Degradation of Wheat Germ Agglutinin and Amylase-Trypsin Inhibitors During Sourdough Fermentation

by

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Abstract

Non Celiac Wheat Sensitivity (NCWS), an intolerance to the ingestion of wheat products, has increased considerably during the past few years. In sensitive individuals, NCWS manifests by intestinal and extra intestinal symptoms in different ways. Two wheat protein fractions have been linked to NCWS, amylase-trypsin inhibitors (ATI) and wheat germ agglutinin (WGA). Physicians recommend that individuals with NCWS adhere to a gluten free diet. However, gluten free diets are often associated with a reduced diversity of products, a higher price and lower sensory and nutritional quality. Thus, it was the objective of this study to explore the possibility of using sourdough fermentation to reduce the bioactivity of these two proteins linked to NCWS in wheat bread.

White pastry flour was used to analyze ATI and whole wheat flour for WGA experiments. The analytical techniques used to determine the fate of ATIs and WGA through the fermentation were size exclusion high performance liquid chromatography (SEC-HPLC), and enzyme-linked immunosorbent assay (ELISA). During fermentation, the pH of the dough decreased to 3.9 ± 0.2, which promoted the degradation of ATI from oligomers into monomers; ATI monomers are less harmful when consumed. WGA is also modified during sourdough fermentation depending on the reducing capabilities of the strains used. Initially, commercial whole wheat flour contained 6.6 µg ± 0.7 of WGA per gram. After 24 h fermentation, doughs fermented with *Latilactobacillus sakei* TMW 1.22 contained 2.7 µg ± 0.4 of WGA per gram of flour, while the doughs fermented with *Fructilactobacillus sanfranciscensis* DSM20451 and *F. sanfranciscensis* DSM20451 Δ*gshR* contained 4.3 µg ± 0.3 and 6.5 ± 1.8 µg, respectively. The WGA-SEC chromatograms show 3 peaks for doughs fermented with *F. sanfranciscensis* DSM20451 Δ*gshR* while the chromatograms with the isogenic strain *F. sanfranciscensis* DSM20451 show a more complex profile with 5 peaks,

one of them from a very large molecular size molecule. The concentration of WGA is lower after fermentation with lactobacilli that have high reducing capacity. Clinical studies are required to determine the safety of consumption and the possible reduction in adverse symptoms, but this is a step towards finding new options to incorporate into the diet of NCWS individuals.

Preface

This thesis is an original work by Luis Eutimio Rojas Tovar.

The section related to Amylase Trypsin Inhibitors isolation, labeling and detection by HPLC was done in collaboration with Dr. Xing Huang from the University of Helsinki, Finland.

This section is being reviewed for publication after major revisions. The title of the manuscript is "The fate of alpha-amylase/trypsin inhibitors (ATI) in sourdough and bread making" and the authors are: Xin Huang, Detlef Schupan, Luis R. Tovar, Victor F. Zevallos, Jussi Loponen and Michael Gänzle. The contributions are: X.H., D.S., J.L. and M.G. designed research; X.H., L.R.T. and V.F.Z. performed research; X.H., D.S., V.F.Z., J.L. and M.G. analyzed data; and X.H., D.S., J.L. and M.G. wrote the paper. I contributed to experiments that generated data shown in Table 1 The pH value and bacterial cell count after fermentation in the mini-dough system and Figure 5 Size-exclusion chromatogram of fluorescein-labelled ATI in bread-making of this publication manuscript; these are included as Table 1 and Figure 5 of this thesis.

Results from Wheat Germ Agglutinin detection by ELISA were poster presented at the 5th International Symposium on Gluten-Free Cereal Products and Beverages held in Leuven, Belgium from June 26th to June 28th, 2019.

Dedication

To those who have believed in me through all these years.

Thank you, I owe you everything.

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List of Abbreviations

ATI	Amylase-Trypsin Inhibitors
BSA	Bovine serum albumine
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
FODMAPs	Fermentable oligo-, di-, and monosaccharides and polyols
GSH	Glutathione
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
HRM-qPCR	High Resolution Melting Quantitative Polymerase Chain Reaction
kDa	Kilodalton
LAB	Lactic acid bacteria
mMRS	Modified de Man, Rogosa, and Sharpe
NaCl	Sodium chloride
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PBS	Phosphate buffer saline
RCF	Relative centrifugal force
SEC	Size Exclusion Chromatography
SDS	Sodium dodecyl sulfate
SD	Sourdough
TFF	Tangential flow filtration
WGA	Wheat germ agglutinin

1 Introduction.

1.1 Overview.

Bread has been consumed by humans for 14,400 years or more (Arranz-Otaegui, 2018). The fact that this food product has existed and developed through ages serves as a testament of its importance in the human diet. The most common cereals used for bread production are wheat and rye. Wheat products dominate the market and consumers' preference. However, in recent years, because wheat has been related to several health disorders, the consumer's perception of wheat products has changed (Shahbandeh, 2019). Gluten proteins from wheat and corresponding proteins from rye and barley trigger celiac disease, an illness that causes intestinal inflammation and reduces nutrient absorption in the intestine (Schuppan & Gisbert-Schuppan, 2019). Other wheat components also cause negative intestinal effects and discomfort to sensitive individuals. That is the case for the disorder referred to as Non-celiac wheat sensitivity (NCWS), which differs from celiac disease as there are no specific biomarkers for NCWS. Wheat components related to NCWS are fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs), amylase-trypsin inhibitors (ATIs), and wheat germ agglutinins (WGA) (Potter et al, 2017; Catassi et al, 2017; Schuppan et al, 2015; Zevallos et al, 2017). NCWS, which is also referred to as non-celiac gluten sensitivity, involves a wide variety of possible symptoms, including bloating, diarrhea, nausea, intestinal damage. Extra intestinal symptoms have also been described, such as tiredness, headaches, joint pain, anxiety, among others (Potter et al, 2017). Individuals affected by NCWS are normally prescribed a diet which is free of wheat products (Pinto & Verdu, 2018). It is difficult to diagnose NCWS given the variety of symptoms that patients can manifest, and in some instances, it overlaps with irritable bowel syndrome (IBS) because of the shared symptoms between the two (abdominal pain, bloating, diarrhea and constipation) and the lack of specific

biomarkers (Catassi et al, 2017). Diagnosis of NCWS is based on a process of elimination, by ruling out celiac disease and wheat allergies (Schuppan et al, 2015).

NCWS has been present for many years. Ellis and Linaker mentioned in 1978 (Ellis and Linaker, 1978) with a 43 year old female patient whose symptoms disappeared after adopting a gluten free diet. At this time though, they believed gluten was the problem. Even though the possibility of a new illness was acknowledged, it did not receive much attention at that time. 30 years later, interest resurfaced; however, due to the lack of biomarkers, some researchers and scientist questioned its existence (Biesiekierski et al, 2012; Pinto-Sanchez & Verdu, 2018). On their book "Wheat Syndromes", Schuppan & Gisbert-Schuppan (2019) established that there is scientific evidence defining NCWS which includes two inflammatory conditions: atypical wheat allergy and ATI-sensitivity. Atypical wheat allergy manifests symptoms –diarrhea, constipation or abdominal pain– within a few hours of consumption. ATI-sensitivity refers to the activation of the toll-like receptors (TLR4) present in the intestine by ATIs. The stimulation of TLR4 receptors is of low consequence to healthy individuals, however in patients with mild to severe chronic illnesses it potentiates pre-existent inflammation (Schuppan & Gisbert-Schuppan, 2019). One of the main characteristics that defines NCWS is that individuals improve once they adopt a gluten free diet, even when gluten is probably not the molecule that triggers their symptoms. Gluten containing cereals –wheat, rye and barley– are high in FODMAPs and also contain ATIs, thus the improvement when a gluten free diet is adopted. Another important characteristic to distinguished NCWS from other digestive disorders is the extraintestinal symptoms that patients manifest (Schuppan et al, 2015).

Sourdough was chosen as the method to modify/degrade WGA and ATI in wheat bread products for the following reasons. Firstly, in most cases, sourdough process involves longer

fermentation times in comparison to yeast bread settings. This longer procedure provides more time for wheat aspartic proteases to act upon WGA and ATIs. Besides time, acidic pH –commonly found in sourdoughs– is necessary for the activation of wheat aspartic proteases (Hammes & Gänzle, 1998; Capuani et al, 2013). Secondly, the ability of bacteria to decrease the redox potential of the dough system can be an important factor when highly disulfide cross-linked proteins are involved. ATI monomers possess 5 intramolecular disulfide bonds, while WGA monomers have 16 intramolecular disulfide bonds. With this background, the glutathione reductase enzyme present in many strains of heterofermentative lactobacilli can be an important factor to regenerate reduced glutathione that can disrupt disulfide bonds and consequently promote proteolysis (Jänsch et al, 2007; Gänzle et al, 2008; Loponen et al, 2008). Other lactobacilli also contribute to the disruption of disulfide bonds by other mechanisms that have not been described yet, but that researchers have found evidence of (Serata et al, 2012; Jänsch et al, 2007). Additionally, it is common in sourdough fermentations that more than one bacteria participate, usually bacteria and yeast grow together, which increases probabilities for proteolysis. Thirdly, sourdough degrades other molecules, like FODMAPs (Gobbetti et al, 2019; Loponen & Gänzle, 2018); thus, finding additional benefits can be very appealing for bakers and consumers. Fourthly, consumers have a positive opinion about sourdough (Statista Research Group, 2019), and although the evidence is only anecdotal, many claim that they feel better consuming sourdough compared to consuming regular yeast bread. The results of this study might help to support their claims scientifically.

Sourdough can be an alternative to gluten free diets to reduce symptoms associated with NCWS. Sourdough fermentation was used in bread production through centuries of history in ancient civilizations, and it's currently regaining its importance in the bread market (do Nascimento et al, 2018; Cappelle et al, 2013). Sourdough is fermented with lactic acid bacteria

(LAB); yeast can also be part of the fermentation (Hansen, 2012). The cell counts of metabolically active LAB and yeast range from 10^7 to 10^9 CFU/g and 10^5 to 10^7 CFU/g respectively (Corsetti, 2013). The pH values of the sourdough are 4.5 or lower, as a consequence of the production of lactic acid. Acetic acid is also produced because heterofermentative lactic acid bacteria are generally prominent members of sourdough microbiota. Factors that can vary during fermentation are: temperature, water to flour ratio, use of a starter or culture, time, source of the flour, number of propagation steps, phages, oxygen concentration, among others (Hammes & Gänzle, 1998). These conditions determine the microbial ecology and select for organisms that dominate the fermentation, and therefore the characteristics of the final product. Lactic acid bacteria in sourdoughs include strains of the genera *Lactobacillus*, *Companilactobacillus, Lacticaseibacillus*, *Latilactobacillus*, *Lactiplantibacillus*, *Furfurilactobacillus, Limosilactobacillus*, *Fructilactobacillus, Levilactobacillus* and *Lentilactobacillus*, which were all included in the genus *Lactobacillus* until April 2020 (Zheng et al, 2020) as well as *Weissella*, *Leuconostoc*, *Lactococcus, Enterococcus,* and *Pediococcus* (Gänzle & Zheng, 2019; De Vuyst et al, 2017). Once the sourdough has been started, bakeries propagate the dough for years, decades or centuries to use it for daily bread production. To feed it, bakeries use a portion of the previous day's sourdough and mix it with new dough, a process called backslopping, which has been practiced at least since year 74, as documented by Pliny the Elder (Pliny the Elder, 74). Another option is to add fresh ingredients to an existing sourdough to propagate it (Corsetti, 2013).

Sourdough is classified in four different types. Type I sourdough produces enough CO_2 for leavening of bread without the addition of baker's yeast, *Saccharomyces cerevisiae*. In this case, the microbiota needs to remain highly metabolically active and multiple refreshments are needed before bread production. *Fructilactobacillus sanfranciscensis* (previously *L. sanfranciscensis*) is

the most common microorganism found in this type of sourdough. Other examples are: *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, *Companilactobacillus alimentarius* and *Furfurilactobacillus rossiae*. Yeast also participate in the fermentation; *Kazachstania humilis*, *Kazachstania exigua,* and *Saccharomyces cerevisiae* are the species that are most frequently identified. Type II sourdoughs are produced by long fermentation periods; thus, the acidification level is much higher than Type I sourdoughs. These acidic doughs are not used for leavening; they help to acidify the dough or are used as dough improvers. These doughs are more flexible, as they require less refreshments, and they tend to be liquid, which is very useful in industrial settings. The use of baker's yeast to achieve leavening is required. Bacteria present in type II include *Limosilactobacillus* species, particularly *Limosilactobacillus reuteri, Limosilactobacillus panis, Limosilatobacillus fermentum* and *Limosilactobacillus pontis*; these typically occur in association with *Lactobacillus* species (previously *L. delbrueckii* group) (Gänzle & Zheng, 2019). Type III sourdoughs are stabilized by processes like drying or pasteurization, which increase the uniformity of end products, and also reduce transportation costs when the sourdough is produced in a different location from the bakery. This type of sourdough is very convenient for industrial manufacturers as well. The definition of Type 0 sourdoughs applies to sponge doughs, when bakeries use *Saccharomyces cerevisiae* to ferment some of the bread flour before the final dough with all ingredients is mixed. This pre-fermentation can vary in length, thus allowing the development of LAB if the fermentation time exceeds 8 – 12 h (Corsetti, 2013; Gänzle, 2014; Decock & Cappelle, 2005).

There are many benefits associated with the use of sourdough in bread production, for instance, reduced content of FODMAPs (Loponen & Gänzle, 2018), lower glycemic index compared to yeast bread (Redhed Miller, 2018; Gobbetti et al., 2019), richer taste (Zhao & Gänzle,

2016; Brandt, 2007), stronger aroma (Hansen & Schieberle, 2005; Cho & Peterson, 2010) increased shelf life (Torrieri et al, 2014; Moore et al, 2008), clean ingredient labels (do Nascimento et al, 2018; Quattrini et al, 2019), reduced sodium (Nogueira et al, 2015), bakery products with higher fiber content (Katina et al, 2005), reduced anti-nutritive factors like phytic acid (Najafi et al, 2012; Corsetti & Settanni, 2007), increased mineral bioavailability (Poutanen et al, 2009), potential antioxidant and antihypertensive effect (Catzeddu, 2019), among others. It can also be used to avoid wasting industry byproducts, like wheat germ, a byproduct of the milling industry (Zhao et al, 2020).

There are two types of lactic acid bacteria based on their carbohydrate fermentation patterns: heterofermentative and homofermentative. Homofermentative bacteria metabolize hexoses through glycolysis (Embden-Meyerhof-Parnas pathway) producing mainly lactic acid. Heterofermentative bacteria on the other hand, follow the pentose phosphoketolase pathway producing lactic acid too, but also CO_2 and ethanol or acetate in significant amounts (Von Wright & Axelsson, 2012). In this study emphasis is made on heterofermentative lactobacilli because of their ability to reduce the redox potential of the dough. Some heterofermentative strains synthetize glutathione reductase, an enzyme that reduces oxidized glutathione (GSSH) to glutathione (GSH) (Calberg & Mannervik, 1985; Vermeulen et al, 2006). Other heterofermentative strains synthetize cystathionine γ-lyase, an enzyme that produces L-cysteine, ammonia and α-ketobutyrate (De Angelis et al, 2002). The importance of reducing conditions on protein degradation relies on the stability of proteins with disulfide bonds on their secondary, tertiary and/or quaternary structure. Reducing agents like glutathione (GSH) can disrupt disulfide bonds, decreasing protein stability and allowing proteolytic enzymes access to the protein. Some homofermentative strains have the

opposite effect, producing H_2O_2 during growth, which helps to strengthen the proteins (Vermeulen et al, 2006).

