DISSERTATION

MOLECULAR AND SOCIOCULTURAL EXPLORATION OF SOURDOUGH: IMPACTS ON GLUTEN SENSITIVITY AND BREAD CHARACTERISTICS

Submitted by

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ABSTRACT

MOLECULAR AND SOCIOCULTURAL EXPLORATION OF SOURDOUGH: IMPACTS ON GLUTEN SENSITIVITY AND BREAD CHARACTERISTICS

Sourdough is a bread product fermented by communities of wild bacteria and fungi known as a starter culture. Previous work has examined the effects of specific starter organisms on bread quality, but the relationships between whole microbiomes and dough/bread physicochemical properties are currently unknown. The objective of this study was to investigate the relationship between physicochemical properties of sourdough breads and the microbiomes of their starter cultures. Twenty sourdough starters with characterized microbiomes were used to produce wheat-based dough and bread. The chemical properties (pH, titratable acidity, free amino acids, Aw) of dough and physical properties (loaf volume, crust color, texture) of the breads were compared to a control fermented with baker's yeast. The degradation and toxicity of gliadin resulting from fermentation with the sourdough samples was also studied *in vitro*. Results indicate that sourdough-fermented breads produced under real-world conditions are distinct from yeast-fermented bread in terms of physicochemical parameters and proteolysis, which may exert downstream effects on the inflammatory capacity of gluten. We also investigated the beliefs and behaviors of gluten-sensitive sourdough consumers and professional sourdough bakers. We found that commercial sourdough is not reported to relieve gluten-mediated symptoms for consumers diagnosed with celiac disease (CD) or non-celiac gluten sensitivity (NCGS), but undiagnosed (UD) gluten-sensitive consumers may benefit from it. We also determined that sourdough bakers act as brokers of health advice in the gluten-sensitive community.

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DEDICATION

This work is dedicated to Tom Stout, who gave me a well-deserved C and also some important words of advice in junior year AP Chemistry, and who has been my friend ever since. Thank you for 22 years of inspiration.

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1.1 Summary

Sourdough bread is a traditional fermented food that, in recent years, has gained popularity as a potential processing strategy to produce bread for individuals with gluten sensitivity. Gluten, a protein found in wheat, barley and rye can cause digestive and systemic symptoms in individuals with celiac disease (CD) or non-celiac gluten sensitivity (NCGS). Some studies have shown that fermentation with sourdough-resident microorganisms can reduce the levels of gluten in bread and can degrade immunogenic gluten epitopes, making wheat bread a more tolerable option for individuals with gluten sensitivities. Additionally, the fermentation process can degrade other wheat proteins to improve bread digestibility and potentially alleviate symptoms of gluten sensitivity. The potential benefits of sourdough fermentation to mitigate gluten toxicity in CD and NCGS have been demonstrated through in vitro and ex vivo trials, with limited supporting clinical data. Further research is needed to fully understand the potential benefits of sourdough for individuals with gluten sensitivities, but it holds promise as an option to alleviate symptoms in this population.

1.2 Introduction and Scope

The umbrella of gluten-related disorders, including celiac disease (CD) and non-celiac gluten sensitivity (NCGS) cause pain and discomfort to sufferers upon consumption of the wheat gluten protein, for which a gluten-free diet is the only known treatment.[1] One dietary option that has been explored to alleviate symptoms of gluten sensitivity is fermentation of wheat dough by sourdough organisms. Sourdough bread is an ancient food made by the fermentation of dough

with wild Lactobacilaceae and yeast. Sourdough bread is leavened with "starter cultures", or communities of naturally occurring bacteria and yeast which are maintained, portioned, and shared among bakers. These communities comprise what is known collectively as the 'the microbiome', which is responsible for the fermentation of carbohydrates in flour to produce the carbon dioxide that causes bread dough to rise. Microbial communities also produce acids and enzymes that affect bread flavor, texture, and shelf-life. In the United States, sourdough bread and other fermented foods and beverages account for a growing market category. Interest in sourdough bread among amateur bakers increased significantly over the course of the COVID-19 pandemic, with "sourdough bread" being the most searched recipe on Google in 2020.[2] Consumers perceive sourdough bread as a high-value, innovative functional food, and are willing to pay up to 3 times more for artisanal sourdough breads compared to commercial breads.[3] Sourdough bread also appeals to consumers due to its "clean" label (i.e., fewer and simpler ingredients) and perceived health benefits, especially with regard to gastrointestinal health.[4]

Sourdough fermentation has been explored for its potential abilities to "detoxify" gluten in bread products through microbially-driven proteolytic activity to create products that are safe for individuals with CD and other gluten intolerances. CD and NCGS affect approximately 7% of the global population and, at present, can only be managed by adherence to a gluten-free diet.[5] Certain strains of lactic acid bacteria (LAB) isolated from sourdough starters have been shown to degrade immunogenic peptides from wheat and rye,[6–10] with carefully curated LAB cultures able to reduce gluten content of wheat flour below 20 ppm, which is the legal limit for labeling a food as "gluten-free".[11] However, the success of sourdough fermentation as a strategy to detoxify gluten appears to depend heavily on study design, with variation in outcomes between studies with different designs for fermentation (i.e., pre-fermented flour vs. a typical sourdough

fermentation), different fermentation conditions (time, temperature, dough yield) and different models of disease (i.e., *in vitro* testing vs. clinical trials).

The inconclusive findings of these studies limit the potential for this strategy of gluten detoxification to be implemented or relied on by food producers and consumers alike. Despite this, there are indications that sourdough has already gained some acceptance as a potential therapy for gluten sensitivity at the consumer level. It is a common theme on popular consumer blogs,[12] culinary websites[13] and even in academic articles.[14,15]

An additional layer of difficulty with studies investigating sourdough fermentation as a method to reduce the toxicity of gluten-containing foods is that most are carried out using fermentation and processing parameters inconsistent with those likely to produce high-quality bread. The literature has recorded a link between sourdough fermentation and a number of organoleptic, rheological, textural, nutritional, and preservation-related benefits in the resulting bread. However, these studies are rarely conducted using the same conditions as those focusing on sourdough fermentation as a therapeutic tool, so it remains unclear whether sourdough fermentation is a technology that can be used to make desirable, high-quality bread that also offers therapeutic benefit for individuals with gluten sensitivities.

The objective of this review is to analyze current literature focused on detoxification of gluten via sourdough fermentation based on study design and disease model and to identify areas which require further studies before sourdough fermentation can be considered and implemented as a commercial strategy for producing breads and other fermented baked goods with reduced gluten immunogenicity. To conduct this review, literature databases were searched with a range of keywords and phrases relating to bread: “sourdough”, “baking technology”, “gluten”, “specific gluten sensitivities”, “therapies for gluten sensitivity”, and the intersection of these

concepts. Additional relevant articles were identified from references or citations in papers resulting from these searches. Where possible, articles published in the past 10 years were favored, but older, foundational publications were also included when necessary. Also included are limited references to popular media and news items when culturally or empirically applicable.

1.3 Bread Technology

1.3.1 *History*

Humans have been making bread for 20,000 years.[16] Cereal grains are among the oldest domesticated plants and wheat (*Triticum aestivum*), in particular, likely co-evolved alongside humans.[17] Bread wheat was developed at the dawn of agriculture from emmer and einkorn wheat grasses[18] near the Caspian sea, where it remained for several thousand years, gradually speciating. From there it spread across Europe and Asia approximately 7,000 years ago.[19] Bread production was a possible motivator for human Neolithic settlement, and even today phrases such as "to break bread" evoke a strong sense of community.[16] Consider the prodigious use of bread as religious symbolism and its many representations as metaphor in art.[15] Bread is a key element in major cultural traditions, such that the tradition of bread-making (specifically, baguettes) was recently added to UNESCO's list of Intangible Cultural Heritage.[20]

Historically, the concept of "bread" and all its corresponding social implications is widely understood to correspond with leavened loaves.[16] For most of human history, sourdough was the only method of bread leavening. It was likely first developed in Egypt, where the terms for *bread* and *ferment* are both associated with the Arabic word meaning *life*.[21] Although sourdough was the original bread leavening technique, baker's yeast (*Saccharomyces cerevisiae)* became available as a leavening method in the 1600s[22] and achieved industrial scale use in the

19th century, when it nearly replaced sourdough due to its rapid fermentation time and predictable outcomes.[23] Recently, sourdough has recaptured consumer interest, rising to Google's most searched recipe of 2020.[2] Consumers are also motivated by health considerations towards sourdough, which they perceive to be healthier than baker's yeast-leavened bread options.[24]

Bread is considered the oldest man-made staple food in the human diet and has been the most regularly consumed food worldwide for several thousand years.[25] Still today, bread accounts for approximately 20% of dietary calories and protein globally.[18] The importance of bread as a food staple is expected only to increase in the future, as growing over-reliance on meat leads to likely food shortages, causing population-dense areas to lean heavily on plant food staples for calories and nutrients.[26] Increasing the accessibility of these nutrients and the digestibility of cereal-based staples for all members of the growing population is an important sustainability goal as well as a human health concern.

1.3.2 Bread Production

The objective of breadmaking is to convert cereal flour into an appetizing and aerated (leavened) product. To achieve this, the steps of breadmaking are roughly similar across recipes and bread types, although the specific process varies from baker to baker.[27] First, ingredients (usually flour, water, salt, and a leavening agent such as baker's yeast) are mixed together and then energy is applied in the form of kneading to develop a network of gluten proteins. Next, dough undergoes a bulk fermentation process, in which gases are produced and retained by the dough mass.[28] Leavening is achieved in this step when CO_2 generated by yeast becomes trapped by a network of gluten protein.[29] After bulk fermentation, the dough is divided, and each piece is shaped. After shaping, the dough undergoes a "proofing" step in which it is leavened a second

time, this time in its final shape to redistribute CO_2 gas bubbles and replace some of the gases that were pushed out during shaping. After proofing, the dough is baked.[27]

A bread product leavened by a wild, synergistic consortium of yeast and bacteria is known as "sourdough". Sourdough organisms ferment flour carbohydrates to CO_2 gas and other byproducts. The mixture hosting the organisms is known as a *starter* and is combined with additional water and flour to make dough, with the starter acting as a leavening agent.[30] Four types of sourdough are discussed the literature. Type I sourdough is used by commercial or artisanal bakeries, and it involves maintaining a wild sourdough culture using a backslopping process. The term backslopping refers to adding a small amount of active ferment to new substrate (flour and water) to contribute microbes for the next stage of the fermentation process.[31] In Type II sourdough, fermentation is achieved through inoculation with a defined sourdough starter culture rather than through a wild starter maintained through backslopping. Type II cultures are liquid and therefore pumpable for use in an industrial bakery. Type III sourdoughs are also intended for industrial use; like Type II sourdoughs, Type III uses defined cultures, but instead of remaining liquid, Type III sourdoughs are dehydrated for convenience, stability, and consistency. While drying techniques vary, spray-drying and drum-drying are common. Type III sourdoughs allow for easy introduction of sourdough flavor to an industrial breadmaking process.[32] Type IV sourdoughs combine techniques of Type I and II, in that they are begun with a defined starter culture and then subsequently propagated through backslopping. These doughs are most often produced for laboratory studies,[30] but are also used by some artisanal bakeries.[33] One important distinction among sourdough types is that Type I sourdoughs are leavened only through the action of the microbes present in the sourdough starter, while Type II and III sourdoughs require the addition of baker's yeast for leavening, while the sourdough is

used as a flavor enhancer or to improve the properties of the dough, but not as a leavening agent.[32] Type IV sourdough mixtures vary in their requirements for the addition of baker's yeast depending on how they are being used.

When an artisanal or home baker wishes to bake leavened bread using a starter, this process typically involves a Type I sourdough prepared from a starter that has been maintained through continuous backslopping. Sourdough starters may be propagated in this way through feeding on a regular schedule for years or even generations.[22,34] The ideal concentration of starter used as inoculum to the new ferment is 10-40% per feeding cycle. Above 40% addition will over-acidify the starter and favor the over-growth of yeasts, while too little inoculum favors over-growth of LAB (especially *Lactobacillus sanfranciscensis*) which grow rapidly and outcompete yeast symbiotes.[30] In feeding a starter, the baker adds flour, water, and occasionally other ingredients.[35] In doing so, the baker contributes an additional microbial load such that over time the strains that are best adapted to the locally specific ecosystem emerge in the starter.[31]

1.3.3 Consumer Perception of Sourdough vs. Baker's Yeast-Fermented Bread

Despite first losing ground to yeast-leavened bread in the 19th century the 19th century due to the convenience factor of packaged baker's yeast,[23] sourdough has regained popularity in recent decades. Many consumers find that sourdough exhibits favorable organoleptic qualities,[36] and indeed they are willing to pay more for a sourdough product.[3] Some work has shown that consumers are sensitive to the acidity in wheat sourdough products, with many consumers preferring a milder sourness;[37] even small differences in sourdough pH are detectable by taste.[38] Part of consumer attraction to sourdough is not its acidity but its overall flavor complexity; it has been shown to contain more volatile flavor-active compounds than standard bread.[35]

Sourdough is also often perceived as a healthier alternative to yeast-leavened bread.[24] In

addition to its proven benefits, such as improved glycemic response and mineral bioavailability,[15] some consumer-produced media such as blogs and social media espouse less definitive claims, such as that sourdough alleviates symptoms of gluten sensitivity, a theme that is reflected even in some academic articles.[14,15] A 2017 survey of 1134 consumers showed that most associated sourdough bread with being healthy. For this belief they ascribed rational such as such as sourdough's fiber content, satiation, glycemic properties, and being "good for the stomach."[24]

Finally, some consumers associate sourdough with clean-label status.[23] As a trend towards "natural" and "clean-label" food sweeps the industry, consumers increasingly show interest in foods free of so-called chemical preservatives such as calcium propionate, mono- and diglycerides, and potassium sorbate which are often added to bread to inhibit microbial spoilage.[39] Sourdough fermentation can delay bread spoilage without the need for preservatives (or at lower concentrations) due to the production of antimicrobial or antifungal compounds[40,41,42] and organic acids[43] associated with extension of shelf-life,[41,42,44,45] making sourdough an attractive alternative to commercial breads for label-conscious consumers.

1.3.4 Sourdough Processing Parameters and Quality

A number of processing decisions can affect the quality outcomes bread leavened with sourdough starter. This is partially due to an interaction between selection pressures in the environment of the sourdough starter, which favor certain organisms rather than others (for example, heterofermentative LAB vs. homofermentative LAB, or exopolysaccharide-producing strains vs. non-productive strains). Similarly, the conditions used for bulk dough fermentation during bread production further affect the growth, performance, and enzymatic activity of those

organisms.[28]

First, exogenous factors affecting starter propagation and management can affect bread quality. Feeding frequency and holding temperature have been found to change the dominant organisms present in a sourdough starter.[46] Dough yield (the ratio of water to flour) of the starter has been shown to influence its aroma and texture properties, mostly by affecting the molar ratio of lactic to acetic acid known as Fermentation Quotient (FQ).[28] The percentage of backslopping inoculum can also affect the starter microbiome. As inoculum percentage increases, pH of the mixture decreases, affecting organism growth rates and creating pH-mediated selection pressures on the community structure.[47] The flour and water used to mix the starter are also important contributors to ultimate bread quality, since they are both a supply of nutrients, potential contaminants, and sources of organisms that could shift the ecology of the starter over time.[47,48]

Similarly, processing decisions made during sourdough bread production, especially the bulk fermentation step, can affect bread quality. Significant decisions a sourdough baker can make in terms of quality outcomes include how much starter inoculum to include in the dough,[21] feeding frequency,[36] fermentation time,[49] and fermentation temperature.[32] Other processing steps (such as kneading time or technique) may also influence quality, but are related to bread technology generally and are not unique to sourdough.[27]

In sourdough baking, a portion of starter is mixed with flour, water, and salt to produce dough. The starter confers moisture as well as microorganisms that leaven and acidify the dough and release enzymes that produce other downstream effects, including but not limited to texture, shelf-life, flavor, aroma, and nutrient availability.[50] The percentage of starter used in the dough recipe varies widely between bakers, with one large-scale review finding values from 5% to 50% starter inclusion in dough formulas.[47] A maximum value of 20% starter in the formula is

typical.[28] Starter incorporation percentage can affect properties of the resulting bread. For example, a starter inoculum of 20% (as opposed to 40%) resulted in crisper crust, softer crumb, larger bubble size, and lower perceived chewiness. The 20% inoculum sourdough also resulted in improved shelf-life properties compared to both 40% sourdough and baker's yeast-fermented bread over 72 hours of storage.[51] Another study showed that 20% sourdough inoculum improved volume compared to higher inoculum concentrations, but also resulted in lighter crust color.[52]

Depending on desired outcomes, bread formula, and equipment, bulk fermentation temperature vary from approximately room temperature to around 40 °C, with a median temperature of 30 °C. Fermentation temperature has an impact on fermentation rate, acidification rate, and bread flavor, aroma, and texture properties.[30] Higher temperatures (above 30 °C) stimulate LAB and are therefore favorable for gluten breakdown, acidification, and flavor formation, while temperatures below 30 °C favor yeast and may result in less acidification and distinct dough rheology from higher temperature ferments.[28,47] Lower temperature ferments also lead to greater phytase activity due to the optimal temperature of the phytase enzyme.[53] This may have potential implications for mineral bioavailability due to liberation of cations from sequestration by phytic acid.

Another important factor is dough fermentation time, which can vary widely. One review of studies covering Italian sourdoughs found a range of 8 hours to 48 hours of fermentation time, with temperatures ranging from 20-46 °C.[54] Longer fermentation time has been shown to decrease dough elasticity[55] and improve overall rheology of the dough up to a certain point determined by the intersection of fermentation time and temperature.[56] One study found that sourdough fermentation at both 25°C and 35°C improved texture properties compared to non-sourdough bread for the first several hours, and that at 25°C this improvement continued with up

to 24 hours of fermentation time. Fermentation time decreases dough pH and allows for an increase in microbial populations.[49] All of these are factors that ultimately impact downstream bread quality outcomes such as flavor, aroma, texture, rise volume, and nutrient availability.

Because sourdough is a complex system governed by multiple interactions, it is challenging to isolate the impact of each processing parameter individually. In reviews and meta-analyses tracking one or more of the aforementioned starter or dough fermentation conditions, it is difficult to draw conclusions because each parameter varies independently. For example, as discussed above, temperature is known to exert strong selection pressures on the sourdough microbiome as well as influencing the growth rate, enzyme activity, and metabolite production of all microbes present. This influence can be exerted through multiple steps of the bread production process, including the holding temperature of the starter, whether or not the starter is subjected to occasional cold storage, the feeding temperature of the starter, the temperature of the water added to the starter, the dough fermentation temperature, and the proofing temperature before baking. Causal interactions become even more difficult to track when multiple parameters are considered. For example, interactions between dough yield and temperature have been shown to influence sourdough aroma, and interactions between temperature and water content have been shown to impact yeast growth.[28] However, it is impossible to imagine that any factor affecting yeast growth does not also influence aroma, so it is clear that separating pairs of interactions neatly with a link to individual outcomes provides a limited picture of the complex network of interactions in a sourdough system.

