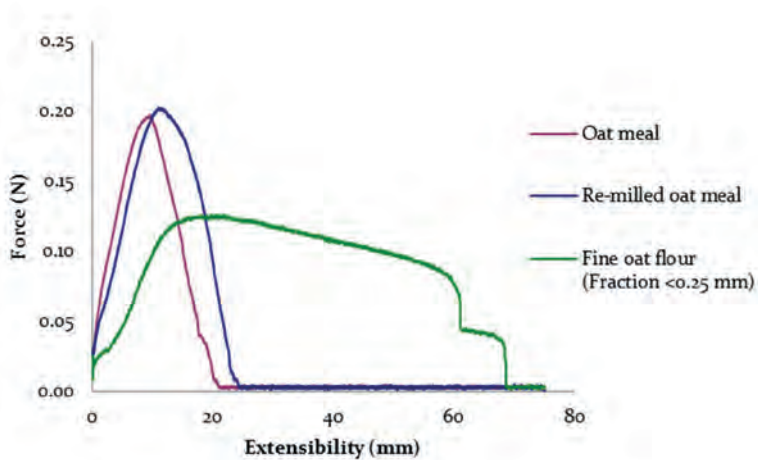
**Figure 2.** Extensibility of oat dough made of non-kilned grains, IR-kilned grains, and steam-kilned grains. IR kilning harmed the extensibility properties of the dough by increasing its stiffness and by causing a complete loss of its extensibility.

The water absorption was affected by the re-milling process. Re-milled oat meal needed 10% more water to reach the peak consistency. However, the Farinograph behavior of oat meal and re-milled oat meal was similar (Figure 3). The extensibility properties of the dough of oat meal and re-milled oat meal were similar (Figure 4). This implies that changing the size distribution of the material did not affect rheology. The particle size distribution is presented in Table 1. In contrast, removing all the bran from the meal to prepare a dough with fine flour (<0.25 mm) resulted in a less stiff and much more extensible dough in comparison to oat meal (Figure 4).

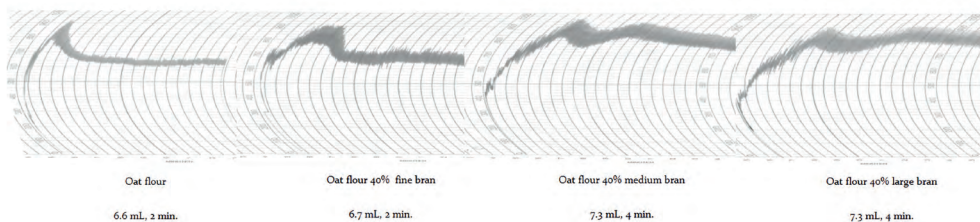
Replacement of 40% of fine flour with bran affected the water absorption and the Farinograph behavior of the flour. No difference was observed with addition of fine bran, while medium and large bran (Table 2) increased water absorption by 10% to reach the consistency of 850 BU, and the peak time from 2 to 4 min (Figure 5). The dough extensibility of oat flour was impacted negatively by the addition of bran. Fine bran fraction had a smaller impact than the medium and large bran fractions (Figure 6).



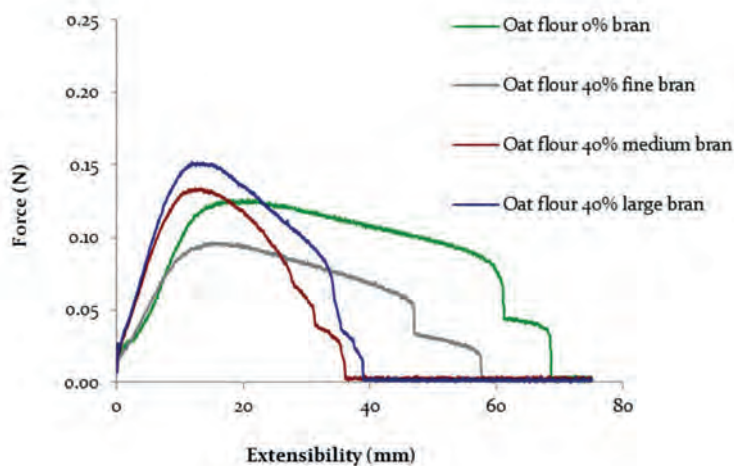
**Figure 3.** Farinograph behavior of oat meal and re-milled oat meal. The particle size distribution did not affect peak time and stability consistency (but re-milled oat meal had absorbed 10% more water).



**Figure 4.** Extensibility of dough made of oat meal, re-milled oat meal, and of the finest sieved fraction of oat meal (<0.250 mm).



**Figure 5.** Farinograph behavior of doughs made of oat flour (<0.25 mm) and of mixtures of 60% oat flour and 40% fine, medium and large bran.



**Figure 6.** Dough extensibility of fine oat flour (<0.25 mm) and of mixtures based on 60% fine flour and 40% bran particles of various sizes: fine (<0.25 mm), medium (0.30 – 0.50 mm), and large (>0.50 mm).

## DISCUSSION

As oat bread applications are being explored it is important to establish whether kilning and milling methods are also suitable for bread-making purposes. We tested two kilning methods, steam and infrared (IR) kilning, and the effect on particle size distribution on oat dough making properties, assessed by Farinograph behavior and by dough extensibility rig. The experiments were performed using the standard dough system developed by Londono et al. (2014), which contained 12.8% gluten.

IR harmed the dough making properties of oat flour completely. IR-kilned grains produced a very stiff and non-extensible dough (Figure 2). This means that IR kilning is not suitable for bread applications as extensibility is an important characteristic related to bread quality. It is known that IR and steam kilning treatments can change the shape of starch granules and affect the protein network (Hu, et al., 2010). The negative effect of IR kilning in oat grains might be comparable to the damage that heat causes to wheat grains, which also is characterized by a negative impact on gluten-forming properties (Schofield et al., 1983) and on baking quality (Ghaly and Taylor, 1982). The purpose of the heat treatment in wheat grains is just decreasing moisture of avoid damage during storage (Bruce, 1992), but in oats the main purpose is to inactivate enzymes and thus to prevent lipid oxidation which would otherwise cause a rancid taste. Oats are also heat-treated to prevent developing a particular taste and aroma (Moltenberg et al., 1986). According to our results, the steam kilning method used in this study would be

appropriate for bread applications. However, more research is needed to standardize the process to establish the most suitable conditions to stabilize oat grains. Steam (hydrothermal) treatments to oats have also been reported as detrimental for dough rheology of composite wheat-oat mixtures (Zhang et al., 1998).

Reducing the particle size of oat meal did not affect its dough making properties. Both doughs were stiff and short. In contrast, removing all the bran from the oat meal resulted in a more extensible and less stiff dough (Figure 4). From this we can conclude that oat dough rheology is negatively impacted by the bran. This could be due to the large particle size of the bran as it could cause physical disruption of the protein network (Noort et al., 2010), or to the chemical composition of the bran (Wang et al., 2004). Our results indicate that beta-glucans are the oat component responsible for the negative impact of bran on the rheology of our dough system. Beta-glucans, which are mainly present in the bran fraction of oats, increase dough stiffness and reduce dough extensibility (Chapter 4). There was no difference between adding high viscosity beta-glucans to oat flour at a concentration of 5% (w/w) and using whole grain oat meal (which had a beta-glucan content of 5.5%). Replacing 40% of the bran-free oat flour with bran of various sizes (Table 2). Large and medium bran fractions affected dough properties in a larger extent than fine bran fraction (Figure 6). The main difference among the bran fractions was their beta-glucan content: large and medium bran fractions contained ca. 8% beta-glucans, while fine bran fraction contained 1.6% only (Table 2). Wheat bran has also a detrimental effect on dough bread-making performance, but in contrast to oat, fine bran is more harmful than large and medium size bran (Zhang and Moore, 1999). These findings are important because they point out the need of fractionation of the milled oat material. Whole grain oat meal is not suitable for bread applications because of its high content of beta-glucans. However, fractionation is an interesting alternative that would allow producing low beta-glucan oat flour for bread applications next to bran which can be used to enrich other food products.

## CONCLUSION

The current kilning and milling methods used to process oats are not suitable for bread applications. IR kilning harms completely the dough making properties of oat grains, giving as a result a very stiff and short dough. Steam kilning was better. Oat meal is not a proper material for bread applications because its high beta-glucan content interferes with dough rheology. Fractionation could be used to adjust this.

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# CHAPTER 6

## Effect of whey protein particles on dough-making properties of oat flour

### ABSTRACT

There is an increasing demand for good quality oat bread for people that suffer from celiac disease or that want to benefit from the healthy compounds present in oats. However, to obtain an oat bread of similar quality and texture to wheat bread, it is necessary to improve the rheological properties of oat flour dough relevant for leavening. Whey protein particles (WPP) were proven to be successful for enhancing viscoelastic properties of wheat starch dough, allowing loaves with specific volumes of ca 3.7 mL/g. Here we studied whether WPP could have the same positive effect on oat flour dough. The effect of WPP was tested at two WPP concentrations (2.2 and 2.0% w/w) and at two dough consistencies (500 and 700 BU). WPP affected dough mechanical properties of oat flour by increasing its resistance to extension and its gas retention capacity. Incorporation of WPP to oat flour improved crumb texture. However, in our small scale baking experiments, WPP did not increase loaf specific volume and had a negative effect on gas production. WPP are promising to be used as a structuring agent in oat flour and could lead to improvements on loaf specific volume and texture, but the process should be optimized.

Keywords: Gluten-free, Celiac disease, bread quality

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## INTRODUCTION

There is an increasing demand for good quality and healthy gluten-free products to diversify the diet of people that suffer from celiac disease, and also to cater demands for gluten-free products in the general consumer population. Gluten-free breads are generally based on starches of low nutritional quality, and it is estimated that – as a consequence – 20–38% of celiac patients have nutritional deficiencies (Barton et al., 2007; Thomson, 2000). Oat-based bread is an interesting alternative to enrich the gluten-free diet because oat is a good source of B complex vitamins, proteins, fat, minerals, and soluble fibre (Butt et al. 2008). Unfortunately, the baking quality of oat bread is poor in comparison to wheat bread regarding loaf volume and texture. This inferiority has been attributed to the absence in oat flour of gluten-like components capable to form a strong network to retain the gas produced by the yeast during the proofing and baking processes. In wheat flour, gluten proteins form a network that confers unique viscoelastic properties to wheat dough. Therefore, baking quality of wheat flour is mainly determined by its gluten content and its composition (Bushuk, 1998).

The formulations for gluten-free oat bread have generally been based on batter systems containing about 120% water (Hüttner et al. 2010, Hüttner et al. 2011). These formulations rely on the use of emulsifiers and hydrocolloids to stabilize gas cells during proofing and baking (Hüttner, et al., 2011). Despite the use of additives, the quality of the resulting breads is still low in comparison to wheat bread regarding specific volume and texture. Also, because of the high water content of the final bread, these breads typically suffer from low shelf life and a poor microbiological quality in comparison to a dough based bread.

Oat fibre consists for a large part of soluble beta-glucans. There is evidence that these beta-glucans play a role in the technological properties of oat flour. The behaviour of beta-glucans in dough systems depends on concentration and viscosity type (Chapter 4). Those factors are affected by the amount of water present in the system and related to the molecular weight of the beta-glucans, respectively (Colleoni, et al., 2002). Londono et al (Chapter 4) have shown that beta-glucans influence both the viscous properties of dough systems and their extensibility properties. Interestingly, when present at low concentrations (<2%), these were improved. Gas retention is related to gas cell stability; both bulk viscosity and surface elasticity play a role here (Kloek et al., 2001). The main mechanism by which beta-glucans contribute to gas cell stability is viscosity. Viscosity on its own does not suffice to stabilize the small gas cells in dough that are required for a fine crumb structure of the final bread (Kloek et al., 2001). In contrast to wheat,



the proteins of oat flour are not able to provide sufficient elasticity. So, the limitation of oat flour with regard to specific volume and texture is related to elasticity. Whey proteins have been demonstrated to be a promising ingredient to improve viscoelastic properties of gluten-free formulations, especially when applied in the form of whey protein particles (WPP) (Riemsdijk, et al., 2011). Whey proteins are globular proteins isolated from liquid whey –a by-product from the cheese industry. The particles result from a process of self-aggregation of whey proteins induced by heat followed by a cold gelation process in the presence of a biopolymer (Riemsdijk, et al., 2011). When added to wheat starch, WPP acted as a gluten substitute, forming a network capable to hold gas and to make the dough expand, leading to the production of loaves with a specific volume of 3.7 mL/g (Riemsdijk et al., 2011); a value that falls within the specific volume range of regular wheat bread which is from 3.5 to 4 mL/g (Belitz et al., 2004). This was a relevant finding because for first time such large volume loaves were obtained with a gluten-free formulation based on a system closer to a dough than to the commonly used batter system. From a practical point of view this is important, because bakers are more inclined to work with dough systems than with batters, because dough is easier to handle and allows them to use the existing technology and equipment developed for wheat.

Londono et al. (Chapter 3) demonstrated that it is possible to reconstruct the extensibility profile of a weak wheat flour using oat flour and vital gluten. Knowing that, we aimed here to investigate whether addition of WPP to oat flour based dough could also lead to improved bread-making properties. This is by no means trivial, since oat flour contains many other components in addition to starch, that could affect the function of WPP in dough. For this we replicated the wheat starch system used by Riemsdijk et al. (2011), and then we replaced wheat starch by oat flour. We characterised the dough mechanical properties –extensibility, gas retention capacity, and loaf specific volume- both under the original conditions and then we adjusted the conditions (amount of water, dough consistency, WPP concentration) to obtain a dough that was easy to handle.