1.2 Protein degradation during wheat sourdough fermentation.

The composition of wheat is on average (w/w) 14 % water, 63-72 % starch, 7-15 % protein, 4.5-5 % nonstarch polysaccharides, and 2-3 % lipids. From the protein fraction, 70 to 80 % is composed of storage proteins, gliadins and glutenins, while 15 % are albumins, and 3 to 5 % globulins (Finnie & Atwell, 2016). During sourdough fermentation, the metabolic activity of lactobacilli or cereal enzymes modify major components of wheat flour. Lactobacilli's interaction with proteins is strain dependent; few species of lactobacilli have extracellular proteases bound to their cell wall, but most lactobacilli express only intracellular peptidases (Savijoki et al, 2006). Thus, it is most common that protein modification is affected indirectly by LAB by two main mechanisms. The first one is the acidification of the dough. Wheat aspartic proteases are active at pH values between 3.5 and 4 (Vermeulen et al, 2005); this pH is achieved in most sourdough fermentations (Lönner & Preve-Åkesson, 1988). Proteinases supply peptide fragments that are small enough for LAB transport systems that transfer peptides into the cell for further metabolism, but they do not degrade proteins outside the cell (Gänzle et al, 2008). Thiele et al (2004) studied gluten depolymerization and hydrolysis of gluten during sourdough fermentation and demonstrated a clear effect of pH on proteolytic activity in chemically acidified wheat doughs.

The second key mechanism where LAB influence protein activity is their contribution to reduce the dough's redox potential. Low values of redox potential indicate reducing conditions, in which disruption of protein disulfide bonds is more likely to happen. Some heterofermentative lactobacilli express glutathione reductase; this enzyme regenerates reduced glutathione (GSH) through the fermentation. Glutathione plays a major role disrupting intermolecular and

intramolecular disulfide bonds of proteins. This phenomenon is especially important in wheat doughs, where the formation of the gluten macropolymer relies heavily on the aggregation of proteins by the formation of intermolecular disulfide bonds between high molecular weight glutelins (Lindsay & Skerritt, 1999). When reducing agents are used, the dough weakens as gluten depolymerizes (Wieser, 2007). Disrupting disulfide bonds causes proteins to lose stability and get more exposure to proteolytic enzymes, i.e. wheat aspartic proteinases, leading to their degradation (Gänzle et al, 2008). Loponen et al (2008) analyzed egg white protein degradation during sourdough fermentation. They observed that heterofermentative microorganisms expressing glutathione reductase degraded ovotransferrin extensibly, something that did not happen in chemically acidified controls or in doughs fermented with the homofermentative lactobacilli. Not all lactobacilli have the same redox regulating system. Jänsch et al (2007) observed that *Limosilactobacillus reuteri* affected the dough's thiol balance with a different system that was not related to the glutathione reductase. They noticed that cystine was involved, even though the mechanism of action was undetermined.

To track protein degradation through sourdough fermentation, size exclusion high performance liquid chromatography (SEC-HPLC) with a fluorescence detector (FLD) was selected as the analytical technique to be used. The principle behind this set up was to fluorescent-label the target protein –ATIs or WGA– with fluorescein isothiocyanate (FITC) and then reintroduce the fluorescent protein conjugate into the dough. Size exclusion chromatography, also known as gel filtration, is a common method to separate biopolymers according to their hydrodynamic volume and molecular weight. In this type of chromatography, the difference in affinity between mobile phase and stationary phase is not the key element for separation, thus, the mobile phase can be accommodated to best carry the analyte without modifying resolution. In a

simplified model, its separation principle relies on the difference of time that it takes the solutes to travel to the porous matrix of the column; this time is ideally governed by the differences between the solute dimensions and the pore dimensions (Hagel, 2011). In this experiment, the matrix of the column is a mixture of cross-linked dextrans and cross-linked agarose with a fractionation range of 10 kDA to 600 kDA (GE Healthcare, 2012). The advantages of this technique are that it is relatively simple, fast, and using fluorescent conjugates, it is possible to track only the molecule of interest without interference from other molecules present. The disadvantages are its low selectivity when the molecular weights are close, the low sensitivity of the FLD and the low precision between replicates, which makes quantification difficult. In addition, when labeling proteins for fluorescence detection, the concentration of the fluorophore should be low otherwise they might aggregate or self-quenching effects can occur (Hermanson, 2013). FITC binds covalently to lysine residues of the proteins. The SEC-HPLC-FLD set up has been used by other researchers like Thiele et al (2003) who used it to determine gluten hydrolysis in fermentation. Tagging proteins with fluorophores is common practice; sometimes, tagging affects the target molecule. In this case, tagging WGA with FITC changes its binding kinetics (Yin et al, 2013). Tagging of proteins with FITC potentially also alters the response to proteolytic degradation, however, past studies with FITC tagged gluten proteins suggested that fluorescence labeling did not interfere with degradation of wheat gluten by aspartic proteases (Thiele et al., 2003).

1.3 Amylase-Trypsin Inhibitors (ATIs).

ATIs are globular proteins that represent 2 to 4 % of the total protein content in wheat (Schuppan et al, 2015; Dupont et al 2011); they are present in other plants as well, i.e. rye, corn, and barley. ATIs have a three dimensional structure abundant in α-helices and a rich content of cysteine residues, which bestow them with high stability against degradation and processing

(Prandi et al, 2013; Breiteneder & Radauer, 2004). They are part of the defense mechanisms of plants against insects, and their concentration varies depending on wheat species and environmental factors, ranging from 0.22 to 1.11 mg of ATI per gram of flour (Prandi et al, 2013). ATIs are albumins, soluble in water (Laatikainen et al, 2017), salt solutions and in chloroform/methanol (CM) mixtures, therefore they are referred as CM proteins sometimes. ATIs occur as noncovalently linked monomers and dimers, or tetramers (Shewry et al, 1984; Zevallos et al, 2017). They inhibit exogenous enzymes like trypsin and α-amylase without inhibiting endogenous enzymes of the germinating kernel (Judge & Svensson, 2006). ATIs are classified according to their electrophoretic mobility and size in three subfamilies, 50-60 kDa, 24-30 kDa, and 12-15 kDa (Tatham & Shewry, 2008). Each monomer is composed of units that go from 120 to 160 amino acid residues (Breitender & Radauer, 2004). The ATIs comprise a family of about 11 related proteins, some examples are the subtypes 0.28, 0.53, 0.19, CM16, CM17, CM1, CM2, CM3, CMX1, and CMX3, among others. The names of the different subfamilies are derived from their electrophoretic mobility relative to bromophenol blue. Fractions dimeric 0.19 and tetrameric CM3 represent more than 50% of the total ATIs (Zevallos et al, 2017). ATIs have a tight secondary structure with five intramolecular disulfide bonds, which makes them very resistant to degradation and stable at extreme pH values (Schuppan et al, 2015). Fraction 0.19 is a homodimer with 124 amino acid residues. Its 10 cysteine residues form disulfide bonds in pairs Cys6-Cys52, Cys20-Cys41, Cys28-Cys83, Cys42-Cys99 and Cys54-Cys115. This pattern is identical to the one found in ATI fragment 0.28 (Oda et al, 1997). ATIs have strong homology among them, which probably means that they originated from a common ancestral gene. For example, comparing the primary structure of fragments 0.19 and 0.53, differences were found only in seven amino acid residues

out of 124 used for comparison. The authors mentioned that inhibitors from the same class are more closely related compared to inhibitors belonging to other classes (Maeda et al, 1985).

ATIs are involved in allergic responses to wheat, like baker's asthma, which is caused by inhalation of cereal flours (Tatham & Shewry, 2008). They are also related to NCWS because they stimulate the toll-like receptor (TLR)4-MD2-CD14 complex causing pro-inflammatory reactions in the intestine of sensitive individuals (Schupan et al, 2015; Zevallos et al 2017). Zevallos et al (2017) reported that fractions 0.19 (dimer) and CM3 (tetramer) exhibited around 5-fold higher biological activity when compared to the fraction 0.28 (monomers); in other words, these fractions cause more problems to sensitive subjects. They also reported that the average ATI daily consumption falls in the range of 500 mg to 1,500 mg per day per person. Additionally, Junker et al (2012) discovered the importance of the ATIs secondary structure, where the TLR4 stimulating activity disappears when the disulfide bonds are reduced.

1.4 Wheat Germ Agglutinin (WGA).

WGA is one of the most studied plant lectins. It was discovered by Joseph Aub –a member of the biomedical community from the Massachusetts General Hospital in Boston– in 1963, due to its ability to agglutinate malignant cancer cells without affecting normal cells (Sharon & Lis, 2007). WGA is located in the germ of the wheat grain and is found as a dimer with a size of approximately 35 kDa at pH values between 3.5 and 7.4 (Baieli et al, 2012). Each monomer is constructed of an average of 171 amino acid units; they are rich in cysteine residues, thus 16 disulfide bridges are present on its structure (32 in the dimer), which produce a very stable conformation (Wright & Raikhel, 1989). There are 3 isolectins, WGA1, WGA2 and WG3. WGA has specificity to bind N-acetyl glucosamine and its β4-linked oligomers; other substrates are N-acetylgalactosamine, even though its affinity is less in this case, and N-acetylneuraminic acid.

WGA acts as a defense mechanism against external threats, like other plants, insects and pathogens (i.e. fungi) (van Buul & Brouns, 2014; de Punder & Pruimboom, 2013). It forms homodimers, and it has low solubility in water or neutral buffers, but its solubility increases in acid solutions (Baieli, 2012). Each WGA monomer has 4 subunits with a domain (binding site) each: A, B, C, and D. WGA monomers associate in a head to tail conformation, pairing the subdomains in an A-D and B-C combination. (Sharon & Lis, 2007). The content of WGA in wheat varies according to variety. Kronis and Carver (1982) reported a yield of about 400 mg of WGA per kilogram of wheat germ, Nagata and Burger (1974) reported amounts from 100 to 200 mg per kilogram of wheat germ, and Catassi et al (2017) reported 100 to 500 mg / kg of wheat germ which in flour is approximately 4 mg / kg in white flour and approximately 30 mg per kg in whole wheat flour. WGA's amino acid composition has been reported in several manuscripts (Rice & Etzler, 1975; Allen et al, 1973; Kronis & Caraver, 1982; Wright et al, 1984; Wright & Raikhel, 1989; Sharon & Lis, 2007). There are small amino acid variations among the three different isolectins. Isolectins WGA I and WGA II are the most abundant, together they make up for 80 - 90 % of the total WGA mixture. WGA II is the most abundant of all three. (Rice & Etzler, 1975; Kronis & Carver, 1982). WGA is stable at a wide range of pH (Nagata & Burger, 1974). WGA's isoelectric point is at pH 8.7 ± 0.3, except for isolectin IIb which is at 7.7 ± 0.3 (Rice and Etzler, 1975).

The stability of WGA due to disulfide bonds has been documented by different researchers. Rice and Etzler (1975) and Allen et al (1973) reported the inability of SDS to dissociate WGA dimers into subunits in the absence of reducing agents. Erni et al (1980) treated WGA with different amounts of reducing agent dithiothreitol (DTT) followed by carboxymethylation with iodoacetamide and discovered two WGA varieties, one, unmodified or slightly modified, but fully

active and the other one, almost fully carboxymethylated with no binding capacity left. Matucci et al (2004) tested WGA's thermal stability and found its inflection point at 65 °C. At 10 min at 90 °C, WGA loses about 80 % of its binding capacity; at 30 min at 80 C, WGA loses more than 90 % of its binding capacity.

There is some controversy related to WGA's health effects in humans. Pusztai et al (1993) conducted an experiment including WGA on rats' diet and found that it has anti-nutritional effects because it decreased their growth. They also reported that WGA's high stability caused a portion of the ingested protein to pass intact through the gut's wall. However, the concentrations used on their experiment were much higher than what is commonly found in nature. Dalla Pellegrina et al (2005) conducted a trial on human epithelial cell layers using differentiated Caco2 cells. Caco2 cells are cells used as model of the intestinal barrier (Sambuy et al, 2005). They reported that WGA was not toxic to the differentiated cells, but that it increased the epithelium's layer permeability to small molecules. The study concluded that the risk at the concentrations present in regular meals is minimum, but that it should be taken into consideration if WGA is used as a drug carrier, because in that form WGA would be administered in a pure form and in higher doses. On a second trial, Dalla Pellegrina et al (2009) assessed the impact of WGA on the immune system by using Caco-2 cells again and making an *in vitro* simulation with immune cells. On this second experiment it was discovered that at nanomolar concentrations WGA can promote the biosynthesis of pro-inflammatory cytokines. Additionally, antibodies that target WGA have been found in human serum (Tchernychev & Wilcheck, 1996). These effects may relate to WGA's contribution to NCWS (Nijeboer et al, 2013). However, a definite conclusion has not been reached yet due to the lack of studies *in vivo* (Gabor et al, 2004; Dalla Pellegrina et al, 2005; van Buul & Brouns, 2014).

1.5 Hypothesis and objectives.

Sourdough process contributes to protein degradation (Gänzle et al, 2008; Di Cagno et al, 2002; Spicher & Nierle, 1988). Using this evidence it was hypothesized that heterofermentative lactobacilli reduce the content of WGA and ATIs in wheat sourdoughs. The main objective of this research was to determine the impact of sourdough fermentation in the content of ATIs and WGA in wheat sourdoughs. The findings of this research are intended to be the first step to identify if sourdough process can be an alternative to produce wheat bread that can be safely consumed by individuals with NCWS.

2 Materials and methods.

2.1 Bacterial strains and culture conditions.

The strains used in these experiments were *Latilactobacillus sakei* TMW1.22 (LTH677, isolated from fermented sausage), a homofermentative microorganism (Ammor et al, 2005); *Fructilactobacillus sanfranciscensis* DSM20451 (isolated from sourdough), a heterofermentative microorganism, and *Fructilactobacillus sanfranciscensis* DSM20451 *ΔgshR*, an isogenic derivative without the ability to regenerate reduced glutathione (Vermeulen et al, 2006; Jänsch et al, 2007). A chemically acidified dough using lactic acid (DL-lactic acid 90 %, Sigma-Aldrich, Canada) and acetic acid (acetic acid glacial 99.7 %, Sigma-Aldrich, Canada) at a 4 to 1 ratio targeting a pH of 4 in the sample was used as a control.

The media used to grow the strains was modified De Man, Rogosa and Sharpe (mMRS). The formula for the media per liter is 10 g of maltose, 5 g of glucose, 5 g of fructose, 10 g of peptone, 5 g of yeast extract, 5 g of beef extract, 4 g of K_2HPO_4, 2.6 g of KH_2PO_4, 3 g of NH_4Cl, 0.5 g of L-cys HCl, 1 g of tween, 50 mg of $MnSO_4$, 100 mg of $MgSO_4$, 10 g of malt extract and 15 g of agar. The strains were cultivated at 30 °C under anaerobic conditions. Media used to cultivate *F. sanfranciscensis* DSM20451 *ΔgshR* contained 10 ppm of erythromycin.