Overall, existing data demonstrate that microbiome ecology and performance, and the downstream effects on bread quality, are influenced by various combinations of these technological parameters. However, further work is needed to fully understand these complex

systems and the relationships that exist between individual processing parameters and quality- and health-related outcomes.

1.3.5 Sourdough Microbiome Roles and Interactions

Sourdough is created when wheat and/or rye flour is mixed with water and fermented spontaneously by a consortium of lactic acid bacteria (LAB) and yeasts, which influence the resulting properties of the baked goods. The dough generally contains 10^7 to 10^9 cfu bacteria per gram and 10^5 to 10^7 cfu yeast per g.[28] While sourdough starters were previously thought to consist only of yeast and LAB, many of them also contain acetic acid bacteria (AAB), which slow the fermentation and contribute a "vinegar" aroma when present.[34] The specific organisms in a given starter vary considerably due to factors like geographic location,[57] starting ingredients, and baker's skin flora[58] although these abiotic factors account for just a fraction of overall microbial diversity.[34] Each sourdough starter's microbiome is a product of its unique environment and processing conditions.[58] LAB species typically found in sourdough include *Lactobacillus sanfranciscensis*, *Lactobacillus pontis* , *Lactobacillus brevis* , *Lactobacillus fermentum* , *Lactobacillus fructivorans*, while common yeasts include *Candida milleri* , *Candida holmii*, *Saccharomyces cerevisiae,* and *Saccharomyces exiguus*.[59] Some pairs of organisms are commonly co-occurring due to traits that make ideal synergistic pairings, such as *L. sanfranciscensis* and *Candida humilis;* while *C. humilis* is maltose-negative, *L. sanfranciscensis* consumes maltose preferentially through expression of the enzyme maltose phosphorylase, which splits the maltose molecule into units of glucose and glucose-1-P. The glucose-1-P can be metabolized without further use of ATP by *L. sanfranciscensis*. Meanwhile, the excess glucose is available for consumption by other organisms in the system, including *C. humilis,* which in turn

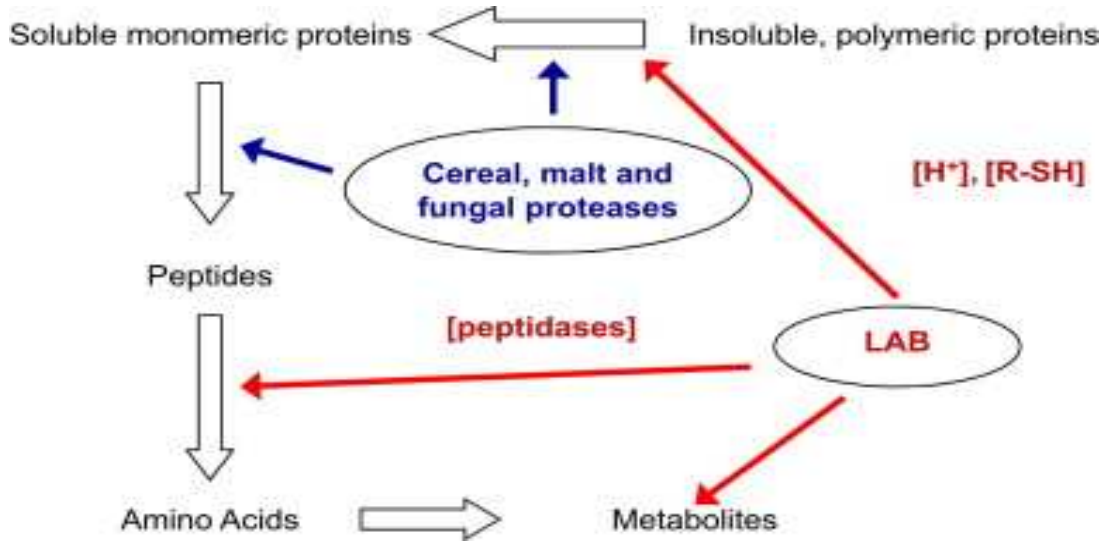

Figure 1.1: Synergistic interaction of yeast and LAB in sourdough. From Gänzle (2014).

liberates amino acids for bacterial metabolism.[60]

Regardless of the specific microbes present, a sourdough starter is characterized by the symbiosis of one or more yeast species and one or more LAB.[30] Yeast proteases autolyze proteins to low molecular weight peptides that can be assimilated by the LAB.[61] LAB are dependent upon yeast to make peptides available, as their metabolism requires peptide uptake despite not excreting exogenous proteases. After the first-stage protein breakdown by yeast proteases and endogenous cereal proteases, metabolic uptake of the resulting peptides by LAB occurs, further breaking down these peptides to constituent amino acids. In turn, LAB participate in the synergistic relationship by releasing organic acids to drop dough pH, favoring growth conditions for yeast and supporting optimal conditions for yeast enzyme activity for yeast enzymes as shown in **Figure 1.1**.[61] LAB also hydrolyze maltose, releasing glucose which can be consumed by maltose-negative yeast as described above.[30] A similar relationship occurs in the metabolism of other carbohydrates. For example, yeasts are able to liberate fructose from more complex carbohydrates, but do not preferentially ferment it. Therefore heterofermentative LAB

use the liberated fructose as an additional electron receptor to generate ATP.[47] In these ways, the symbiosis between sourdough yeast and LAB is maintained as long as the appropriate ratio of LAB to yeast is maintained. This ratio is generally from 100-1000:1[36] and the purpose of feeding the starter at regular intervals is to maintain this ratio as well as to replenish the food source of the organisms.[59]

1.3.6 Microbiomes as Drivers of Differences in Sourdough Bread

Previous research indicates that the composition of a sourdough microbiome likely affects its quality, including flavor, aroma, texture, shelf-life, and nutritional properties.[50] However, most studies correlating quality outcomes to the sourdough microbiome have been conducted on lab-curated, single-organism ferments or co-cultures of yeast and LAB that do not reflect the rich species diversity in wild or commercial sourdough starters.[62] Others use processing steps (such as creating cytoplasmic extract, or CE, to concentrate enzymes from starter organisms) inconsistent with how sourdough is produced in a commercial setting.[63] While a few commercial sourdoughs have been studied in this context, this work has been done on mostly uncharacterized starters,[64] meaning that it is difficult to tie quality outcomes to specific composition of the sourdough microbiome. However, a recent study of over 500 globally sourced sourdough starters found that all were microbially distinct and exhibited some physical and quality differences, including dough rise volume and volatile organic compound profile. Although few quality outcomes were measured in this study, their differences were tied to the composition of the starters' microbiomes.[34] This lends the assertion that the microbiome may be a driver of other differences between sourdough breads vs. yeast breads, and sourdoughs vs. other sourdoughs, such as changes in gluten structure and immunoreactivity of gluten. However, these studies are subject to the same limitations mentioned above, namely focusing on curated

co-cultures or uncharacterized starters.

Despite the need for more work directly comparing the quality and functional outcomes of fermentation with different sourdough microbiomes, some trends have emerged indicating that sourdough overall shows some benefits compared to dough leavened with baker's yeast, known as "straight dough". First, sourdough is considered to have preferred organoleptic properties compared to straight dough. The volatile aroma profile of sourdough bread is more complex than that of baker's yeast-fermented bread, and volatile compounds generally appear at higher concentrations. Sourdough has been described as more aromatic and rich-tasting compared to non-sourdough bread.[35]

From a nutritional perspective, sourdough exhibits better nutrient bioavailability and a reduction in antinutritional factors such as the mineral-binding phytate complex. Endogenous cereal phytases have improved activity at low pH, such as that of sourdough, and sourdough fermentation has been shown to increase phytase activity approximately tenfold.[65] Since about 70% of phosphorous in wheat is bound up in the phytate complex, phytase activity is desirable since it leads to a reduction in this anti-nutritional complex and improved nutritional availability of minerals and micro-nutrients.[66] Sourdough fermentation has also been shown to reduce the content of acrylamide, a possibly carcinogenic compound that can form during baking.[67] This is likely due to the lower pH of the processing conditions but also by bacterial catabolism of asparagine.[68] Next, sourdough may be more digestible than non-sourdough bread. In small trials with sourdough vs. yeast-leavened bread or croissants, sourdough stimulated the appetite more than yeast-leavened products and resulted in less feelings of fullness and satiety in participants.[62,69] The body may process sourdough more easily in other ways, too. Sourdough fermentation may improve glycemic control for diabetics consuming bread products due to

fermentative consumption of monosaccharides and oligosaccharides in the dough.[70] Reduction of carbohydrate FODMAPS (fermentable oligosaccharides, disaccharides, monosaccharides, and polyols) is a known outcome of sourdough fermentation[71] and this may be useful not only for diabetics but to minimize symptoms of irritable bowel syndrome and NCGS.[72,73]

Another benefit of sourdough relative to yeast-leavened dough is the production of exopolysaccharides (EPS). Sourdough's desirable texture and viscosity compared to straight dough is due in part to the effect of EPS produced by LAB. These bolster the elasticity of the dough, some of which is compromised due to gluten breakdown. Some of these EPS have been investigated for possible prebiotic activity.[74,75] Sourdough EPS are of interest because it has been shown that EPS produced *in situ* by microorganisms have greater effect on bread texture and rheology than the same concentration of exogenous EPS added to a bread formula.[76,77] Sourdough-fermented breads also exhibit greater overall stability[45,76,78] and hinderance of staling in comparison to yeast breads.[78–80] The mechanisms for shelf life extension are largely through mold inhibition,[30] delayed fat rancidity,[81] and improved moisture retention and delay of crumb firming.[82]

Many of the benefits and functional properties of sourdough can be attributed to the organic acids that are produced by sourdough microbes. For example, the pH decrease achieved by the distribution of aids in the dough improves gas retention and water binding of gluten and starch, induces swelling of pentosans, and inhibits endogenous flour amylases.[7] Some functional properties of sourdough can be attributed both to the metabolism of the microorganisms and to the pH decrease achieved by the diffusion of organic acids which are the byproduct of this metabolism. For example, while the acidic environment lowers glycemic index through the formation of low-digestibility resistant starch and through α-amylase inhibition, organisms also

contribute to lower glycemic index by metabolism dough maltose, glucose, and fructose.[50] Low pH also contributes to breakdown of the phytate complex through endogenous flour enzymes, leading to phytic acid breakdown and mineral release [83]; microorganisms present in some sourdough fermentations have also demonstrated phytase activity.[50] **Figure 1.2** summarizes these benefits and their principal mechanisms.

1.3.7 Gluten Chemistry and Functional Importance to Bread

Gluten is the storage protein complex in wheat and other members of the Triticeae family.[84] The gluten complex consists of hundreds of distinct but related proteins.[85] These storage proteins appear to have no enzymatic function, but only store accumulations of nitrogen, carbon, and sulfur for the plant seedling in deposits known as protein bodies located within the plant endosperm.[86] These fractions are collectively referred to as prolamins[85] and are organized

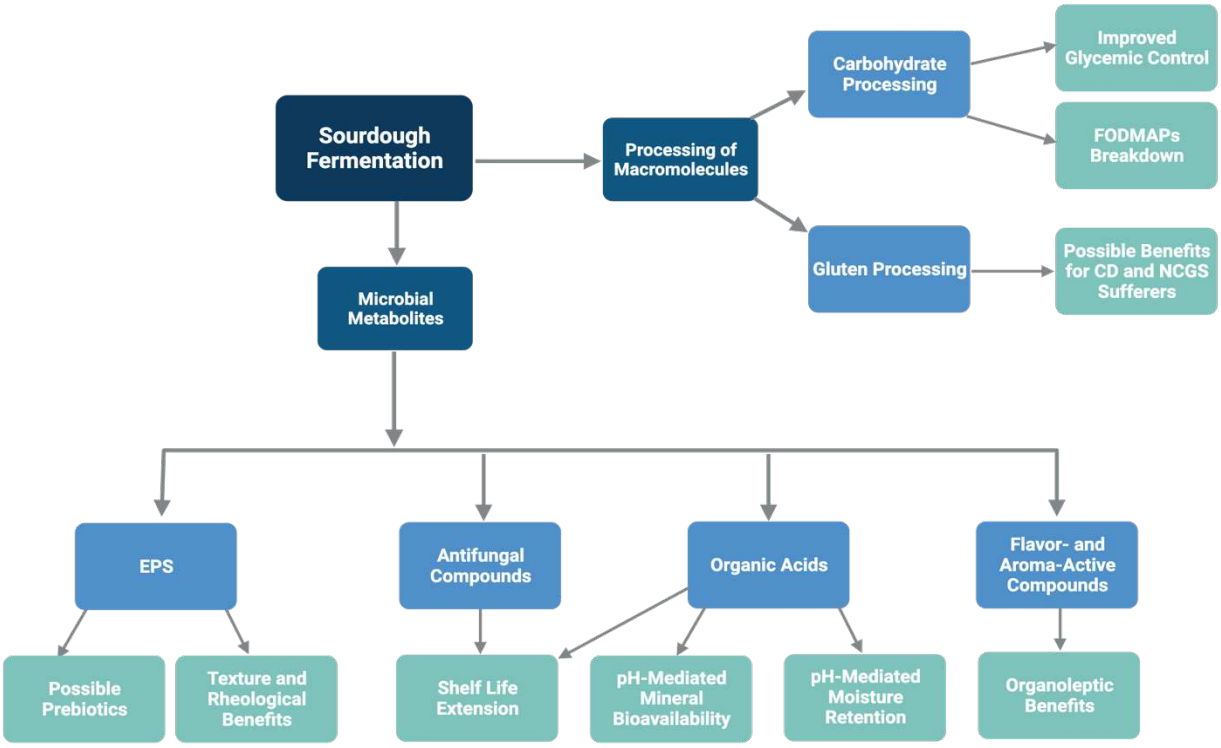

Figure 1.2: Benefits of sourdough fermentation and their simplified pathways. Created using BioRender.

as described in **Figure 1.3**.[87] Prolamins are further classified based on their sulfur content; sulfur-rich prolamins are enriched with sulfurous amino acids cysteine and methionine, and therefore demonstrate enhanced capacity to form disulfide bonds. All prolamins are rich in proline and glutamine, and can be classified into fractions known as gliadin and glutenin, each comprising approximately 50% of the protein complex by mass. Glutenin confers strength and elasticity to dough, while gliadin gives dough its viscosity and extensibility.[88] Notably, gliadin contains the greatest concentration of immunogenic epitopes shown to affect patients with CD.[89] The glutenin fraction is further divided into high- (HMW) and low-molecular-weight (LMW) subunits, while the gliadin fraction is divided into α/β, γ, and ω groups according to their amino acid profiles and solubility.

The presence or absence of sulfur residues in prolamins is relevant as it pertains to the ability of the gluten complex to participate in disulfide bond cross-linking, which strengthens the

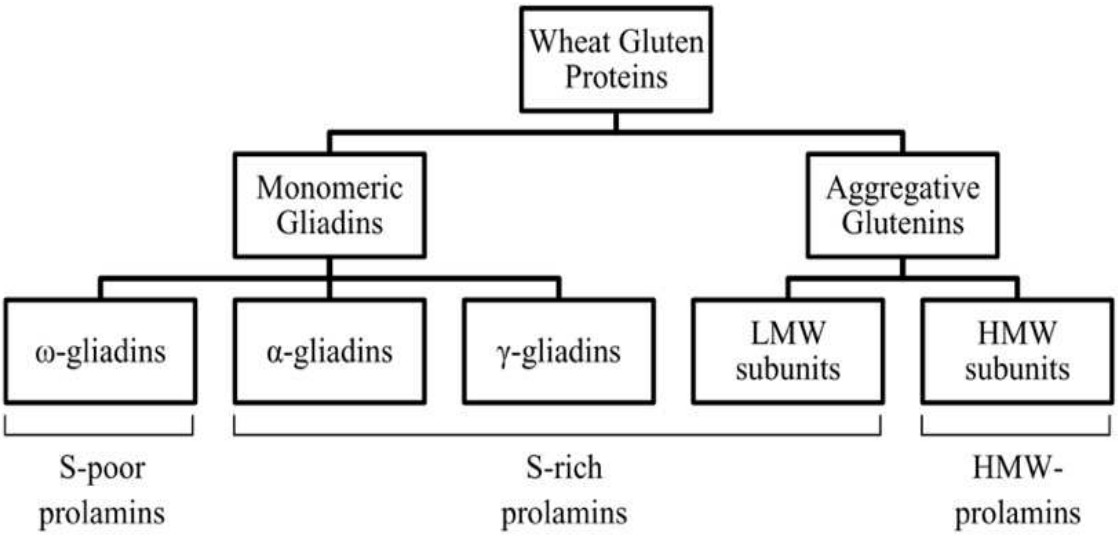

Figure 1.3: Classification of prolamin subunits from wheat. From Lindsay & Skerritt (1999).

gluten complex and relates to improved baking properties of the wheat used.[88] The elasticity of the glutenin fraction is thought to be conferred by hydrogen-bond interactions between glutamine residues of adjacent spiral motifs of HMW glutenin subunits, plus hydrophobic interactions between adjacent tyrosine residues. However, too much disulfide cross-linking may detract from dough elasticity properties by tightening the overall structure.[88] The proteins involved in the gluten complex are brought together when the ground endosperm of a grain (flour) is combined with water and then worked by mixing and/or kneading, allowing for oxidation of sulfhydryl groups and subsequent inter- and intramolecular interaction. These interactions directly affect the functional properties of dough including stiffness, elasticity, and gas retention.[90]

Broadly, gluten proteins are heat-stable with prolific functionality as binding, moisture-retention, emulsifying, extension, and gelling agents, and as fillers. For these reasons, they are often used as additives even in non-bread products such as spices, meats and meat substitutes, dressings, confectionary, and even pharmaceuticals.[85] However the primary functionality of gluten in food comes from its role as a gas-trapping agent in leavened bread, where it is required for bread to rise. Gluten proteins largely determine the properties and overall quality of wheat flour by forming an extensive, gas-trapping network through disulfide and hydrogen bonding. The texture and functional properties of this gluten network are not replicable in dough made from gluten-free flour.[29] Although gluten is a necessary functional component of leavened bread products, it is the immunogenic trigger in CD pathology[91] as well as a number of known allergens, and it is among the causal mechanisms of NCGS.[92]

1.3.8 Gluten-Free Grains and Benefits of Sourdough Fermentation

Bread typically requires gluten in order to rise, relying on its gas-trapping ability.[29]

Because gluten-free grains such as quinoa, buckwheat, or teff do not contain gluten, other additives are needed to obtain the expected quality attributes of gluten-containing bread. These additives may include hydrocolloids or enzyme preparations. Alongside additives, sourdough fermentation is also a common technology used to improve the quality of gluten-free doughs.[93] Although sourdough fermentation typically occurs in gluten-containing wheat or rye doughs, other flours can be used, including gluten-free flours or pseudo-grains such as quinoa, buckwheat, or chia.[30]

Sourdough fermentation can be applied to gluten-free or non-wheat products, like those typically consumed by CD or NCGS patients, to improve their organoleptic and functional properties. First, sourdough fermentation delays staling and prolongs crumb softness of gluten-free breads and also decreases their brittleness and firmness over time.[80] Anti-fungal compounds produced during sourdough fermentation also act as mold inhibitors and staling retardants in gluten-free breads, especially where the strain *L. plantarum* FST 1.7 is present.[94] Also, sourdough-fermented gluten-free bread can achieve equivalent loaf volume to reference breads.[79] This is likely due to EPS production, which affects dough rheology and provides structure in the absence of gluten. EPS-producing LAB have are beneficial to sorghum bread texture, and they also increase gas production during proofing, probably due to prebiotic stimulation of yeast metabolism, yielding improved rise.[95] Finally, because sourdough fermentation is known to have positive effects on flavor, it may be used with gluten-free flour such as teff or buckwheat to target consumer acceptance.[96]

1.4 Gluten Sensitivities

"Gluten sensitivity" can be defined as an adverse reaction stimulated by the ingestion of gluten-containing foods. There are a number of distinct diagnoses, including celiac disease (CD),

non-celiac gluten sensitivity (NCGS), and irritable bowel syndrome (IBS). The prevalence of gluten sensitivities worldwide is difficult to estimate due to the absence of a widely agreed upon definition of the term within the clinical community and population data relying on self-reporting. Pathologies such as NCGS and IBS are estimated to affect up to 20% of some geographically specific populations.[97] One UK-based survey found that 13% percent of participants reported having some type of gluten sensitivity. The rest of the world is estimated to have a self-reported gluten-sensitivity rate of around 10%.[98]

In comparison to other gluten sensitivities, there is a greater amount of population data characterizing the prevalence of CD. Appearing in greater prevalence in whites than in Black or Hispanic populations,[99] CD occurs at a rate of about 1% in the U.S. and Europe.[100] Other studies have noted its prevalence at 1.5% in both Saudi[101] and Finnish school children,[102] and approximately 3% in Finnish adults over the age of 52.[103] The highest known rate of CD in the world is 5.6%, among the Saharawi people of the Western Sahara.[104] Based on stored serum studies, incidence of CD has increased nearly fivefold globally since the 1950s.[105] The mechanisms behind this surge in cases have not yet been identified. Some evidence suggests an increase in environmental triggers to the disease, as CD pathology is known to include both genetic and non-genetic risk factors. Indeed, only 3% of genetically predisposed individuals develop CD[106] and studies in monozygotic twins display a concordance rate up to 85%.[107] Other research indicates that recent advances in agricultural practices, such as the use of nitrogen-based fertilizer, may be driving up protein (and therefore gluten) content in cereal grains, or that the use of shorter-acting leavening agents such as baker's yeast may have decreased gluten breakdown.[108] Some authors hint at contributing sociological factors: due to bread's unique place as a symbol in certain religious traditions, such as Catholic communion, it may historically have

been socially proscribed to report contraindications to bread consumption.[15] As religiosity diminishes it may have become more acceptable to report symptoms in some cultures.