## **MATERIALS AND METHODS**

### ***Materials***

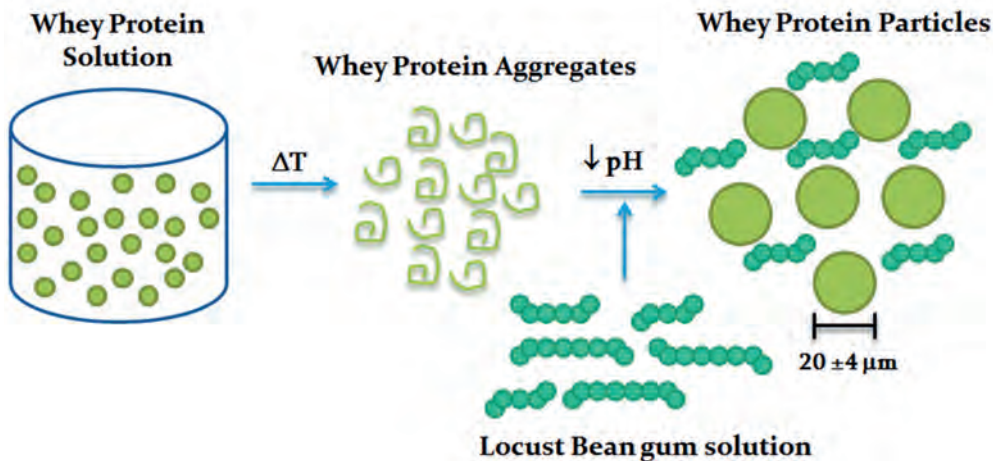
Preparation of WPP was done using whey protein powder with a protein concentration of 90% w/w (Davisco Food International Inc, USA.), Locust bean gum (Danisco Holland BV, The Netherlands) and Glucono-delta-lactone (GDL) (Sigma Chemicals, The Netherlands). All chemicals were of analytical grade.

For the dough preparation we used wheat starch (Sigma Chemicals) to replicate the system developed by Riemsdijk et al. (2011). Commercial oat meal (De Vlijt, the Netherlands) was used as a source of oat flour. Dried active bakery yeast (Bruggeman Instant, Belgium, Lot 50109T2) and D-glucose (Sigma) were used for the gas holding capacity tests. Salt (Merck) was added to all mixtures.

### *Preparation of whey protein particles (WPP)*

WPP as in Riemsdijk et al. (2011). First, the whey protein solution (6.5% w/w) was prepared by stirring for 2,5 h at 68°C to promote formation of small protein aggregates (Alting and Hamer, 2000). The Locust bean gum solution was prepared by stirring for 1 hour at 80°C.

After preparation, both solutions were allowed to cool down to 25°C and mixed to give a final concentration of 3% whey protein and 0.45% Locust bean gum. Glucono-delta-lactona (0.2%) was added to the final solution to decrease the pH gradually to 5.2. This step is known as a cold gelation process and leads to the formation of WPP. After addition of GDL, the WPP suspension was stirred for one hour and incubated at 4°C for 16h. The scheme of the process is presented in Figure 1.



**Figure 1.** Scheme of the formation process of whey protein particles (WPP) (after Riemsdijk et al., 2011). In the first step, whey proteins form protein aggregates induced by heat (T). The second step consist of a self-aggregation process of the protein aggregates induced by a pH reduction in presence of a Locus bean gum solution. The final size of WPP is  $20 \pm 4 \mu\text{m}$ .

### ***Characterisation of WPP***

For determining the structure and functionality of the WPP, the suspension was centrifuged for 15 minutes at 2000 x g to recover the particles formed; the supernatant was discarded. Next, excess Locust bean gum was removed by washing with distilled water followed by centrifugation. This washing step was repeated twice. Finally, distilled water was added to re-suspend the particles and to adjust the concentration to 6.5% (w/w) whey protein.

The shape and size of WPP were confirmed using a single lens microscope (20x), and a confocal laser scanning microscope (CLSM) using images made at 20x. Rhodamine B (M=479 g/mol, Merck, Germany) was used to label the proteins. The dye (0.2% w/v) was prepared using distilled water as solvent. The size distribution was established by analysing 5 images using Image-J software.

The functionality of WPP was checked by measuring the shear stress and the apparent viscosity of the suspension. The measurements were performed in a Haake RheoScope1 (Thermo Electron Corporation, Germany) by applying shear rate sweeps at 25°C in a cone/plate (angle 1°/diameter 75 mm). First the sample was equilibrated for 15 min and then the shear rate was increased from 1 to 300s<sup>-1</sup> in a logarithmic scale (21 steps, 10 measurement points, 10 sec.). The functionality of our WPP was also checked in the WPP-wheat starch system to compare their behaviour with the reported by Riemsdijk et al. (2011).

### ***Flour mixtures and dough preparation***

All experiments were performed using the sieved fraction of commercial oat meal (< 0.250 mm). In the context of this paper, this fraction is referred to as oat flour. When WPP were incorporated in oat flour under the same conditions used for wheat starch: 2.5% whey protein and 300 BU consistency (Riemsdijk et al., 2011), this resulted in a sticky dough that was difficult to handle. Consequently, the conditions were adjusted by reducing the amount of water in the system to obtain a dough easier to handle. This was done by reducing the amount of WPP suspension added to the system to increase dough consistency to 500 and 700 BU. This also decreased the dough whey protein concentration from 2.5%, used in the wheat starch-WPP system, to 2.2 and 2.0%, respectively. A control made of pure oat flour was included for each consistency treatment.

The dough was prepared according to the AACC method 54-21 using a 10 g Micro-Farinograph (Brabender instruments, Germany, Mod.-No. 8 110). NaCl (1.3% flour basis) was added to all mixtures (Method 54-21, AACC) and it was pre-mixed with the

flour for one minute before adding the WPP suspension, or the water, in the case of the controls. Mixtures were mixed until the stability consistency was reached (typically between 2 and 2.5 minutes). Each dough was prepared in triplicate.

### ***Evaluation of dough mechanical properties***

The effect of WPP on mechanical properties of oat flour was evaluated by extensibility, gas retention and baking tests. The variables considered were: maximum resistance to extension and mm extension at maximum resistance, dough specific volume gain and loaf specific volume.

### ***Extensibility test***

The dough extensibility properties were measured as described by Londono et al. (Chapter 3). After mixing the dough was incubated for 20 minutes at 30 °C and 85% HR. Then, the dough was moulded into a ball and pressed between two oiled grooved Teflon bases to make dough strips. The strips were allowed to relax again for 40 minutes within the grooved bases in a plastic container at 24 °C and 80% relative humidity. Subsequently, maximum resistance to extension (g), extensibility at maximum resistance (mm) and total extensibility (mm) of each strip were measured using a Texture Analyser fitted with the SMS/Kieffer Extensibility Rig (Stable Micro systems). The standard settings for wheat flour were used according to the instructions of the manufacturer: Measure force in tension; return to start; 2.0 mm/s pre-test speed; 3.3 mm/s test speed; 10.0 mm/s post-test speed; 120 mm distance; 5g trigger force; 200pps data acquisition rate. Each dough treatment was made in triplicate and from each dough 7-9 strips were obtained to perform the test. Thus, the resulting graph is the average of 21-27 measurements.

### ***Gas holding capacity***

To evaluate the effect of WPP on the gas retention capacity of oat flour, we measured first the gas production over time, the specific volume gain at a fixed amount of gas produced (20 mL), and dough specific volume gain over time. Dried active bakery yeast (1.8%) and D-glucose (1.0%) were added to the flour and pre-mixed for one minute before incorporating the WPP suspension under dough preparation. The dough was prepared in the Farinograph as described.

Gas production was measured using the method described by Peighambardoust et al. (2010). Dough pieces of 5 g were placed in a closed Erlenmeyer connected by a hose to a graduated cylinder filled with acidic water (pH=2.0). The temperature of the whole

system was kept at 40°C with the help of a water bath. A graduated cylinder (250 mL) was used to measure the volume of the gas by water displacement (see Peighambardoust et al., 2010). The volume displaced was measured every five minutes for a period of 200 minutes. The experiment was replicated five times per dough formulation.

The same procedure was followed to measure the dough volume at a fixed amount of gas produced, but the dough was taken out of the system once the 20 mL gas had been produced. The volume of the dough was measured in a graduated cylinder filled with n-Hexane (Sigma) and this was used to calculate the dough specific volume.

To measure the volume increase over time the dough was divided into five pieces of 2.7 g; one piece was used to measure the initial volume, and the other four were placed in a climate chamber at 35°C and 85% RH for proving. The volume of the dough balls was measured every 15 minutes. In total 4 measurements were made per dough formulation during 60 minutes of proving time, and the procedure was made in triplicate. From the data, specific volume and volume gain were calculated.

### ***Baking: loaf specific volume***

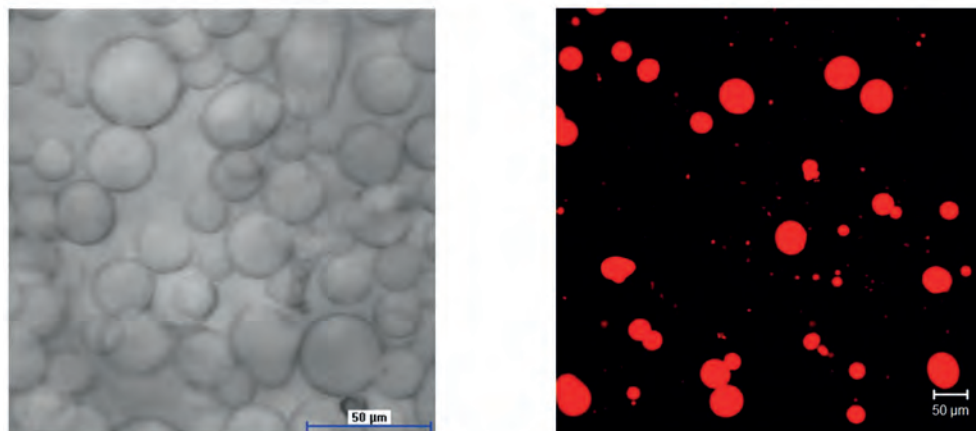
The baking experiments were performed following the same protocol that was used for wheat starch/WPP breads (Riemsdijk et al. 2011), with the exception of the proving time. The amount of flour for all the treatments was scaled up from 10 to 50 g, and the same was done with the other ingredients. The dough was prepared using the 50 g Farinograph mixer head (Brabender instruments, Germany). After mixing the dough was split into two dough balls of 30 g each and placed in baking tins separated by an aluminium piece that divided them into two compartments of equal dimensions. The dimensions of the baking tins were 18 cm<sup>2</sup> (length top), 15 cm<sup>2</sup> (length bottom), and 3 cm height. The baking tins containing the dough balls were placed at 35°C and 85% RH for 30 minutes. Then, they were put into a home baking machine (Princess, Silver Breadmaker, type 1935) at 200 °C for 35 minutes. Per mixture, a total of six loaves were baked.

## **RESULTS**

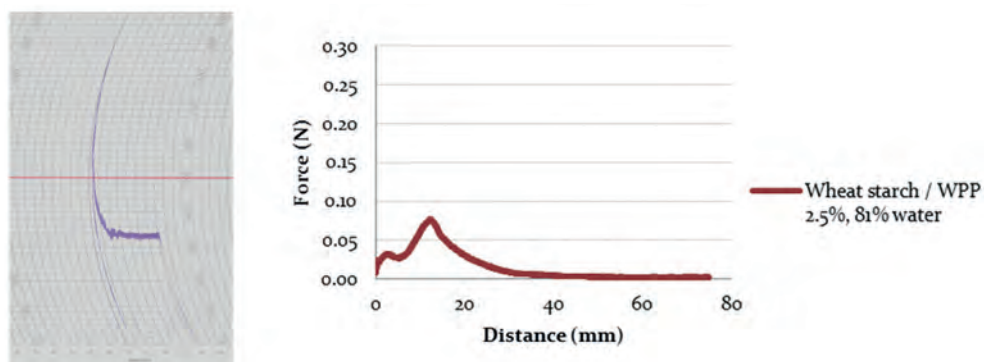
### ***Structure and viscoelastic properties of WPP***

The rheological measurements performed under the same parameters used by Riemsdijk et al. (2010), confirm the viscoelastic properties of the WPP produced for this study and their functionality on wheat starch. WPP presented the expected spherical

shape and their average size was  $18 \pm 4 \mu\text{m}$  (Figure 2). WPP also behaved as expected when incorporated in wheat starch at the same conditions as used by Riemsdijk et al. (2010): WPP 2.5% whey protein, 81% water, and 300 BU consistency (Figure 3).



**Figure 2.** Structure of WPP obtained on a single lens microscope (left) and on a Confocal Laser Scanning Microscope -CLSM- (right). Rhodamine B (0.2%) was used to label the proteins to distinguish them from the Locus bean gum. The average size of the particles was  $18 \pm 4 \mu\text{m}$ .



**Figure 3.** Farinogram and extensibility measurements that resulted from incorporation of WPP into wheat starch. The system behaved in accordance to the findings of Riemsdijk et al. (2011).

### ***Dough texture and Farinograph behaviour***

***Under the conditions used for wheat starch (300 BU, WPP 2.5% whey protein):*** Incorporation of WPP suspension to oat flour at the same conditions used for wheat starch resulted in a sticky system difficult to handle; no dough development was observed. While under these conditions the oat flour-WPP system received 70% water,

the control made of pure oat flour required an addition of only 58% water (w/w) to reach the same consistency. The system was closer to a batter than to a dough.

***After modifying the conditions:*** In order to obtain a dough that could be handled, the amount of WPP suspension added to the system was decreased with the purpose of reducing the water content in the system, but as a consequence of this, the WPP concentration also decreased. Thus, the effect of WPP was not tested at the conditions used for wheat starch, but at two higher consistencies with their respective lower whey protein concentrations: i) 500 BU consistency and WPP 2.2% whey protein; ii) 700 BU and WPP 2.0% whey protein. These conditions resulted in a dough with a good texture.