Carbohydrates were autoclaved separately to avoid Maillard reactions during the process. Moreover, erythromycin was dissolved in 0.5 mL of 70 % ethanol and added to the media after autoclaving (to avoid its degradation due to high heat).

2.2 Confirmation of strain identity by PCR.

The identity of *L. sakei* TMW 1.22 and *F. sanfranciscensis* DSM20451 was confirmed by sequencing the 16S ribosomal RNA genes of the strains. DNA was extracted from an overnight culture grown in mMRS medium using DNeasy Blood and Tissue Kit (Qiagen, USA) following manufacturer's instructions. The extracted DNA was amplified using PCR with Universal 16S rRNA primers (forward 616V, 5'-AGA GTT TGA TYM TGG CTC-3'; reverse 630R, 5'-CAK AAA GGA GGT GAT CC-3') (Juretschko et al, 1998). DNA was purified using GeneJET Gel Extraction and DNA Cleanup Micro Kit (ThermoFisher, USA) following manufacturer's instructions. Purified DNA was then sent to the Sanger sequencing service at the Molecular Biology Services Unit, Department of Biological Sciences at the University of Alberta, Edmonton, Alberta, Canada. The results were then compared to the BLAST database for confirmation of the strain.

F. sanfranciscensis DSM20451 *ΔgshR* was confirmed by PCR using primers that targeted the glutathione reductase gene (gshR), which is absent in the *ΔgshR* strain. DNA was extracted using same methodology as mentioned on the previous paragraph, then PCR was run using primers (forward, 5'-ATT GCA AGG CTC GGG ATT AG-3'; reverse, 5'-GTC ATC CGA AAC CAG TCA GTA G-3') targeting the deleted gshR gene designed based on the gene DQ866807.1 from the gene bank (https://www.ncbi.nlm.nih.gov/nuccore/DQ866807.1). In this PCR experiment, *F. sanfranciscensis* DSM20451, *F. sanfranciscensis* DSM20451*ΔgshR*, and a negative control were run simultaneously. The amplified DNA from the PCR then was run in an agarose gel. Results

confirmed the mutant's identity being positive for *F. sanfranciscensis* DSM20451, and negative for *F. sanfranciscensis* DSM20451 *ΔgshR* and negative control. Additionally, *F. sanfranciscensis* DSM20451 and *F. sanfranciscensis* DSM20451 *ΔgshR* were plated on mMRS agar with erythromycin; the only strain that grew under these conditions was *F. sanfranciscensis* DSM20451 *ΔgshR*.

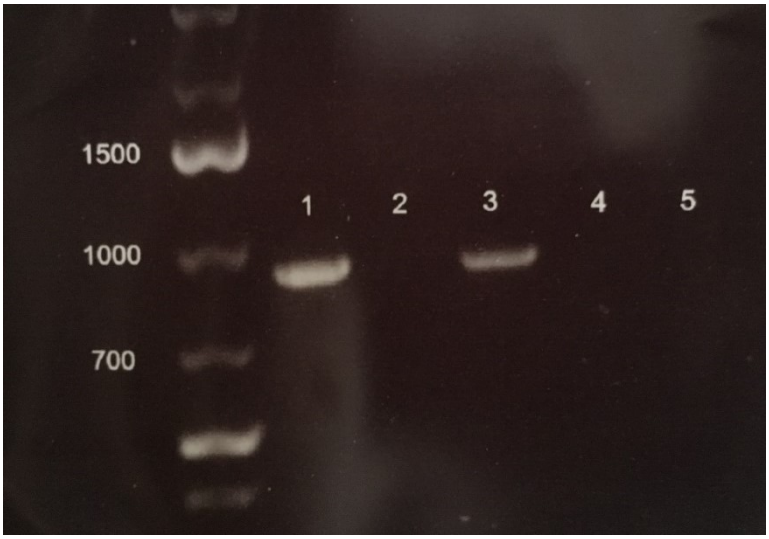

Figure 1. Gel showing PCR results using primers that targeted the gshR gene in *Fructilactobacillus sanfranciscensis.* Samples 1 and 3 are *F. sanfranciscensis* DSM20451; samples 2 and 4 are *F. sanfranciscensis* DSM 20451 *ΔgshR*. Sample 5 is a negative control.

2.3 Inoculum preparation for sourdoughs.

L. sakei TMW 1.22, *F. sanfranciscensis* DSM20451 and *F. sanfranciscensis* DSM20451 *ΔgshR* were taken from the -80 °C and streaked on mMRS agar. Plates were incubated at 30 °C for a period between 24 and 48 hours under anaerobic conditions. Then, single colonies were removed from the plates, inoculated into mMRS broth, and incubated at 30 °C for 24 h. This initial broth was used to subculture the broth used to inoculate sourdoughs in a 1:1 ratio (1 mL of cultured broth per gram of flour). Inoculated broth was incubated at 30 °C for 24 h. Culture was centrifuged at 3,878 RCF (relative centrifugal force) for 3 min. Supernatant was discarded, and sterile tap

water was used to wash the culture. Here and in other parts of the experiment, sterile tap water was used instead of sterile deionized water because sterile tap water avoids the hypo-osmotic shock that bacterial cells would experience if there were exposed to sterile deionized water instead. Tap water is used in common baking practice, and the variability that its mineral composition might have is not significant compared to the minerals and molecules present in the dough system. Moreover, Minervini et al (2019) found that tap water source has very little influence on the microbial composition of mature sourdoughs. Continuing with the procedures of this thesis, tap water was sterilized in the autoclave, and one batch of sterile tap water was used for each experiment. Culture washing procedure was repeated twice. After centrifuging following the second wash, supernatant was discarded and cultured was reconstituted with sterile tap water to the final desired volume (as mentioned above, 1 mL of culture per g of flour).

2.4 Analysis of modifications of fluorescent labeled Amylase-Trypsin Inhibitors (ATIs) by SEC-HPLC.

The analysis of the Amylase-Trypsin Inhibitors (ATIs) was done in collaboration with Dr. Xin Huang from University of Helsinki, Finland and it is in the process of being published.

ATI labeling with FITC. ATI tetramer (60 kDa) was isolated by Dr. Xin Huang. Then, ATIs (20 mg/mL) in 0.1 M sodium bicarbonate buffer were mixed with DMSO containing 10 mg/mL of FITC for two hours in the dark. PD-10 desalting columns were used to remove the excess of FITC. ATI-FITC conjugate was stored protected from light at 4 °C.

Sourdough and bread dough preparation with ATI-FITC conjugate. Mini sourdoughs were prepared by mixing 1.5 g white wheat flour (Fazer, Finland) with 1.5 g sterile tap water containing the bacterial culture or a rye sourdough starter obtained from Fazer, Finland. Mini sourdoughs were fermented for 24 h at 30 °C. Once the sourdough was ready, the rest of the ingredients to

prepare mini bread were added: 3.5 g white wheat flour, 1.5 g water that included 1 % dried yeast (active dry yeast, Fleischmann's, Canada), 160 mg sucrose and 160 mg salt. After mixing, dough was fermented for 2.5 h at 30 °C. Then dough was "baked" in a water bath for 8 minutes at 95 °C (due to the small size of the dough, oven baking was not the best option to follow). ATI-FITC conjugate was added in the sourdough stage or at bread dough stage at 1 % of wheat flour protein (flour contained 12.5 % of protein in this experiment). Two sets of experiments were conducted: on the first set, ATIs were added at the sourdough mixing stage, while on the second set, ATIs were added during the bread dough mixing stage. Samples from the first set were collected at different points of the process as follows: A1, after sourdough mixing; A2, after sourdough fermentation; A3, after bread dough fermentation; A4, after baking and A5 is the same as A2, but baked. Samples from the second set collected at: B1, after bread dough mixing; B2, after bread dough fermentation and B3, after bread baking. Figure 2 summarizes the process. A yeast bread was prepared too, without bacterial culture, using 5 g of flour, 2 g of water, 1% (m/m) active dry yeast and 6 mg of ATI-FITC conjugate. The rest of the process was the same as with the mini bread described above.

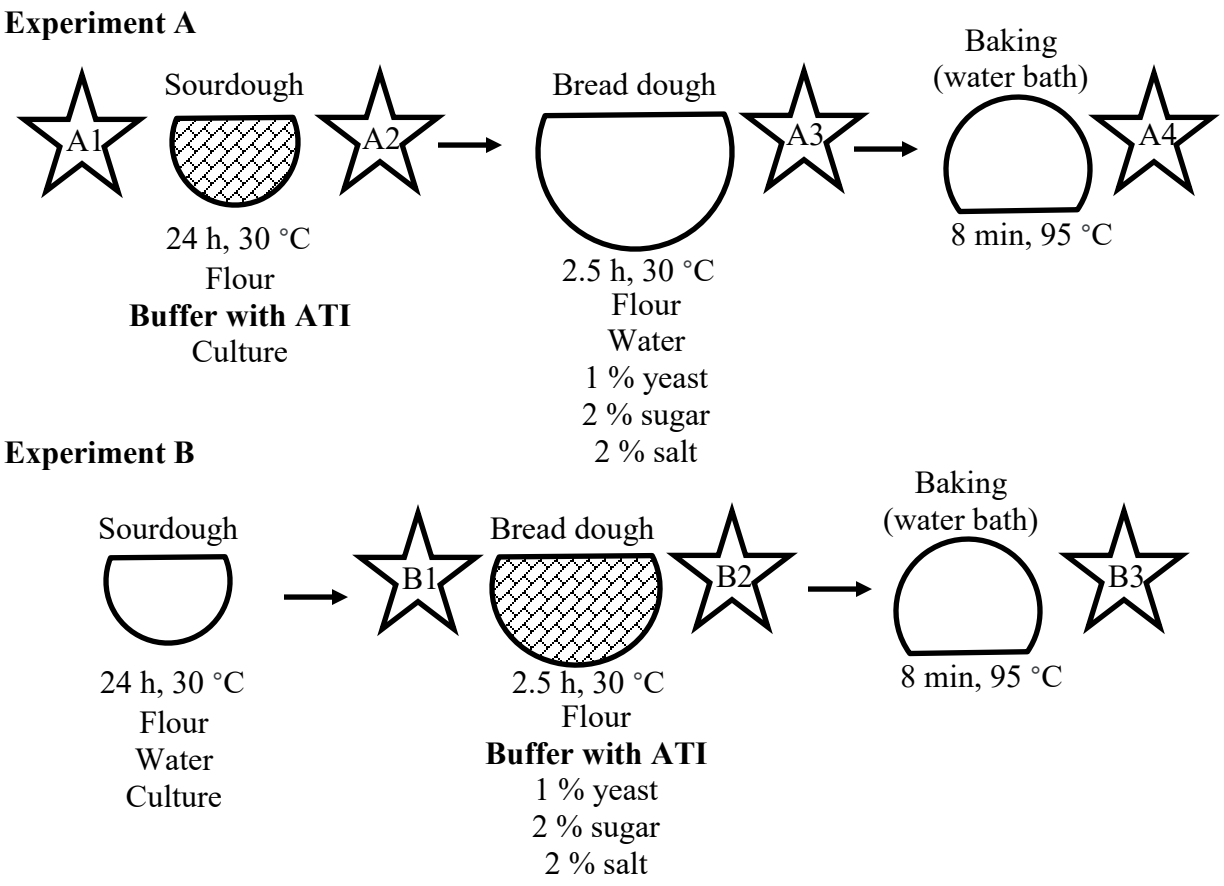

Figure 2. Diagram showing the process used to prepare ATI-FITC inoculated mini-doughs. Stars indicate when samples were taken and their identification code. A5 is A2 baked.

SEC-HPLC. Samples were freeze-dried overnight and grounded using mortar and pestle. ATIs were extracted with 1 mL of 100 mM ammonium acetate pH 6.9 from 100 mg of sample. Supernatant was filtered against 0.45 μm membrane, and then analyzed using a Superdex peptide 10/300 GL size exclusion column (GE Healthcare, USA) in a HPLC system (Agilent Technologies 1200 series, Germany) using its fluorescence detector (Excitation = 488 nm, Emission = 530 nm). The flow rate was 0.5 mL/min and mobile phase was 100 mM ammonium acetate at 25 °C. The standards for this experiment were BSA (66 kDa), Lysozyme (14 kDa) and Glutathione (0.307 kDa). The software to process the data was Chromoleon version 6.8 (Thermo Fisher Scientific).

HRM-qPCR confirmation of fermenting strains. To confirm that the fermentations were made by the strains used as inoculum, high resolution melting quantitative PCR (HRM-qPCR) was performed following the conditions described by Lin and Gänzle (2014). Thirty miligrams of freeze-dried sourdough sample were mixed with 2.5 mL of sterile 0.8 % wt/vol NaCl solution. The mixture was centrifuged at 500 gravity for 5 min and supernatant was collected. The supernatant was centrifuged at 5000 gravity for 15 min to harvest the cells. DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, USA). For the PCR, a Rotor-Gene Q (Qiagen, USA) and Type-it HRM PCR Kit (Qiagen, USA) were used. The primers for the Experiment were Universal Primers targeting 16S rRNA (forward, 5'-TCC TAC GGG AGG CAG CAG T-3'; reverse, 5'-GGA CTA CCA GGG TAT CTA ATC CTG TT-3'). The conditions for the HRM-qPCR were denaturation 5 min at 95 °C, then 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s and extension at 72 °C for 10 s. For the final stage, temperature went from 65 °C to 90 °C at 0.1 °C/step with 2 s holding at each step. The software used to process the data was PeakFit Software (Systat Software Inc., USA).

2.5 Sourdough preparation to analyze WGA.

For this experiment 3 types of flour were tested. Two pure wheat cultivars and commercial whole wheat flour. The pure wheat cultivars were: Red Fife and Brennan. Wheat grains from the pure cultivars were ground using a 0.5 mm screen on a Retsch ZM200 ultra centrifugal mill (Retsch, Germany). Pure cultivar flour was stored at -20 °C.

Sourdoughs were prepared as follows: whole wheat flour and sterile tap water including the culture were mixed in a ratio 1:1 using a sterile tongue depressor. Four different sourdoughs were prepared: one per strain (*L. sakei* TMW 1.22*, F. sanfranciscensis* DSM20451, and *F. sanfranciscensis ΔgshR*), and a chemically acidified dough prepared with a mixture of acetic acid

and lactic acid in a 1:4 ratio targeting a final pH of 4. Sourdoughs were incubated at 30 °C for 24 h. Samples were taken at 0 h (right after mixing) and 24 h to determine: pH, cell counts and WGA content. Samples were stored at -80 °C for further analysis. Three independent replicates were prepared per sample to make the results statistically significant. Independent experiments mean that 3 different microbial colonies were taken from the mMRS plate and used to prepare 3 independent sourdoughs; replicates were prepared one per day.

To ensure that the inoculum dominated the fermentation microbiota throughout the process, two strategies were used: backslopping sourdoughs or using a larger initial inoculum. Backslopping was a way to introduce bacteria already adapted to the dough, which would make it more competitive against the bacteria and fungi present in the flour. To backslop a sourdough, a fresh sourdough was prepared as described above. After 24 h, a fraction (in a 10% ratio) of this sourdough was collected and used in a fresh dough. For example, to prepare 10 g of backslopped sourdough, 1 g of sourdough + 4.5 mL of sterile tap water + 4.5 g of flour were mixed. After mixing them, they were incubated for 24 h at 30 °C. In the experiments were this technique was used, 2 backslopping cycles were used.