Because NCGS is often self-diagnosed before individuals present at a physician's office, and because there are no formal diagnostic criteria for NCGS, it is difficult to track the incidence of this condition.[109,110] Estimates for prevalence of NCGS are usually derived from populations who report observance of a gluten-free diet;[105] these estimates vary widely from 0.6-6% of the population[92] with most studies estimating no higher than 5%.[111] Convoluting assessments of NCGS prevalence is the fact that the gluten-free diet has benefited from a perception of wellness advantages,[112] such that many adherents of a gluten-free diet may not actually experience symptoms of gluten intolerance. Instead, consumers may embark on a gluten-free diet due to a belief in its ability to promote weight-loss or overall well-being without advice from a medical professional.[92] One common form of NCGS is irritable bowel syndrome (IBS), which is thought to affect approximately 11% of the world's population,[97] but it is difficult to understand the degree to which this population overlaps with those who suffer from other forms of NCGS.

1.4.1 Celiac Disease

Wheat gluten was first identified as the trigger for CD in 1950 by Willian Dicke.[91] In this chronic condition of the small intestine the antigen, gluten, triggers an autoimmune response in susceptible individuals. From the perspective of patient experience and symptoms, consuming gluten often leads to gastrointestinal distress. From an immunopathology perspective, gluten consumption leads to increased intestinal barrier permeability, inflammation, infiltration of lymphocytes to the intestinal epithelium, and circulating antibodies to endogenous tissue transglutaminase II (TG2) and gluten protein. Long-term exposure to gluten can lead to extensive damage to intestinal barrier, resulting in loss of intestinal barrier architecture observed as villous

atrophy and crypt hyperplasia.[113] This loss of villous structure can lead to malabsorption and malnutrition.[100]

CD symptoms can also manifest in other body systems. In fact, more than 80% of patients report non-gastrointestinal symptoms. These may include migraines,[114] anemia, osteoporosis, and overall self-reported decreased quality of life.[115] There is also evidence that CD can result in psychological and neurological problems due to toxic gliadin peptides crossing the blood brain barrier and binding to receptors in the brain.[1] CD is also typically found to occur in conjunction with other autoimmune diseases such as Type I diabetes, thyroiditis, and dermatitis herpetiformis.[115]

The causes of CD have been strongly linked to genetic factors, especially the HLA-DQ2/8 gene, with nearly 95% of CD patients exhibiting the HLA-DQ2 phenotype and virtually all the remainder positive for HLA-DQ8.[116] As **Figure 1.4**[117] depicts, other factors can include environmental components and non-HLA genetic drivers.[117] Serological diagnostic criteria for CD include (1) at least one copy of either the human leukocyte antigen HLA-DQ2 or HLA-DQ8 gene variant, and (2) circulating autoantibodies to the TG2 enzyme after exposure to gluten.[115] Although a tentative diagnosis can be made on the basis of clinical and serological data, intestinal biopsy is the principle diagnostic tool.[118] Although a gluten-free diet can be an effective treatment to CD if it is enacted quickly enough, up to 25% of adult CD patients experience persistent symptoms despite abstaining from gluten even when biopsies show intestinal healing.[119] In other cases, the cell loss from enterocyte apoptosis may exceed the rate

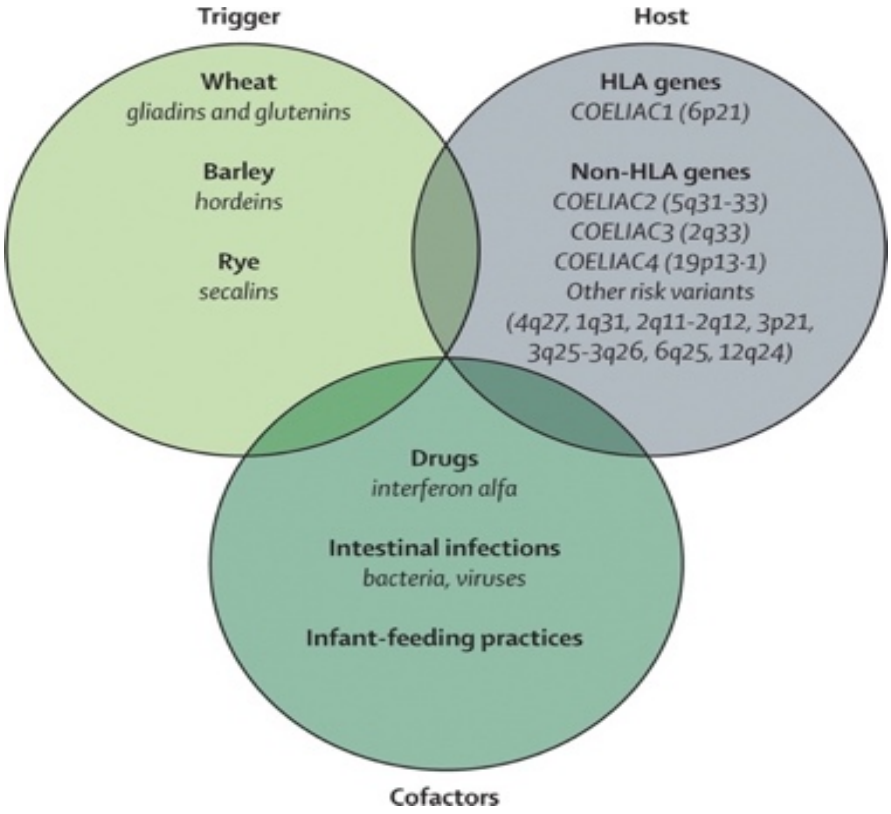

Figure 1.4: Drivers of celiac disease. From Di Sabatino & Corazza (2009).

of cell proliferation due to crypt hyperplasia, so villous atrophy may ultimately be impossible to overcome for some patients.[120]

The pathogenic cascade of CD is complex. The gliadin component of gluten is sparingly digested by stomach and intestinal enzymes due to a high proportion of proline and glutamine residues which confer partial resistance to digestion and to the rate-limiting effects of low-efficiency COOH-terminal degradation.[121] Many of the resulting peptides are known to act as antigenic triggers.[122,123] As a result of their resistance to digestion in the intestinal tract and by brush border enzymes, these peptides can cross the intestinal epithelial barrier intact and trigger a both innate and adaptive immunological responses in the lamina propria.[124]

The role of zonulin, an epithelial tight-junction protein, has also been investigated for its

part in CD pathology. In non-susceptible individuals, zonulin regulates tight junctions and prevents the passage of these poorly digested protein fragments from crossing the intestinal epithelial barrier. However, individuals susceptible to CD experience a degree of barrier irritation upon exposure to gliadin peptides leading to zonulin-mediated tight junction disruption. The hypothesized mechanism is binding of the target chemokine receptor CXCR3, along with the adaptor protein MyD88, by gliadin. The recruitment of this complex appears to stimulate zonulin upregulation, which has been observed in patients experiencing celiac pathology.[125]

Once gliadin enters the lamina propria, the inflammatory response of intestinal epithelial cells signals for the infiltration of intestinal epithelial lymphocytes, initiating the innate immune response, as well as production of enzyme tissue transglutaminase II (TG2). TG2 is a multi-functional enzyme typically associated with wound healing. However, TG2 has a high affinity for the deamidation of glutamine residues located one amino acid away from proline (i.e., Q-X-P) which is a highly conserved pattern in gluten, especially gliadin. For this reason, TG2 deamidates the neutral glutamine residues in gliadin peptides to charged glutamic acid residues, which in turn increases the binding affinity of MCH class II surface receptors on antigen-presenting cells (described below) for these peptides, initiating the adaptive immune response.[105]

The HLA gene, located on the class II region of chromosome 6,[101] encodes for cell surface receptors on antigen-presenting cells, macrophages, dendritic cells, and B-cells.[115] In individuals with the HLA-DQ2 or HLA-DQ8 alleles, the receptors express in a shape that binds these deamidated gliadin peptides[101] resulting in activation of gluten-specific $CD4^+$ T-helper cells which act as effectors of an inflammatory cascade including IFN-γ and IFN-21,[113]

ultimately leading to tissue damage through degradation of matrix proteins.[105] Antibodies against the TG2 enzyme itself are also formed during this process, which is thought to occur when gliadin peptides bind to MHC class II receptors concurrently with TG2. Thus, both the deamidated gliadin peptides and TG2 are antigenic triggers for the body's immune response.[105]

The innate immune response of CD, initiated at the onset of gliadin translocation to the lamina propria, is propagated by increased epithelial lymphocytes which express Natural Killer

cell receptors NKG2D and CD9/NKG2A. These receptors recognize the stress-response proteins MICA and MICB and the HLA-E receptor on epithelial cells and are upregulated in the presence of IL-15.[105] Recognition of stress-response proteins produces a cytolytic response that eliminates enterocytes exhibiting stress signals.[113] Unlike other markers of CD, these increased intraepithelial lymphocytes maintain full activity even upon total gluten withdrawal and are correlated with refractory CD and enteropathy-associated T-cell lymphoma.[126] Some evidence suggests that non-gluten wheat proteins, such as inhibitors of amylase and trypsin that act as pest resistance molecules, may play a role in this innate response.[127]

For several decades, investigation into CD pathology has been carried out via human and animal cell lines. It has been found that digests of the prolamin (gliadin) fraction from wheat, barley, and rye, which are known to be poorly tolerated by CD patients, inhibit development and differentiation of rat small intestine, cause agglutination of undifferentiated human myelogenous leukemia K562(S) cells, and demonstrate various pathogenic effects in the human colorectal

adenocarcinoma Caco-2 cell line. In Caco-2 cells, wheat prolamin digests induce decreased cell

viability and differentiation and impaired metabolic processes of differentiated cells. For

these

reasons, the effect of wheat, rye, and barley prolamin digests on these cell lines has been considered to give useful insight into the possible pathogenic effects of these prolamins in *in vivo* systems.[135]

Table 1.1 summarizes the peptides thought to have some effect, either toxic or protective, on CD pathology. Peptides with toxic activity in celiac patience have several features in common. First, they are at least partially resistant to digestion by human gastric, pancreatic, and intestinal enzymes. Second, they are recognized and processed to partial deamidation by the human endogenous enzyme TG2. Also, this deamidation process enhances their binding affinity for the HLA-DQ2/DQ8 receptor. Third, they have strong polyproline II (PPII) helix character.[136] Notably, the α_2-gliadin 33mer and γ_5-gliadin 26mer gluten fragments have been specifically attributed to the inflammatory response elicited in CD patients, as both contain multiple toxic epitopes.[137]

1.4.2 Non-Celiac Gluten Sensitivity

Patients who do not meet diagnostic criteria for CD but whose symptoms cease upon adherence to a gluten-free diet are said to exhibit non-celiac gluten sensitivity (NCGS), of which there appear to be many forms. These patients report withdrawal of symptoms upon cessation of gluten intake but show negative biopsies and serum tests for CD. In a clinical setting, diagnosis of NCGS is made on the basis of ruling out wheat allergy and CD despite the occurrence of wheat-correlated gastrointestinal symptoms.[138] NCGS is characterized by both gastrointestinal and non-gastrointestinal symptoms much like those described by CD patients, but there are currently no biomarkers for the condition. The typical biomarkers of CD, such as TG2 and anti-

endomysial IgA and IgG, are at normal levels in NCGS patients. IgG antibodies against native

Table 1.1: Principal immunogenic peptides relevant to celiac disease pathology.

Peptide	Sequence	Activity	Source
α2-gliadin 57-89; "33mer"	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF Including one copy of PFPQPQLPY, three copies of PQPQLPYPQ, and two copies of PYPQPQLPY (see below)	CD4+ T-cell induction in all celiac patients	[123]
α2-gliadin 62-75	PQPQLPYPQPQLPY	Stimulation of CD4+ T-cells on intestinal biopsy	[128,129]
α9-gliadin 57-68	QLQPFPQPQLPY	Stimulation of CD4+ T-cells in nearly all celiac patients and intestinal biopsy	[130]
α-gliadin 31-43	LGQQQPFPPQQPY	Agglutination of K562(S) cells; IFN-dependent inflammation	[122,131]
α2-gliadin 51-54	PSQQ	Agglutination of 100% K562(S) cells at a concentration of 2X '31-43' peptide	[122]
γ5-gliadin 228-236	PQQPYPQPQPQ	CD4+ T-cell clone stimulation	[128]
γ5-gliadin 138-153	QPQQPQQSFPQQQRPF	CD4+ T-cell clone stimulation	[128]
γ5-gliadin 115-123	PQQSFPQQQ	CD4+ T-cell clone stimulation and on intestinal biopsy	[132]
γ5-gliadin 60–79	LQPQQPFPQQPQQPYPQQPQ	CD4+ T-cell clone stimulation	[129]
γ5-gliadin 102-113	FSQPQQQFPQPQ	CD4+ T-cell clone stimulation	[129]
γ5-gliadin 66-78	FPQQPQQPYPQQP	CD4+ T-cell clone stimulation	[129]
α9-gliadin 60-68	PFPQPQLPY	CD4+ T-cell clone stimulation	[133]
α2-gliadin 62-70	PQPQLPYPQ	CD4+ T-cell clone stimulation	[133]
γ5-gliadin 58-84; "26mer"	FLQPQQPFPQQPQQPYPQQPQQPFPQ	Enhanced T-cell antigenicity	[134]
1157.5 Da peptide from durum wheat gliadin	QQPQDAVQPF	In the presence of PT- gliadin, prots against agglutination in K562(S) cells and against apoptosis in Caco-2 cells	[120]

gliadin have been found in nearly 50% of NCGS patients. There is some evidence that the condition may also be related to the innate immune response caused by non-gluten wheat peptides such as amylase trypsin inhibitor (ATI)[127,139] or possibly to the ingestion of fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPS) in cereals.[71] Wheat germ agglutinin is an endogenous wheat protein thought to play a role in NCGS symptoms; this protein has been shown to increase cell permeability and release of pro-inflammatory cytokines in Caco-2 cell models.[138] Some NCGS patients are thought to bear the HLA-DQ2 or -DQ8 haplotype,[140] but the widespread prevalence of this genetic marker (30-40% of global population)[106] weakens the evidence for this to be used as diagnostic criteria without further differentiating markers like those observed in CD.

While many symptoms of CD and NCGS overlap (bloating, fatigue, and abdominal discomfort) there are some distinguishing features. NCGS does not activate basophils and biopsies of NCGS do not show an inflammatory response to gliadin *in vitro*, whereas these are each characteristic of CD.[140] Another common method of clinical assessment for NCGS involves asking the patient to follow a gluten free diet for a period of time and then reintroducing of gluten to evaluate the effects. The assessment is based on the patient's rating of their own symptoms. However, because these analyses are not conducted blind, patient bias and psychosomatic effects may affect reporting and therefore diagnoses.[1]

1.4.3 Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is a gastrointestinal condition that affects a large portion of the population and often goes undiagnosed. Like NCGS, the heading under which it is often included, IBS has no biomarkers, so it is diagnosed clinically. Rome III criteria are typically used to diagnose IBS. Under these criteria, first, a person must have at least three days per month in

the past 12 weeks of continuous or recurrent abdominal pain in which relief occurs with defecation; stool frequency and form must be altered. Second, the onset of symptoms must occur more than six months before diagnosis. There are three types of IBS: IBS-D, in which the patients experience predominantly diarrhea, IBS-C, constipation, and IBS-M in which patients suffer both symptoms.[97]

A gluten-free diet has been found to relieve symptoms in some IBS patients.[111] However, it is not clear that the gluten protein is responsible for symptoms in all patients. As discussed previously, other proteins may be responsible for symptoms in non-CD patients who experience sensitivity when consuming wheat products.[1] FODMAPs, which are present in wheat, have also been shown to cause gastrointestinal symptoms in many people with IBS.[72] Previous work has shown that the reduction of FODMAPs in bread by sourdough fermentation can allow some people with IBS to consume wheat bread with no symptoms.[139]

1.5 Existing and Experimental Therapies for Gluten Sensitivities

Currently, the only treatment for CD is lifelong adherence to a gluten free diet[100] and a gluten free diet is also a behavioral and diagnostic marker of NCGS.[1] However, novel therapies are being explored to avoid the outcomes of accidental exposure or to allow sensitive individuals to consume wheat products. The following section reviews the efficacy and limitations of the gluten-free diet, followed by a brief review of dietary, horticultural, and processing strategies to mediate inflammatory and immune responses to gluten. Pharmacological and immunomodulatory strategies also exist; these will be reviewed only briefly, as they have been the subject of extensive research in their own right and are outside the scope of this review as they do not relate to diet or food processing. Strategies to manage gluten-mediated symptoms

based on modification of the gluten protein itself will be reviewed in greater detail, as this relates most to the food processing-based techniques of interest to this dissertation.