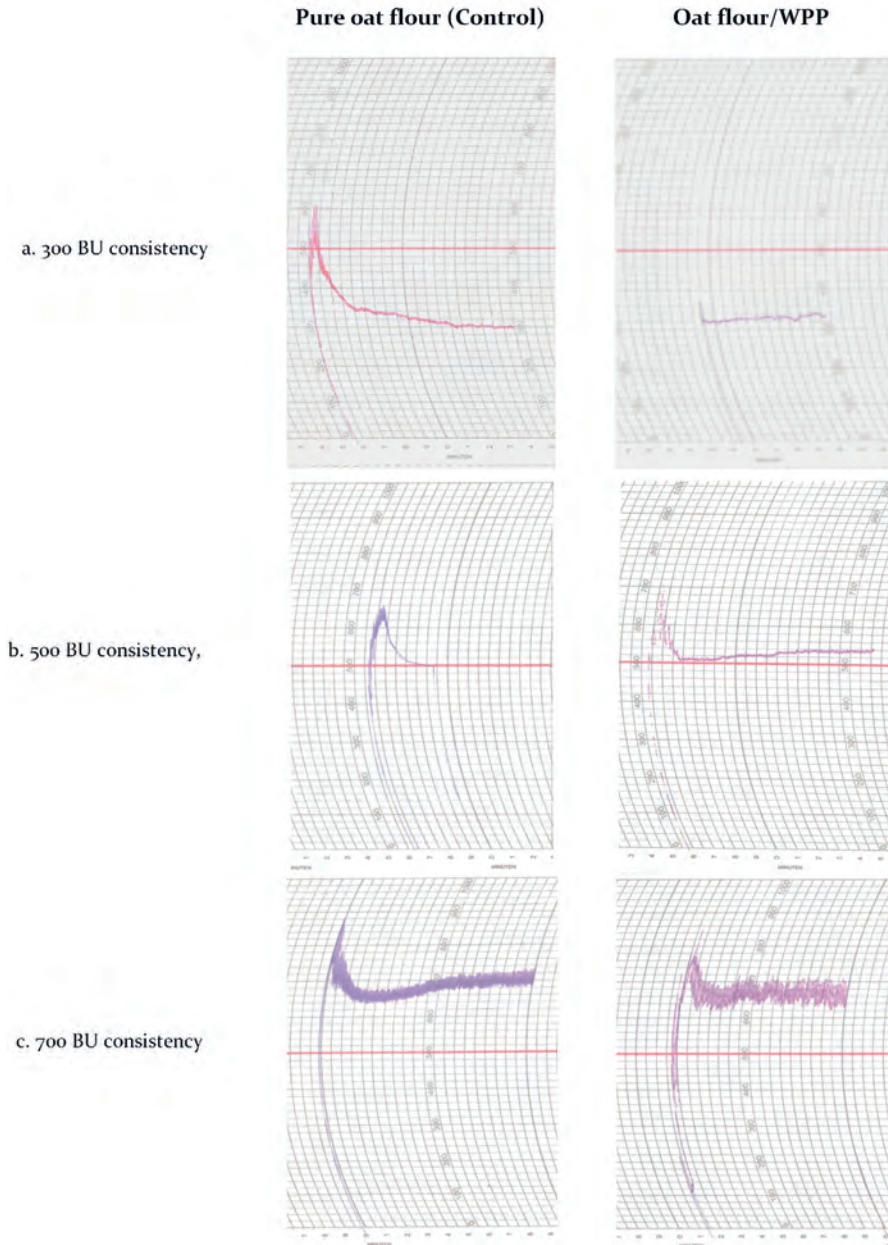
No dough development was observed at 500 BU consistency (Figure 4b). In contrast, at 700 BU there was dough development, visible as the occurrence of a peak consistency and a later stabilization, showing some width in the oscillations of the mechanical pen (Figure 4c).

The Farinograph curves of pure oat flour at both conditions tested showed that consistency has a large effect on dough development (see controls in Figure 4). So, the Farinograph behaviour observed in oat flour/WPP at 700 BU is the result of an interaction between water availability (which governs consistency), water binders like beta-glucans, and WPP.

### ***Effect of WPP on dough extensibility***

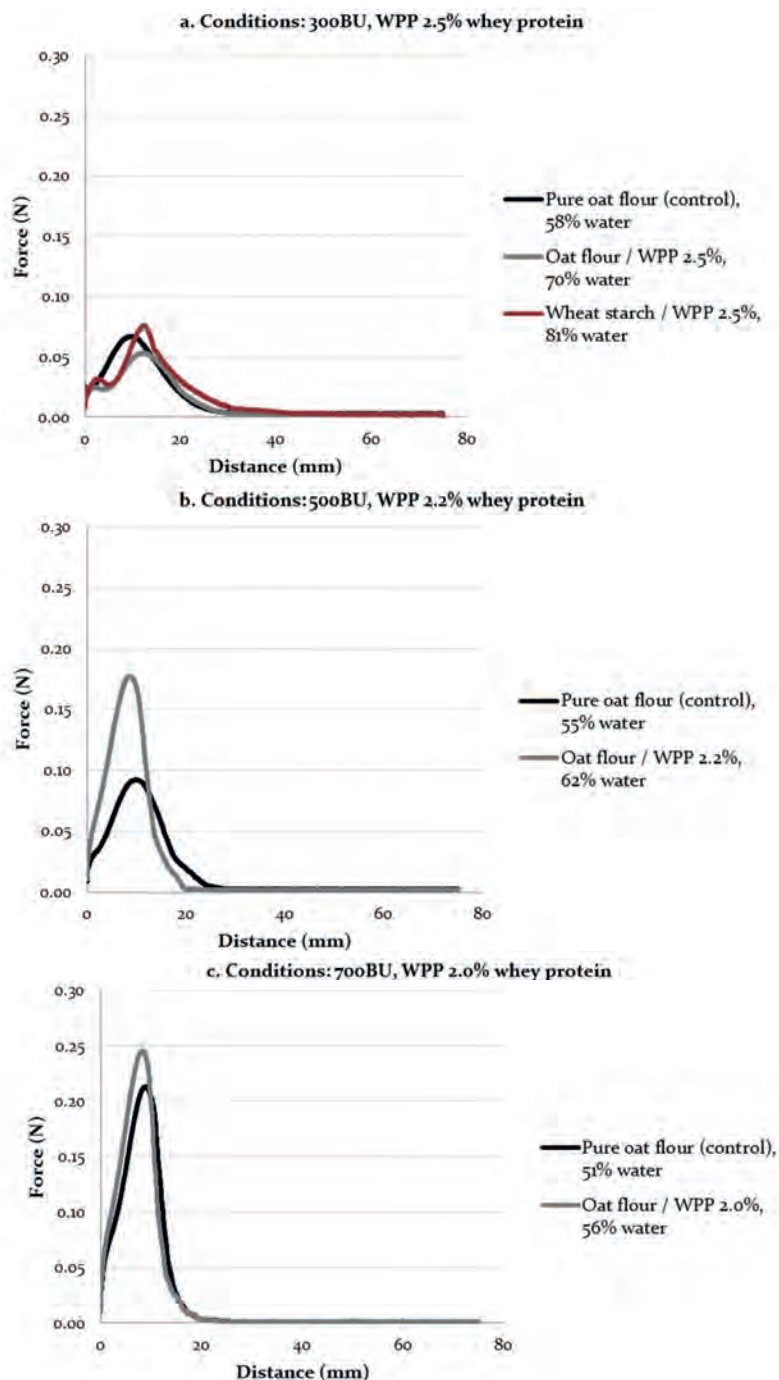
Incorporation of WPP at the conditions used for wheat starch resulted in a loss of resistance to extension in comparison to the control made of pure oat flour. The dough presented a flowing behaviour that can be observed as a delay to begin with the extensibility test and as an initial bend in the graph (Figure 5a). In contrast, incorporation of WPP to oat flour at the adjusted conditions increased the maximum resistance to extension of the dough from 9 to 17 g at 500 BU (Figure 5b), and from 21 to 24 g at 700 BU (Figure 5c). Incorporation of WPP did not produce significant changes in extensibility at maximum resistance.





**Figure 4.** Farinograph curves of pure oat flour (control) and oat flour containing WPP at three conditions: 300 BU and WPP 2.5% (a); 500 BU and WPP 2.2% (b); and 700 BU and WPP 2.0% (c). In the farinogram the 'x' axes represents time, and the 'y' represents consistency; the scale goes from 0 to 1000 BU, and the red line indicates the 500 BU consistency. Reduction of water in the system to increase consistency from 500 to 700 BU improved dough development as it can be observed in the controls (left). Incorporation of WPP at 700 BU improved dough development and increased the strength of the network in comparison to pure oat flour at the same consistency (c).



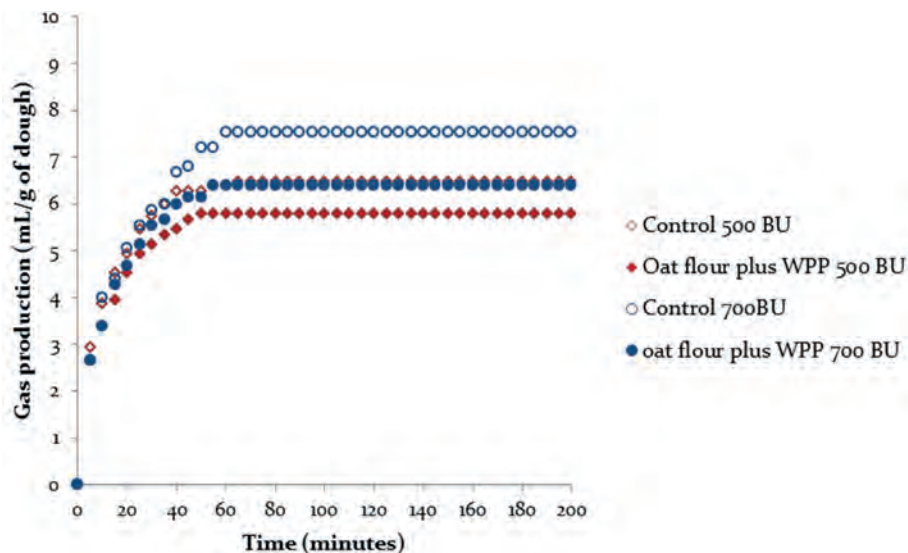


**Figure 5.** Dough extensibility of oat flour and oat flour plus WPP at different conditions: at 300 BU consistency and WPP 2.5% whey protein (a); at 500 BU and WPP 2.2% whey protein (b); at 700 BU and WPP 2.0% whey protein (c). Wheat starch plus WPP was also included in (a) for comparison.

## Effect of WPP on gas holding capacity

### Gas production

The gas holding capacity was tested at 500 and 700 BU. Total gas production was higher at 700 BU consistency than at 500 BU (Figure 6). In both cases, gas production was lower in the dough containing WPP than in the controls made of pure oat.



**Figure 6.** Gas production over time at 500 and 700 BU consistency. At both consistencies oat dough containing WPP produced less gas than the corresponding pure oat flour control. Each data point corresponds to the average of five measurements per dough formulation.

### *Dough specific volume at a fixed amount of gas produced (gas holding capacity)*

Because of the differences in gas production observed, we tested the gas holding capacity of the dough at a fixed amount of gas produced. The volume of the dough was measured after 20 mL of gas production (16-18 minutes of proofing). This volume was used to calculate the specific volume and the specific volume gain. At 500 BU no difference between the specific volume and specific volume gain between the control and the WPP-containing dough (Table 2) was observed. In contrast, at 700 BU the specific volume and the specific volume gain were 21% and 41% lower in the dough containing WPP than in the control, respectively (Table 1).

**Table 1.** Effect of WPP on specific volume and specific volume gain at 20 mL of gas production at 500 BU and 700 BU; the time required was 18 min and 16 min, respectively. The experiment was replicated five times per dough mixture.

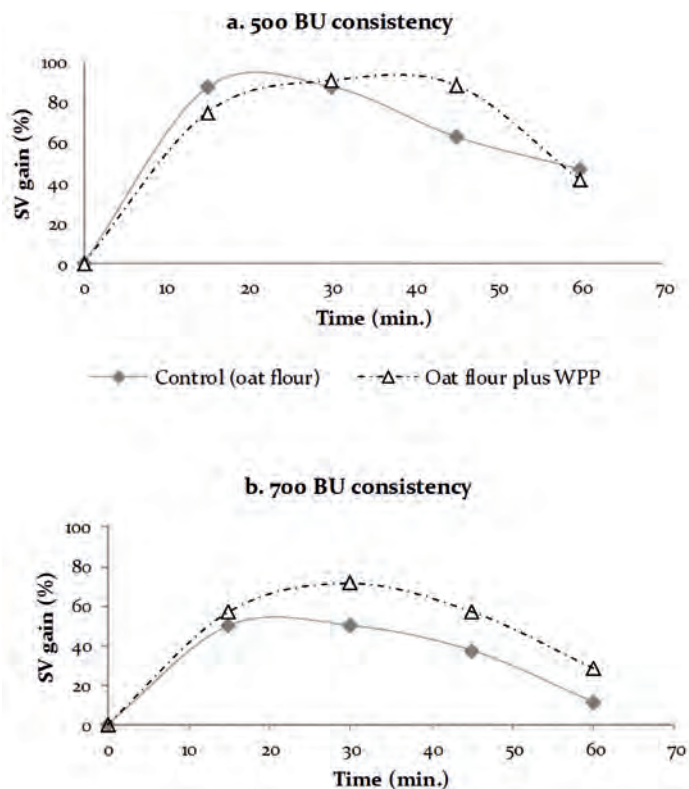
Experiment/ consistency	Mixture	SV At 20 mL gas production mL/g	SV gain At 20 mL gas production %
500 BU	Control	1.61	101
	Oat flour plus WPP 2.2 %	1.60	100
700 BU	Control	1.68	98
	Oat flour plus WPP 2.2 %	1.32	58

### *Dough specific volume gain over time*

The specific volume was monitored during 60 minutes, and this information was used to calculate the specific volume gain in relation to the initial volume. The largest initial specific volume after mixing was observed in the control made at 700 BU (1.0 mL/g), which was significantly larger than the dough containing WPP at the same consistency, and that of the control and the dough containing WPP at 500 BU consistency. The values for these treatments ranged between 0.78-0.8 mL/g of dough.

All mixtures reached a maximum dough specific volume of 1.5 mL/g. Incorporation of WPP increased the specific volume gain of the dough at both consistencies tested, but the effect was more evident at 700 BU (Figure 7).

The data shown in Figure 7a and b clearly show differences in gas cell stability. For both conditions, gas cell stability is improved when WPP is present. At 500 BU, gas cell stability is significantly better than at 700 BU.