In the doughs were a larger inoculum was used, 2 or 3 times the initial broth volume were used. Namely, if 5 g of flour were going to be used, then instead of preparing only 5 mL of culture in mMRS, 10 mL (2x) or 15 mL (3x) were prepared, incubated, centrifuged, washed and reconstituted to a final volume of 5 mL. These 5 mL would be mixed with 5 g of flour, which would be the equivalent of a 10 mL or 15 mL culture in 5 g of flour, doubling or tripling the amount of bacteria used initially.

To prepare chemically acidified doughs, each different flour used in the experiments was tested individually for its buffering capacity. In other words, these doughs were prepared by

mixing: whole wheat flour + sterile tap water + 1:4 Acetic acid with lactic acid mix; the amount of acid mix needed was determined based on the quantity required to achieve a final pH of 4. Different flours required different amounts of acid mix to reach that pH.

To check the possible impact of proteases on the integrity of WGA, protease from *Aspergillus* oryzae (Sigma Aldrich, USA) was used in one set of sourdoughs. Fifteen grams of sourdough were mixed with 25 µL of protease solution (protease's activity >= 500 amino peptidase units per gram) and incubated for 24 h at 30 °C.

To determine pH of the sourdoughs, 1 g of sourdough was diluted with 9 mL of sterile deionized water and mixed thoroughly. Then the measurement was taken with the pH meter. To obtain cell counts in the sourdoughs, 1 g of sourdough was diluted with 9 mL of sterile deionized water and mixed thoroughly. Serial dilutions were prepared from this mixture using sterile peptone saline solution (10 g of Peptone + 9 g NaCl per liter of solution). 100 µL of the first dilution were taken and added to 900 µL of sterile peptone saline solution, then mixed using a vortex. This process was repeated 4 or 5 times, as needed. Plating was done using Whitley Automatic Spiral Platter (Don Whitley Scientific, England). Plates were incubated at 30 °C for a period between 24 h and 48 h. As mentioned earlier, *F. sanfranciscensis ΔgshR* was plated on media with erythromycin. The initial target population to inoculate sourdoughs was 10^8 CFU per g of sourdough.

The identity of the bacteria that dominated the fermentation was confirmed by comparing colony morphology from initial cell count plates with the morphology from the plates after fermentation. For the experiment with ATI-FITC conjugate, HRM-qPCR was used as an additional confirmation technique.

2.6 WGA analysis by size exclusion high performance liquid chromatography (SEC-HPLC).

This experiment is similar to the one used to analyze ATIs using protein tagged with FITC. The general purpose was to prepare sourdoughs using WGA tagged with fluorescein isothiocyanate (FITC), and then separate the proteins using a size exclusion column (Superdex, GE, USA) and analyze them with the fluorescence detector installed in the High Performance Liquid Chromatography (HPLC) device (Agilent 1200 Series, Agilent Technologies, USA). This process required several steps which will be explained in the following paragraphs.

Purification of WGA. The purification protocol used was described by Baieli et al, 2012. The chitosan beads used were the CH250. The chitosan used was from Sigma Aldrich, Canada of molecular weight ranging from 190 kDa to 375 kDa. As a summary, 200 g of wheat germ from the local store were agitated for 1.5 h with 600 mL of hexane. Then hexane was removed and another 600 mL of hexane were added and mixed again for 1.5 h. Hexane was removed, and wheat germ was left on the fume hood to ensure that no hexane remained. Afterwards, 1.6 L of 0.05 M HCl were added with stirring for 4 h at room temperature. pH was adjusted to 7, and mix was centrifuged at 1,396 RCF for 15 min. The supernatant was collected and concentrated using a minimate tangential flow filtration (TFF) capsule (Pall, USA) with a molecular weight cut off of 3 kDa. CH250 beads were added to the concentrate and left with stirring overnight at room temperature. Beads were collected and washed with 20 mM Tris-HCl buffer, pH 8.5, with 0.15 M NaCl. After washing, WGA was eluted with 1 M acetic acid with stirring. Buffer was exchanged on eluted sample to PBS (0.01 M Phosphate, 0.15 M NaCl, pH 6.8) using centrifugal filtration (Amicon Ultrafree MC with molecular weight cut off 10 KDa, Milipore, USA). SDS-PAGE was used to confirm identity of the protein extract.

Even though the purification was successful, WGA was purchased from Sigma Aldrich (USA) because of the amount of WGA needed to conduct all the tests.

WGA labeling with FITC. The labeling protocol was based on Sigma-Aldrich FITC labeling protocol and Hermanson, G.T., 2013. A 0.1 M sodium carbonate solution, pH 9, was prepared fresh. WGA was dissolved in 1.5 mL of the carbonate solution (3 different amounts were tested, 2 mg, 20 mg and 15 mg of WGA). Samples were centrifuged to ensure that protein was dissolved. In darkness, FITC was dissolved in 1 mL of dimethyl sulfoxide (DMSO), again, 3 different amounts were tested (1 mg, 10 mg and 1.5 mg of FITC). Slowly, DMSO solution was added to the carbonate solution containing WGA (0.25 mL every 15 min). When all the DMSO was added, solution was left in darkness, at 6 °C, for 8 h with very gentle agitation. Subsequently, NH_4Cl was added to a final concentration of 50 mM to stop the reaction. The mix was left for 2 h in the dark, at 6 °C with gentle agitation. PD-10 Desalting columns (GE Healthcare, USA) were used to collect the WGA-FITC conjugate and remove the rest of the salts. The protein conjugate was protected from light and stored at 4 °C.

Sourdoughs with WGA-FITC conjugate. Sourdoughs were prepared with the addition of WGA-FITC conjugate. The amount of conjugate used was the necessary needed to achieve a final concentration of 250 μg of WGA per gram of sourdough. For example, in the last test, 0.35 mL of WGA-FITC conjugate were mixed with 2.65 mL of sterile tap water containing the bacterial culture and 3 g of whole wheat flour. After that, the procedure was the same as the one described in section 2.5.

Extraction of WGA. The procedure to extract WGA was mostly based on Baieli et al, 2012. As a summary, 1 g of sourdough was taken from -80 °C storage, thawed at room temperature and mixed with 8 mL of 0.05 M HCl. Afterwards, samples were placed on agitation at 300 RPM at 25

°C on an Innova 44/44 R agitator shaker (New Brunswick Scientific, USA) for overnight extraction. pH was adjusted to 7 with NaOH, and samples were centrifuged at 1396 RCF for 15 min. Supernatant was collected and stored at -80 °C. WGA was in the supernatant.

SEC-HPLC. After samples were extracted, they were filtered (13mm Fisherbrand syringe filters, 0.2 μm, Nylon) and placed in HPLC vials. The experiment was conducted using column Superdex 200 Increase (GE Healthcare, USA) with a separation range from 10 kDa to 600 kDa, The HPLC settings were: mobile phase: 0.1 M sodium phosphate, 0.15 M NaCl, 0.05 M N-acetyl glucosamine, pH 7.0; flow rate of 0.5 mL/min; temperature of 25 °C; injection volume of 100 μL; fluorescence excitation wavelength of 488 nm and fluorescence emission wavelength of 530 nm; run time 75 min.

Standards of different molecular size were used to determine elution time based on size. The standards used were: bovine serum albumin (BSA, 67 kDa), β-lactoglobulin (35 kDa), lysozyme (14 kDa), vitamin B12 (1.3 kDa) and glutathione (0.3 kDa). A flour sample without WGA-FITC conjugate was tested too as a blank.

WGA standard was run in three separated conditions, one as mentioned above; the second one diluting it in SDS sample buffer, which has the following formula (4x): 1 mL of tris (1 M, pH 6.8), 0.4 g of SDS, 2 mL of glycerol, 1 mL of β-mercaptoethanol, and 5 mL of deionized-ultra filtered water. After mixing sample with SDS buffer, the mixture was heated to 95 °C for 7 min. The mobile phase used for this run was 0.1 M sodium phosphate, 0.15 M NaCl, 0.1 % SDS, pH 7 and an injection volume of 10 μL; the last settings used for the WGA standard were using a mobile phase of 0.1 M sodium phosphate, 0.15 M NaCl, 0.05 M N-acetyl glucosamine, pH 7.

2.7 WGA determination using antibodies (ELISA).

Extraction of WGA. The procedure to extract WGA was the same as mentioned earlier, except that for the first set of fermentations, hexane was used to remove lipids. Briefly, 1 g of sourdough was taken from -80 °C storage, thawed at room temperature and mixed with 3 mL of hexane. The mixture was placed on agitation at 300 RPM at 25 °C on an Innova 44/44 R agitator shaker (New Brunswick Scientific, USA) for 1.5 h. Samples were centrifuged at 1,396 RCF for 3 min. Hexane was discarded and the procedure was repeated one more time. After removing hexane the second time, samples were placed to dry in the fume hood for 1 h. Then the rest of the process followed the acid extraction described earlier.

In the last ELISA, the steps involving hexane were removed because the possibility of continuous bacterial activity during extraction was a concern.

Quantification of WGA with ELISA. ELISA (Enzyme-linked immunosorbent assay) kits to detect and quantify WGA were purchased from MyBioSource (Cat.No. MBS9311666, California, USA). The tests were performed following the supplier's instructions (MyBioSource, 2020). Briefly, all samples were brought to room temperature naturally (no heating baths or other devices were used). Extracts from sourdoughs were diluted 100 times using deionized water previous to use in ELISA wells. This dilution was based on the expected WGA content on the sourdough based on literature findings because the ELISA kit's detection range is between 0.25 ng WGA/mL to 8 ng WGA/mL. Each sample was tested in two ELISA wells (duplicates). A standard curve was prepared every time an ELISA was performed. A blank was tested every time as well using "sample diluent" including in the kit instead of sample. To start the test, 50 µL of each sample were added to the ELISA wells. Then 100 µL of HRP-conjugate were added to each well. Afterwards, the plate was incubated for 60 minutes at 37 °C. After incubation the wells were washed 4 times with washing solution included in the kit. When washing was finished, 50 µL of

chromogen solution A and 50 µL of chromogen solution B were added to each well. Plate was protected from light and incubated again at 37 °C for 15 min. Then, 50 µL of stop solution were added and optical density was read at 450 nm.

Using the standard curve, an equation was obtained which was used to calculate the WGA concentration of the samples.

Influence of oxidizing and reducing agents on WGA. To determine the possible effect of a reducing vs an oxidative environment on the ELISA detection method, a test was performed without fermenting. For this test, commercial flour and Red Fife flours were used. Three different samples were prepared: 1) 0.5 g flour + 0.5 mL sterile tap water, 2) 0.5 g flour + 0.5 mL of 5 % H_2O_2, and 3) 0.5 g flour + 0.5 mL of 0.01 M glutathione (GSH). After mixing samples were collected and stored at -80 °C to analyze their WGA content with the same methodology mentioned earlier in the section of extraction of WGA.

Preparation of bread. In order to analyze the effect of sourdough fermentation on the WGA content in sourdough bread, Type I sourdough bread was prepared using *F. sanfranciscensis* DSM20451 based on the methodology described on Tang, et al (2017). Ten grams of whole wheat flour were mixed with 10 mL of sterile tap water containing the culture. The mix was fermented for 12 h at 32 °C. After that, 20 g of whole wheat flour and 20 mL of sterile tap water were added and fermented under same conditions for 2.5 h. Then, the rest of the ingredients were added: 70 g of whole wheat flour, 30 mL of sterile tap water, 2 g of salt, 2 g of sugar, and 0.5 g of yeast and mixed until development for 7 min. Dough had 1 h of floor time at 32 °C. Afterwards, 150 g of dough were weighted, rolled and set into a bread pan. Doughs were proofed at 32 °C for 90 min and baked at 177 °C for 15 min. A control bread was also prepared using straight dough process and no lactobacillus strain was added, only baker's yeast. The formula for the control bread was

100 g of flour, 2 g of salt, 2 g of sugar, 0.5 g of yeast and 60 g of sterile tap water. These ingredients were mixed together and processed in the same way as the way as sourdough bread after all ingredients were mixed.

After baking, bread was cooled at room temperature for 1 h. Then, samples were collected, freeze-dried, ground and stored at -80 °C for further analysis using the extraction procedure described in section 2.5 and the ELISA analysis described in section 2.6.

Determination of interference of the bread matrix on WGA quantification by ELISA. After testing the baked product, a possible interference with the antibody detection using ELISAs was detected. Thus, two experiments were performed to determine the possible sources. In the first experiment, one sample was tested twice, undiluted and diluted 100 times. These same two, were spiked with 2 ng of WGA/mL. On the second experiment, one sample was diluted using two different diluents, PBS and sample diluent included in the ELISA.

3 Results.

3.1 Fate of ATI during sourdough fermentation and bread making.

High resolution melting quantitative polymerase chain reaction (HRM-qPCR) of fermentations with ATI-FITC conjugate. HRM-qPCR was used to confirm that the fermentation was made by the same strain as the one used to inoculate the sourdough. Figures 3 and 4 show the melting curves of the sourdoughs used for the ATI experiment versus the pure cultures. The peaks of the pure culture are very close to the sourdough peaks, confirming that the fermentation was performed by the same strain.

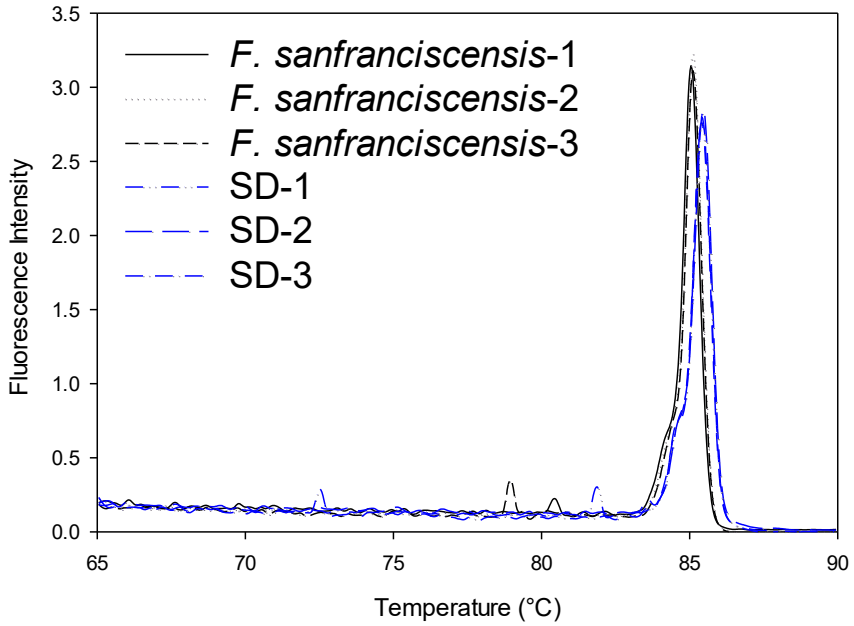

Figure 3. HRM-qPCR curve from 3 replicates of sourdough (SD) fermented with *F. sanfranciscensis* DSM20451 compared with 3 replicates of a pure culture of the same strain.