1.5.1 Pharmacological Modulation of Immune Response

While attempts have been made to use steroids as a therapy for CD or refractory CD, they have been mostly dismissed due to apparent side effects and also due to the fact that early studies showed a relapse of symptoms upon steroid withdrawl.[141,142] Results of more recent studies indicate that steroids may be promising as an adjunct therapy, though trial sizes have been small and side effects have been recorded.[143,144] One recent trial found no usefulness for the steroid studied (budesonide) as an adjunct therapy for newly diagnosed CD patients[145] but an anti-TNFα antibody known as Infliximab has been able to help several patients with refractory CD that is unresponsive to steroids.[146–149]

Vaccines for CD have also been attempted. NexVax2, a vaccine including five immunogenic gluten epitopes recognized by gluten-specific T-cells has been shown to be safe but also ineffective[150] and to have some undesirable side effects.[151]

Interleukin blockers have been investigated for potential treatment in autoimmune disorders [152]. IL-15 signaling has been the target of attempts to modulate CD, because IL-15 is upregulated in patients with active CD.[153] The monoclonal antibody PRN-015 (previously known as AMG 714) binds IL-15 and inhibits its functions, cutting shorts its downstream signaling capabilities.[154] This therapy is undergoing clinical trials[155] and is not currently in use among CD patients.[156] Tofacitinib is another small molecule, administered orally, that can inhibit the IL-15 signaling pathway. It has been shown to revert enteropathy in mice that over-express IL-15[157] and human trials are ongoing.[158]

In addition to Tofacitinib, several synthetic peptides have shown promising

immunomodulatory activity, albeit through distinct mechanisms. Some have been tested as HLA-DQ blockers, which inhibit gluten peptides from binding to the HLA-DQ receptors, have been attempted for use as CD therapies. These small peptides can prevent binding of gliadin peptides to HLA-DQ receptors through competitive inhibition, thereby avoiding a gluten-mediated inflammatory cascade. One synthetically derived peptide was found to have 50-times better finding affinity to HLA-DQ receptors than an immunogenic α-gliadin peptide.[159] Other synthetic peptides have shown promise acting through different mechanisms. Larazotide, a small synthetic peptide derived from a toxin of *Vibrio cholera*, has been found to prevent the disruption of tight junction proteins at the intestinal barrier and has shown some promise in clinical trials.[160]

1.5.2 Gluten-Free Diet

A recommendation of lifelong gluten abstinence has been the standard course of treatment for gluten sensitivity since the 1950s.[113] Even minor exposure (10 mg/day) to gluten can cause mucosal damage in CD patients, and for this reason accidental exposure is a major concern.[137] Unfortunately, the social and economic costs of a gluten-free diet are high, and gluten avoidance is difficult. Gluten is commonly used as an additive in many foods that would be surprising to the average consumer, such as preserves, meat, and seafood. This, along with its prevalence in staple products like bread and pasta, contributes to a daily gluten intake of 5-20 g/day in a typical Western diet.[85]

For patients with CD and NCGS, a gluten-free diet represents a path toward wellbeing. The response-eliciting dose of gluten for individuals with CD is between 10 to 100 mg.[161] In August of 2013, the US Food and Drug Administration and the Codex Alimentarius Commission issued a rule governing the voluntary use of the label “gluten-free”, which states that foods

bearing this label may not be derived from a gluten-containing grain and may not contain more than 20 ppm (mg/kg) of gluten.[162] Other bodies have moved to bring their own standards in line with this ruling to reach greater global consensus.[85] Gluten-free production is a significant challenge to the baking industry, as a lower limit of 20,000 ppm gluten has been identified as the lowest possible concentration of gluten that allows for making bread without the addition of other structure-enhancing additives.[163] For reference, a typical slice of bread is estimated to contain about 4 g of gluten.[85]

Gluten frequently contaminates gluten-free products, even sometimes products targeted specifically to the treatment of CD, such that even those individuals on a diet of gluten avoidance may ingest between 5 to 50 mg/day.[164] A fully gluten-free diet is lower in both sensory and nutritional quality than equivalent standard diets,[138] as many gluten-free foods have lower nutritional value than wheat-based alternatives.[119] Not only is a gluten-free diet lower in overall ingredient variety[165] but gluten-free foods are also frequently deficient specifically in calcium, iron, magnesium, zinc, and vitamins D, B12, and folate.[166]

Social, economic and behavioral patterns all play a role in gluten-sensitive individuals' likelihood to adhere to a gluten-free diet.[167] Gluten-free foods are less available in supermarkets and restaurants and more costly where they are available.[119] This is especially true in less developed areas. Because gluten-free options are limited in restaurants, a strict gluten-free diet may limit social interactions or travel.[168] CD and NCGS patients on gluten-free diets are excluded from the social, religious, and symbolic interactions governed by gluten-containing bread. Making and eating the leavened bread that are staple foods in many culinary traditions is a way of participating in communal human cultural heritage[169] that nearly 80 million people with CD[170] and around 800 million with NCGS[97] are currently unable to share. Gluten-restricted diets

limit adherents from participating in cultural traditions stretching back generations and severs them from support networks[171] and engagement with social activities.[168]

For these reasons, there is an urgent need for low-cost technologies to improve access to high-functionality reduced-gluten foods, or foods with lower gluten immunogenicity, while still maintaining desirable sensory properties.

1.5.3 Selectively Bred and Genetically Modified Wheat

Selectively bred and genetically modified wheat with reduced immunogenicity has been studied as a potential therapy for CD. Wheat with alternative or modified forms of the gluten protein has been found to provoke less cell damage and toxicity responses in *in vitro* and *ex vivo* assays.[172–174] For example, it has been known for some time that PT digests of tetraploid durum (pasta) wheat exhibit reduced gluten toxicity compared to those of hexaploid bread wheat, the most commonly used wheat in the food industry.[175] Other studies have examined wheat varieties with different ratios of α, β, γ, and ω gliadins. Gliadins decrease in toxicity in the order α > β > γ > ω, and indeed wheat with all gliadin fractions as opposed to only α and β showed lower toxicity in in vitro organ culture.[173] However, the attempt to manipulate gliadin fractions can impair baking properties. The deletion of α-gliadins in an attempt to reduce toxicity decreased immunostimulatory epitopes but also led to loss of mechanical and rheological properties, whereas the deletion of loci coding for γ- and ω- gliadins removed immunostimulatory epitopes without affecting dough quality.[174]

1.5.4 Gluten Sequestration

Targeting gluten (or gliadin) to inhibit HLA-DQ receptor binding has been another widely studied area of therapy for CD. Several natural and synthetic molecules have been shown

to bind to gliadin, modifying it so that it is resistant to digestion and limiting the release of peptides that would trigger an immune response, thereby attenuating symptoms. Molecules able to bind gliadin in this way include hydroxyethyl methacrylate, the synthetic polymer sodium-4-styrene sulfonate (poly(HEMA-co-SS)),[176] and green tea polyphenols.[177]

The prevention of gliadin deamidation by TG2 has also been explored; *in vitro* administration of a TG2 blocker, lysine methyl ester, has been shown to prevent gliadin deamidation, which would reduce the affinity for binding by HLA-DQ2 MHC class II receptors, but is it unknown whether this is a viable strategy beyond *in vitro* applications.[178] Overall, gluten sequestration appears to be a promising method to hinder gluten's ability to engage the inflammatory processes, but requires further research in more complex model systems and/or clinical settings.

1.5.5 Enzymatic Modification of Gluten-Containing Food Products and Ingredients

Enzymatically modifying gluten proteins in food may allow CD patients to consume gluten-containing food products with reduced symptoms. This can occur by consuming supplemental enzymes to assist with gluten digestion, or by applying gluten-degrading enzymes to a food during production to "pre-digest" the gluten protein before it reaches the consumer.

Oral enzyme therapies are considered to be an encouraging therapeutic development in the treatment of CD and NCGS. These therapies involve the co-ingestion of gluten-containing foods alongside enzymes that can survive gastric-duodenal conditions to degrade the gluten before it can act as an antigenic trigger.[137] For example, prolyl endopeptidases (PEP) have been used to detoxify gluten by cleaving proline bonds in food models such as beer.[179] Other gluten-degrading enzymes, such as subtilisin and cysteine proteases, have been studied to similar effect. While these therapies would not allow CD patients to consume large quantities of gluten, they

may enable ingestion of up to a few grams of gluten per day, which would be far more sustainable than the current standard.[137]

From the food processing perspective, the pre-digestion of gluten proteins in a food product occurs in a similar manner, though the sources of the enzymes may differ. In oral therapeutic applications, enzymes are typically isolated and purified, while food processing applications find these enzymes produced directly in the food product. For example, enzymes native to wheat, some of which are expressed during germination, are naturally able to hydrolyze gluten,[180] though it should be noted that the extent to which this may affect gluten-mediated inflammation and immune responses is unknown. More substantial evidence for processing-derived gluten degradation exists in discussions surrounding fermentation, as several strains of LAB are known to produce the aforementioned PEP enzymes and cysteine proteases. Due to the prevalence of LAB in sourdough starter cultures, sourdough fermentation has been explored as a food processing strategy with potential to reduce gluten toxicity. Thus, the remainder of this review will focus on flour fermentation by sourdough organisms as a method of enzymatic processing of gluten with intent to reduce gluten toxicity in CD and other gluten sensitivities.

1.6 Sourdough Fermentation as a Processing Tool for Celiac-Safe Food Products

Sourdough fermentation has been widely investigated for gluten proteolysis. Indeed, it has been shown that fermentation of wheat flour with sourdough-associated organisms can degrade immunostimulatory[7,8,6] gluten fragments and reduce gluten content as measured by standard commercial methods.[7,10] However, all of these studies have investigated this approach using isolated cultures of LAB or isolated microbial enzymes rather than studying sourdough fermentation as a food processing method. That is, these studies do not take into account the whole food system and entire sourdough consortia and are thus not reflective of commercially

available sourdough microbiomes.

Despite the limitation of using pre-selected sourdough-resident isolates rather than whole consortia, these studies have still demonstrated that sourdough-associated LAB, either alone or in conjunction with fungal proteases, can lower gluten content in wheat or rye flours,[7,10] degrade CD-associated immunogenic gluten peptides,[7,9,10,63,181] produce "gluten-free" wheat flour (< 20 ppm gluten),[63,182,183] and detoxify contaminated gluten-free flour.[9] They also produce a reduced toxicity response *in vitro*[9,10,163,184] and *ex vivo* based on jejunal[185] and duodenal biopsies collected from CD patients.[186] Though few studies address how these fermentation-based detoxification methods affect people with CD and other gluten sensitivities, the clinical trials that have been published demonstrate that LAB-detoxified gluten-free flour[9] and wheat flour with gluten hydrolyzed by LAB can be safely fed to some CD[11,186,187] or NCGS patients.[62,69,188,189]

1.6.1 Sourdough and Degradation of Immunogenic Peptides

The proteolytic activity of sourdough organisms is a distinguishing feature of sourdough in comparison to straight dough leavened with baker's yeast. Fluorescent labeling of wheat proteins shows that in comparison to straight dough, fermentation with LAB decreases the concentration of HMW peptides and increases the concentration of dipeptides and free amino acids.[8] The LAB present in sourdoughs can break down both wheat glutelin and rye secalin.[7,10] However, pH-mediated secalin breakdown has also been shown to occur in chemically acidified rye doughs that do not contain microorganisms, as rye and other grains contain endogenous proteases with optimal pH levels below that of straight dough fermentations.[190]

Fermentation with sourdough organisms has been shown to hydrolyze immunogenic peptides.[6,7] Enzyme preparations from common sourdough-associated LAB display greater

gliadin hydrolysis compared to chemically acidified dough.[7] Examples of sourdough-adjacent microorganisms and their enzymes shown to hydrolyze gluten are shown in **Table 1.2**. Enzyme exudates are also able to hydrolyze the 31-43 fragment of gliadin[7,198] as well as α_2-gliadin 57-89 (33mer), which is known for a high prevalence of immunostimulatory epitopes within its structure.[63] Evidence suggests that gluten breakdown begins at approximately 6 hours of sourdough fermentation[75] and that short fermentations do not generate significant changes in gluten content.[199]

Table 1.2: Some gluten-modifying enzymes and their associated organisms.

Organism	Enzyme	Reference
Fungi		
Aspergillus niger	Aspergillopepsin	[191]
Aspergillus oryzae	Dipeptidyl peptidase (DPP)-IV	[192]
Bacteria		
Bacillus spp.	Subtilisin, thermolysin	[193,194]
Lactobacillus spp.	Iminopeptidase, DPP, prolyl endopeptidase, prolidase, prolinase	[195,196]
Bifidobacterium spp.	Prolyl endopeptidase	[197]

Sourdough fermentation can be applied in conjunction with other technologies to increase proteolysis. Along with wheat germination, which activates endogenous wheat proteolytic enzymes, sourdough fermentation was able to reduce gliadin content up to 95%; the two applications together were more effective than either used alone.[200] Similarly, sourdough LAB fermentation has been combined with fungal proteases (standard dough improvers consisting of enzymes extracted from *Aspergillus oryzae* and *Aspergillus niger*) to increase gluten breakdown and hydrolysis of toxic peptides.[181] Dough improvers dramatically improve the effectiveness of protein breakdown, with some studies suggesting that fungal proteases are necessary for

extensive protein hydrolysis in sourdough.[201] Other non-LAB sourdough-associated microorganisms such as *Bacillus* spp. can also effectively carry out gluten hydrolysis. Multiple *Bacillus* isolates were found to degrade the 33-mer and to reduce the content of wheat flour dramatically, although no isolate is known to render wheat dough gluten-free on its own.[202] However, other studies have shown that sourdough organisms can be used to render the gluten content of wheat flour below 20 ppm or to decontaminate gluten-free flours that have been contaminated by wheat or gluten.[63,182]

Foods, including grains, that have been tested and shown to contain less than 20 ppm of gluten can be labeled as gluten-free. However, some non-gluten-containing grains such as oats and buckwheat occasionally become contaminated gluten through harvest or processing.[203] At this time, sourdough fermentation has not been shown to eliminate gluten or toxic gliadin epitopes to the extent that it renders a product safe for patients with CD, although it may reduce NCGS symptoms.[138]

In the first study to demonstrate that sourdough LAB can detoxify contaminated gluten-free flours, wheat flour was mixed with gluten-free flours in a 3:7 ratio and fermented with *Lactobacillus alimentarius* 15M, *Lactobacillus brevis* 14G, *Lactobacillus sanfranciscensis* 7A, *Lactobacillus* hilgardii 51B, and their cytoplasmic extracts (CE). Gluten was found to be almost entirely degraded in the resulting dough after 24 hours according to a peptide profile obtained by RP-FPLC.[9] A liquid ferment of durum wheat was later carried out with the same LAB strains and their CE. The resulting dough was spray dried, then mixed with gluten-free buckwheat flour at a 3:7 ratio and used to make pasta. A commercial R5 ELISA assay showed that gliadin content in the resulting pasta was reduced around 80% compared to that made with unfermented durum wheat. Although the resulting product contained gluten at above safe levels (1045 ppm) the

authors proposed further trials may result in a product reaching safe levels of gluten.[204] In a later study, *L. sanfranciscensis* 7A, LS3, LS10, LS19, LS23, LS38 and LS47, *L. alimentarius* 15M, *L. brevis* 14G, and *L. hilgardii* 51B plus two fungal proteases were used to render a standard wheat flour below 10 ppm. The detoxified flour was used to make pasta with a palatability roughly comparable to commercial durum wheat pasta according to assessment by a trained sensory panel.[205]

In another study demonstrating the potential food safety application of sourdough organisms, *L. sanfranciscensis* LS40 and LS41 and *L. plantarum* CF1 were employed against a *S. cerevisiae* control to test gluten detoxification in gluten-free flour that had been "contaminated" with 400 ppm gluten to mimic processing contamination. A 16-hour fermentation with sourdough-associated LAB resulted in a final gluten concentration below 20 ppm despite the initially high levels of purposeful gluten contamination.[182]

Still other research has shown that sourdough-associated organisms can be used to render wheat flour gluten-free. One study showed that *L. alimentarius* 15 M, *L. brevis* 14G, *L. sanfranciscensis* 7A, *L. hilgardii* 51B were sufficient to reduce levels of gluten in lightly contaminated gluten-free dough to safe levels, but with the further addition of six highly proteolytic strains of *L. sanfranciscensis*, the organism mixture was able to reduce full wheat flour to "gluten-free" levels of 12 ppm. When the 33-mer peptide was incubated with these organisms in a buffer system, HPLC ESI-ion trap MS showed its complete degradation after 18 hours.[181] The same results have been demonstrated in durum wheat. A cytoplasmic extract from *L. sanfranciscensis* 7A, LS3, LS10, LS19, LS23, LS38, and LS47, *L. alimentarius* 15M, *L. brevis* 14G, and *L. hilgardii* 51B, alongside fungal proteases, reduced durum wheat semolina flour to below 20 ppm gluten.[63] Separately, durum wheat flour was rendered gluten-free (below

10 ppm) with these same LAB strains plus fungal proteases and used produced bread at an industrial-capacity bakery. Comparing commercial standard durum wheat flour and to five commercial gluten-free flours, the rendered gluten-free bread performed favorably in terms of sensory and nutritional characteristics.[183] These studies show that some sourdough-associated organisms are strongly proteolytic against proline-rich substrates such as gluten, and that these organisms can potentially be used to aggressively degrade gluten to levels that could be safe for gluten-sensitive individuals while preserving the technological properties of the resulting pasta products. This technique has also been applied to wheat-based bakery products but either they have been processed differently than traditional wheat-based baked goods[186] or the quality of the resulting bakery items was not described or assessed.[187]

1.6.2 Indicators of Reduced Immunogenicity from Sourdough-Fermented Grain

The previous section examined *in vitro* studies investigating the proteolytic capacity of certain sourdough-associated organisms. The above section has covered studies that discuss a change in the concentration of gluten or certain gluten epitopes following treatment with certain sourdough-resident organisms. Now, this review turns to studies that investigate changes in immunogenicity of gluten fermented with sourdough-associated organisms. Immunogenicity is determined by the ability of the gluten or gluten peptides to stimulate a T-cell response. During processing by fermentation, gluten peptides undergo conformational changes that can affect their immunogenicity in ways that do not necessarily track changes in quantity.[206]

1.6.2.1 In Vitro and Ex Vivo Trials

Because a minimum quantity of gluten is necessary for achieving the desirable baking

properties of bread, it is important to examine how sourdough processing affects not only the quantity, but also the toxicity of gluten. Fermentation by sourdough-associated LAB can reduce the immunogenicity of wheat based on *in vitro* cell models of CD and *ex vivo* studies using biopsies collected from individuals with CD.