**Figure 7.** Effect of WPP on gas retention capacity of oat flour, expressed as the proportion of specific volume (SV) gain over time at two consistencies and protein concentrations: (a) 500 BU consistency and 2.2 % whey protein, and (b) 700 BU consistency and 2.0 % whey protein.

### *Loaf specific volume*

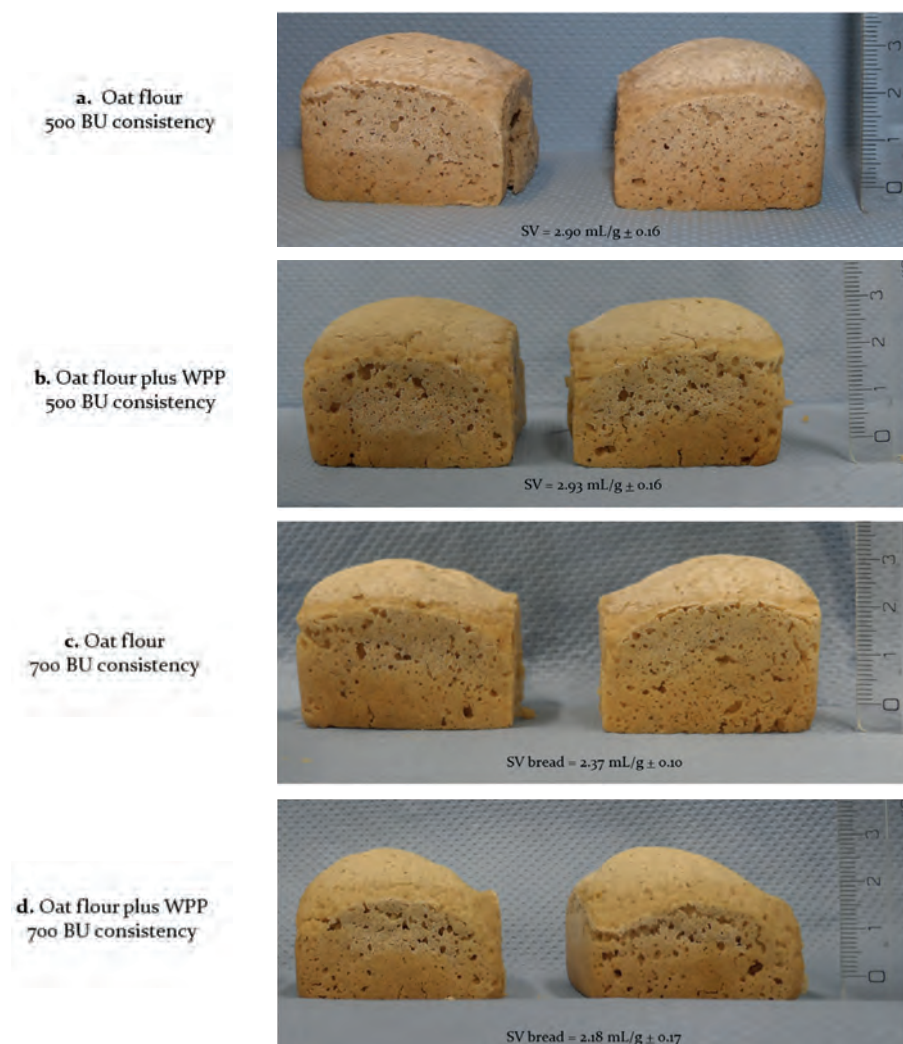
Oat flour based dough were prepared with or without added WPP at both 500 and 700 BU and proofed for 30 min. Then, miniature loaves (30 g dough) were baked as described in materials and methods. Characteristics of the resulting breads like weight and specific volume were measured. Crumb structure was qualitatively assessed.

We observed clear differences as the result of dough consistency: the loaf specific volume was considerably lower at 700 BU as compared to 500 BU (Table 2, Figure 8). The inclusion of WPP did not result in a significant improvement at both dough consistencies.

WPP did not have an effect on bake loss (Table 2). Markedly, we observed that WPP doughs produced breads with a better crumb structure than non WPP breads (Figure 9).

**Table 2.** Effect of WPP on baking on bake loss and loaf specific volume.

Consistency BU	Mixture	Bake loss <sup>1</sup> %	SV dough mL/g	SV bread mL/g
500	Oat flour (control)	29.1 ± 1.3	2.24 ± 0.10	2.90 ± 0.16
	Oat flour/ WPP	29.8 ± 0.5	2.26 ± 0.08	2.93 ± 0.10
700	Oat flour (control)	25.9 ± 1.0	1.88 ± 0.07	2.37 ± 0.10
	Oat flour/ WPP	25.0 ± 0.3	1.75 ± 0.13	2.18 ± 0.17

<sup>1</sup> water loss after baking.**Figure 8.** Loaves made of mixtures with or without WPP. Presence of WPP did not have any effect on loaf specific volume (SV) at 500 BU but had a negative effect at 700 BU. Increasing consistency also had a negative effect on SV.



a. Oat flour



b. Oat flour /WPP



**Figure 9.** Crumb appearance of bread slices of oat flour with or without WPP. Incorporation of WPP improved crumb texture at both consistencies tested. The picture shows the bread slices made at 500 BU consistency: (a) pure oat flour; (b) oat flour/WPP.

## DISCUSSION

Since the low volume and reduced texture of oat bread are attributed to the poor dough mechanical properties of oat flour, we set out to study whether whey protein particles (WPP) could improve the mechanical properties of oat flour dough. WPP were proven to be successful in improving the specific volume of wheat starch-based breads. The main question behind our study was if WPP could perform in the same way in the presence of other components different to starch. While wheat starch can act as a kind of inert filler in the continuous matrix or as a part of the protein-starch network (Bloksma, 1990), with the use of oat flour other components enter the system, some of which have a great capacity to bind water, e.g. beta-glucans, and may interact positively or negatively with WPP.

Our approach was first to replicate the results that Riemsdijk et al. (2011) obtained by incorporating WPP into wheat starch. By doing this we confirmed the functionality of the WPP produced for this study. Once the functionality was assured, we proceeded to replace wheat starch with oat flour in the system under the same conditions, but this resulted in a sticky dough difficult to handle because of an excess of water in the system. Consequently, we had to adjust the conditions in order to improve dough texture. We did this by reducing the amount of WPP suspension added to the system to increase the consistency of the oat flour-WPP dough system (from 300 BU, originally used in wheat starch, to 500 and 700 BU). However, the implication of this approach was the reduction of the WPP concentration. Therefore we could not compare the effect of incorporating WPP to oat flour at the same WPP concentration that resulted in an increased specific volume of wheat starch breads: while 2.5% WPP were used in wheat starch by Riemsdijk et al. (2011), we used 2.2 and 2.0 % WPP. The reason for having done it in such a way, is that the WPP suspension is very difficult to manipulate, we could not vary the amount of water to improve the dough texture without varying also the WPP concentration. An alternative to cope with this was to use WPP in the form of powder instead of suspension, but that was out of the scope of this research as it implied additional experiments to assure the particles did not lose functionality. The baking experiments were performed under a miniaturized approach (30 g dough) using a home baking machine. However, although we acknowledge that there are better instruments for baking, the reproducibility of our results was high and the conclusions are reliable. Moreover, as we followed the baking protocol used to test the effect of WPP on wheat starch (Riemsdijk et al., 2011), the results of the two studies could be compared to assist the analysis.

For the first time we demonstrated that it is possible to obtain an oat bread from pure oat flour based on a dough system and, more significantly, with a superior loaf specific volume than that reported for batter systems, typically between 1.14 and 2.40 mL/g (Huttner et al., 2010; Hager et al., 2012), although higher volumes are currently possible in batter systems. The specific volume of our pure oat flour breads was 2.90 mL/g (Table 2). There are two important factors to consider when comparing our baking results with the results obtained in other studies: the type of material (meal or flour) and the amount of water used in the formulation. We used a sieved fraction of oat meal (<0.250 mm) while most bread applications of oats rely on the use of whole grain oat meal. We decided to use the fine fraction to avoid the interference of fibres in the protein network (Noort et al., 2010). Londono et al. (Chapter 4) found that beta-glucans - contained mainly in the oat bran - impact negatively the extensibility properties of oat dough. This fine fraction used for the experiments accounted for 30% of the oat meal only, which makes our breads very costly. This can be corrected by adjusting the milling settings to produce flour instead of meal, and by fractionating the material into bran and flour. The bran can be treated as a by-product because it is a good source to extract beta-glucans to enrich other food products, or even to add it as a topping to oat bread. Regarding the amount of water used in formulations there is also a large difference between batter systems and our dough system: while we added 55% water to obtain a dough system (500 BU), batters account for 95-120% water (Huttner et al., 2010; Hager et al., 2012). This reduction of water would improve the shelf life and the microbiological quality in the final bread.

WPP affected the mechanical properties of oat flour dough. WPP increased the resistance to extension and the gas retention capacity of the dough (Figure 5), although the gas production in the WPP mixtures was lower than in pure oat flour (Figure 6). In wheat, this is considered a sign of the strength of the protein network (Gan et al., 1990). However, despite WPP made the dough network stronger, the improvement on loaf volume observed on wheat starch did not occur on oat flour; the effect of WPP was neutral at 500 BU and detrimental at 700 BU with respect to the controls (Table 2). A clear improving effect on crumb structure was observed in oat bread with the incorporation of WPP at both consistencies tested (Figure 9). While incorporation of WPP to wheat starch resulted in loaves with specific volumes of 3.7 mL/g (2.3 mL/g mixture) (Riemsdijk et al., 2011), incorporation to oat flour at 500 BU consistency resulted in loaves of 2.93 mL/g (2.26 mL/g mixture). The appearance of the oat-WPP and wheat starch-WPP breads was very similar regarding height and shape to the wheat starch loaves, the main difference was related with the loaf weight: our oat flour/WPP loaves were heavier than wheat starch/WPP loaves because they retained 17% more water.



The results observed at 500 BU are promising because although no improvement in loaf specific volume occurred, the optimization of the process could lead to better results. First, we did not have the same WPP concentration, so maybe 2.2% is not enough to have an effect on specific volume. Second, we used the same proving and baking conditions in the baking experiments for the controls and for the WPP mixtures, but as the gas production in WPP mixtures is lower, the processes should be optimized. Riemsdijk et al. (2011) did not observe differences on gas production in wheat starch-WPP mixtures with respect to the control.

Beta-glucans are the most likely component responsible for the negative effect on loaf specific volume observed with incorporation of WPP to oat flour at 700 BU. The reduction of the amount of water in the system increases the concentration of beta-glucans, and also of other components. Beta-glucans play a key role in the viscoelastic properties of oat flour, their functionality depends on concentration in the dough system (Chapter 4). This is confirmed with the behaviour of pure oat flour (controls), because the only reduction of water added to the flour to increase consistency from 500 to 700 BU, increased viscosity and reduced loaf specific volume in 18% (Table 2). The dough network becomes stronger at higher consistencies limiting the expansion of the dough, because when beta-glucans are partially hydrolysed they interact with each other forming macromolecular networks (Doublier and Wood, 1995).

Another remarkable factor is that the loaf specific volume of our breads prepared with a dough consistency of 500 BU, whether with and without WPP, were also superior than the maximum of 2.39 mL/g reported for other gluten-free formulations that relied on additions of emulsifiers, gums, and hydrocolloids such as beta-glucans (Lazaridou et al., 2007; Demirkesen et al., 2010; Nunes et al., 2009). The use of oat flour for gluten-free bread applications has the advantage that oat flour itself already contains beta-glucans.

## CONCLUSION

WPP affect the dough mechanical properties of oat flour by increasing its resistance to extension and its gas retention capacity. Incorporation of WPP to oat flour is promising to improve crumb texture, but optimization of the process could lead also to improvement of loaf specific volume. Beta-glucans are the most likely oat component that interact with WPP in the network and this interaction is water dependent.

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# CHAPTER 7

## General discussion

The motivation of this thesis was the need for alternatives to wheat based products for people suffering from celiac disease (CD) and for consumers in general. We are not yet at the point where the technological properties of wheat in bread applications can be replaced, not even equalled, but our work has contributed to increasing our knowledge in that direction. The selected oat (*Avena sativa*) as a target crop to replace wheat is an attractive alternative as it contains healthy components that can enrich the diet of people. Oat is the closest relative to wheat that is considered gluten-free and that can be consumed by most people suffering from CD. This close taxonomic relation might also be meaningful from a technological point of view. Oat is a robust crop that does not need much agronomical input for production, although its relatively low yield, in comparison to for example winter wheat, is a clear downside.

This thesis is divided into two parts, one with focus on safety and the other on technology. In the first part (Chapter 2), the aim was to clarify the safety of oats for CD patients. The second part, consisting of Chapters 3-6, contributed to establishing the fundamentals of using oats for gluten free bread applications based on a dough system instead of the commonly used batter system. First, a standard test was developed allowing the study of the dough-making properties of oat flour (Chapter 3). Subsequently, this test was used to analyse the role of beta-glucans in dough rheology (Chapter 4), and to study the effect of the kilning and milling processes on dough-making properties (Chapter 5). Finally, the effect of whey protein particles on improving the viscoelastic properties of oat flour was studied (Chapter 6). In this General Discussion I will briefly recapitulate the main findings and will discuss the most relevant issues regarding the merits of our approach and the relevance of our findings. The discussion is organised along a number of questions such as consumers, whether CD patients or not, and people from the food industry could pose.

## Part I: Safety of oats for celiac disease patients

### *Are oat products safe for people suffering from celiac disease?*

First and foremost, normal oat products in supermarkets are generally contaminated with gluten, from wheat, rye, or barley, to levels that are largely sufficient to induce symptoms in CD patients. Hence, the question of safety of oats concerns only to oat products from a guaranteed gluten-free production chain. A review of scientific literature based on clinical and follow-up studies concluded the general safety of oats for people diagnosed with CD (Pulido et al., 2009). The most recent evidence supports the long-term safety of oats (irrespective of oat varieties) in Finland. In this study, in which 106 CD patients were involved, the consumption of up to 100 g oats per day for up to eight years did not result in small-bowel mucosal villous damage, inflammation, or gastrointestinal symptoms (Kaukinen et al., 2013). None of these patients had to discontinue the study due to symptoms. The mucosal morphology of the patients who had consumed oats in larger amounts and over a longer period was even better than in patients who consumed low amounts of oats for a short period or did not consume oats at all.