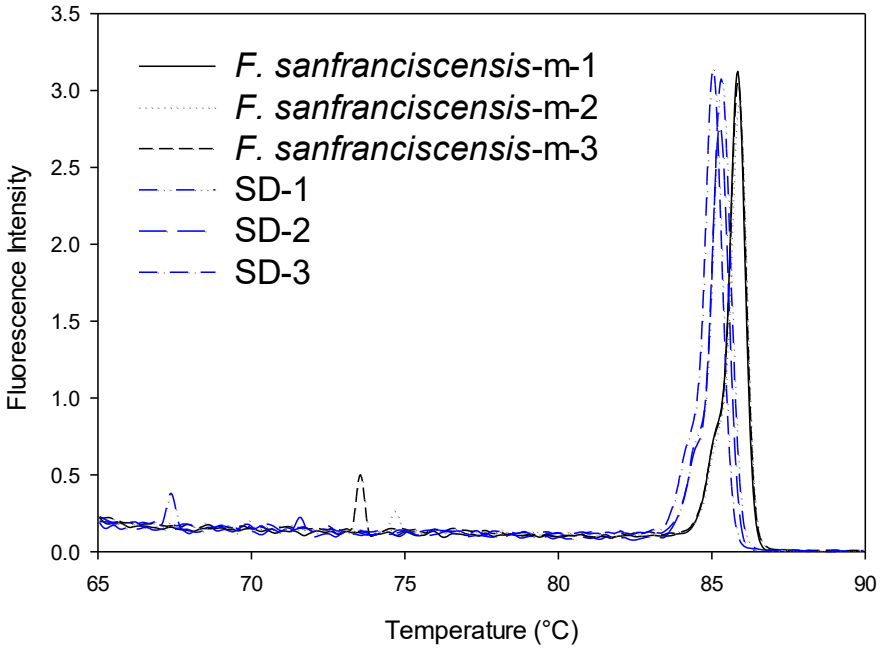

Figure 4. HRM-qPCR curve from 3 replicates of sourdough fermented with *F. sanfranciscensis* DSM20451 *ΔgshR* compared with 3 replicates of a pure culture of the same strain.

Determination of modification of fluorescence labeled ATI during sourdough fermentation and bread making with SEC-HPLC. Table 1 shows cell counts, pH values, and the peak ratio results obtained from the fermentations made using mini doughs inoculated with ATI-FITC conjugate. The data demonstrates that the abundance of the ATI tetramer is lower when added in the sourdough stage in comparison to its addition later, in the bread dough mixing process. This result is reasonable, as more time increases the chances of molecular interaction, and thus degradation. The effect of pH is illustrated when comparing chemically acidified dough at pH 5 with the chemically acidified dough at pH 4> The only change observed with the pH 5 dough occurred during baking, while with the pH 4 dough ATI changes through the whole process. Additionally, the wheat sourdough values are very similar to the values obtained with the chemically acidified pH 4 dough; this backslopped wheat sourdough, with a microbiota already adapted to the dough environment, is able to decrease the pH fast to values where the wheat proteases become active, thus producing similar results as the chemically acidified dough. Values on Table 1 also confirm that there is no difference between *F. sanfranciscensis* DSM20451 and its isogenic counterpart *F. sanfranciscensis* DSM20451 *ΔgshR*. This result suggests that the disruption of disulfide bonds does not influence ATI depolymerization.

Table 1. pH value and bacterial cell count after fermentation in the mini-dough system, and ATI tetramer degradation throughout the bread-making process. The degradation of ATI tetramers is expressed as the ratio of the peak height of the ATI tetramer divided by the peak height of the ATI monomer in size-exclusion chromatograms.

	Bread system	pH	CFU g^{-1} log10	A1	A2	A3	A4	B1	B2	B3
I	*F. sanfranciscensis*	4.01±0.05	8.37±0.05	1	0.62	0.62	0.56	1	1	0.61
II	*F. sanfranciscensis ΔgshR*	4.07±0.04	8.71±0.03	1	0.64	0.63	0.55	1	1	0.63
III	Control, pH 5.0			1	1	1	0.61	1	1	0.60
IV	Control, pH 4.0			1	0.26	0.38	0.42	1	0.63	0.61
V	Wheat sourdough	3.69±0.04	9.57±0.19	1	0.25	0.27	0.36	1	0.64	0.57

Note: For ATI dosed at the sourdough stage, A1: after sourdough mixing (control); A2: after lactobacilli fermentation; A3: after yeast fermentation; A4: after baking; For ATI dosed at bread dough stage, B1: after bread dough mixing; B2: after yeast fermentation; B3: after baking.

Figure 5 shows examples of chromatograms obtained from the ATI-FITC mini doughs. The changes on the main peak show the status of the ATI tetramer through the fermentation. On figure 5A –yeast doughs– no appreciable changes can be seen, only after baking. On figure 5B – chemically acidified pH 5– very similar results as on 5A, no major changes before the baking step. Figure 5C –rye starter wheat sourdough– shows the most significant changes from all samples; the peak shifts to the right, showing a decrease in size of the ATI-FITC conjugate.

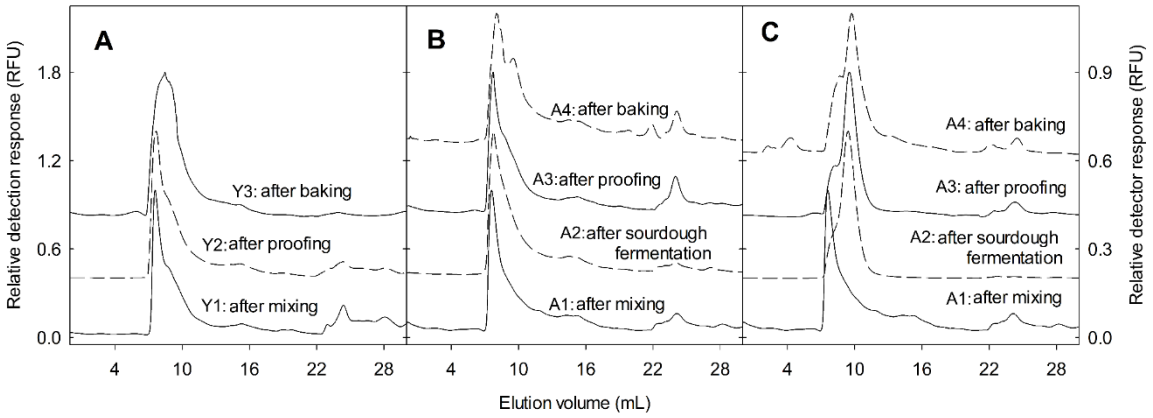

Figure 5. Size-exclusion chromatogram of fluorescein-labelled ATI in bread-making. Panel A. Yeast-fermented bread; Panel B. (III) chemically -acidified dough at pH 5.0; Panel C. (V) Rye starter-wheat sourdough. Fluorescence labeled ATI was added to bread dough (Panel A) or to sourdough (Panels B and C). ATI was extracted after mixing of sourdough, after sourdough fermentation or after mixing of bread dough, after proofing, and after baking. Fluorescence labeled ATI was separated by size exclusion chromatography coupled to a fluorescent detector set at excitation 488 nm and emission 530 nm. Chromatograms were normalized to the highest peak intensity in each chromatogram, and were offset by 0.4 RFU. The elution volume of the molecular marker bovine serum albumin (66 kDa) was 7.25 ml, of lysozyme (14 kDa) was 9.86 ml, and of glutathione (307 Da) was 17.35 ml. The Roman number and the sample letters are same indicated in Table 1.

3.2 Fate of WGA during sourdough fermentation and bread making.

Wheat germ agglutinin (WGA) was also analyzed using SEC-HPLC after fermentation of sourdough with addition of fluorescence-labeled WGA. Table 2 shows the standards used as a reference to know the molecular size of samples on the HPLC chromatograms. WGA is included on the table, even though, on its native form, it does not follow a "normal" SEC profile (very wide peak), most likely because the lectin domain of WGA interacts with the solid phase of the dextran column dextran. Therefore, modifications on the HPLC settings were tested. Figure 6 illustrates three different elution profiles of WGA, the peak that is narrow and elutes fastest is when WGA is denatured; the other two peaks show WGA elution on its undenatured/native form. N-acetyl neuraminic acid was added to block the 8 binding sites present in the WGA dimer, however, no inhibition effect appears on the chromatogram; it is the same elution profile as pure native WGA.

N-acetyl glucosamine was used with the same purpose; nevertheless, this time, instead of using it directly in the sample, it was used on the mobile phase, supplying abundance of WGA's substrate through the whole chromatographic run. N-acetyl glucosamine shifted the elution volume of WGA but did not elute at the volume corresponding to the molecular weight of the standard of the same size (Table 2), possibly because of light interaction with the column still present. The fastest elution of WGA occurred only after the protein has been denatured. Surprisingly, β-mercaptoethanol, SDS and heat did not fully separate the WGA dimer; the peak at 15 mL corresponds to a size of 35 kDa, which is where most of the WGA eluted at; a peak shoulder was observed at 17 mL elution volume, which corresponds to the molecular weight of the monomer of 17 kDa. This result testifies to the high stability attributed to WGA in literature.

Table 2. Standards used during SEC HPLC experiment with column Superdex 200 increase.

Standard	Molecular size (kDa)	Elution volume (mL)
Bovine serum albumin (BSA)	67	13.83
β – Lactoglobulin	35	15.08
Lysozyme	14	18.81
Vitamin B12	1.3	20.5
Glutathione	0.3	19.3
Wheat germ agglutinin (WGA)	35	21.2

Note: Each standard was run alone, so elution times would be determined.

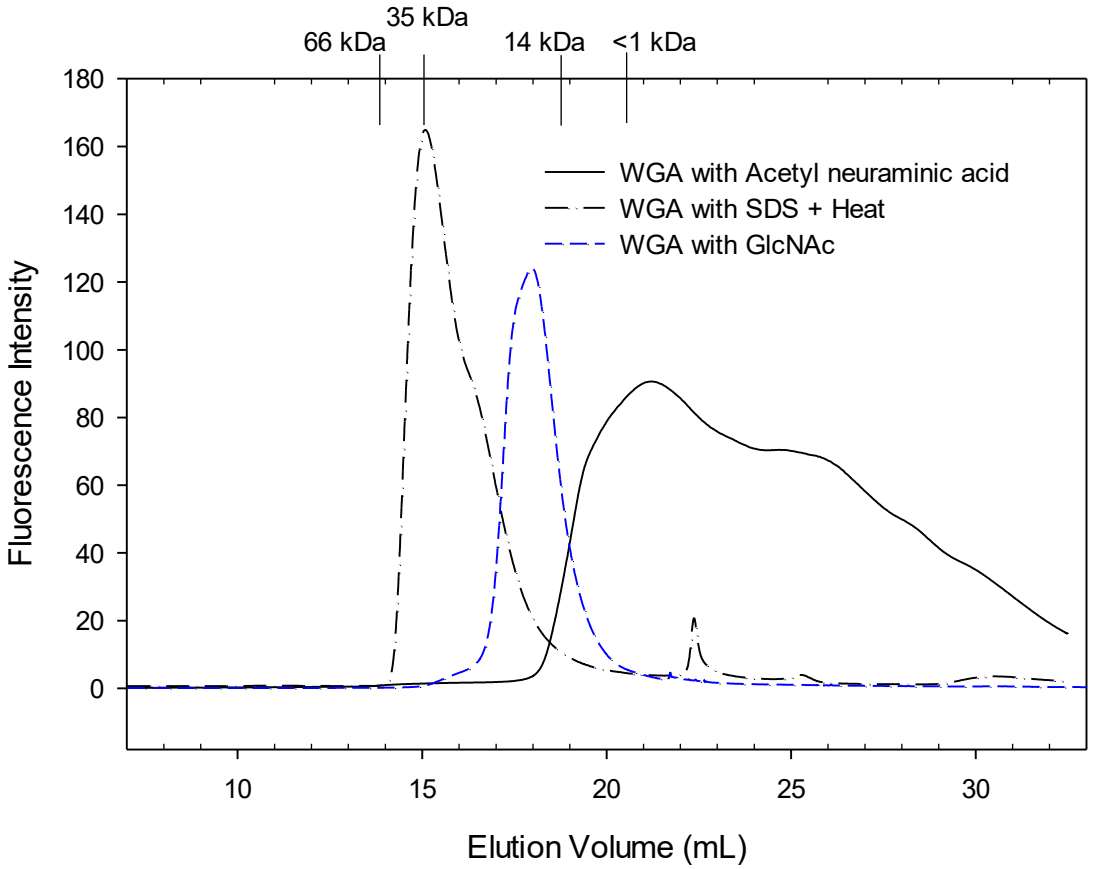

Figure 6. WGA under three different conditions: WGA with N-acetyl neuraminic acid, WGA with N-acetyl glucosamine and WGA after heating with SDS buffer at 95 °C for 7 min. Arrows indicate the molecular weight of standards eluting at that volume. The chromatogram of WGA without any additional chemicals is almost identical to the one shown here with Acetyl neuraminic acid.

Overall, the results obtained with fluorescence labeled WGA show high variability, some samples more than others; this prevents the use of the data quantitatively, thus the data will be used only qualitatively. In the chemically acidified doughs there is not much variation with the 24 h samples, but the 0 h samples lack uniformity. Nevertheless, information can be gathered out of them. The first conclusion is that there are changes in WGA with chemically acidified doughs. The major peak is observed at 18 mL (WGA dimer), but there are peaks after that, which means that the WGA was broken down into monomers and smaller fragments.

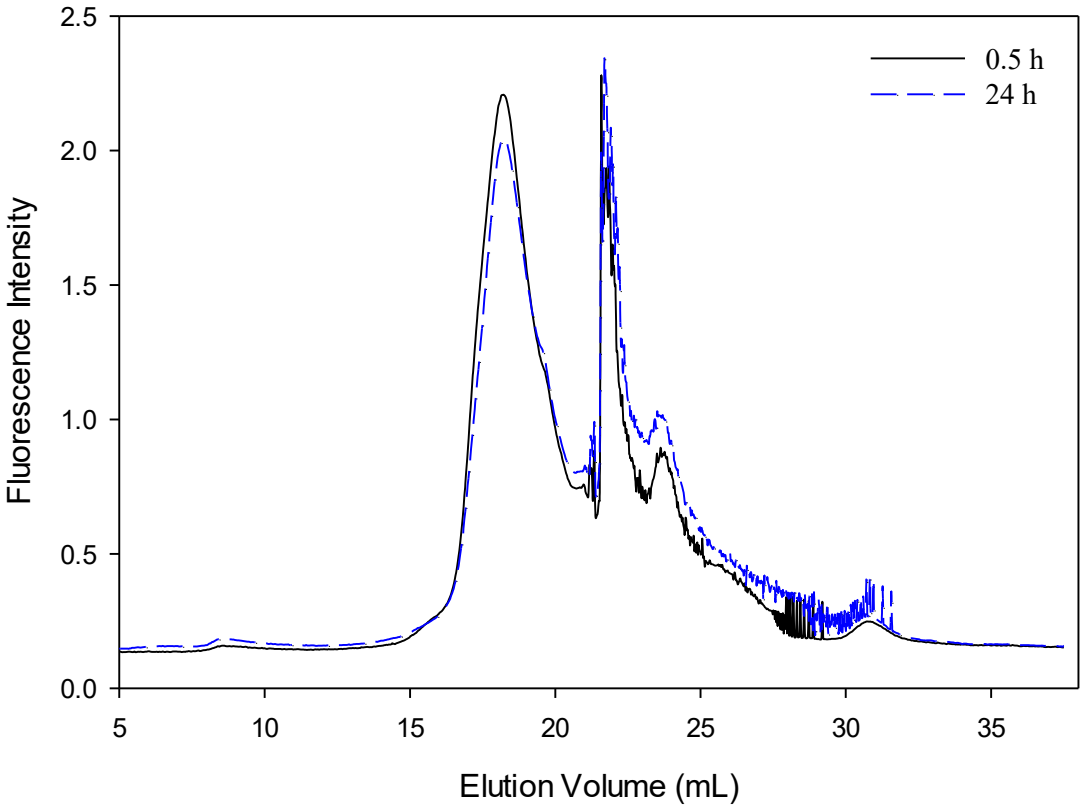

Figure 7. Chromatogram of fluorescence labeled WGA extracted from chemically acidified doughs after 0.5 h and 24 h of fermentation.