Enzyme preparations of sourdough-associated LAB showed strong hydrolytic abilities on toxic PT digest of gliadin according to negative agglutination tests on K562(S) cells (a suspension of cells isolated from the bone marrow of a chronic myelogenous leukemia patient), indicating a reduced presence of celiac-relevant antigens in the enzyme-digested gliadin.[7] After 24 hours of fermentation, enzyme preparations of these same LAB, alongside fungal proteases, were able to entirely hydrolyze gluten in a flour made with 30% wheat. The resulting pepsin-trypsin (PT)-digested protein extract showed a minimal agglutinating activity (the protein concentration required to induce agglutination) on K562(S) cells 250 times higher than non-sourdough fermented doughs.[9] These same strains of LAB, when applied to rye flour, where able to entirely hydrolyze rye secalins such that no prolamins were recognized by R-5 Western analysis after 48 hours of fermentation. A PT-digest of protein extract from these *in vitro* fermentations showed a decreased tendency to agglutinate K562(S) cells and a decreased toxicity on Caco-2 cell cultures. In this study, toxicity was indicated by a slowing of cell growth after exposure to the agonist, an increase in caspase-3 (a protease enzyme that induces apoptosis), and elevated levels of nitric oxide (a molecule associated with inflammation and alterations in barrier integrity). The same gliadin applied to CD jejunal biopsies showed no increase in CD3+ intraepithelial lymphocytes (indicative of immune activation and inflammatory activity) or expression of the Fas receptor (associated with apoptosis regulation) compared to controls.[10]

A 10^9 cfu/ml slurry of the established probiotic VSL #3, which contains some organisms

associated with sourdough, was able to completely degrade the 33-mer as well as the toxic gliadin epitope at 62-75 from a starting concentration of 750 ppm of each peptide. Wheat flour treated with VSL #3 and a PT digest did not cause an increase in intraepithelial lymphocytes when applied to celiac jejunal biopsies, and it also showed decreased agglutination activity on K562(S) cells. The cocktail was more effective than any individual strain acting alone.[185]

Wheat flour rendered gluten-free (below 10ppm) by the action of LAB and fungal proteases was tested in a small pilot study of volunteers and on duodenal biopsies of ten young celiac patients. PT-digested proteins from the wheat flour applied to the biopsy samples did not cause release of IFN-g mRNA at levels greater than control.[186] Similar results were found for durum wheat flour rendered gluten-free through the same combination of LAB and fungal proteases. Durum wheat flour is more resistant to protein hydrolysis and so it required a higher concentration of fungal proteases and a longer fermentation time. However, after 72 hours the durum wheat flour was detoxified to below 20 ppm. When applied to duodenal biopsies of celiac patients, PT digests from this durum wheat flour induced IFN-γ and IL-2 expression on duodenal biopsies of celiac patients at levels comparable to negative control.[63]

Some additional organisms have also been investigated. The sourdough-resident organisms *Enterococcus mundtii* QAUSD01 and *Wickerhamomyces anomalus* QAUWA03 were found to degrade gliadin and phytic acid in six strains of wheat better than baker's yeast controls. When applied to Caco-2 cells, gliadin treated with these organisms showed improved cell response in terms of better tight junction formation and lower trans-epithelial electrical resistance (TEER).[184]

In a study investigating the intersection of decreased immunogenicity and organoleptic properties, sourdough fermentation was used to achieve bread of intermediate gluten content.

With approximately 72% the gluten content of typical straight dough, this sourdough fermented dough had eight times higher minimum agglutinating capacity on K562(S) cells compared to full-gluten bread, but it behaved similarly to full-gluten bread in terms of structural, rheological, and chemical properties.[163]

Finally, *ex vivo* trials also demonstrate potential for CD and NCGS patients through the mechanism of sourdough-mediated amylase trypsin inhibitor (ATI) degradation. ATI can induce an innate immune response in CD and NCGS patients and, like gluten, is resistant to gastrointestinal digestion. A recent study demonstrated that sourdough fermentation degraded tetramers of ATI while baker's yeast fermentation did not. The sourdough-fermented sample reduced the release of pro-inflammatory cytokines MCP-1 and TNF-α on the human monocytic cell line THP-1.[207] In fact, the extent of gluten hydrolysis varies more among sourdough-associated organisms than does the extent of ATI hydrolysis, which was universally extensive among 87 isolates recently tested.[208]

These several studies indicate that many sourdough-resident LAB exhibit strong proteolytic action against wheat and rye proteins and can be used to process flour in such a way that it appears to stimulate fewer gluten-mediated symptoms in *in vitro* and *ex vivo* assays. However, in most of these studies, organisms were selected on the basis proteolytic activity after passing screening tests for action against proline-rich substrates or ATI, depending on the study. Only one of the aforementioned studies[207] used a symbiotic consortium of sourdough yeast and bacteria, while the others utilized lab isolates, enzyme extracts, or curated groups of LAB only. As such, it is still unknown whether these conclusions can be extended to wild or commercial

sourdough cultures.

1.6.2.2 Clinical Trials

Although *in vitro* and *ex vivo* evidence may be promising, clinical trials in humans are the only way to ensure the safety of sourdough as a processing tool for gluten reduction or detoxification. Some of the work described above has been extended to clinical trials in humans to examine the effects eating flour treated by fermentation with sourdough organisms. In this way, it is possible to determine if products made from the treated flour trigger gluten-mediated symptoms in gluten-sensitive individuals. If not, it is good evidence that the gluten has been detoxified or degraded to irrelevant quantities.

When *L. alimentarius* 15M, *L. brevis* 14G, *L. sanfranciscensis* 7A, and *L. hilgardii* 51B and their CE were used to "detoxify" a 3:7 ratio of wheat to gluten-free flours (imitating a scenario in which the gluten-free flours had been contaminated), the resulting bread was tested in 17 CD patients for 2 days. The patients had all tested negative for anti-TG2 antibody and had been following a gluten-free diet for at least 3 months. Participants were randomly given 80g of one of the breads (either the sourdough treatment or a non-sourdough control) each day for two days. Six hours after eating the bread each day they ingested a rhamnose, lactulose, and sucrose solution that allowed researchers to measure intestinal permeability. When fed baker's yeast-fermented bread, 13 of the participants showed a rhamnose/lactulose absorption pattern that indicated an increase in intestinal permeability, but they showed no difference from baseline when fed the sourdough-fermented breads.[9]

A small pilot study later examined the safety of feeding CD patients wheat flour processed with these organisms (*L. alimentarius* 15M, *L. brevis* 14G, *L. sanfranciscensis* 7A, and *L. hilgardii* 51B plus fungal proteases). The study used these organisms to reduce wheat

flour to gluten levels below 10 ppm according to R5 antibody-based sandwich and competitive ELISAs, then the authors investigated whether CD patients could safely consume baked goods made from the sourdough-treated flour.[186] This treated flour was spray-dried and used to prepare baked goods which were then subjected to trial in 8 young CD patients (in remission) for 60 days, with hematology, serology, and intestinal permeability measurements taken before and after the trial. Results indicated that the participants did not show a gluten response to the treated flour; the young celiac patients fed the sourdough-fermented wheat dough did not produce IFN-γ mRNA at levels greater than those shown in duodenal mucosa from healthy subjects. However, study authors recommended a larger clinical trial before a former recommendation is made on the use of this technology for gluten reduction in flour for CD patients.[186]

In another 60-day trial, a small cohort of 16 CD patients were divided into three groups. Members of each group were fed 200 g daily either bread fermented with baker's yeast; bread fermented with *L. alimentarius* 15M, *L. brevis* 14G, *L. sanfranciscensis* 7A, LS3, LS10, LS19, LS23, LS38, LS47 and *L. hilgardii* 51B; or bread fermented with all of these LAB and plus fungal proteases. All patients fed baker's yeast bread developed symptoms and increased levels of anti-TG2 antibodies and anti-endomysial antibodies, greater lymphocyte infiltration, and mucosal atrophy—all markers of CD. In contrast, patients fed on the dough fermented by LAB only showed no clinical symptoms, but three of the four showed changes in intestinal mucosa, including subtotal atrophy and an increase in intra-epithelial lymphocytes. Meanwhile those fed on the dough fermented with LAB and fungal protases showed no symptoms or indications of CD.[187]

Later, another small cohort of CD patients was fed a diet of 200 g/day of wheat flour with protein partially (n=2) or fully (n=5) hydrolyzed via LAB fermentation and fungal proteases.

Only *L. alimentarius*, 15M, *L. brevis* 14G, *L. sanfranciscensis* 7A, and *L hilgardii* were used to prepare extensively hydrolyzed flour, while six strains of additional heavily proteolytic *L. sanfranciscensis* (LS3, LS10, LS19, LS23, LS38, and LS47) were added to prepare the fully hydrolyzed flour. Patients consumed about 200 g daily of baked goods made with either the fully or partially hydrolyzed flour. Participants consuming the diet of fully hydrolyzed flour had no clinical complaints, no increase in TG2 levels, and no change in Marsh grades of small intestinal mucosa throughout the 60-day study. The two patients consuming a diet of partially hydrolyzed wheat flour similarly had no clinical complaints, but both developed subtotal atrophy.[11]

In a shorter six-day trial of 20 participants, those who consumed baked goods produced with flour hydrolyzed by fermentation with fungal proteases and *L. alimentarius*, 15M, *L. brevis* 14G, *L. sanfranciscensis* 7A, and *L hilgardii* plus the proline-targeting strains of *L. sanfranciscensis* (LS3, LS10, LS19, LS23, LS38, and LS47) did not exhibit an increase in natural gluten reactive T-cells in the blood even in the presence of high concentrations of gliadin. T-cells that reacted with gluten were found to be elevated in those consuming natural wheat flour baked goods, as is expected with the consumption of gluten in celiac patients.[209]

Other trials indicate that fermentation by particular sourdough LAB may help relieve symptoms of NCGS/IBS. In a crossover study in 87 IBS patients consisting of two four-week treatment periods, participants were asked to eat 7-8 slices per day of a commercially supplied sourdough-fermented rye bread high- or low-FODMAP rye bread of unknown microbial composition. Study authors claim that a fructan-consuming Lactobacillus strain was present in the formula for one of sourdough starters, causing it to result in low-FODMAP bread; this strain is not named. In a randomized, double-blind crossover study, the 87 participants received either the low-fodmap sourdough rye bread or regular sourdough rye bread, with a washout period of 2

weeks in between. Low-FODMAP sourdough bread caused statistically lower symptoms of flatulence, abdominal pain, stomach rumbling, and overall symptom experience. However, compliance and bread intake varied over participants and the study did not control the background diet.[189]

In a later study, partially gluten-reduced dough fermented by *L. sanfranciscensis* 7A, LS3, LS10, LS19, LS23, LS38 and LS47, *L. alimentarius* 15M, *L. brevis* 14G, and *L. hilgardii* 51B plus fungal proteases was made into wheat bread and durum wheat pasta and tested in a randomized double blind clinical trial against bread and pasta fermented with baker's yeast alone. Participants were assessed for reduction of symptoms of IBS, with ten participants in each group. Half of the patients showed a decrease of at least 30% in VAS (Visual Analogue Scale) and/or IBS-SS (Irritable Bowel Syndrome Severity Score) scores. No differences were found in Hospital Anxiety and Depression scores or quality of life between the two treatments.[188]

Beyond the effects observed on gluten and CD, sourdough fermentation has been shown to render breads more digestible, at least in healthy volunteers. In one crossover study in 17 healthy adults, commercially produced sourdough-fermented croissants conferred improved post-prandial gastrointestinal function over yeast-fermented croissants from the same source. Sourdough croissant consumption reduced feelings of hunger, fullness, nausea, bloating and abdominal discomfort compared to yeast pastry consumption. In this study, microbial composition of the commercial sourdough was evaluated by not reported.[69] In another study of 36 healthy volunteers, bread made from flour fermented with *L. plantarum* CR1, *Lactobacillus rossiae* CR5 and *S. cerevisiae* E10 compared favorably to straight bread in terms of appetite stimulation and satiety reduction. Sourdough bread also spent less time in the orocecal transmit route, with longer fermentation having an indirect relationship to length of time spent in transit.

Sourdough breads had the lowest values for post-prandial glucose responses and the highest free amino acid levels and ingestion of sourdough correlated with longer circulation time of amino acids in blood plasma. Although these measurements were taken in healthy volunteers, results indicate encouraging possibilities for individuals suffering from NCGS.[62]

These studies show promising results for the potential of sourdough fermentation as a therapy for CD by gluten reduction or by breakdown/modification of immunogenic epitopes and for NCGS by gluten and FODMAP reduction, ATI degradation, and increased digestibility. An eventual commercial application of this concept might involve identifying fermentation organisms and conditions under which the toxicity of gluten (or other components) for CD/NCGS patients is reduced while gluten quantity sufficient to achieve good bake quality is simultaneously maintained.

1.6.3 Indications of Caution for Sourdough as a Treatment Option for Gluten Sensitivity

Despite these encouraging results, not all studies endorse the therapeutic possibilities of fermentation with sourdough organisms. In a study investigating TG2 binding potential of gliadin from sourdough breads compared to gliadin from standard wheat breads, it was found that fermentation with *L. plantarum* reduced overall interaction between TG2 and gliadin by decreasing transamidation of the peptide. However, it also alters the conformation of the peptide such that existing QLP binding motifs (which make gliadin peptides attractive binding targets for HLA-DQ molecules) on the 33-mer peptide are more exposed. Crucially, this means that fermentation with *L. plantarum* could increase wheat toxicity to CD patients even if it reduces gluten content overall. Other *lactobacilli* decreased QLP binding availability but not significantly.[210]

Although the complete mechanism of benefit that sourdough may have for NCGS/IBS

patients is unknown, some research has pointed in part to that improved digestion and post-prandial response.[62,69] Yet, other studies have been unable to demonstrate positive post-prandial response from sourdough. In 24 healthy adults recruited for a randomized crossover trial of sourdough-fermented, yeast-fermented, and unfermented rye breakfast crispbreads, results showed that sourdough crispbread consumption reduced hunger and desire to eat. However unfermented crispbread resulted in the lowest insulin response and no significant difference was found in postprandial glucose response between the three treatments.[64] Furthermore, in a trial testing sourdough-fermented wheat bread administered to IBS sufferers for one week, patients experienced no significant change in symptoms. Although the sourdough was lower in FODMAPs and ATI than control bread, this had no impact on gastrointestinal symptom scores or inflammatory marker levels. In fact, the consumption of the sourdough bread caused an increase in non-gastrointestinal symptoms.[139] Another crossover trial of industrial white or artisanal sourdough bread consumption in ten subjects did not find a significant difference in any clinical parameters, including glycemic response and microbiome changes.[211]

Other studies simply indicate that conclusions based on one sourdough microbiome may not be translatable to other microbiomes. For example, when sourdough's ability to break down wheat germ agglutinin, a protein thought to contribute to NCGS symptoms, was investigated, results were heavily dependent on the presence of specific organisms and could not be extrapolated to all sourdough fermentations.[138]

In the only attempt to so far to examine consumer-ready sourdough, Ogilvie et al. [212] examined a single artisanal sourdough culture for its proteolytic ability compared to a rapid baker's yeast fermentation. Both breads were mixed and fermented according to standard protocols with the same raw ingredient ratios and then baked into breads; gluten was isolated

from the breads and subjected to INFOGEST digest. Total gluten concentration was compared along with concentration of six reactive immunogenic peptides, including the 33-mer. Baking decreased levels of α/β- and γ-gliadin, but not ω-gliadin, in both bread types. The authors postulate that during fermentation, α/β -gliadin and γ-gliadin, the sulfur-rich components, do not degrade but rather undergo disulfide cross-linking which increases their later digestion steps and degradation in baking. Meanwhile sulfur-poor ω -gliadin does not undergo cross-linking and therefore remains unchanged during baking. Levels of α/β- and γ-gliadin decreased more in the fast fermentation baked bread (85% and 89% respectively) than in the sourdough bread (a 60% and 55% decrease), meaning that more gliadin was extracted after baking from the sourdough than from the fast ferment control. The immunogenic peptides appeared in both doughs during the intestinal phase of digestion. Thereafter, sourdough fermentation slightly decreased levels of five of the six immunogenic peptides, but these five peptides (including the 33-mer) underwent a greater degree of breakdown in the fast ferment with baker's yeast. Neither ferment reduced concentration of the sixth peptide significantly. Based on this study, the authors suggest that sourdough likely contains similar levels of immunogenic peptides as bread fermented with baker's yeast, although they warn that other cultures must be screened.[212]

1.7 Gaps in the Literature

While *in vitro*, *ex vivo*, and clinical studies mentioned have shed some light on the potential for sourdough fermentation of wheat bread products as a technology to alleviate symptoms of gluten intolerance, studies so far have focused heavily on isolated or optimized cultures and in some cases on enzymatic preparations from these cultures. Yet it is unknown if results attributed to the highly curated cultures that have been studied so far under laboratory conditions are translatable to fermentations performed by mixed cultures in a commercial setting.

At least some gluten peptides, possibly immunogenic in nature, are likely to remain in non-optimized commercial fermentations.[212] Therefore, current data is only sparingly useful to health practitioners without translation to models more representative of food systems likely to be encountered by real consumers. Furthermore, few studies have addressed the effect of sourdough fermentation on features of gluten processing, such as deamidation, or structural changes which might expose new binding epitopes.

Secondly, most studies carried out so far have focused on time, temperature, or culture conditions intended to maximize gluten breakdown with only limited reference to the resulting bread (or pasta) organoleptic quality. Because gluten is essential for the functionality of wheat products, these two goals are often in opposition. However, if a microbiome could be identified that has potential to reduce gluten toxicity without degrading gluten quantity below the lower limit of 20,000 ppm that allows for making bread of acceptable quality,[163] it would be a therapy with immediate commercial utility.

Instead of limiting investigation to a select, curated group of organisms, the next wave of research should investigate fermentations performed by commercially viable mixed sourdough cultures of the type that are used to prepare bread products for sale to the public. These studies should investigate not only overall gluten content as a measurable outcome of sourdough fermentation but also the presence/absence of specific immunogenic epitopes as well as functional indicators of gluten's toxicity and ability to participate in a pathogenic cascade, for example by TG2 binding affinity.

Further research should also take advantage of work that has already proven the ability of sourdough-associated organisms to break down gluten at elevated fermentation times and temperatures by attempting to adapt these conditions to a commercial breadmaking process.

Because many of these studies have been conducted at fermentation time/temperature conditions that are within the range of recorded sourdough baking practices but outside the empirical norm, it remains to be seen how fermentation conditions targeted to reduce gluten immunogenicity might simultaneously affect other aspects of bread quality, and what role the sourdough microbiome might play in these outcomes.

1.8 Conclusion

Under certain conditions, sourdough fermentation has been demonstrated to be an effective tool for the breakdown of gluten, the digestion of toxic gliadin epitopes, the detoxification of gluten from wheat and contaminated gluten-free flours, and the mediation NCGS drivers such as FODMAPs and non-gluten peptides. Further work is needed to validate these studies in clinical settings. The true efficacy of sourdough as a commercial-scale tool for the mediation of symptoms of celiac disease and NCGS remains to be seen and requires research into consumer-ready cultures and products, which is so far lacking in the literature.