Nevertheless, 'general' safety is a somewhat ambiguous term that leaves room for debate. We have recently seen that long term and large scale oat consumption studies that support oat consumption by CD patients (Kaukinen et al., 2013; Gatti et al., 2013;) are being published next to non-clinical studies that claim reactions of oats to antibodies or T cells and/or that warrant caution (Comino et al., 2011; Mujico et al., 2011). The claim of general safety of oats for the majority of CD patients leaves the question open about what happens with the minority to whom the consumption of oats is not safe, or for whom this remains unclear. In addition to this, it is a fact that some patients present complaints or some discomfort after eating oats, but the causes of these symptoms are not clear, and might not be even oat-specific.

A typical characteristic of clinical studies is that a number of patients drops out from the studies before they are concluded. Some patients argued that they did not feel well when challenged with oats, others simply dropped out without giving a clear explanation. So, if we assume that gluten contamination was rigorously avoided in these trials, there are three possibilities:

1. The drop-outs did not like the taste or smell or mouth-feel of oats;
2. They suffered from microbial and physiological changes in their intestine due to the adaptation to the high fibre content of oats (Mälkki, 2004) (both 1. and 2. may similarly occur within a population of healthy individuals that had never

consumed oats before); and

3. Some CD patients had T cells capable of reacting to peptides present in avenins, which form the prolamin fraction of oats.

The evidence for the latter comes from Arentz-Hansen et al. (2004), who studied celiac disease patients who clinically suffered from oat intolerance. Three patients had specific avenin-reactive T cells and the authors established T cell clones which were reactive with the Av-alpha-9A and Av-alpha-9B peptides and which were not cross-reactive with homologous wheat, barley and rye epitopes. These peptides were thus annotated as true avenin epitopes and named DQ2.5-ave-1a and DQ2.5-ave-1b (Sollid et al. 2012). We have not found further studies that describe more patients that react specifically to these avenin epitopes, so it remains unclear how many people, other than the three identified, have oat avenin-specific T cells. Also, knowing the range of sensitivities to gluten that occur in the CD patient population, it is likely that sensitive individuals either did not volunteer or were excluded from the clinical studies reported.

In general, very little was known about oat avenins when we started with this research. Questions that were addressed in this thesis are: (i) do oat avenins always contain the two avenin-specific epitopes; (ii) do other CD epitopes or variants thereof, as known from wheat, rye and barley (Sollid et al., 2012), also occur in avenins; and (iii) what would be the variation among oat varieties regarding these epitopes?

The first question was addressed using a novel approach. Rather than testing a series of economically available hexaploid oat varieties or breeding lines – which will always be non-exhaustive, thus keeping people wondering about all untested varieties –, we determined the sequence variation in all three genomes of oat across the genus *Avena*, by testing accessions of various oat species with an A, C and/or D genome at diploid, tetraploid, as well as hexaploid level. The avenin genes from these *Avena* species were cloned and sequenced. Based on this sequence information, *in silico* predictions were made on the presence of the two avenin epitopes DQ2.5-ave-1a and DQ2.5-ave-1b, on the presence of the annotated epitopes from wheat, rye and barley (Sollid et al. 2012), and on all possible variants with up to three amino acid differences, which potentially could be detected by monoclonal antibodies raised against the intact epitopes from wheat, rye and barley. T cells raised against wheat, barley or rye epitopes may also detect variants, although these cells of the immune system tolerate fewer changes, and a single amino acid change may already abolish the recognition (Mitea et al. 2010; Salentijn et al. 2012). The presence of the two specific avenin epitopes was confirmed within a conserved domain of two of the four types of avenins present in all *Avena* species examined. Since all oat species have avenins with these sequences, it is also unlikely that individual varieties exist in which the epitopes do not occur in

some avenins. However, it is possible that the number of genes varies, or that they are expressed at lower or higher levels, thus varieties could differ in the amount of avenin proteins containing these epitopes.

Furthermore, none of the avenin sequences contained intact epitopes from wheat, rye and barley that trigger the disease in 1% of the world population, but we found five variants with two and three amino acid substitutions in peptide fragments that were predicted to be resistant to proteolysis with trypsin and chymotrypsin in the gastro-intestinal track, but not to pepsin. If this *in silico* prediction reflects the situation *in vivo*, these variants cannot trigger a T cell cross-reaction, but if some resist *in vivo* proteolysis, they could give rise to cross-reactivity. In fact, it would be highly informative to test these five variants for their ability to elicit stimulation (i.e. cross-reactivity) of corresponding epitope-specific wheat gluten reactive T-cell clones. It is important to note that the two established specific avenin epitopes differ from the homologous DQ2.5-glia- $\alpha$ 1b epitope by 4 out of 9 core residues. Interestingly, the gliadin variant Av-gamma-2b reported by Vader et al. (2003) as cross-reactive with gluten T-cell clones, was predicted to be sensitive to proteolysis and therefore, unlikely to be able to trigger any *in vivo* response.

So, based on the collected scientific evidence presented, it is not possible to claim that oats are safe for the entire CD population because of the proven existence of avenin specific T cells in some CD patients, and because cross-reactivity with some of the variants of gluten epitopes found in oat avenins cannot be ruled out completely. It remains unknown how many patients have T cells that react to the specific two avenin sequences or that can cross-react with gluten epitope variants present in oat avenins. However, empirical evidence is coming from Scandinavian countries, where 70% of the people with CD consume oats without complaints (Peräaho et al., 2004). It is believed that the actual frequency is significantly higher (Mälkki, personal communication), but this needs further corroboration.

***Are monoclonal gluten antibody signals that demonstrate the presence of CD related epitopes of clinical relevance in oats?***

The monoclonal antibodies R5 and G12, commonly used in commercial kits to detect gluten in wheat, rye and barley, give signals in oats, and these signals vary in strength among oat varieties (Mujico et al., 2011; Comino et al., 2011). These oat samples were guaranteed to be pure. However, intact CD gluten epitopes are absent in oats. According to *in silico* predictions, there are five imperfect variants of these epitopes that do occur, but they are sensitive to proteolysis by pepsin, trypsin and chymotrypsin in the gastrointestinal tract.



Consequently, the signals of the antibodies R5 and G12 can only be meaningful for oats if they would detect the two specific avenin epitopes, but the recognition sites of the antibodies do not match with the epitope sequences. Based on our avenin sequences the signals generated with the antibodies R5 and G12 are unlikely to present specific binding. Therefore, the detection of signals towards oat protein extracts, and the differences in staining intensity among oat varieties, do not have clinical relevance until proven otherwise.

The question that remains is what these antibodies do detect. The intact amino acid motifs for recognition by the antibodies, which are five and six amino acids long, are shorter than the T-cell epitopes, which are nine amino acids long. The antibodies recognition motifs do not occur in oat avenins, but some variants exist with one (R5) and two amino acid substitutions (G12). These variants may lead to cross-reactivity. Cross-reactivity with motifs that do not directly relate to CD epitopes has no clinical relevance. Therefore the R5 and G12 antibody signals in oats, and the differences reported among oat varieties, should not be interpreted as a sign of immunogenicity. Thus, a call for a screening of oat varieties based on these antibodies, such as Comino et al. (2011) did, is meaningless. In fact, it is even to be considered counterproductive to look for oat varieties without R5 or G12 staining, as one of the consequences of this complete lack of correlation is that absence of staining does not mean that a variety lacks the amino acid motifs corresponding to the two avenin epitopes that do exist, so lack of staining would give a false sense of absolute safety.

### ***Are there additional possible sources of epitopes?***

That possibility exists. First, probably not all epitopes that trigger CD are known. Second, this thesis has greatly expanded the available sequence information on avenins in the three genomes of the genus *Avena*, but still some genes may have remained undetected. Currently oat is being sequenced, and the complete genome sequence will shortly make it possible to screen for other prolamin-like genes in oat. A preliminary look of two relevant contigs of avenins that resulted from the genome sequencing of an oat variety (courtesy of Tim Langdon, Aberystwyth University, Wales), indicates that the inference we made about the number and diversity of oat avenins holds well. The sequenced variety contains four different avenins, one from each of the four groups of genes that we distinguished in Chapter 2, plus two additional truncated genes, which may either represent two additional genes from these groups, or pseudogenes. In wheat, the prolamin gene families have expanded much more, leading to many more prolamins genes and a higher prolamin protein level in the grains, but also to many pseudogenes (90% of the alpha-gliadins (van Herpen et al. 2006), 50% of the gamma-



gliadins (Salentijn et al. 2012). More EST and RNAseq libraries will be published during the coming years and that information offers the possibility to cross-check oat for the possible occurrence of any new CD epitope that may be identified in the near future.

### ***I am diagnosed with CD, should I eat oats?***

One important reason for people with CD to consume oats is that they can also have nutritional deficiencies due to the damage of the villi in the small intestine and, in addition to that, a non-fortified gluten-free diet has a reduced nutritional quality (Thomson, 2000). Oats contain iron, vitamins and minerals that patients are deficient of (Butt et al., 2008). The high content of soluble fibres are also beneficial for them as soluble fibres help to prevent the development of diabetes.

A person diagnosed with CD would therefore benefit from oat consumption, as long as he or she does not belong to the small group of people with CD carrying T cells that react to the two avenin epitopes. But it is important that CD people buy gluten-free labelled oat products because normal oats found in supermarkets are likely contaminated with gluten. Unless precautions are taken, fields of oats can easily become contaminated with rye, barley or wheat. Also, oat grains are typically processed and milled in installations that are used for other cereals. The current European Regulation and the US-FDA allow oats to be labelled as gluten-free if these have been specially produced, prepared and/or processed in a way to avoid contamination with wheat, rye, barley, related species such as spelt, and derived species such as triticale. The gluten content of such gluten-free oats must not exceed 20 ppm (EC Regulation 41/2009; Food and Drug Administration, 2013). The Federal Register of the USA collected and carefully judged data from reliable sources, commented these, and concluded that the proportion of individuals with celiac disease who cannot tolerate oats in daily amounts of about 50 g, or less, is probably very low, possibly below 1 percent [ $<1\%$ ] of the population of individuals with CD. The US-FDA clearly aims at self-responsibility and takes a pragmatic approach towards oat consumption by celiac patients.

When a CD patient chooses to move to an oats containing diet, it is recommended to gradually introduce oats in the diet. The intake should stop for some days if the person experiences some discomfort, and try again later while keeping track of any effects. This is because the discomfort can also be a relatively harmless response of the intestinal microflora to the high content of fibres in oats (Mälkki, 2004). When people switch to a diet with large amounts of fibres, it may take some time for the microflora to adapt.

### ***Why eat oats if I am not diagnosed with CD?***

Non-CD people, which is the great majority of the population, also may have health benefits from oats consumption. The high content of soluble fibres (beta-glucans) helps to decrease cholesterol and post prandial glucose (PPG) levels in the blood (Othman et al. 2011). This is suggested to decrease the risk of getting cardiovascular disease and diabetes type II ((Behall et al., 2004; Mathews, 2011; Othman et al. 2011). There are also some studies that suggest that the intake of dietary fibre may play a role in prevention of obesity (Ludwig et al., 1999; Anderson, 2003).

Cardiovascular disease is now the major cause of death in United States (Behall and Hallfrish, 2011). Oat consumption is considered part of a prevention strategy and management of this disease, as a reduction of 1% in serum cholesterol could result in a reduction of 2-3% in the observed rate of the disease (Behall and Hallfrish, 2011). The recommended intake of oats to accomplish 3 g of beta-glucans per day is estimated to lower serum cholesterol by 5-15% (Ludwig et al., 1999; Anderson, 2003). Overall, increasing oat consumption would positively impact health.

## **Part II: Technological properties of oat**

### ***Why are there so few oat baking products in the supermarkets?***

Oat products can be found in the supermarkets mainly in the form of breakfast cereals, porridges, and meal. The supply chain begins with the type of varieties grown, followed by the methods used to stabilise oats (enzyme inactivation), and for processing (such as flaking and milling). This supply chain is totally geared towards these applications, which raises the question whether the current settings are suitable for bread-making applications and whether they limit their expansion.

There is a consensus regarding the fact that oats are technologically limited but, in fact, this thesis shows that the potential of oats has not been sufficiently explored. In the case of baking applications - which is the most important application of cereals in consumer products -, the main reason given to explain the small number of commercial products is the lack of gluten. Quoting Webster and Wood (2011), which is the most important compilation of oat literature available: *“Since oat proteins do not have gluten-forming characteristics, oats cannot be used as sole grain in yeast-raised bread”*. This type of strong statements in the most complete book on the scientific knowledge about oat has prevented researchers and bakers of trying to make oat bread.

There are two factors influencing bread quality that need to be considered. One factor is the composition of the material used and the functionality of its individual components, and the other is related to optimising the bread-making process itself (kilning, milling, formulation, mixing, baking). Both factors remain largely unresolved for oat due to very limited research. This stands in contrast to wheat where a vast amount of research supports the composition of wheat regarding baking quality and on optimal bread-making technology, and further research is ongoing.

### ***Why use a dough system instead of a batter system?***

It seems to be also accepted that it is only possible to make oat bread based on a batter system. The amount of water added to gluten-free formulations is double the amount typically used for wheat. However, it is not clear how this amount was defined. For example, Hager et al. (2012) used 95% water in the formulation to make oat bread and mentioned that it was established by empirical trial and error testing because - according to them - “the Farinograph method is not applicable to gluten-free systems”. Huttner et al. (2010) used 120% water but no references are provided to clarify the criteria behind the decision. As far as we know, there are no studies supporting the conclusion that a batter system is better to make oat bread than a dough system.

As an important first step, we demonstrated in this thesis that it is possible to use pure oat flour to make yeasted bread using a dough system instead of a batter system, and that the Farinograph type Z-blade mixer can be used to prepare the dough just as it is used for wheat flour, although more research is necessary to establish the optimal consistency for oat flour (Figure 1). Regarding the amount of water used in formulation, in our dough system we used 55% water (w/v), about half of what is used normally in batter systems (Huttner et al., 2010; Hager et al., 2012). This reduction has advantages because having less water would improve the shelf life and the microbiological quality in the final bread.