Even with the variability on chemically acidified doughs at time 0.5 h, it seems that the fermentation does not change WGA in these doughs (Figure 7).

L. sakei TMW 1.22 appears to reduce the content of the WGA dimer, as there is a wide area after the main peak at 18 mL. All chromatograms from fermentations where LAB participated show a peak at 30 mL (Figure 8).

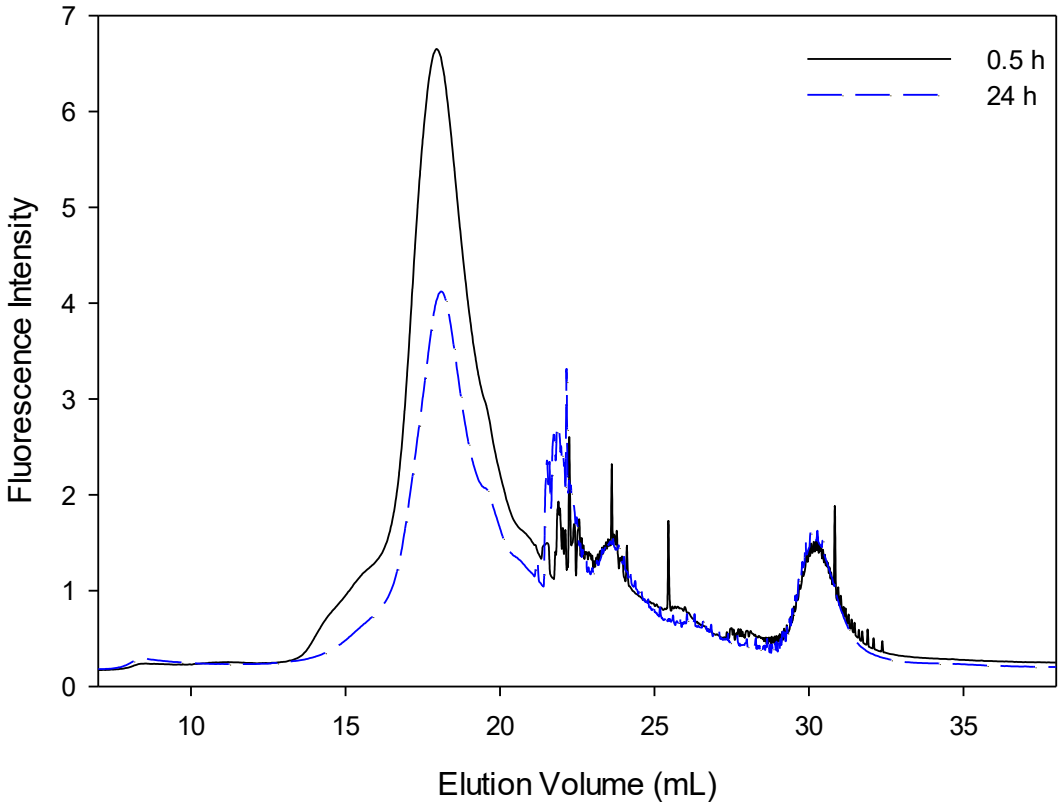

Figure 8. Chromatogram of fluorescence labeled WGA extracted from doughs fermented with *L. sakei* TMW 1.22 after 0.5 h and 24 h of fermentation.

In the chromatograms from doughs fermented with *F. sanfranciscensis* DSM20451 *ΔgshR* the results look very similar when comparing 0.5 h samples vs 24 h samples, both have exactly the same three peaks at the same elution times. Additionally, a large peak at 30 mL can be observed (Figure 9). This peak appears in all samples, except the chemically acidified doughs. At this elution volume, only very low molecular weight molecules should be found (anything after 22 mL should be smaller than 1 kDa).

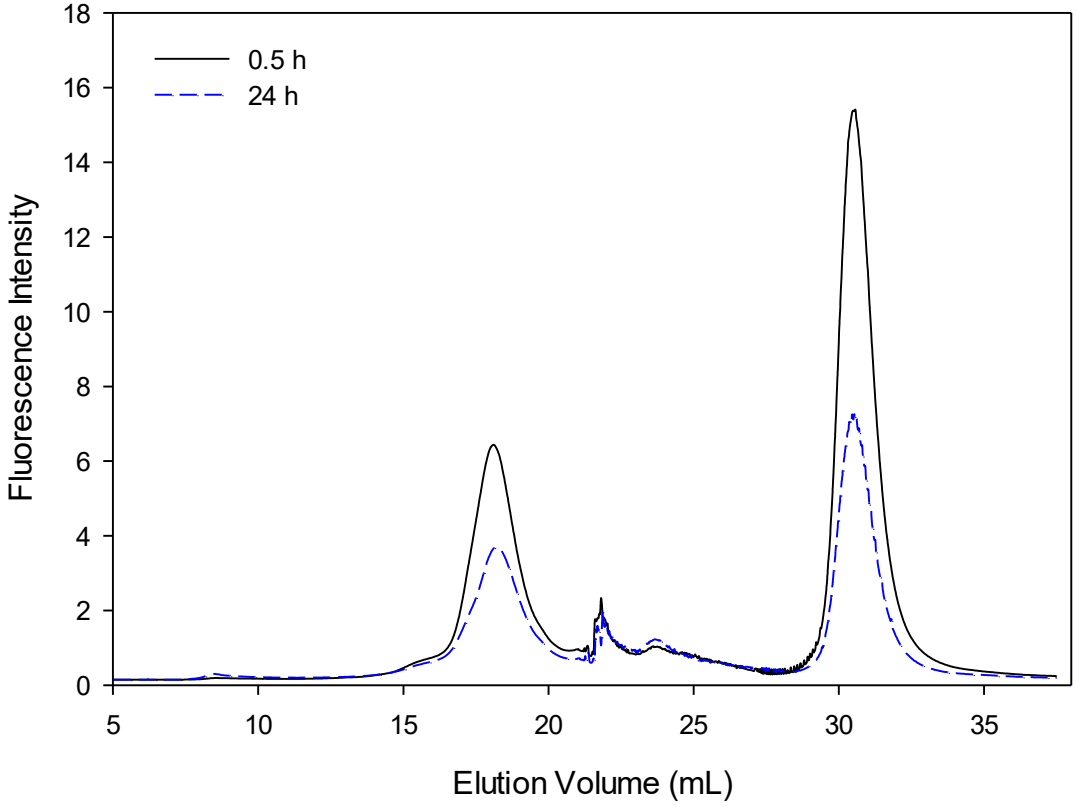

Figure 9. Chromatogram of fluorescence labeled WGA extracted from doughs fermented with *F. sanfranciscensis* DSM20451 Δ*gshR* after 0.5 h and 24 h of fermentation.

The chromatograms from *F. sanfranciscensis* DSM20451 doughs show a large molecular peak at about 8 mL (Figure 10). This could mean that WGA was probably incorporated into the gluten macropolymer. The exact size of this heavy molecule is unknown, however as it elutes right after the void volume of the column its molecular weight is higher than 600 kDa.

Comparing the results between both strains of *F. sanfranciscensis*, *F. sanfranciscensis* DSM20451 has a more complex elution profile, indicating that more changes happen to WGA during fermentation with this strain.

Overall, fermenting with the sourdough strains used on this experiment reduces the content of the WGA dimer; chemically acidified samples show similar trends, but with a less peaks on the chromatogram.

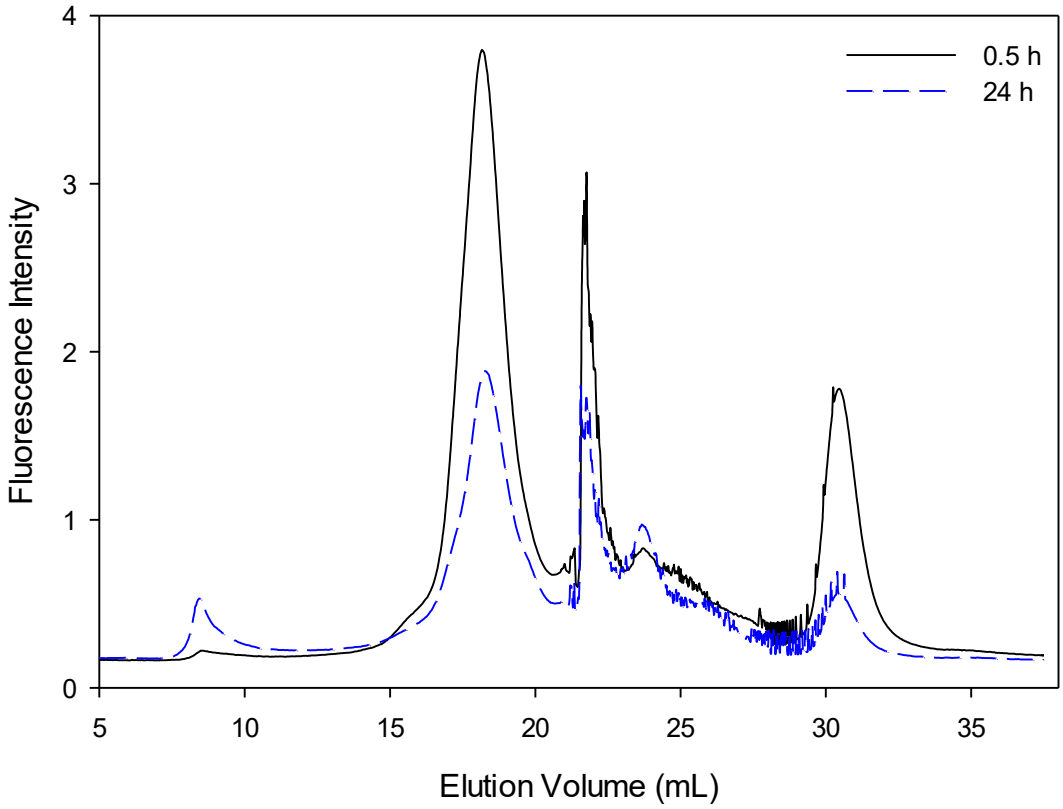

Figure 10. Chromatogram of fluorescence labeled WGA extracted from doughs fermented with *F. sanfranciscensis* DSM20451 after 0.5 h and 24 h of fermentation.

In theory, only one peak should be seen in the 0.5 h samples, the 35 kDa peak. However, this is not the case in any of the 4 different dough tests. This probably means that some of the main factors afecting WGA act very fast. The 0.5 h doughs should be 0 h. However, it took time to prepare the doughs, collect samples and finally freeze them. Perhaps this time, around 30 min, was all it took for the first reactions to occur.

One possible explanation for the variation could be that, after fermentation, once WGA has been subject to proteolysis, it is uncertain if the resulting peptides are soluble in dilute acid. If their

solubility changed, then they probably remain in the precipitate after acid extraction of WGA. Furthermore, if the proteolytic process does not always produce the same peptides, then it is likely that would cause variability. The reason behind this is that not all peptides produced can be detected by the methodology. Only the ones who have lysine bound to FITC will appear on the chromatogram. Any peptide fragment that does not have lysine, or that its lysine is not bound to FITC will remain undetected. Moreover, if the peptides were introduced into the microbial cell, then those are probably not extracted either, due to the biomass remaining in the precipitate.

3.3 WGA quantification using ELISA.

3.31 pH and cell counts on sourdoughs used for WGA quantification with ELISA.

Doughs prepared to analyze WGA with antibodies (ELISA) were monitored to verify microbial growth and acidification by measuring pH and cell counts (Table 3 and Table 4).

Table 3. pH and cell counts of sourdoughs with commercial whole wheat flour (n = 3 biological replicates).

	pH			Cell counts		
Strain	**0.5 h**	**24 h**	**24 h + protease**	**0.5 h**	**24 h**	**24 h + protease**
Lt. sakei TMW 1.22	5.78	4.00	3.99	1.0×10^8	1.1×10^9	1.3×10^9
F. sanfranciscensis DSM20451	5.85	4.14	4.22	7.3×10^7	1.2×10^9	1.2×10^9
F. sanfranciscensis DSM20451 *ΔgshR*	5.75	3.99	4.06	1.7×10^7	1.8×10^9	2.1×10^9
Chemically Acidified	4.04	4.04	4.09	n.d.	n.d.	n.d.

n.d., not determined

Table 4. pH and cell counts on sourdoughs with Red Fife pure wheat cultivar flour (n = 3 biological replicates).

	pH		Cell counts	
Strain	**0.5 h**	**24 h**	**0.5 h**	**24 h**
F. sanfranciscensis DSM20451	6.01	3.84	8.3×10^7	1.1×10^9
Chemically Acidified	4.07	3.93	n.d.	n.d.

n.d., not determined

Every time an ELISA was performed, a set of standards and a blank were run in order to obtain the equation of the regression curve and calculate WGA concentration. Also, each sample was tested in duplicates. The first experiment was conducted using commercial whole wheat flour and backslopped sourdoughs.

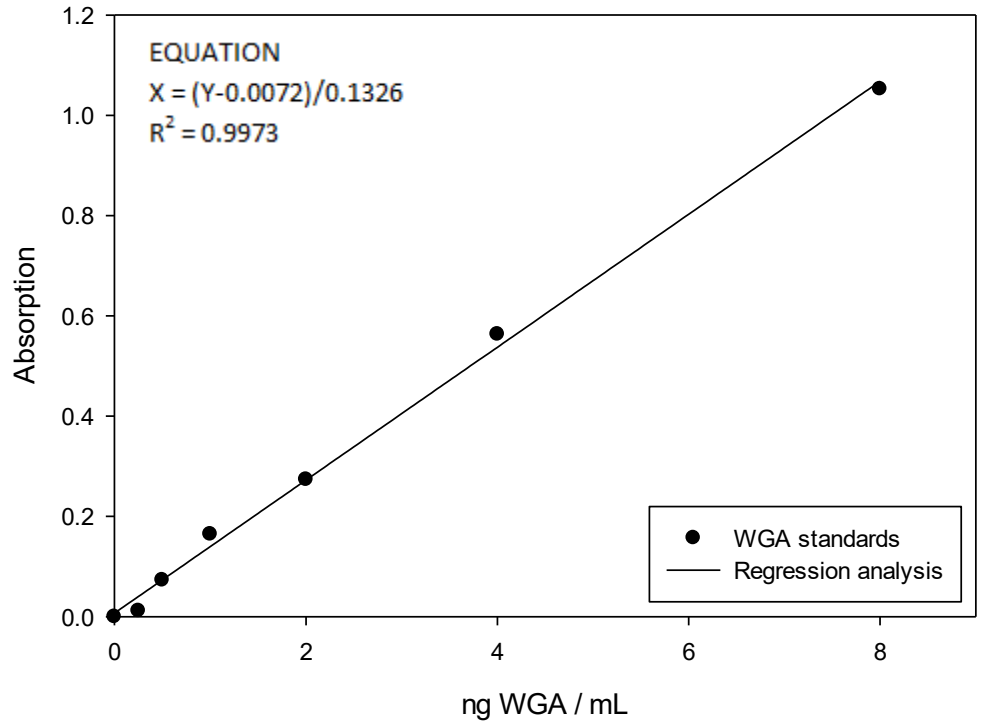

Figure 11. ELISA standard curve used for the samples shown on Figure 12 and Table 3.