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CHAPTER 2: HYPOTHESIS, SIGNIFICANCE & INNOVATION

2.1 Hypothesis and Specific Aims

Fermentation with sourdough microorganisms has been investigated as a strategy for reducing gluten content in wheat and rye[1,2] or degrading immunogenic and allergenic peptides from wheat and rye that provoke symptoms in sufferers of celiac disease (CD) and non-celiac gluten sensitivity (NCGS).[1–5] However, these studies have mainly relied upon single-culture isolates, curated co-cultures, or enzyme extracts, none of which accurately reflect the complex microbiomes used by home or commercial bakers. Consequently, their findings lack applicability to the diverse microbial communities found in the sourdough starters used to make high-quality baked products. Research on intact commercial sourdough microbiomes shows that the community structure of the microbiome affects bake quality attributes of the resulting bread, such as aroma and rate of gas production.[6] Based on these gaps in the literature, we sought to conduct further research on intact commercially viable sourdough microbiomes to explore their impact on both gluten breakdown and bread quality.

To date, the literature available that characterizes relationships between sourdough fermentation and gluten proteins has largely focused on manipulating the fermentation process to maximize gluten breakdown and detoxification, with only sparing attention given to the quality and acceptability of the resulting products. Thus, the potential for practical application by food producers and consumers alike remains limited. Further investigation is needed to determine if sourdough fermentation can be carried out under conditions that detoxify gluten for CD/NCGS therapy while simultaneously maintaining the desirable qualities of the resulting baked goods.

Based on the existing literature, it is not yet known if sourdough fermentation can

achieve good quality bread while also detoxifying gluten to a degree that makes sourdough tolerable for gluten-sensitive consumers. However, public perception has already embraced this hypothesis as fact. Numerous consumer blogs, social media sites, and websites advocate sourdough (both commercially produced and homemade) as a healthier alternative to yeast-leavened bread for consumers with gluten sensitives. Similar beliefs have been advanced by hobbyist and professional sourdough bakers who endorse sourdough as a therapy for gluten-sensitive customers. Despite this, a link has not yet been firmly established between commercially produced sourdough bread designed to optimize bread quality (instead of using lab conditions designed to maximize gluten degradation) and symptom improvement. Therefore, it is essential to gain more clarity about gluten-sensitive consumers' perceptions of sourdough bread and its impact on symptom alleviation.

Based on this background information, I hypothesized that 1) community structure of sourdough microbiomes would be a determining factor in degree of gluten breakdown, loss of gluten toxicity, and overall bread quality, and 2) both bakers and gluten-sensitive consumers would perceive commercial sourdough as healthier and as less likely to trigger gluten-mediated symptoms than yeast-leavened bread.

The hypothesis was tested through the following aims:

1. Investigate the influence of 20 different complex, intact, and commercially viable sourdough microbiomes on bread quality in comparison to a control bread leavened with baker's yeast (**Chapter 3**).
2. Conduct a survey of gluten-sensitive consumers to gather data on their sourdough consumption habits and sourdough's effect on their gluten-mediated symptoms. Discover the role of diagnosis, demographics, sources of information, and social support on

participant's beliefs and habits regarding sourdough bread (**Chapter 4**).

3. Survey commercial sourdough bakers to explore their understanding of sourdough's therapeutic effects and its alignment with gluten-sensitive consumers' beliefs and experiences, and investigate the bakers' role in disseminating accurate or misleading information about sourdough to consumers (**Chapter 5**).
4. Evaluate the toxicity of gluten extracted from bread fermented by 20 different sourdough microbiomes *in vitro* (**Chapter 6**).

2.2 Significance & Innovation

Gluten-related disorders, such as CD and NCGS, exhibit a global prevalence of around 10%[7] and currently can be treated only by compliance with a gluten-free diet.[8] Even for those who maintain diligent gluten avoidance strategies, accidental exposure is a significant concern[9] because gluten frequently contaminates gluten-free grains, leading to possible daily contaminating doses above safe limits.[10] Avoidance is further complicated owing to gluten's widespread use as a functional food additive in preserves, meat, seafood, and more.[11] Additionally, gluten-free foods are harder to find, more expensive, and of lower nutritional value than standard wheat-based products.[12] As such, there is a pressing need for solutions that expand the dietary options of CD and NCGS patients. By developing technologies that improve access to gluten-reduced or lower-toxicity foods with adequate nutritional and sensory properties, we could reduce the burden of millions of people globally who are affected by CD and NCGS.

The approach taken in this study deviates from previous studies focused on sourdough fermentation as a technology to produce CD- and NCGS-safe produces in several key ways. First, it moves away from using lab-designed cocultures or single-organism fermentations and instead focuses on intact, microbially complex, symbiotic starter microbiomes sourced from

home or commercial bakers. While previous studies, which use single-organism isolates or lab-curated co-cultures, are very informative about the capabilities of individual organisms under specific conditions, they provide little insight about real, complex sourdough microbiomes. Commercially viable sourdough starters typically consist of many interacting yeast and bacteria species.[6] More than 50 species of bacteria and 20 species of yeast can fill each available ecological role in a sourdough starter, leading to numerous possible combinations and countless interactions.[13] While previous work has made great strides in determining the capacity of several of these organisms to degrade gluten, it remains unknown whether the same conclusions could be extended to complex and commercially viable sourdough microbiomes, considering their inherent stressors and interactions. This study aims to bridge that gap.

Another innovative aspect of this approach is its focus on the interplay between gluten detoxification and bread quality. While previous research has explored the use of sourdough-associated organisms to detoxify gluten, these studies have rarely followed up to investigate whether the same organisms, process, and conditions can also yield high-quality baked goods with desirable attributes. In the few exceptions where such studies exist, they either focus on non-baked goods such as pasta[14] or they use a process that does not reflect commercial sourdough-making, such as producing yeast-leavened baked goods from LAB-treated flour.[15,16] To date, it is not yet understood if sourdough, as it is typically made by home bakers and commercial bakeries, can simultaneously achieve high-quality wheat bread *and* render wheat gluten more tolerable for gluten-sensitive consumers. The role that microbial community structure might play in these outcomes is also poorly studied. In a novel approach, this study will employ time and temperature fermentation conditions designed to promote gluten detoxification while also aiming to achieve desirable bread products. We will investigate both quality outcomes

and toxicity effects of fermenting wheat flour with 20 different sourdough microbiomes. Our goal is to find correlations between the outcomes of these studies and the community structure of the microbiomes used to ferment the dough.

Lastly, this research addresses the anecdotally supported claim that eating commercial sourdough bread leads to a reduction in gluten-mediated symptoms compared to eating yeast-leavened bread. Despite apparently widespread acceptance of this claim in blogs, social media sites, and even some research articles, it is so far unsupported by data. This study aims to collect the first empirical data on the subject by conducting a survey of over 1,000 gluten-sensitive individuals. On the assumption that sourdough bakers might be important links in the chain of information transmission, we were also interested in exploring the extent to which sourdough bakers discussed the subject of sourdough with their gluten-sensitive customers and also what feedback they received from those consumers on the topic of sourdough. Therefore, we also surveyed a smaller number of professional sourdough bakers. The combination of these two surveys will provide the first ever data on gluten-sensitive consumers' experience with sourdough bread as well as factors that influence their sourdough eating habits an. It will also offer insight into the extent to which sourdough bakers participate in spreading the information and beliefs that their customers hold.

Ultimately, this study will shed light on gluten-sensitive consumers experiences with commercial sourdough bread as well as the feasibility of using microbially complex and commercially viable sourdough microbiomes to produce high-quality baked goods with reduced gluten toxicity. Finally, this study will associate these outcomes to the makeup of the microbial community structure in a way that no previous study has been able to do.

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CHAPTER 3: SOURDOUGH STARTER CULTURE MICROBIOMES INFLUENCE PHYSICAL AND CHEMICAL PROPERTIES OF WHEAT BREAD

3.1 Summary

Sourdough starter cultures comprise diverse communities of bacteria and fungi that drive fermentation to acidify dough and produce the carbon dioxide which contributes to bread rise. While specific genera and species of microorganisms have been identified from starters and associated with specific attributes, the overarching relationship between sourdough starter culture microbiomes and the physicochemical qualities of sourdough fermented dough and bread have not been defined. Sourdough starters (n=20) of known microbial populations were used to produce wheat-based dough and bread which were analyzed for chemical and physical properties. These data were then combined with to the 16S and ITS rRNA sequencing data in order to elucidate relationships between microbial taxa and dough/bread qualities. Sourdough doughs and breads were distinct in most parameters from the Baker's yeast control. Understanding of the relationship between sourdough starter populations and quality parameters can lead to targeted development of sourdough bread products with specific physical and chemical properties.

3.2 Introduction

Sourdough bread is an ancient food product made by the fermentation of dough with naturally occurring "starter cultures", or communities of bacteria and yeasts. These microbial communities of are responsible for the fermentation of carbohydrates in flour to produce the carbon dioxide (CO_2) that causes bread dough to rise. In conventionally produced bread, a single

known strain of yeast (*Saccharomyces cerevisiae;* "Baker's yeast") is added to the dough for leavening via CO_2 production. Sourdough starter cultures, on the other hand, comprise wild yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) endogenous to the flour they were created with and the environments in which they were produced.[1] These wild yeasts produce CO_2 to leaven bread similarly to *S. cerevisiae*, but they and the bacterial communities in the starter are also known to produce a variety of metabolic byproducts that drive quality differences and sensory attributes between conventional dough and sourdough breads.[2]

Organic acids are perhaps the most notable metabolic byproducts of sourdough in comparison to conventional dough due to their noticeable effect on bread flavor. Lactic acid and acetic acid are produced by LAB and AAB, respectively. These acids create a low pH environment, typically between pH 3.5-4.3 in comparison to pH 5.0-5.5 observed in a straight dough.[3] From the perspective of food safety and shelf life, this decrease in pH can limit the growth of spoilage microorganisms over the course of fermentation and during storage.[4] The greater acidity observed in sourdough breads also facilitates enzymatic hydrolysis of proteins and starches by enzymes endogenous to flour and those produced by the starter culture, which can influence the sensory characteristics of bread.[5] Enzymatic hydrolysis of dough proteins liberates free amino acids which can participate in the Maillard reaction, producing colored pigments and volatile aroma compounds which affect crust color and flavor, respectively.[6,7]

Sourdough starters are known to be diverse in terms of their microbial profiles; while core groups of microorganisms such as yeast, LAB and AAB are always present, the precise species diversity and abundance of organisms that appear in sourdough starters can vary widely between individual starter cultures. A recent comprehensive study characterizing 500 sourdough starter cultures collected from North America, Europe and Australasia demonstrated that the

presence of specific LAB and yeasts are predicted by climatic conditions such as storage temperature and seasonality, while others are predicted by origin (i.e., commercial vs. personal), starter age, and flour (i.e., whole wheat vs. not whole wheat).[1] Specifically, *S. cerevisiae* was noted as the most prevalent yeast species across all samples, and LAB populations were often dominated by *Lactobacillus sanfranciscensis* or coordinated populations of *Lactobacillus plantarum* and *Lactobacillus brevis*. While the diversity of flavor and quality of sourdough breads is widely appreciated as a positive attribute of these artisanal products, little is known about how diversity and abundance of microorganisms in sourdough starter cultures impact specific physical and chemical properties of final breads.

The contributions of individual microorganisms towards bread quality have been studied in controlled environments, such as the addition of selected LAB isolates to conventional dough fermentation. For example, doughs fermented with a commercial *S. cerevisiae* and supplemented with select strains of *L. sanfranciscensis* and *L. plantarum* have been shown to slow staling due to their contributions of α-amylase, while *Lactobacillus hilgardii* has demonstrated the same benefit due to the production of EPS.[8] The proteolytic properties of various LAB have been explored similarly.[9] While useful in delineating the functional potential of these isolates, studies such as these eliminate the inherent complexities of sourdough cultures as they exist in homes, bakeries, and food processing facilities; true sourdough starter cultures are diverse, dynamic communities of organisms that interact with one another in a complex growth environment. The importance of this is underscored by the apparent antagonistic effects that some cultures can have on one another; sample doughs fermented with *L. brevis* and *L. plantarum* contained greater final concentrations of starch, maltose, and fructose when *Lactobacillus amylovorus* or *Pediococcus pentosaceus* were added to the ferment, which is likely caused by competition for

substrate and decreased activity of heterofermentative LAB.[10]

Notably, nearly all studies examining the relationship between microorganisms from sourdough starter cultures and bread quality have focused on yeasts and LAB. However, differences in the production of volatile aroma compounds and dough rise in microscale fermentations of real sourdough starters have been shown to be associated not with the predominant yeasts and LAB populations, but by the variable population and abundance of AAB including *Acetobacter.*[1] This suggests that non-dominant microorganisms can influence important quality parameters in sourdough fermented products and demonstrates the need to examine microorganisms that comprise sourdough starter cultures as complete communities rather than isolated strains.

Despite the known contributions of specific bacteria and yeasts to bread quality and the established associations between the abundance and growth of pairs or organisms to one another, it is unknown how the overall microbial consortia of sourdough starter cultures influence bread quality. The objective of the present study was to analyze and compare the physical and chemical properties of dough and breads on the basis of the microbial profiles of the sourdough starter cultures from which they were prepared. We hypothesized that sourdough starter culture microbiomes would drive differences in physical and chemical properties of dough and breads. Twenty previously characterized sourdough starter cultures[1] were used to ferment wheat doughs. The doughs were analyzed for chemical attributes (pH, titratable acidity, water activity, free amino acids,) and subsequently baked into breads, which were analyzed for physical properties (volume, texture, color) relative to a control bread prepared with a commercial strain of *S. cerevisiae*. We demonstrate that significant differences exist between sourdough and control doughs/breads, and also between individual sourdough samples. Hierarchical clustering of

physical and chemical properties of sourdough indicate that sourdough samples can be grouped into six clusters according to the similarity of their physical and chemical properties. *Lactobacillus, Pediococcus, Acetobacter Gluconobacter,* and *Enterobacteriaceae* were predominant ASV mapped to bacteria while *Saccharomyces, Wickerhamomyces,* and *Kazachstania* were predominant genuses in the fungal populations. Correlation analyses suggest that fungal populations could be driving loaf volume and compression while bacterial populations contribute to pH, TA, and loaf volume.

3.3 Materials and Methods

3.3.1 Starter Culture Preparation

Twenty sourdough starters collected from an international survey[1] were generously donated to this study by Dr. Ben Wolfe at Tufts University. These starters were propagated from 5 g aliquots to > 200 g by combining the starter with 10 g of a 1:1 w/w mixture of autoclaved deionized water (dH_2O) and King Arthur brand all-purpose flour (King Arthur Baking Company, Inc., Norwich VT, USA) in loosely capped 50 mL polypropylene tubes for 24 h. The starters were grown to a sufficient volume for use in baking by transferring each starter to an autoclaved glass jars before the immediate addition of 40 g of 1:1 flour and water. This was followed by two subsequent feedings of 120 g and 200 g of 1:1 flour and water after 24 h and 48 h, respectively.

3.3.2 Dough Preparation & Baking

Eight hours after the final propagation step, each starter was used to prepare 1 kg bread dough (65% hydration) by combining 200 g of sourdough starter with 400 g King Arthur all-purpose flour, 225 g autoclaved dH_2O, and 12 g non-iodized kosher salt (Morton Salt, Chicago

IL, USA). A control dough of the same hydration was prepared by combining 665 g King Arthur all-purpose flour, 430 g autoclaved MilliQ water, 12 g non-iodized kosher salt, and 10 g active-dry yeast (ACH Food Companies, Inc., Oakbrook Terrace IL, USA).

All doughs were prepared in sanitized 5-quart bowls attached to KitchenAid mixers (KitchenAid, Benton Harbor MI, USA) and kneaded for 10 minutes with a dough hook attachment. To ensure complete incorporation of ingredients, the sides of each bowl were scraped after 3 and 7 min of kneading. Kneaded doughs were transferred to autoclaved 4-quart glass bowls, covered with plastic film, and fermented in environmental chambers (Caron Products and Services, Inc., Marietta OH, USA) at 35°C and 30% relative humidity for 24 h. The yeast control dough fermented under the same conditions for 2 h, as *S. cerevisiae* produces CO_2 quickly and requires only a short rise time per manufacturer's instructions.

Each fermented dough was separated into three 150 g portions which were formed into loaves and placed in aluminum loaf pans with dimensions 3.5 x 2.5 x 6 inches (MontoPack, USA). All loaves were proofed for 3 h prior to baking at 180°C for 40 min in the center of a conventional oven (General Electric, Boston, MA). The remaining dough was refrigerated at 4°C until chemical analysis, then the remaining portion was lyophilized (Labconco, Kansas City, MO) for gluten digestion analysis. Breads were cooled to room temperature after cooking and analyzed for physical attributes immediately after cooling. A simplified workflow diagram of the

analyses conducted from these samples is shown in **Figure 3.1**.

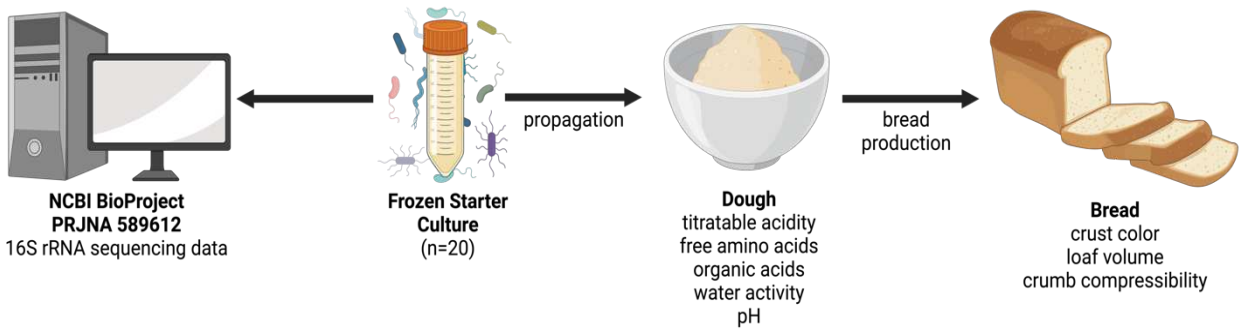

Figure 3.1: Workflow of sample collection and analysis. 20 frozen starter cultures were propagated from 5 g total material to 200 g total material through four feedings of flour and water (1:1). The starter was combined with flour and water to produce a dough, which was kneaded and left to ferment for 24 h at 35°C. Dough samples were reserved for the indicated analyses. The remaining dough was baked at 180°C for 40 min and underwent the indicated analyses immediately after cooling.

3.3.3 Dough Analysis

3.3.3.1 pH and Titratable Acidity

10 g of each dough was weighed and combined with 100 mL of deionized water in a sanitized commercial food blender (Oster, Owosso, MI USA), then transferred to a glass beaker for mixing on a magnetic stir plate. The pH was recorded while mixing using an Accumet AB150 pH meter (Fisher Scientific, Hampton, NH USA). Titratable acidity of the diluted dough solution was measured by titrating 0.1N NaOH into the solution until the pH of the mixture reached 8.4. The volume of the 0.1N NaOH used for titration was recorded and titratable acidity was calculated based on the equivalent weight of lactic acid and reported as MilliEqu NaOH.[11]

3.3.3.2 Water Activity

The bottom of a plastic sample boat was coated with a dough sample and then inserted into a calibrated Aqualab model 3TA water activity meter (Aqualab, Pullman, WA USA). The

meter auto-adjusted to room temperature for measurement.