In addition, the possibility to work with a dough system broadens the spectrum of potential applications of oat because dough is a more familiar and easy to use system for bakers than a batter. The most common gluten-free applications of oats are as flakes for cereal breakfasts, which does not represent a technological challenge. In contrast, bread applications require a system able to expand, to incorporate gas during mixing, and to hold it during proving and baking.

The bread made using oat flour had a loaf specific volume of 2.90 mL/g, which is superior to the specific volume reported for batter systems made of whole grain oat meal, which ranges between 1.14 and 2.40 mL/g (Huttner et al., 2010; Hager et al., 2012). However our dough system and the loaves obtained from it do not reflect the final oat

bread aimed for with respect to optimal quality. We simply demonstrated that there are other ways that could lead to better results and that there is much to investigate to be able to define what actually “optimal quality” means for oat bread. Taking as example rye bread, it does not look at all as wheat bread and nevertheless people find it in the shops, buy it, and eat it as rye bread. So, the fact that oat bread does not comply exactly with the quality standards of wheat bread is not the reason explaining why oat bread is not easy to find in supermarkets and bakeries. Actually, the pure oat bread that we present in Chapter 5, is quite similar in shape and volume to the bread that Riemsdijk et al. (2011) made using a mixture of wheat starch and wheat gluten; the volume per unit of mass was lower, but this was mainly due to the high water retention capacity of oat flour, which resulted in a larger weight and caused a decrease of specific volume (Chapter 5). Our results are promising because even without optimising the process, and without understanding the functionality of all oat components other than beta-glucans, the general appearance of the loaves and their specific volume achieved, lead us to think that there is room for much more improvement. Our oat bread looks similar to the bread that people are familiar with (Figure 1).

We consider the lack of knowledge and the high prices of the grains as the main reasons to explain the limited availability of oat bread, and in general of oat products, in the supermarkets. Oat, as a crop and as a raw material for food industry, has particularities that need first to be understood in order to be able to deal with them in a product development process.



**Figure 1.** Dough system (left) and baked loaf (right) made of pure oat flour.

### ***Why use gluten to develop a standard test if the aim is a gluten-free oat bread?***

One aspect that might be confusing for the reader of this thesis is that on one hand the motivation of the research was to contribute with food alternatives for people that suffer from celiac disease and, on the other hand, we used gluten – the trigger of

CD - to develop a standard test for oat. The readers might argue that this represents a contradiction and that the standard test developed does not fit the context of the research as expressed in the General Introduction.

However, our goal for developing the standard test was to have a standardised dough system that allowed us, in the first place, to study the intrinsic technological properties of oat cultivars and to understand the functionality of various oat components. We used gluten as a means for this, because the properties of gluten are well known and could serve as a model. This approach helped us also to clarify the loss of bread quality reported when oat flour was used to make composite wheat-oat breads (Flander et al., 2011). We demonstrated that beta-glucans interfere with gluten aggregation in a gluten-containing system (Chapter 4), and that the negative effects on dough viscosity and on dough extensibility are the same in the presence and absence of gluten. Removing gluten from the system did not affect the sensitivity and the reliability of the test. Our standard test can therefore be used to standardise the kilning and the milling settings for oat grains, and allows us to study the functionality of components other than beta-glucans, also for all applications in which no gluten is present.

### ***Is there a trade-off between baking quality and health benefits?***

Beta-glucans are the most important oat component, as they are responsible for the approved health claims of oats. Beta-glucans help lowering the postprandial glucose and cholesterol levels in the blood. The positive effects of beta-glucans are related with total content but also with their viscosity, and the latter is determined by molecular weight (MW). The higher the MW, the higher the viscosity and the more beneficial for health (Mälkki, 2004; Wood, 2011).

Beta-glucans impact oat dough rheology by increasing stiffness and decreasing extensibility (Chapter 4). The high molecular weight beta-glucans (high viscosity) have a larger impact than low molecular weight beta-glucans (medium viscosity). Flours with a low content (< 2%) had better extensibility properties than flours containing 5%. We did not perform baking experiments to precisely determine the relation between beta-glucans (content and viscosity) and baking quality, but we expect also a detrimental effect of high concentrations of beta-glucans (Flander et al., 2011). The opposite effects of beta-glucans on health (more is better and longer is better) and baking quality (less is better, shorter is better) should be studied in detail in order to find the right balance between baking quality and health benefits (keeping them at the maximum possible). In addition to technological studies for optimising the beta-glucan content in flour, there are also possibilities to optimise health benefits through product development. For instance, oat bran particles, which contain most of the beta-glucans, could be added as a topping.

### ***Are the current kilning and milling methods suitable for bread applications?***

The current kilning and milling methods used for oats have been used to produce porridges, flakes and meal. We demonstrated that both processes affect dough rheology and, as a consequence, would have an effect on bread quality (Chapter 5). The infrared (IR) kilning method used by a miller in The Netherlands to stabilise oat grain, damages completely the extensibility properties of the grains, which are relevant for bread-making applications. For this purpose, and according to our results, steam kilning seems to be more appropriated. However, more research is necessary to find out which are the most suitable conditions to inactivate the lipase activity in grains intended for bread-making.

Regarding the milling settings, what is common to find in the market is oat meal and not oat flour. Oat meal has a larger particle size and it still includes the bran fraction. The impact of the bran fraction on dough rheology is negative, mainly because of the beta-glucans contained in the bran itself (Chapter 4 and Chapter 5). Our results show that low beta-glucan oat flour is more suitable for bread applications than oat meal. The milling yield is an important factor that can cause reluctance of millers to accept oats, especially for bread applications. The commercial oat meal that we used in some of the experiments (milled at 8000 rpm) was too coarse: about 60% of the particles had a size larger than 0.250 mm. After re-milling at the maximum speed (16.000 rpm) this proportion was reduced to 40%. So, producing oat flour for bread applications won't be attractive if millers have to waste such amount of bran. However, oat bran could be considered as a valuable by-product of the milling process as it can be used to extract beta-glucans for other applications. Thus, a strategic approach to fractionation is an interesting possibility as it would enable millers to produce a fine flour with low content of beta-glucan (suitable for bread applications), as well as bran for beta-glucan extraction or to sell as topping for other food products.

### ***What is the major obstacle for expansion of oats?***

The greatest difficulty that oat faces as a crop is yield. Oat yield is lower than the yield of its major competitor crop, wheat. Worldwide many smaller crops, including oat, are gradually being replaced by high yielding and better valued major crops: maize, soybean, and wheat (see Table 1 in General Introduction). Yield is therefore the most important breeding aim because any improvement would help pushing oat into the market. Important improvements have been achieved in the UK. Despite the lower investments in oat breeding in comparison to wheat, the rate of increase in oat and wheat yield was similar between 1985 and 2005. However, wheat yield is on average still about 3 tons per hectare higher than that of oat (Valentine et al., 2011).

Low yields have several consequences on the dynamics of the market as they tend having an effect on decisions of farmers, food industry and consumers. In addition to the low yields of oat, farmers are more certain growing wheat because wheat has a larger market

demand as it is used for plenty of industrial applications. Thus, to motivate farmers to grow oat, it is necessary to stimulate them with higher prices of the harvest. This discourages food industry of using oats because it is more profitable for them to use less costly crops as raw material for product development. As a consequence, consumers have a reduced variety of products and they have to pay more for them.

The situation becomes more critical when the oat production is tailored for a gluten-free market. Gluten-free oats should be produced in a special way to guarantee that oat is not contaminated with gluten. The gluten-free production chain includes measures such as selection of the field, extensive cleaning of machinery, and exclusive locations for processing and packaging. All these measures substantially increase the production costs through the whole chain.

### ***Which are the most relevant traits to breed for?***

As yield is the main drawback of oat, breeding programs should aim to improve it as much as possible. Yield (kg/ha) is a complex trait that can be disentangled into several factors including grain yield, milling yield, resistance to lodging, and resistance to diseases and pests. Along with breeding for yield it is important to continue studying the functionality of the different oat components to elucidate potential technological applications, and to improve the selection process with the establishment of new criteria. This would allow exploiting the diversity of the available germplasm.

These possibilities exist, but they need investment. The struggle is to stimulate sufficient investment in research and in product development as long as oat is such a small crop. This is in fact an example of a more general problem, because funding largely goes to major crops because the return is visible in a shorter term. Investments in large crops pay off more quickly.

### ***Perspectives for new products***

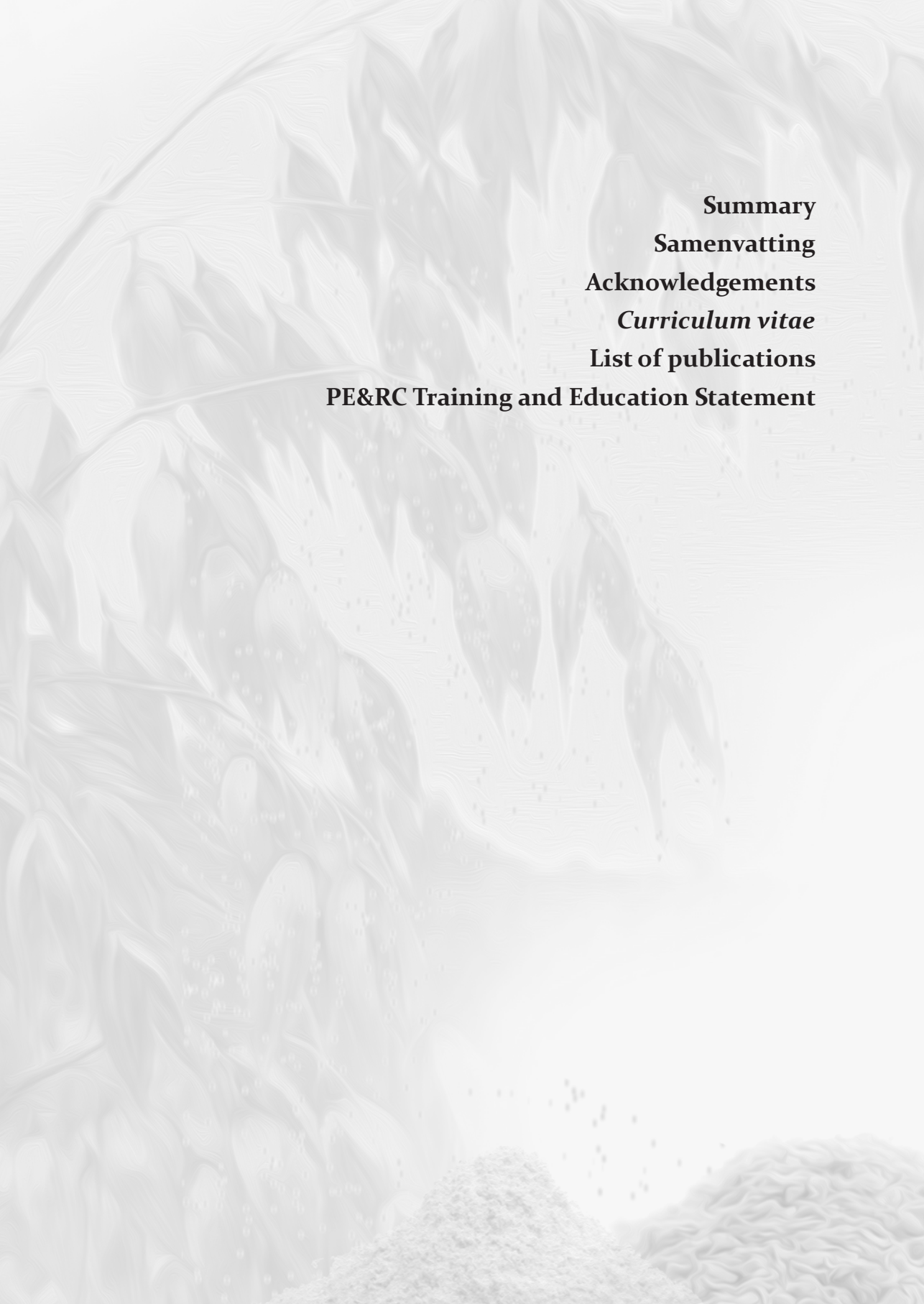
Although it might be difficult to reach exactly the same bread quality standards of wheat using pure oat flour, it does not mean that oat bread, with its own quality particularities, cannot be welcome in the market. Oat bread with its own identity could enter the market to compete with spelt bread or rye bread. The dough system developed here opens new possibilities besides bread. A dough system is also promising for other applications that do not require as much elasticity as bread does. Some of these applications include cookies, crackers, muffins, or pizza base.



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**Summary**  
**Samenvatting**  
**Acknowledgements**  
***Curriculum vitae***  
**List of publications**  
**PE&RC Training and Education Statement**



## SUMMARY

The motivation to perform this study was to generate the fundamentals to use oats for bread-making applications. This will offer consumers a healthier alternative product to wheat bread in their daily diet, because oat foods, especially through their high amount of soluble fibre (notably beta-glucans) contribute to the reduction of blood cholesterol levels and of blood glucose rise after the meal. Oats also have a high content of (poly-)unsaturated fatty acids that contribute to maintaining normal blood cholesterol levels. One specific target group that would benefit from the development of good quality oat bread are people with celiac disease (CD). Oats is widely consumed by them, even though its safety has been subject of some debate for a long time. Two peptides from oat avenins can be recognized as T cell epitopes by few CD patients, and differential signals of gluten-specific monoclonal antibodies and in-vitro T cells to oat varieties have suggested the existence of differences in immunogenicity. These health and food safety issues have been addressed in the General Introduction.

Bread is consumed all over the world. So far, production of large-volume bread is only possible with wheat. The quality of existing oat bread is below to what consumers are used to with wheat bread. This is partly due to the lack of knowledge regarding the functionality of oats for other purposes than porridge and breakfast cereals, which are the most common applications. These applications do not represent a big technological challenge as bread does, because bread-making requires a system able to hold gas during proving and baking. In wheat, this is conferred by gluten proteins that form a viscoelastic network with the capacity to expand and to maintain itself after expansion. Oats lack gluten proteins with network-forming capacity. Current oat bread applications rely on batter systems and on the use of additives to increase viscosity for stabilization of gas cells.