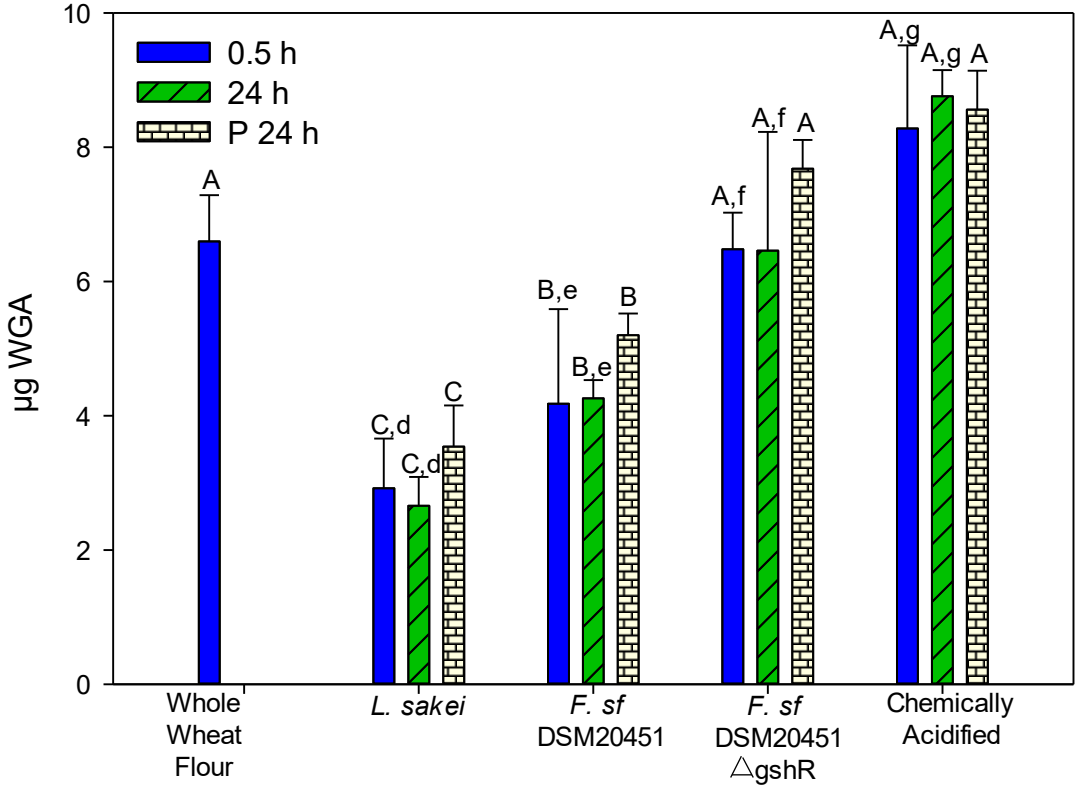

Figure 12. WGA content (µg WGA / g dry matter) of different sourdough samples (n=3 biological replicates) at 0.5 h, 24 h and 24 h with protease from *Aspergillus oryzae* at 30 °C compared to the content in whole wheat flour (control). Data are statistically different unless they share a common superscript ($P < 0.05$).

The strains with the highest reduction in the content of WGA were *F. sanfranciscensis* DSM20451 and *L. sakei* TMW 1.22, indicating a possible correlation between bacteria with the ability to create a reducing environment versus bacteria that lack that capability (Figure 12), since this reduction was not observed with *F. sanfranciscensis* DSM20451 Δ*gshR*. It is remarkable that *L. sakei* TMW 1.22 has the highest reduction, since it does not have the glutathione reductase gene (gshR), thus it has a reduced ability to disturb disulfide bonds that provide stability to proteins compared to strains like *F. sanfranciscensis* DSM20451 that have the gene. The differences

between the two isogenic strains of *F. sanfranciscensis* indicate that glutathione reductase activity affects WGA stability.

Adding protease did not reduce the content of WGA; in fact, the content of WGA in protease treated doughs, with one exception, was higher than their protease negative counterparts. The viscosity of samples with protease was considerably reduced; it was not measured, but it was easily observed that these doughs had a more liquid consistency. A possible explanation is that perhaps protease had enough substrate with the most abundant units of proteins present in the dough (gliadins and glutenins).

The data shown on Figure 12 was analyzed statistically using a two way ANOVA analysis using the Shapiro-Wilk normality test. The first variable analyzed was the different time samples (0.5 h, 24 h and 24 h with protease). On this first part of the analysis, the result showed that the 24 h protease samples are statistically different to the 0.5 h samples. On the second part of the analysis, comparing sample type, chemically acidified samples, and *F. sanfranciscensis* DSM20451 *ΔgshR* are not different than the flour. *L. sakei* and *F. sanfranciscensis* DSM20451 are different from each other and from the flour. The statistical comparison procedure used the Holm-Sidak method.

On the next experiment, hydrogen peroxide and glutathione were added to flour to verify whether oxidizing conditions or reducing conditions affect WGA levels in dough. There was not any difference in the WGA content with these experimental settings (Table 5).

Table 5. Results of Redox test with whole wheat flour from pure wheat cultivar Red Fife

	WGA [c] **µg WGA / g Flour**
Glutathione	3.03 ± 0.03
Hydrogen Peroxide	3.05 ± 0.61
Water	2.97 ± 0.14

Fermentations were also performed using flour from pure wheat cultivar Red Fife for these experiments, the strain *F. sanfranciscensis* DSM20451 also reduced the WGA content compared to the flour control. However, when using Red Fife as substrate, chemically acidified doughs also reduced the WGA content, a result not observed when commercial flour was used (Figure 13).

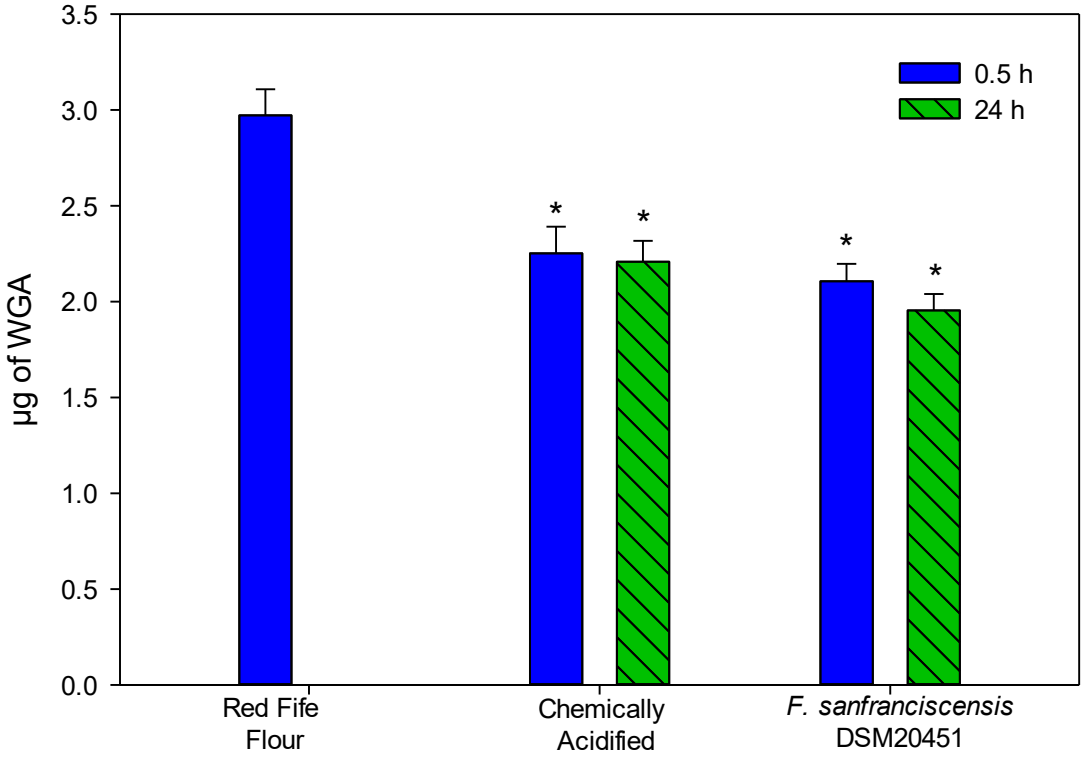

Figure 13. WGA content (µg WGA / g flour) of different sourdough samples with Red Fife wheat pure cultivar (n=3) after 24 h of fermentation at 30 °C compared to the content in flour (control). * Indicates bars that are significantly different to the control ($P < 0.05$)

Table 6. Results of Redox test with commercial whole wheat flour

	WGA [c] µg WGA / g Flour
Glutathione	2.61 ± 0.26
Hydrogen Peroxide	2.65 ± 0.05
Water	2.41 ± 0.03

Commercial flour was also tested with the addition of hydrogen peroxide and glutathione. Results confirmed what was observed using the Red Fife pure cultivar, these conditions did not cause interferences (Table 6).

Sourdough bread was also prepared and tested. Table 7 shows the results of this experiment. The water loss after freeze drying the bread samples was 40.6 % for the sourdough samples, and 39.9 % for the control samples.

Table 7. Results of sourdough baked bread vs straight dough baked bread (using commercial whole wheat flour); n=3 in all samples except on straight dough after proof (only 2).

	WGA [c] **µg WGA / g sample**
Straight dough 0 h (dough)	1.14 ± 0.11
Straight dough after proof (n=2)	1.09 ± 0.02
Straight bread after bake	2.83 ± 0.17
Sourdough bread after bake	0.256 ± 0.014

During this tests, a problem with diluting the WGA extracts was noticed (SD bread was diluted 10 times while control bread was diluted 100 times). In previous experiments, extracts were always diluted 100 fold in two steps, based on the expected WGA content found in literature of 1.25 µg to 5.25 µg WGA / g SD (Nagata & Burger, 1974) to ensure that the sample would be in the range of detection of the ELISA kit. For this experiment, less WGA was expected after baking, so other dilutions were used. However, the results were inconsistent. It was suspicious to get a result 10 times lower in the sourdough bread vs the control. Moreover, the WGA content before baking should be higher than the one present in bread.

Table 8. Comparison of the same sourdough sample with different dilutions using deionized water to identify possible interferences on the ELISA detection method. Two nanograms of WGA per mL taken from the standards included in the ELISA kit were added to the spiked samples.

	WGA [c] ng WGA / mL	**µg WGA / g dry sample**
Straight dough after baking (undiluted)	1.940 ± 0.244	0.0194 ± 0.00244
Straight dough after baking (1/100)	1.311 ± 0.194	1.311 ± 0.194
Straight dough after baking (undiluted – spiked)	0.179 ± 0.115	0.00179 ± 0.00115
Straight dough after baking (1/100 – spiked).	0.501 ± 0.033	0.501 ± 0.033

The results from the diluted and spiked samples were inconsistent (Table 8). None of the spiked samples reflected even the amount added of extra WGA, and the diluted samples did not correlate with the expected content according to the dilution made.

Looking at these results, it was decided that a different diluent was probably needed. Thus, for the next test, PBS and sample diluent were examined (Table 9). The results obtained were not very dependable either; WGA concentrations did not vary according to the dilutions made. The only one that changed properly was the first dilution made with sample diluent.

Table 9. ELISA test using the same sample but different diluents to check possibilities of interference elimination (SD = sourdough).

	WGA [c]	
	ng WGA / mL	**µg WGA / g sample**
SD 0 hr	7.61	0.0761
SD 0 hr with sample diluent (1/10)	< 1	0.07
SD 0 hr with sample diluent (1/100)	< 1	0.86
SD 0 hr with PBS (1/10)	7.61	0.761
SD 0 hr with PBS (1/100)	8.83	8.83

In this assay, the undiluted, purified at the lab, WGA was also tested; the antibodies confirmed the presence of WGA. Nevertheless, the concentration was so high that it saturated the ELISA kit, resulting in a value above detection limit (10.45 ng WGA/mL).

4 Discussion.

In this project, the fate of two different wheat proteins -ATI and WGA- during wheat sourdough fermentation was studied. Protein modifications in sourdough are based on cereal proteases, intracellular microbial peptidases, and disulfide exchange reactions. Both ATI and WGA are highly disulfide linked proteins. The experimental design chosen in this thesis aimed to differentiate between the roles of proteolysis and disulfide exchange reactions.

4.1 **Mechanisms of proteolysis**.

Lactobacilli do not usually have extracellular proteases. Some examples of strains that have extracellular proteinases -referred to as cell envelope proteinases (CEP), cell wall-bound proteinases or lactocepins- are those used as starters for the production of fermented dairy products, including *Lactococcus lactis, Lacticaseibacillus casei, Lactobacillus helveticus,* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Siezen, 1999). Sourdough lactobacilli without extracellular proteases depend on the proteases present in the flour to be supplied with peptides; if proteases are inactivated, their growth is poor and the sourdough does not develop properly, as it was demonstrated by Loponen et al (2007) when they prepared an inactive sourdough-like ferment, and *Levilactobacillus brevis* grew poorly. Zheng et al (2015) analyzed the genome of 174 strains of *Lactobacillus* and *Pediococcus* and found that extracellular proteases were present on strains that grow in milk, but not on strains that utilize different substrates, like cereals (Zheng et al, 2015).

The two strains used in this project, *L. sakei* and *F. sanfranciscensis* do not express extracellular proteases. The genome of *L. sakei* 23K does not encode for extracellular proteinases (Chaillou et al, 2005). It is not the same strain as *L. sakei* TMW 1.22, however, Xu et al (2015) also mentioned that no extracellular protease activity has been reported for *L. sakei* after analyzing the genome of *L. sakei* La22. Vermeulen et al (2005) screened the genome of *F. sanfranciscensis* DSM20451 and did not find genes that code for Prt, the most common extracellular proteinases present in lactobacilli. In addition, they evaluated the proteolytic activity of the strain using casein conjugated with FITC to prepare doughs. In the doughs where the cereal aspartic proteinases were inhibited, *F. sanfranciscensis* was not able to grow. Thus, they concluded that *F. sanfranciscensis* DSM20451 does not have extracellular proteinases (Vermeulen et al, 2005), which was confirmed later by analysis of the genome of the strain (Zheng et al., 2015). Vogel et al (2011) analyzed the genome of *F. sanfranciscensis* TMW 1.1304 and also found no genes coding for extracellular proteases, only intracellular peptidases.

If cells lyse, then intracellular peptidases will be released to the dough. The possible effect of this phenomenon depends on the type of sourdough being used; the longer the fermentation, the more impact that this will have. This process of release of intracellular peptidases by autolysis is desirable in cheese manufacturing (Savijoki et al, 2006) but is likely less important in Type I sourdoughs where fermentation microorganisms are continuously growing and metabolically active.