3.3.3.3 Ninhydrin Assays for Dough Free Amino Acid Levels

Free amino acid concentration was measured in lyophilized dough from each sourdough sample via ninhydrin assay. Dough was first subjected to extraction by dissolving 50 mg of lyophilized powder into 200 μL trichloroacetic acid and 15.5 μL ethanol. The solution was shaken for 60 minutes, then centrifuged at 7700 x g for 15 minutes before reserving the supernatant for analysis. 10 μL of supernatant was mixed with 100 μL of 2% ninhydrin reagent (Santa Cruz Biotechnology, Dallas, TX), heated to 99°C for 10 minutes, then cooled to 4°C. Samples were then combined with 225 μL of 10 mM carbonate buffer (pH 10.0). Absorbance was read at 570 nm. Free amino acid concentration was determined based on a standard curve of L-leucine (Thermo Scientific, Waltham, MA).

3.3.4 Bread Analysis

3.3.4.1 Texture

Two 1-inch slices were removed from the center of each bread load and used for texture analysis. Force of compression was accessed with a TAXT2 texturometer (Stable Microsystems, Goldalming, England) with a one-inch acrylic probe, using a test force of 7 g and a test distance of 6.2 mm. The maximum force of compression was identified and recorded using Texture Expert Exceed Version 2.64 software.

3.3.4.2 Color

A 0.5” sample of crust from the end of each loaf was placed into the sample cup of a

calibrated HunterLab ColorFlex Colorimeter (Model 45/0; Reston, VA). L*, a* and b* values were recorded for each sample. ΔE was calculated using the updated CIE 2000 formula in the ColorTools plugin for Microsoft Excel, which accounts for degree of detectable difference by human evaluators.[12]

3.3.4.3 Loaf Volume

Loaf volume was assessed via rapeseed displacement according to AACC standardized methods, substituting short grain white rice for rapeseed.[13]

3.3.4.4 Microbiome analyses

Raw sequence reads for our donated starter cultures were retrieved from the publicly available data set (NCBI BioProject Accession # PRJNA589612) and analyzed with the R package DADA2 v3.14 pipeline following standard protocol for 16S rRNA v4 and ITS region amplicon sequence variants (ASVs).[14] Low-quality sequence reads were filtered, low-quality bases were trimmed, calculation of error rates were performance, and ASVs were deduced from the remaining sequence reads. Paired-end sequence variants were merged into contigs where contigs <251 bp or >253 bp were removed. Remaining ASVs were assigned taxonomy using the Silva database v132 for bacteria[15] and the UNITE database for fungi.[16] ASVs assigned to mitochondria or chloroplasts were filtered out from the bacterial taxa and reads unassigned at the phylum or class levels. Based on minimum read counts in the 20 sourdough sample microbiomes used, 1800 bacterial reads per sample and 7000 fungal reads per sample were used for downstream analyses. The compositional analysis approach[17,18] detected ASVs with zero count values in a sample and replaced them with a small, non-zero values using the R package vCompositions v1.3.4.106 followed by a center log transformation (CLR). Principal Component

Analysis (PCA) was performed on log-transformed data to visualize microbiota composition by 20 sourdough starter samples. Beta diversity was estimated using Aitchison distances from CLR-transformed data. Taxonomic composition for mapped reads above 1% were plotted using the R package ggplot2 v3.3.5.[19] Each microbiome was assessed for species diversity and richness according to Chao1,[20] Simpson,[21] and Shannon[22] indices. All microbiome analyses were conducted in R v4.1.0.

3.3.4.5 Statistical Analysis

To assess and visualize similarities across samples based on dough and bread properties, we performed a principal component analysis (PCA) and hierarchical clustering based on pairwise distances[23] calculated using Euclidean distances followed by Ward's minimum variance clustering[24] (hclust, method = ward.d2) using the ggplot2 package FactoMineR in R v4.1.0. All statistical analyses and correlations were performed using GraphPad Prism version 10.0 for Mac (GraphPad Software, San Diego, CA USA). A one-way ANOVA followed by a Tukey's HSD was performed to determine statistical significance between group means for measurements of all physical and chemical properties of dough and breads ($p < 0.05$). Correlations were calculated using Spearman's ρ test for non-parametric data.

3.4 Results & Discussion

3.4.1 Chemical Attributes of Dough

For measurement of pH and TA, 10 g of each dough sample was mixed into 100 g of dH_2O. In general, the pH and TA of all sourdough samples were distinct from the yeast control. All sourdough samples measured significantly lower in pH (**Figure 3.2 A**) and significantly

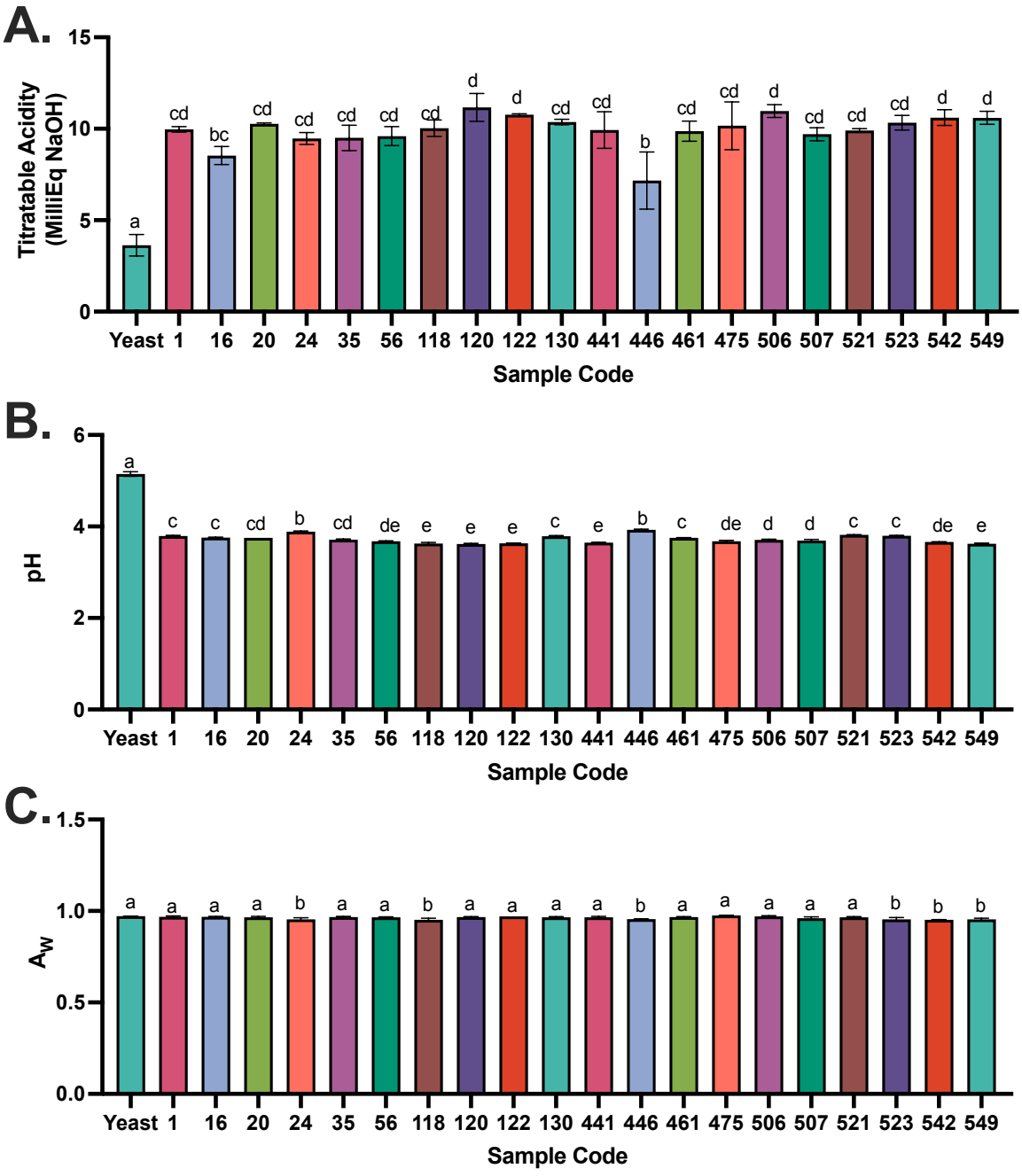

Figure 3.2: Measured characteristics of dough including (A) titratable acidity, (B) pH, and (C) water activity. All tested parameters demonstrated significant differences between sourdough and yeast doughs. Values are expressed as mean ± standard deviation. Different letters within graphs indicate significant differences ($p \leq 0.05$).

higher in titratable acidity than the yeast control (**Figure 3.2 B**). The yeast control demonstrated the lowest average TA, at 3.63 MilliEqu NaOH. The pH range of all sourdough samples was

3.62 to 5.25, which is consistent with the findings of other studies.[26]

Water activity measurements were consistent across sourdoughs starters and between sourdough and yeast control (**Figure 3.2 C**) with an average of 0.964 ± 0.003. Dough samples clustered into two different groups for water activity measurements, and six samples differed significantly in water activity from yeast control. These values are higher than those reported by some other authors,[27] yet some work also reports Aw values in line with these findings[28] since Aw values depend on many processing factors. Differences in Aw may arise from the hygroscopic abilities of exopolysaccharides (EPS) produced by some species of bacteria in sourdough systems, including *L. sanfranciscensis*.[29]

The consistency in water activity measurements was expected, as all doughs were fermented at the same hydration percentage of 65% under the same conditions. Those differences that were observed may have arisen due to the production of water-binding exopolysaccharides which are known to be produced in sourdough systems by some types of lactobacilli.[29]

Free amino acids were measured in dough as an indicator of protein hydrolysis. This study used FAAs as an indicator of protein breakdown, despite the limitation that it does not

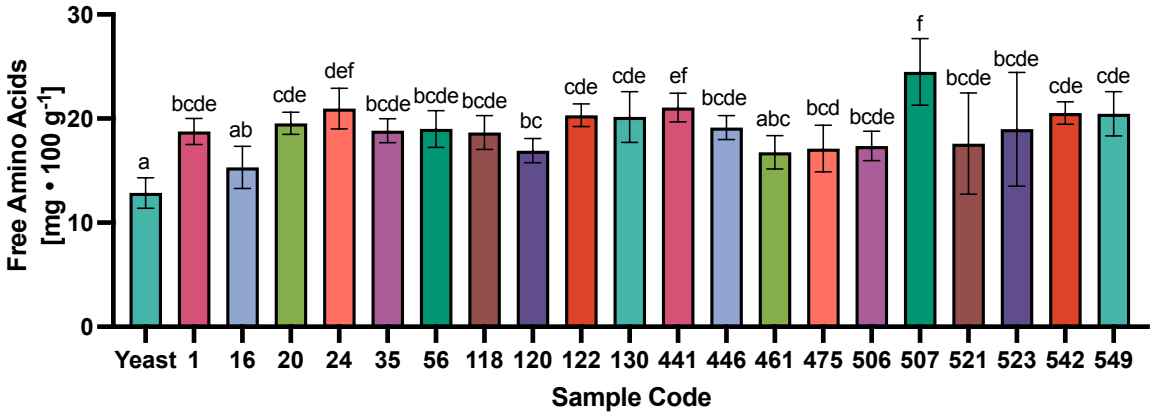

Figure 3.3: Free amino acid content of sourdough and yeast-fermented control dough. 18 out of 20 sourdough samples had a significantly greater concentration of free amino acids. Values are expressed as mean ± standard deviation. Different letters indicate significant differences ($p \leq 0.05$).

account for other products of proteolysis such as di- or tripeptides and smaller peptide chains. Thus, additional tests are needed to determine a more complete picture of the protein hydrolysis profile in a dough sample. However, the concentration of FAAs in a sample does have some meaningful implications for bread quality. The amide groups of free amino acids can participate more readily in the Maillard reaction compared to intact proteins,[30] meaning that color and flavor development may occur more readily in doughs with a higher concentration of FAAs.[31]

The yeast control contained the lowest concentration of free amino acids, while sample 507 contained the greatest. Free amino acid concentration values for all samples fell within the range of 12.85-24.48 mg per 100 g dough. 18 out of 20 contained a greater concentration of free amino acids than the yeast control dough (**Figure 3.3**).

3.4.2 Physical Attributes of Bread

Crumb compressibility is defined as the force in Newtons (*N*) required to compress the crumb to 40% of its original height. This texture measurement gives information about the degree to which the crumb resists compression, which is related to a number of quality factors such as the crumb's density, aeration, moisture level, degree of gluten breakdown, and ratio of organic acids. It also serves as a proxy for sensory evaluation of crumb texture by a human panel. In other studies, crumb hardness (resistance to compression) has been negatively correlated with consumer acceptability[33] making it a useful quality indicator.

Loaf rise volume refers to a volume, usually expressed in cm^3, occupied by the final baked bread loaf, quantifying the crust and the crumb. This is the volume achieved by a loaf following various steps intended to expand the size of the loaf, including kneading, fermenting, proofing, and baking, and it denotes the extent to which a loaf is able to expand to its final baked

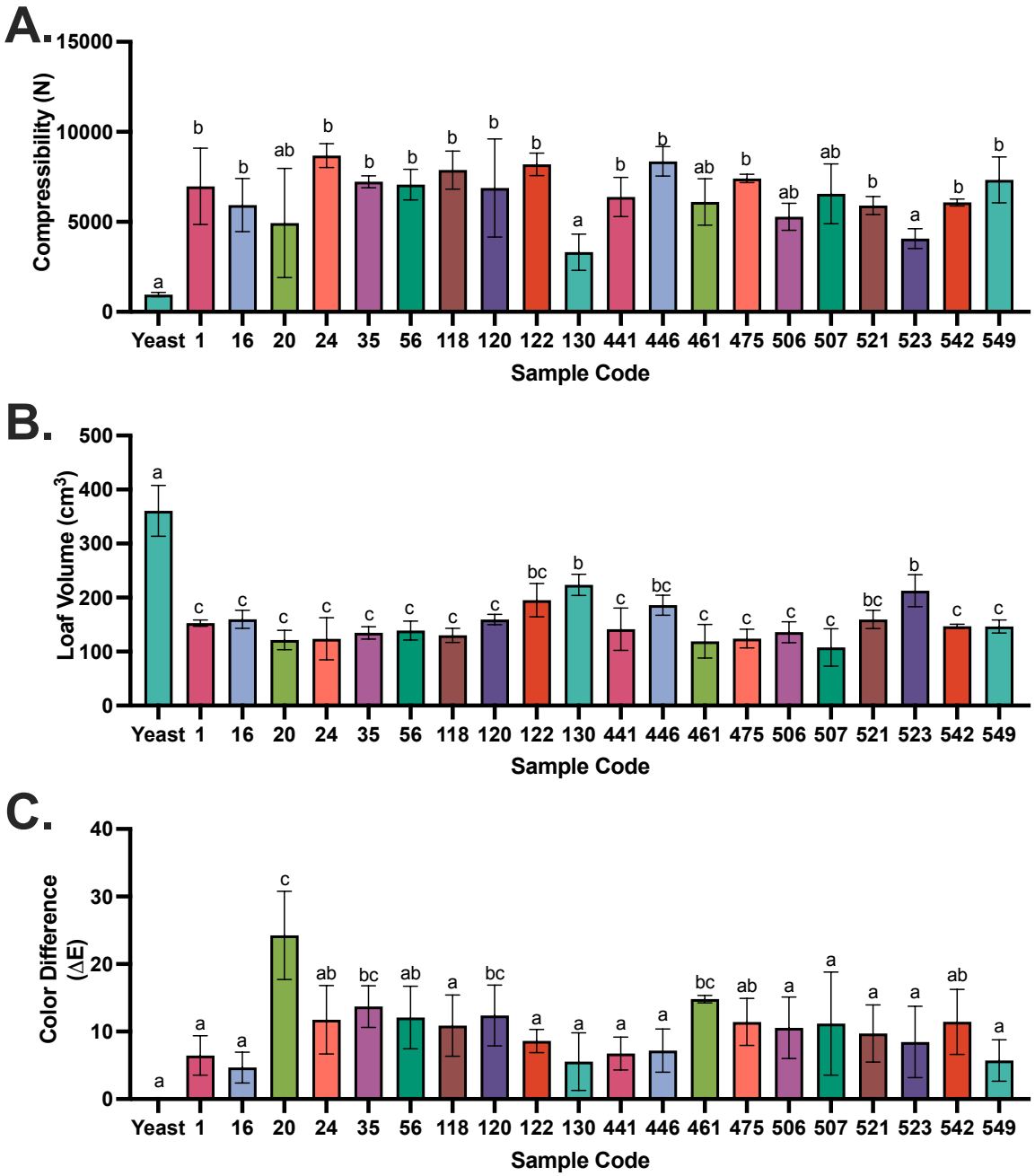

Figure 3.4: Physical properties of baked sourdough breads compared to yeast control. Analyses performed included (A) crumb compressibility, (B) loaf volume, and (C) crust color. Values are expressed as mean ± standard deviation. Different letters within graphs indicate significant differences ($p \leq 0.05$)

volume from the initial volume of unfermented dough. Rise volume is an indicator of the gas trapping ability of the gluten network.[32]

Crust color difference (ΔE) measures the difference in color of each baked sourdough

loaf crust compared to the yeast control bread. The greater the ΔE value, the more dissimilar is the color of the sourdough crust from the yeast control. This number is related to the extent or lack of Maillard and caramelization reactions that occurred on the surface of the loaf during baking, because these reactions produce brown-colored polymers which can be detected by L*a*b* colorimetry.[34] The development of browning pigments is a quality attribute in itself, since crust darkening has previously been correlated with consumer acceptance scores.[33] It is also reflective of other quality features of the dough (such as pH or FAA liberation) or of the sourdough-making process, such as dough hydration, bake time, or bake temperature). Acceleration of browning reactions is driven by higher pH[35,36] and presence of FAAs, although sugars and not FAAs are usually the limiting components of the reaction.[37]

Sourdough samples showed notable difference from yeast control in crumb compressibility (**Figure 3.4 A**), loaf rise volume (**Figure 3.4 B**) and crust color (**Figure 3.4 C**). The yeast control loaf showed significant increase in rise volume ($p < 0.0001$) compared to all sourdough samples. This observation underscores the importance of optimizing fermentation time and temperature parameters to limit the degree of proteolysis in sourdough fermentations. Gas-trapping capability is achieved by a ratio of high molecular weight (HMW) to low molecular weight (LMW) gluten protein units. The HMW units provide strength and elasticity, while the LMW units confer viscosity.[31] While other authors have shown that proteolysis can take place in sourdough fermentations to some extent without adversely affecting bread volume,[38] it appears that in this experiment the extent of proteolysis in all sourdoughs was aggressive enough to diminish the quality of the gluten network relative to control. We observed an inverse relationship between loaf volume and crumb compressibility where the yeast control required the least force for compression while all sourdough samples required more force. Six out of 20

sourdough samples were not statistically different from the yeast control.