This thesis consists of two parts. The first part concerns the safety of oats for people with celiac disease (**Chapter 2**). This was studied by cloning and sequencing avenin genes from 13 *Avena* species with combinations of the three genomes (A, C, D) that are also present in the hexaploid cultivated *A. sativa*. We identified up to 10 avenin genes in a single hexaploid oat plant. Avenin proteins clustered in four groups of which two contained the two avenin CD epitopes. All *Avena* species examined harbored avenins of these two groups, so it is unlikely to find oat cultivars that are devoid of the avenin CD epitopes. None of the internationally agreed gluten CD epitopes from wheat, rye and barley were found to be present in oat avenins. Some epitope variants with two and three amino acid substitutions occurred, but they were predicted to not resist proteolysis in the gastro-intestinal tract and will therefore not be of clinical relevance.

Perfect recognition sites of antibodies R5 and G12 (which are used in commercial gluten detection kits) were also not present in avenins. Thus, monoclonal antibody signals to oat are probably due to cross-reactivity or promiscuous recognition of avenin peptides, and such signals should not be interpreted as differences in immunogenicity of oat varieties for CD patients.

The second part of this thesis focussed on the study of the technological properties of oats. Oats have been used as an addition to wheat-based dough or in an oat-based batter system. However, while for wheat the dough-making parameters necessary to obtain good quality bread have been defined through a long history of research, this is not the case for oats. To fill this gap, this thesis studied the technological properties of oats using a systematic approach. First, we developed a dough testing system that allowed us to assess the dough-making properties of oat flour in a standardized way (**Chapter 3**). For this we used wheat as a model. We reproduced various quality profiles of wheat flour using combinations of oat flour and vital gluten. Then, we selected a dough system made of 87.2% oat flour and 12.8% gluten as our standard dough test system. This dough system was sensitive to differences among oat cultivars.

Thus, having developed a tool that could detect differences regarding dough-making properties among oat cultivars, the next step was to try to explain those differences in terms of compositional factors. We decided to start our exploration with beta-glucans, because these fibres are one of the oat components that attract interest because of their health benefits. We studied the impact of beta-glucans on dough rheology (**Chapter 4**) following two strategies: (i) using the developed standard dough system containing gluten; and (ii) by removing the gluten from the system and replacing these proteins by alternative network-forming compounds. In both systems, beta-glucans affected dough rheology. Increasing their concentration resulted in an increase of dough stiffness and in a reduction of dough extensibility. Beta-glucans negatively influenced the elastic properties that additional wheat gluten conferred to oat dough. Low beta-glucan (<2%) oat flour had better extensibility properties than oat meal dough or oat flour dough enriched with beta-glucans. The effect was governed by its concentration and its molecular weight (which determines viscosity). Medium-viscosity beta-glucans had a less negative impact than high-viscosity (high molecular weight) beta-glucans. Overall, our findings indicate that beta-glucans are a key component determining rheology of oat-based dough systems.

**Chapter 5** addressed the effect of particle size distribution on dough-making properties. We found that oat meal is not the best material for bread-applications because it produces a very stiff and short dough. Re-milling did not change this pattern. In contrast, complete removal of the bran from the oat meal did improve dough-making

properties, which indicated that dough rheology was negatively impacted by the bran. Large and medium size bran particles were more harmful than fine bran particles. Large and medium bran contained 8% beta-glucans, while fine bran contained 1.6% only. We concluded that oat meal is not appropriate for bread applications. Fractionation of the milled product is an interesting alternative to produce low-beta-glucan flour for bread-making purposes, and the bran can be used to enrich other food products with beta-glucans.

This chapter also addressed whether kilning and milling methods applied to oat grains could affect bread-making purposes. Infrared (IR) and steam kilning both affected dough-making properties of oat grains in the standard dough system. The effect of steam kilning was on water absorption only. Non-kilned and steam-kilned grains showed similar extensibility behavior. In contrast, IR kilning affected water absorption and harmed completely the dough extensibility properties of oat grains. Flour from IR kilned grains made a very stiff and short dough. Thus, IR kilning is definitely not suitable for bread applications.

Finally, in **Chapter 6**, we addressed the need for good quality gluten-free oat bread. As further research is required for better understanding of the oat dough system, we studied the rheological properties of oat flour relevant for leavening with gluten alternatives. Whey protein particles (WPP) had appeared to be successful in enhancing viscoelastic properties of wheat starch dough, allowing loaves with specific volumes of ca 3.7 mL/g. We studied whether WPP could have a similar positive effect on oat flour dough. WPP increased the resistance to extension and the gas retention capacity of oat flour dough. However, in our small scale baking experiments, WPP did not increase loaf specific volume and had a negative effect on gas production. On the other hand, WPP improved crumb texture. WPP are promising as a structuring agent in oat dough, but the process should be further optimized.

In the General Discussion we pay attention to the food safety issue of oats for people with coeliac disease. Our analysis across the genus *Avena* of avenin genes and proteins produced an important new and supporting argument to the safety of oats, as they appeared to contain none of the generally agreed celiac disease-related gluten epitopes from wheat, barley and rye. With this analysis we also could explain the positive signals for the presence of gluten (as described in the literature for several oat varieties on the basis of the R5 and the G12 antibody assay and on T cell tests) as being the result of cross-reactivity or promiscuity, without having clinical relevance. The data in this thesis therefore support the advice to gradually introduce the consumption of oats into the daily diet of people with coeliac disease. Further, we discuss the results and the consequences of our technological research on oat flour dough. It appeared that beta-



glucans have a serious negative effect on the rheology of the oat dough, which indicates the need for further research on improvement of the balance between optimum application of beta-glucans for health (high amounts and of high molecular weight is better) and for baking quality (low amounts and of low molecular weight is better). Also the pre-treatment of oat flour (notably kilning and milling) and the application of whey protein particles to replace gluten require further optimization. Here the developed standard oat flour dough model system will be a useful tool.

## SAMENVATTING

Het onderzoek beschreven in dit proefschrift legt de basis voor het maken van brood uit haver. De motivatie voor dit onderzoek is om consumenten een gezonder alternatief te bieden voor tarwebrood, vooral door het hoge gehalte aan oplosbare vezels in haver. De haver-specifieke oplosbare voedingsvezels (beta-glucanen) kunnen bijdragen aan de verlaging van het cholesterolgehalte in het bloed en aan een verminderde stijging van het glucosegehalte na de maaltijd. Ook heeft haver een hoog gehalte aan onverzadigde vetzuren, die bijdragen aan de instandhouding van een normaal cholesterolgehalte in het bloed. Een andere doelgroep voor haverbrood zijn de mensen met coeliakie. Zij kunnen haver goed verdragen, ook al is de veiligheid van haver voor deze mensen een tijd in twijfel getrokken. De reden hiervan waren twee peptiden uit haver-avenines die door T cellen van enkele coeliakiepatiënten herkend konden worden en tot een klinische reactie leidden. Ook bleken gluten-specifieke monoklonale antilichamen en in vitro T celklonen met bepaalde havervariëteiten een reactie te geven die erop leek te wijzen dat deze variëteiten immunogeen zouden kunnen zijn. Deze gezondheids- en voedselveiligheidsaspecten zijn in de General Introduction (Hoofdstuk 1) aan de orde gesteld.

Brood wordt over de hele wereld gegeten. Hoog-rijzend brood kan echter alleen met tarwe gemaakt worden. De kwaliteit van haverbrood voldoet wat dat betreft niet aan de verwachtingen van de consument. Dit is gedeeltelijk het gevolg van gebrek aan kennis over de technologische mogelijkheden van haver buiten de bekende toepassingen als havermoutpap en ontbijtgraan. Deze toepassingen vormen geen technologische uitdagingen in vergelijking tot het maken van brood, dat een systeem vereist om gas vast te kunnen houden tijdens het rijzen en bakken. In tarwe zijn de gluteneiwitten hier verantwoordelijk voor. Zij vormen een visco-elastisch netwerk dat uit kan zetten zonder te breken. In haver ontbreken gluteneiwitten met deze eigenschap. Daarom wordt haverbrood niet uit een deeg maar uit een beslag gemaakt waarbij additieven gebruikt worden als alternatief voor gluten om de gascellen te stabiliseren.

Dit proefschrift bestaat uit twee delen. Het eerste deel gaat over de veiligheid van haver voor mensen met coeliakie (Hoofdstuk 2). Hiervoor hebben we avenine genen gekloneerd en gesequenced uit 13 *Avena* soorten die gezamenlijk de drie genomen (het A, C en D genoom) van het gewas *Avena sativa* vertegenwoordigen. We konden op deze wijze tot 10 avenine genen in een enkele haverplant identificeren. De avenine eiwitten konden in vier gen-clusters gegroepeerd worden, waarbij de bovengenoemde twee avenine-epitopen steeds in twee van deze groepen aanwezig bleken te zijn. Alle onderzochte haversoorten bleken avenines uit deze twee epitopen-bevattende groepen

te bevatten. Het lijkt daarom onwaarschijnlijk dat er haver soorten of variëteiten bestaan waarin zulke avenines niet aanwezig zijn. Verdere analyse van de avenine genen toonde aan dat geen van de internationaal erkende coeliakie-gerelateerde gluten-epitopen uit tarwe, gerst en rogge in de aveninen van haver voorkomen. Epitooptvarianten met twee of drie aminozuursubstituties werden wel waargenomen, maar deze bleken volgens een *in silico* model niet resistent tegen proteolyse in het maag-darm kanaal en ze zullen daarom niet van klinische betekenis zijn. Ook werden geen perfecte herkenningsmotieven voor de R5 en G12 gluten-specifieke antilichamen gevonden. Deze antilichamen worden gebruikt in commerciële testkits voor het aantonen van gluten in voedingsproducten. Dus, mocht er in testen met deze antilichamen bij sommige havervariëteiten een reactie gezien worden dan moeten deze worden gezien als veroorzaakt door kruisreactiviteit of promiscuïteit van de antilichamen. Deze reacties mogen niet geïnterpreteerd worden als immunologisch relevante potenties van deze variëteiten.

In het tweede deel van het proefschrift is aandacht besteed aan de technologische mogelijkheden van haver. Haver wordt gebruikt als toevoeging aan tarwedeeg of in een haverbeslag systeem. In tegenstelling tot haver zijn de technologische parameters om een deeg te maken van tarwe het resultaat van jarenlang onderzoek. Het onderzoek in haver is in deze thesis op een systematische manier aangepakt. Eerst hebben we een deeg-teststelsysteem ontwikkeld waarmee het mogelijk was om de verschillende deegkarakteristieken van haverbloem op een standaard manier te onderzoeken (Hoofdstuk 3). Hierbij is het tarwe-systeem als model gebruikt. We hebben verschillende kwaliteitsprofielen van tarwebloem herhaald in combinaties van haverbloem met vitaal tarwegluten. Hieruit is een haverdeeg standaardmodelsysteem ontwikkeld bestaande uit 87.2% haverbloem met 12.8% gluten. Met dit modelsysteem bleek het mogelijk om kwaliteitsverschillen tussen havervariëteiten aan te tonen.

Met dit model konden we vervolgens op zoek gaan naar de oorzaak van de verschillen tussen de degen van het bloem van de verschillende havervariëteiten. Hierbij zijn we begonnen met het onderzoek naar het effect van beta-glucan, mede omdat dit vezeltype zeer in de belangstelling staat wegens de gezondheidsbevorderende eigenschappen, maar ook om het sterke watervasthoudende vermogen en de mogelijke invloed hiervan op de rheologie van het deeg (Hoofdstuk 4). Hierbij zijn twee strategieën gevolgd: (1) gebruikmakend van het standaardmodelsysteem met toevoeging van gluten, en (2) door vervanging van het gluten door andere visco-elastische netwerkvormende stoffen. In beide systemen bleken beta-glucanen een nadelig effect op de rheologie van het deeg te hebben. Bij toenemende concentraties van beta-glucanen bleek de stijfheid van het deeg toe te nemen en de extensibiliteit (uitrekbaarheid) van het deeg te verminderen. Beta-glucanen bleken ook de aanvankelijk door gluten veroorzaakte elasticiteit van het deeg nadelig te beïnvloeden. Verder bezat haverbloem met een

laag beta-glucaangehalte (<2%) een betere extensibiliteit dan deeg van havermeel of haverbloem waaraan extra beta-glucaan toegevoegd was. Hierbij bleek naast de concentratie ook het molecuulgewicht van het beta-glucaan een rol te spelen; dit molecuulgewicht bepaalt de mate van viscositeit van het beta-glucaan. Medium-visceus beta-glucaan had een minder negatief effect dan hoog-visceus beta-glucaan. In het algemeen bleek uit ons onderzoek dat beta-glucaan een sleutelrol speelt in de rheologie van haverdeegsystemen.

Ook is aandacht besteed aan de invloed van de deeltjesgrootte na het malen op de deegeigenschappen. Volkoren havermeel bleek niet optimaal te zijn voor broodbereiding omdat het zeer stijf en kort deeg levert (Hoofdstuk 5). Opnieuw malen bracht hierin geen verbetering; totale verwijdering van de zemelfractie uit het havermeel deed dat wel. De zemelfractie bleek dus een negatief effect op de rheologie te hebben, waarbij de grote en middelgrote zemeldeeltjes een sterker effect hadden dan de fijne zemeldeeltjes. De oorzaak hiervan was het hoge beta-glucaan gehalte van 8% in de grote en middelgrote zemelfractie, terwijl deze in de fijne fractie slecht 1.6% bedroeg. De conclusie is dat volkoren havermeel niet geschikt is voor het maken van deegbrood, maar dat met fractionering van het meel wel een laag beta-glucaan houdend bloem verkregen kan worden met betere deegeigenschappen. De uitgezeefde zemelfractie met hoog beta-glucaangehalte kan benut worden voor het verrijken van andere voedingsproducten. De gangbare methoden voor eesten en malen van haver zouden verder gestandaardiseerd moeten worden ten behoeve van de productie van deeghaverbrood.