As bacteria do not contribute to the initial degradation of protein molecules, the proteolytic system from the flour is responsible for protein degradation. Wheat flour contains different kinds of proteases. Whole wheat flour has more proteases than “white” flour, which is derived mainly from the endosperm. One of the proteases in wheat is the serine carboxipeptidase D (*CPW-II*), an

exopeptidase with an optimum pH from 4 to 5.5 that has preference for basic groups and releases C-terminal amino acids. It also hydrolyzes peptides with bulky hydrophobic or aromatic side chains (Remington, 2013). The most important group however, is the aspartic proteinases. Most, if not all, are endopeptidases (Rawlings & Barret, 2013). It has been demonstrated that some associate with gluten during mixing (Kawamura & Yonezawa, 1982; Bleukx & Delcour, 2000). Their optimum pH is acidic, and they are located in different parts of the grain (endosperm and bran). They seem to have specificity to hydrolyze peptide bonds adjacent to arginine, lysine, phenylalanine, leucine, tyrosine and tryptophan as two independent experiments proved (Kawamura & Yonezawa, 1982; Bleukx et al, 1997).

The first step for protein catabolism is the disruption of the quaternary structure of the protein. Both ATI and WGA have a very stable quaternary structure. LAB contribute to that process by decreasing the redox potential of the dough. Jänsch et al (2007) tracked the level of free thiols in the dough through the fermentation. They determined that the levels of free thiols increased when fermenting with *F. sanfranciscensis* DSM20451, while the levels decreased on their chemically acidified dough. Vermeulen et al (2006) discovered a similar phenomenon during fermentation. Free thiol levels increased while fermenting with *F. sanfranciscensis* TMW1.53, but decreased with *L. sakei, Schleiferilactobacillus perolens* and with their chemically acidified control. Xu et al (2018) observed similar results when fermenting with strains that express the glutathione reductase enzyme. Capuani et al (2012) demonstrated that even at the beginning of the fermentation there can be significant differences between the starting redox potential in the dough depending on the strain used. They tested 4 strains and found that each one produced a unique redox value even when fermentation was starting. This finding shows that redox reactions happen really fast. *Weissella cibaria* had the highest reducing capacity, while *Pediococcus pentosaceus*

had the lowest. On their experiment, high redox potential values meant more oxidative conditions, while low redox potential values meant more reducing conditions (Capuani et al, 2012). The redox potential is so closely related to the fermenting strain in a specific matrix that it can help to determine problems during fermentation when deviations occur (Capuani et al, 2013).

Reducing compounds (i.e. glutathione) impact protein degradation (Thiele et al, 2004; Loponen et al, 2008). In most cases, they favor protein degradation because disruption of disulfide bonds causes protein depolymerization and unfolding. In wheat and rye doughs, low redox potential values are needed for proteolysis because the highly disulfide-linked gluten macropolymer is insoluble and thus inaccessible for proteases. In other cereals like buckwheat, the relationship between reducing agents and protein degradation differs (Capuani et al, 2013). One explanation is related to the fact that enzymes are also proteins with or without disulfide bonds; if disulfide bonds are present on the enzyme's structure, then disturbances on them can provoke the loss of the enzyme's structure and consequently the loss of their capability to bind to their substrate and perform their specific function.

Addition of external proteases promotes proteolysis. Malt is a frequent ingredient added to the dough for this purpose (Mäkinen & Arendt, 2012; Bonomi et al, 2014). Fungal proteases are also common, mainly proteases from *Aspergillus oryzae*, i.e. flavourzyme from Novozymes, and *Aspergillus niger* (De Angelis et al, 2010; Rizzello et al, 2007). *A. oryzae* is a major producer of proteases; it has 135 proteinases genes. Most secretory proteinase genes function in acidic pH, and include serine type carboxypeptidase, pepstatin-insensitive proteinase, aspartic proteinase and aorsin (Machida et al, 2005). Rizello et al performed experiments that demonstrated the effectiveness of fungal proteases used in conjunction with LAB to degrade gluten (Rizzello et al, 2014). Calasso et al (2018) also used a combination of LAB and proteases from both *A. niger* and

A. oryzae to produce reduced gluten bread and pasta to test with IBS patients. They found that this could be a promising area of research to offer safe wheat products to them. Loponen et al (2009) executed a similar experiment with rye malt in rye sourdoughs; according to their results, they were able to hydrolyze more than 99.5 % of the prolamin fraction.

4.2 ATI degradation during preparation of sourdough bread.

In this study, only the tetramer ATI was used (fractions CM3 and CM2). These reaffirmed the importance of pH on proteolytic activity in a wheat dough matrix. LAB decreases the pH to values around 4, at these conditions, wheat aspartic proteases are active and degrade proteins. LAB utilizes the peptides released by wheat proteases and uses them to produce amino acids and flavoring compounds. This mechanism has been widely documented in literature (Rollán et al, 2010; Gänzle, 2014; Gobbetti et al, 2014; Thiele et al, 2002; Vermeulen et al, 2005).

Protein aggregation of quaternary structure may additionally impact the biological activity. In many cases, the interactions between monomers change when the pH around changes. In nature, there are many examples of proteins that exist in different aggregation forms at different pHs. WGA is one of them. At pH values above 3.5 it is a homodimer, while below 2.2, it exists as a monomer (Baieli et al, 2012). β-lactoglobulin is another example, between pH 5.2 to 7 it is a dimer, while at values from 3.5 to 5.2 it is an octamer, and above 8 it is a monomer (Kelly et al, 2009). Information about aggregation status and pH for ATI was not available in the literature. ATIs' oligomers are not linked by disulfide bonds; they are non-covalently bound (Oda et al, 1997).

There was no apparent difference between *F. sanfranciscensis* DSM20451 and its isogenic mutant lacking the glutathione reductase gene. Previous research showed that the ability of *F. sanfranciscensis* DSM20451 to regenerate reduced glutathione through the fermentation had an important effect disrupting proteins' disulfide bonds, and thus promoting proteolysis (Loponen et

al, 2008). ATIs' structure has intramolecular disulfide bonds present; thus, it was hypothesized that the mutant would have less impact on the protein's structure due to its inability to regenerate reduced glutathione during fermentation.

ATI degradation by lactobacilli has not been studied widely. Caminero et al (2019) performed an experiment where rats were fed a diet containing ATI and gluten. The study evaluated the ability of lactobacilli to degrade gluten and ATI. They claim that the strains used in their experiment degraded ATIs and gluten, even though the evidence to support this is not clear. They conclude that bacteria helped to reduce ATIs' toxic effect on sensitive mice.

Laatikainen et al (2017 and 2016) studied the impact of sourdough process on FODMAPs and ATI and consequently on patients with IBS and/or NCWS. Their sourdough fermentation time was around 12 h. However, they did not specify which strain was used to perform the fermentation, and they did not prepare a chemically acidified control. The type II sourdough used in this study was fermented with *Limosilactobacillus* species and *Lactobacillus* species (Loponen and Gänzle, personal communication). Laatikainen et al (2016) performed a clinical trial with rye sourdough bread, this study was focused on FODMAPs. On this study the severity of symptoms decreased when patients consumed the low FODMAP bread. However, the first study used two different sourdough breads, one prepared with regular sourdough and a second prepared with a fructan degrading *L. crispatus*, which was not specified on the paper. They attributed the differences to the *Lactobacillus* strain used. Laatikainen et al. (2017) performed another clinical trial using wheat bread and observed that ATI was degraded more intensely from tetramer to monomer on sourdough samples compared to their yeast control. Most of the remaining ATI were of size around 12 kDa. FODMAPs were also lower in sourdough bread. Despite the strong reduction of FODMAPS, sourdough bread was not tolerated better than yeast bread. IBS patients still

manifested adverse effects after sourdough bread consumption. A higher number of subjects, longer treatment time, and a design to prevent the nocebo effect –which means that when patients expect negative results, they experience negative symptoms just because they expect them– could help to obtain more reliable results about the possible differences between sourdough bread and regular yeast bread. Alternatively, improved symptoms after consuming rye but not wheat may indicate that wheat components other than FODMAPs remained active; moreover, rye ATI have not been related to adverse symptoms.

The results of the present study are in accordance with some of the results in the experiments performed by Laatikainen et al (2016 and 2017) and Caminero et al (2019). ATIs are modified by sourdough fermentation; sourdough favors the degradation from tetramer to monomer. The process effects on ATIs vary depending on the experimental settings, like strain used, hours of fermentation, LAB present in the dough or in the host's microbiome. More studies are needed to determine the effect in NCWS or IBS patients.

4.3 WGA and sourdough.

WGA's role in NCWS is less clear, and information on degradation during sourdough is not available. The concentration of WGA in white flour is low, approximately 4 mg per Kg of flour (Catassi et al, 2017), and bread with whole wheat is consumed less than white bread. A report from Agriculture and Agri-Food Canada showing Canadian market bakery trends established that 1,909 products were produced using regular wheat flour compared to only 330 products with whole wheat flour (Agriculture and Agri-Food Canada, 2013). One study relates to WGA's thermal stability. It is important because their anti-nutritive potential depends on their biological activity and on their biochemical characteristics. Matucci et al (2004) used ELISA to screen for presence of WGA in foods; they also studied WGA's thermal stability. They discovered that highly heat

treated foods like pasta had a sharp reduction on active WGA concentrations. Their thermal analysis showed that after 65 °C WGA loses activity considerably; the efficiency of the process also depends on the time the temperature is maintained. Rizzello et al (2010) analyzed the effects of sourdough on nutritional characteristics of wheat germ; however, they did not screen specifically for WGA, assuming that the baking heat treatment would be enough to almost fully eliminate WGA's bioactivity.

On the present study, combining the results from both experimental techniques, SEC-HPLC and ELISA, used to analyze WGA through fermentation, it can be concluded that sourdough reduces the concentration of WGA in whole wheat doughs depending on the strain's ability to reduce the redox potential of the system. The exact mechanism of action is unknown, as *L. sakei* most likely does it with a different system than *F. sanfranciscensis*. This discovery agrees with WGA's highly disulfide cross-linked structure (32 disulfide bonds per dimer). Reviewing the results of other researchers that also used SEC-HPLC to track fluorescent protein conjugates and protein degradation, in most cases, results are also used qualitatively only. Moreover, variability is mentioned, but it is less than what is observed on this experiment (Bleukx et al, 1997; Thiele et al, 2004; Stromeck et al, 2011). Thus, it is probably related to the individual settings of this test.

One possible explanation of this variability between replicates could be the change in solubility during WGA extraction from sourdough. The procedure used was adapted from Baieli et al, a protocol designed to extract WGA from ground wheat germ (Baieli et al, 2012). Starting from a dough is very different than starting from flour. During dough mixing, WGA can interact with all the molecules present and form new complexes (Cauvain, 2015; Grosch & Wieser, 1999). One example, the possibility of removing lipids from flour previous to dough mixing was considered. However, this option was discarded as preparing a dough with no lipids will make it

very difficult for bacterial growth and would probably hinder fermentation. Consequently, using hexane in the dough to remove lipids was not as efficient as using it in the wheat germ flour. Finally, lipid extraction was eliminated from the protocol due to the possibility of bacterial growth during the 3 h that the process would take.

On this analysis, there are differences between the results obtained by *F. sanfranciscensis* DMS20451 and its isogenic strain missing the glutathione reductase gene. This result goes in accordance with WGA's highly disulfide-linked structure. ELISA results point towards a higher degradation by the wild strain. The SEC-HPLC experiment confirms that more complex reactions happen with the wild strain which is consistent with the peaks shown on the chromatograms. The high molecular peak seeing on the chromatogram could be evidence that the disulfide bonds of WGA are reduced, and thus available to complex with cysteine amino acids present in gluten.

5 Conclusion: Significance to NCWS and IBS.

Reviewing the hypothesis and objective stated at the beginning, and looking at the data obtained, it can be concluded that lactobacilli –both homofermentative and heterofermentative– reduce the content of WGA and ATIs in wheat sourdoughs. Table 1 shows how ATI is converted from a tetramer, fractions CM2 and CM3, to monomer; the settings of the experiment determine the degree of disaggregation. For example, longer fermentation –experiment A– increases the amount of tetramer transformed to monomer, compared to shorter fermentation –experiment B. This modification to smaller molecules reduces ATIs' bioactivity. Zevallos et al (2017) determined that fractions 0.19 (dimer) and CM3 (tetramer) had about 5 times higher biological activity than the 0.28 monomers. Another consideration is that ATIs' secondary structure is required to stimulate the TLR4 receptors; reduction of disulfide bonds removes their TLR4 stimulating capacity (Schuppan et al, 2015). On these experiments it is not known if the disulfide bonds were

reduced, however, it is likely that *F. sanfranciscensis* DSM20451 disrupted at least a portion of them. This possibility can be analyzed in future studies. Additionally, ATIs' effects are dose dependent (Schuppan et al, 2015); thus, even partial degradation might have enough impact to reduce immune response in NCWS patients.

In the case of WGA, it is known that its binding capacity is required to maintain its negative biological effects (van Buul & Brouns, 2014; Dalla Pellegrina, 2009). Thus, looking at WGA's thermal stability mentioned in the introduction section, in bread, WGA should present a very low risk, as Rizzello et al (2010) mentioned, the thermal process should inactivate most of WGA's activity. The internal temperature of the dough exceeds 90 °C for more than 10 min –total baking time is frequently above 20 min in average– in a usual bread loaf baking profile (Therdthai et al, 2002). Besides the temperature inside the oven, the cooling time should also be considered, which translates to more time at high internal temperature.

In this project, data shows that WGA concentration is reduced during sourdough fermentation. Part of the protein changes to monomers, and the chromatograms of *F. sanfranciscencis* DMS20451 and its isogenic mutant strain provide evidence that points towards disruption of disulfide bonds. In this state, it is likely that during baking, the protein will not retain any significant activity. Evaluation of baked samples, which was not achieved by experiments described in this thesis, would complement greatly the experimental design shown here.

Putting together all the modifications determined for ATIs and WGA during sourdough fermentation, both protein fractions are likely to be less bioactive to NCWS patients. The next step to support this hypothesis would be to design experiments to test that. It can start with *in vitro* tests using Caco2 cells or immune cells that have TLR4 receptors, and observe how they react when

exposed to sourdough samples vs yeast bread samples. Then after that, if results look promising, evaluate the possibility of performing clinical trials with volunteer subjects.

Besides bioactivity trials, I would have liked to analyze fresh WGA sourdough samples using SEC-HPLC-FLD. The final protocol for this experiment was developed after a couple of runs, thus part of the samples were frozen and thawed a few times before the final chromatographic run. During the first tests, results were more precise, an attribute lost on the last run. Using the data to analyze peak sizes and areas could have provided more helpful information as to the type of modifications happening to the protein. I would also add baked WGA samples to the HPLC experiment. The result on Figure 6 of WGA standard with SDS and heat treatment shows how WGA's elution profile changes considerably due to heat.

As a final note, there is still a lot of work left to prove solidly that wheat sourdough bread can be safely consumed by NCWS patients. At this point though, results look promising. This study, added to the reduction in FODMAPs reported in other experiments (Laatikainen et al, 2016; Loponen & Gänzle, 2018), is a step towards finding alternatives to treat patients other than just following a gluten free diet, which is the solution offered by health care professionals. Perhaps industry will raise to the challenge and improve the quality of gluten free products, but until then, using sourdough might offer another option so people can continue including wheat products on their diet. Sourdough has many uses and benefits, as it was discussed earlier. The more researchers study it and learn about it, the more they discover about new benefits (Gobbetti et al 2019). Fermentation was how ancient cultures used to remove anti-nutrients and improved the quality of the food they consumed (Gänzle, 2020); without fermentation, our society as it is today would not exist. It seems that it is time to return to our origins. Perhaps the answer was found thousands of years ago; we just did not notice.

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