Vervolgens is het standaard deegteststelsel gebruikt in experimenten om de effecten te onderzoeken van de gangbare eest- en maalmethoden van haverkorrels (Hoofdstuk 5). Stoom-eesten beïnvloedde de waterabsorptie, maar veranderde de extensibiliteit niet. Infrarood-eesten daarentegen had effect op zowel de waterabsorptie als de extensibiliteit. Het resulteerde in een zeer stijf en kort deeg. Dit maakt toepassing van infrarood-eesten definitief ongeschikt bij broodbereiding.

Tot slot is aandacht geschonken aan het maken van een glutenvrij haverbrood van goede kwaliteit (Hoofdstuk 6). Hoewel verder onderzoek naar het haverdeegstelsel voor het verkrijgen van meer inzicht in de technologische aspecten noodzakelijk is, kon het model toch gebruikt worden voor onderzoek naar de rheologie waarbij gluten in het deeg vervangen werd door alternatieve ingrediënten. Wei-eiwitdeeltjes (WPP) waren al doeltreffend gebleken in het verbeteren van de deegeigenschappen van tarwezetmeel, leidend tot specifieke rijsvolumes van 3.7 mL/g. We hebben hier onderzocht of een vergelijkbaar positief effect ook met haverbloem verkregen kon worden. Inderdaad bleek WPP de existentie-weerstand en het gasvasthoudend vermogen in haverbloemdeeg te

verhogen. Hoewel in onze kleinschalige bakexperimenten een negatief effect op de gasproductie werd waargenomen en het specifieke rijsvolume niet toegenomen was, bleek de structuur van de korst verbeterd te zijn. WPP zijn daarom veelbelovend als structuurverbeteraar in deeg gemaakt van haverbloem, maar het proces moet nog wel verder moet worden geoptimaliseerd.

In de General Discussion komen we uitgebreid terug op de veiligheid van haver voor mensen met coeliakie. De analyse van het genus *Avena* wat betreft avenine genen en eiwitten leverde hierbij een belangrijk nieuw en ondersteunend argument, want geen van de algemeen erkende coeliakie-gerelateerde epitopen uit tarwe, gerst en rogge werden aangetroffen in avenines. De positieve signalen voor de aanwezigheid van gluten, die in de literatuur voor enkele haverrassen beschreven zijn op basis van de R5- en G12-antilichaamtest en in T-cel testen, kunnen verklaard worden door kruisreactiviteit en promiscuïteit, en hebben geen klinische relevantie. De data ondersteunen het advies om de consumptie van haver geleidelijk te introduceren in het dagelijks dieet van mensen met coeliakie. Verder bespreken we de resultaten en de consequenties van het technologisch onderzoek aan haverbloemdeeg. Het bleek dat beta-glucaan de rheologie van het deeg ernstig verstoort. Hier is duidelijk verder onderzoek nodig om tot een betere balans te komen tussen de toepassing van beta-glucaan voor gezondheid (veel en hoogmoleculair is beter) en voor bakkwaliteit (weinig en laagmoleculair is beter). Ook de voorbehandeling van haverbloem (eesten en malen) en de toepassing van wei-eiwit ter vervanging van gluten vragen nog om verdere optimalisatie. Hierbij zal het hier ontwikkelde standaard haverbloemdeeg-systeem goede dienst kunnen bewijzen.

## ACKNOWLEDGEMENTS

It was almost six years ago when I came to the Netherlands to start with my PhD program. I had some difficulties and I had to stop once, but I started again and here I am graduating. Sometimes it looked so far away... I imagined myself as an elderly person writing the general discussion of this thesis. The decision of starting again was difficult to make because I felt exhausted. That was a tough period for me because I was far from that sweet and warm place called “home”, feeling that a PhD was perhaps not something for me. My self-confidence was a little bit harmed. There, at home, everything appeared to be always calm despite the bombs exploding outside. This is just to illustrate how the place where I grew up (Medellín) used to be. But I am not going deeper in the circumstances that brought me to be the person that will graduate, although certainly that context influenced substantially the way how I dealt with the whole PhD process, which includes my relationship with my supervisors and with every person involved.

I would like to write some lines to say “thanks a lot” to all people that in some way have contributed to my personal development. The woman that leaves Wageningen University is very different to the one that once came with a bag full of hopes, squeezed by a huge poncho given by her father (because he said it was important for the winter). I learned in Wageningen that science is wonder and that wonder is not fiction. Science was like a fantastic story to me in my developing world. In Wageningen, fantasy became somehow real, and science stopped being the image of Neil Armstrong landing on the moon. I would like to take advantage of this important moment to acknowledge people for whom I might not find the precise words to express my gratitude. Some will never read how much I appreciate the little things they did for me. Now, when I look back, I realise those little things appear huge.

First of all, I want to thank my supervisors because they were a key factor for my success to obtain the Doctor of Philosophy title (that is how it is and how it should always be). René Smulders was always supportive and enthusiastic to hear about all the aside projects and dreams that I built up in parallel to my PhD. During this time I discovered passions that were sleeping in my inner self: I started to write my opinions about different subjects and send them to politicians and journalists in Colombia. I was surprised to realise that my opinions were actually read, and some appeared even in the newspapers! I shared those things with René and he celebrated them. Moreover, I appreciate his help with the writing when writing happened to be difficult and painful for me because of health concerns. I really value his consideration about my health and about my dreams (most of them related with my home country; which is

like the town Macondo from “One hundred years of solitude”. Of course we have there yellow butterflies, wonderful landscapes, and amazing people, but also solitude -as a metaphor-.

It is important to feel that your work make sense for somebody; with Luud Gilissen I always had that feeling and that confidence. He was the connexion with society that sometimes is missing in science. He made me get closer to the celiac disease patient society, and the bakers and millers. Thanks to Luud I became a little bit fanatic to labels from food and non-food products. I started to read even the shampoo labels looking for the “hidden gluten”. It was amazing to see how committed he was with the subject. I could imagine him eating oats every day to accomplish the recommended daily beta-glucan intake. In addition, I acknowledge him for a book that he selected as a present for me. I enjoyed it a lot. Books are the best present that people could give me, but I understand that selecting a book for someone might be difficult. However, with that present, I realised that Luud knew something about my interests, despite we did not take so much time to share personal views about life and society.

Sometimes I said to people that my boss was man 2 m high and 4 m kind. That is how I described Richard Visser. I want to thank Richard for having accepted me in his department, even though I had this “failure” PhD story on my shoulders. Something that I found admirable about Dutch culture was the lack of prejudices (at least that was my perception when I came to the Netherlands). At the beginning I felt that Dutch people did not like to have too much contact with me because of something very silly: they did not ask “how are you?” That question is the way Colombians say “hello”, although we might not want to hear the truth. I was surprised because when I had problems in my first PhD project, I received messages of support from people that I even did not know. Some said that if something went wrong, it did not necessarily meant that I was not able to succeed in another project. With Richard I found the same support, always a smile, and also very good remarks that helped me to improve the quality of the research. Once he said: “I have noticed that you are a kind of people’s person”. I think that is exactly what I am. Therefore, I want to thank him also for his comment.

Now it is the time to acknowledge Rob Hamer. It was always a pleasure to meet him. I encountered many difficulties in my PhD project related to the fact that I was working on food technology in the department of plant breeding. Rob was always very busy, but he managed to generate gaps in his agenda to discuss with me, and to arrange things in his department so that I could work. It is weird what I am about to express: I particularly liked when his secretary Nicole sent me a message to cancel one of our meetings. She was so kind and so respectful that made me feel I was not a PhD student, but the



Ministry of something. I guess that is how respect feels like. Respect is what Rob always inspired me and gave me. During the whole process I felt his appreciation and interest for my work, he tried to provide me the tools that allow me to follow the right path. His questions and remarks opened new routes that motivated me to keep the spirit up -which in my opinion is one of the hardest things of the PhD-. His pragmatic view forced me to have my feet on the ground and to avoid taking unnecessary steps. We were quite a good team. I also want to acknowledge his attention to read my manuscripts. His corrections were very precise and encouraged me to try to write better every time. And last: I want to thank Rob for a sentence I will never forget: “whatever you do, you need to learn to take distance”.

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Last but not least: Thanks Dad for lending me your poncho!

Por último: ¡Gracias Papá por prestarme tu ruana!

## CURRICULUM VITAE



Diana M. Londoño was born on July 8<sup>th</sup> 1981 in Antioquia, Department of Colombia. She obtained a degree in Agronomy from the Universidad Nacional de Colombia (Medellín) and she specialized in Phytopathology. In January 2002, she moved to Urabá, a region located in North of Colombia, to perform her BSc thesis in the Bananas Research Centre (Cenibanano). In Cenibanano she studied the population dynamics of pathogenic nematodes and their relation with root damage in banana plants. As an agronomist, she worked for three years (2004-2007) as technical assistant in the production of cut flowers, she was responsible for designing the strategy to produce

Hortensias (*Hydrangea macrophylla*) to export to US in a farm of 15 ha. In September 2007 she got a grant from the Flemish Interuniversity Council (VLIR) to study Master of Science in Nematology in Ghent University (Belgium).

From there she looked for the way to perform the MSc thesis in Wageningen University in the Biometris department. She was accepted to study the resistance and tolerance of a widely cultivated variety of *Chrysanthemum morifolium* to the pathogenic nematode *Meoloidogyne hapla*. The purpose of the research was to elucidate the reproduction capacity of the nematode on the cultivar. For this project she got the support of a *Chrysanthemum* breeding company (Deliflor). After obtaining the MSc degree, Diana continued working for one and half years in the department of Biometris on the development of mathematical models to predict population dynamics of pathogenic nematodes and yield loss in potato crops. The aim of the project was to support the decisions of farmers regarding crop rotation and nematicide application.

In July 2009 Diana started a PhD in the department of Plant Breeding of Wageningen University. The core responsibility was to study the safety and the technological properties of oat (*Avena sativa*), with the aim to provide good quality food products for people suffering from celiac disease. In the first part of the project she clarified the celiac disease safety of oats by cloning avenin genes and by screening the translated proteins. She developed a standard test to study the contribution of different oat components to the technological properties of oat flour, and to screen oat varieties according to their bread-making potential. She used different techniques and platforms related with genomics, proteomics, food technology, and bioinformatics. She defended her thesis in June 2014.

Along with his scientific drive and interest in agriculture, Diana is a committed person with society. She is involved in several projects related with children education and empowerment of people from rural areas in Colombia. She is currently busy with a group of friends on the setting up of a public library in the village where she spent most of her life. The library is called “la Casita de la cultura rural” (House of the rural culture). La Casita will open its doors in July 2014 to offer cultural activities to 30 children that attend the school of the village and their parents. This project has been planned and will be executed in collaboration with the teacher from the school and the Secretary of Education of San Vicente (Municipality), where Diana is also advisor *ad honorem*. Diana is also mentor of bachelor students from the Universidad Nacional de Colombia.

**LIST OF PUBLICATIONS**

- Londono, D.M., Van 't Westende, W.P.C., Goryunova, S., Salentijn, E.M.J., Van den Broeck, H.C., Van der Meer, I.M., Visser, R.G.F., Gilissen, L.J.W.J., Smulders, M.J.M. (2013) Avenin diversity analysis of the genus *Avena* (oat). Relevance for people with celiac disease. *Journal of Cereal Science* 58: 170–177. <http://www.sciencedirect.com/science/article/pii/S073352101300057X>
- Londono D.M., M.J.M. Smulders, R.G.F. Visser, L.J.W.J. Gilissen, R.J. Hamer. Development of a standard test for dough-making properties of oat cultivars. *Journal of Cereal Science* 59: 56-61. <http://www.sciencedirect.com/science/article/pii/S0733521013001689>
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- Londono D.M., M.J.M. Smulders, R.G.F. Visser, L.J.W.J. Gilissen, R.J. Hamer. Understanding the role of beta-glucans in dough systems. (submitted to journal of cereal science).
- Gilissen LJWJ, HC van den Broeck, DM Londono, EMJ Salentijn, F Koning, IM van der Meer, MJM Smulders (2012) Food-related strategies towards reduction of gluten intolerance and gluten sensitivity. Pp 29-35 in Proceedings of the 25th meeting of the Working Group on Prolamin Analysis and Toxicity (Peter Koehler, ed.), September 2011 in Fellbach, Germany. [http://www.wgpat.com.ar/proceeding\\_25th.pdf](http://www.wgpat.com.ar/proceeding_25th.pdf)
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## PE&RC TRAINING AND EDUCATION STATEMENT

With the training and education activities listed below, the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)



### Review of literature (4.5 ECTS)

- Celiac disease : triggering molecules

### Writing of project proposal (4.5 ECTS)

- Oats as alternative for wheat for CD patients

### Post-graduate courses (6.6 ECTS)

- Multivariate analysis; PE&RC (2009)
- Basic statistics; PE&RC (2009)
- Linear mixed models; PE&RC (2009)
- Introduction to R for statistical analysis; PE&RC (2009)
- Applied bioinformatics in plant sciences; Athens, Greece (2010)

### Laboratory training and working visits (4.5 ECTS)

- Bread quality of oat cultivars; Department of Food and Nutrient Sciences, University College Cork, Ireland (2010)

### Invited review of (unpublished) journal manuscript (1 ECTS)

- Journal of Cereal Science: dough rheology and quality of gluten-free bread (2014)

**Deficiency, refresh, brush-up courses (1 ECTS)**

- Principles of plant breeding (2012)

**Competence strengthening / skills courses (3.6 ECTS)**

- Scientific publishing; WGS (2012)
- ILP: information literacy including EndNote introduction; WGS (2012)
- TWP: techniques for writing and presenting a scientific paper; WGS (2013)
- P&TM: project and time management; WGS (2013)

**PE&RC Annual meetings, seminars and the PE&RC weekend (1.2 ECTS)**

- PE&RC Days (2009-2012)

**Discussion groups / local seminars / other scientific meetings (2 ECTS)**

- Celiac disease consortium meeting (2009-2012)

**International symposia, workshops and conferences (9 ECTS)**

- Xth International gluten workshop; Clermond-Ferrand, France (2009)
- Second international symposium on gluten-free products and beverages; Finland (2010)
- Cereals Europe spring meeting; Germany (2011)
- AACC International annual meeting; Floriade (2012)
- Cereals Europe spring meeting; Belgium (2013)

**Supervision of MSc student**

- Effect of whey protein particles on dough-making properties of oat flour



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