All samples were assessed for color change against the crust color yeast control. In comparison to the yeast control, which is set to 0 in these analyses, samples scoring < 1 were ranked as "not perceivably different" from the yeast control while samples scoring between 3 and 6 were deemed "acceptable for commercial production". Samples scoring greater than 6 represent samples where the color is different from the yeast control.

3.4.3 Taxonomy of Sourdough Starter Culture Microbiomes

Taxonomic composition for mapped reads above 1% were plotted for the twenty sourdough samples using the R package ggplot2 v3.3.5.(**Figure 3.5**).[19] All samples showed greater diversity of fungal amplicon sequence variants (ASVs) than bacterial ($p < 0.0001$). Fungal microbiomes show relative abundance distributed across a greater number of ASVs in each sample. Samples represented an average of 16.55 fungal ASVs and only 5.25 bacterial ASVs. At the genus level, we observed five genera represented across 20 samples: *Acetobacter,*

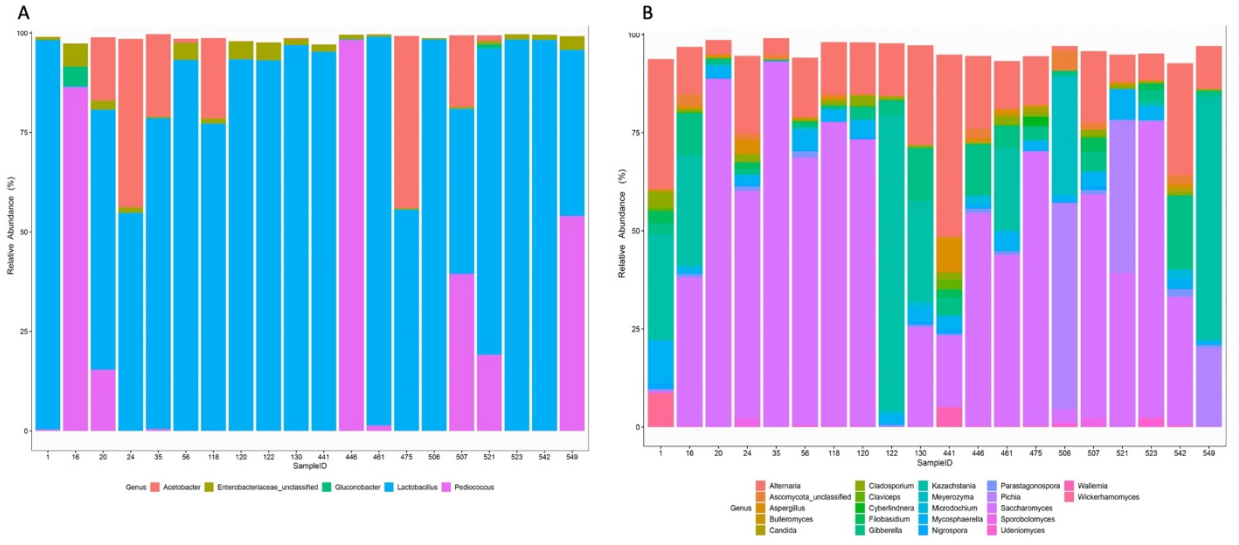

Figure 3.5: Relative abundance of (A) bacterial composition and (B) fungal composition of sourdough samples. Taxa present at a relative abundance above 1% are shown at the Genus level.

Enterobacteriaceae, Gluconobacter, Lactobacillus, and *Pediococcus*. In samples 542, 523, 506, and 461, and 1, *Lactobacillus* comprised over 98% of all bacterial reads; it comprised over 90% for samples 56, 120, 122, 130, and 441. Meanwhile, *Pediococcus* entirely dominated sample 446, constituting 99.1% of reads.

Previous studies have shown that while the cell count increases from flour to sourdough, the species diversity decreases as the sourdough environment selects for just a few species.[40] Heavy dominance by *Lactobacillus* in sourdough starter cultures has been noted across current literature.[41] Dominant *Lactobacilli* found in sourdough tend to be heterofermentative;[42] in the present study heterofermentative species *L. sanfranciscensis* and *L. brevis* dominate many of the samples, but homofermenters such as *L. plantarum* and *Pediococcus damnosus* dominate others nearly to exclusion.

In contrast, we observed 21 fungal genera across these samples. Only sample 35 showed dominance over 90% by a single fungal ASV, with *Saccharomyces* making up 94.7% of reads. Despite this, *Saccharomyces* was the dominant yeast overall in the starters, making up more than 50% abundance in 10 of the 20 samples and absent from only 2 samples (1 and 122). Previous studies have observed that some fungal and bacteria species out-compete one another or demonstrate negative co-occurrence; one of these pairs is *S. cerevisiae* with *L. sanfranciscensis*, while another is *S. cerevisiae* with *Kazachstania humilis.*[1] Therefore, the dominance of *L. sanfranciscensis* in sample 1 may explain the low abundance of *S. cerevisiae*. However, samples 130, 523, and 542 are also almost entirely dominated by *L. sanfranciscensis*, yet all three contain *S. cerevisiae*. On the other hand, sample 122 contains no *L. sanfranciscensis* but it does contain a high abundance of *Kazachstania* spp. which may explain the absence of *Saccharomyces* ASVs.

There were several other genus-level fungal reads that occurred in a majority of samples.

For example, *Nigrospora*, *Filobasidium*, *Cladosporum* and *Aspergillus* were detected in 19 out of 20 samples and *Parastagonospora* and *Fusarium* were found in 18 of 20 samples. All 20 samples contained *Mycosphaerella* and *Gibberella* at very low abundance, and *Alternaria* was present in all samples at abundances ranging from 1.1% to 47%. Notably, each of these fungi are plant pathogens capable of growing on wheat and have been detected in whole wheat breads using culture dependent and independent techniques.[44]

3.4.4 Hierarchical Clustering of Sourdough Samples Reveals Distinct Groups

We assessed the similarity of sourdough samples to one another based on taxonomic features as well as physical and chemical properties using Euclidean distance (**Figure 3.6 A-C**). Hierarchical clustering is a technique that aims to gather samples into clusters based on their

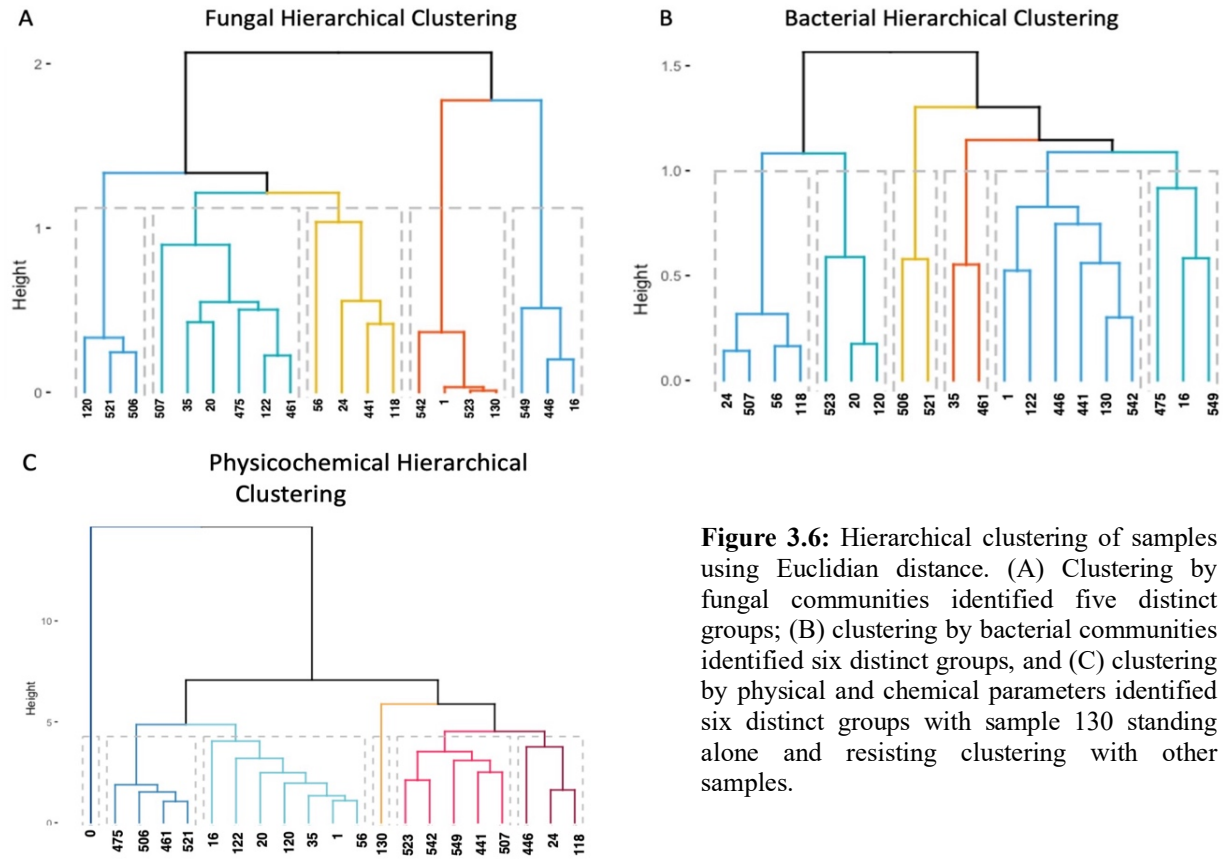

Figure 3.6: Hierarchical clustering of samples using Euclidian distance. (A) Clustering by fungal communities identified five distinct groups; (B) clustering by bacterial communities identified six distinct groups, and (C) clustering by physical and chemical parameters identified six distinct groups with sample 130 standing alone and resisting clustering with other samples.

similarity to other samples in the same cluster. Samples are compared to other samples by visualizing each sample as a point in *n*-dimensional space. Each of the *n* dimensions comprises a measured parameter (for example pH, or loaf volume) with the vector centered at zero, and the sample sits at the point represented by its measurements on each vector of the *n* dimensions. Then, the Euclidean distance between points in *n*-dimensional space is calculated, and samples are grouped in clusters according to the points with the shortest Euclidean distance between them. The bottom-up pairwise approach employed here utilizes Ward's method,[39] which merges each pair of points (or later, each pair of clusters or clusters and points) that will result in the least increase in post-merge within-cluster variance. These clusters are represented in a dendrogram, which represents clusters as "branches" off a central point and individual samples as "leaves" at the end of the branches. The height of the branches is representative of the Euclidian distance at which the samples or clusters were merged, and therefore it indicates the relative overall similarity of the samples or clusters.

We observed that samples clustered at the shortest Euclidian distance when grouped by fungal communities (**Figure 3.6 A**), and by the greatest Euclidian distance when grouped by physicochemical parameters (**Figure 3.6 C**) with bacterial community grouping by intermediate distance (**Figure 3.6 B**). This suggests that samples are more similar in their fungal composition than in bacterial composition or in their physical and chemical quality parameters. In terms of fungal composition, samples 1, 523, and 130 form an especially tight cluster, with very short branches indicating short distances between points. 130 and 523, which group very closely together, are also notable for both falling on the extreme high end of the sourdoughs' performance in rise volume and at the extreme low end of the sourdough samples for force required for compression, in the same statistical group and the yeast control bread. Meanwhile

bacterial composition forms the samples into six clusters, two of which contain only two samples and cannot be linked to other clusters. Branches diverge at similar heights for each cluster, and in contrast to **Figure 3.6 A**, the dendrogram for bacterial composition does not exhibit any extreme closeness in grouping (**Figure 3.6 B**).

Figure 3.6 C demonstrates hierarchical clustering of samples by the outputs of their physicochemical properties. While the microbiomes of starters within each clade is distinct, these clusters are formed only on the basis of similarity in dough and bread quality outputs. The 20 samples in this study fall into five clades, the smallest of which consists of only one sample and the largest of which is made up of approximately 1/3 of the sample set. Clustering of this type may allow researchers to select a member of each clade to subject to further testing (for example, screening for immunogenic epitope degradation) in the expectation that testing one member of each clade would represent the diversity of the entire sample set.

3.4.5 Relationships Between Physicochemical Properties and Starter Culture Microbiome Diversity Richness

Relationships between the physical and chemical properties of 20 sourdough samples and their bacterial and fungal microbiomes were assessed by Spearman's ρ correlations (**Figure 3.7**). The variable correlation plot shown in **Figure 3.7** demonstrates the relationships between physicochemical variables in dough and bread. Titratable acidity is driven in the same direction as water activity; both are driven the opposite direction as pH. In the same way, there is a relationship between loaf volume and color difference, which are correlated with one another but in the opposite direction as force of crumb compression. No strong relationships are apparent between the chemical outputs of fermented dough (pH, water activity, and titratable acidity) and

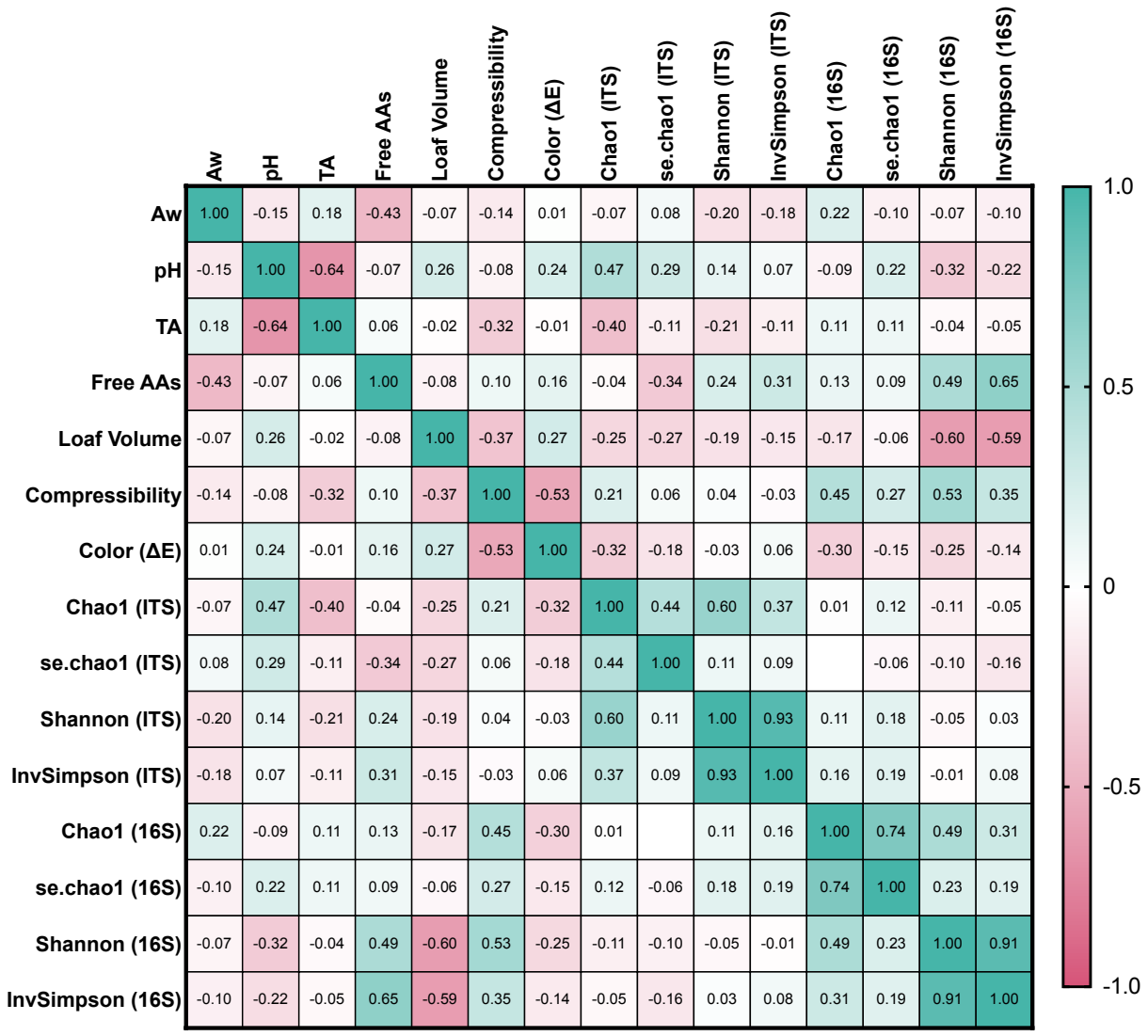

Figure 3.7: Correlation matrix of physical and chemical properties of sourdough samples and indices of microbiome diversity calculated for fungal (ITS) and bacterial (16S) populations.

the physical properties of baked bread (crust color change, loaf rise volume, and crumb compression) although weak relationships do exist. For example, loaf volume bears a weak positive relationship with dough pH, and crumb compressibility a weak negative relationship to dough TA.

Previous work has shown at as pH drops, endogenous cereal proteases achieve increased activity and contribute to the proteolytic breakdown of gluten by microorganisms.[51] Because a gluten network is required to trap evolved gases and allow bread to rise, it follows that low pH

would likely correlate with a shift towards poor rise volume. However, our data did not identify any significant relationship between pH and FAA content or between pH and dough/gliadin hydrolysis (data not shown) and it showed only a very weak correlation (0.26) between pH and rise volume (**Figure 3.5**). This may be due to the presence of interfering compounds such as exopolysaccharides (EPS) that can be produced by some strains of *Lactobacillus* present in sourdough. These compounds have been shown to have a relationship to many quality parameters of bread and sourdough, including rise volume.[52]

The richness and diversity of the bacterial and fungal microbiomes were expressed using Chao1, Simpson, and Shannon indices. The Chao1 index estimates the total number of species in a habitat based on the number of observed species in a sample.[20] It is shown alongside the standard error of the index, denoted here as "se.Chao1", which indicates the uncertainty associated with the Chao1 index. The Shannon index is the product of a calculation to estimate the abundance and evenness (equal distribution) in a sample;[2] a higher Shannon index suggests a more diverse community with more even distribution of species. The Simpson index takes into account both species richness and abundance but with greater weighting on abundance of species.[21] Here, the inverse of the Simpson index (denoted InvSimpson) is used; higher InvSimpson indicates greater species diversity, while lower InvSimpson suggests lower species diversity.

No strong correlations (either positive or negative) existed between fungal and bacterial biodiversity indices, nor fungal biodiversity and quality parameter. However, several statistically significant correlations were identified between quality variables and bacterial diversity indices. In terms of physical characteristics of bread, greater bacterial diversity as determined by Shannon index was associated with greater compressibility, while loaf volume was inversely

associated with bacterial diversity as indicated by both Shannon and InvSimpson indices. In terms of chemical characteristics, the concentration of free amino acids in the dough was correlated with greater bacterial diversity as defined by Shannon and InvSimpson indices. These findings suggest that despite fungal populations of sourdough demonstrating greater overall diversity, the diversity of the bacterial populations have a greater influence on the quality outcomes of the breads they are used to produce.

Lactobacilli in sourdough systems have been shown to release free amino acids through the action of peptidases.[48] Our findings reflect previously published data showing that free amino acid levels are lowest in dough fermented by yeast alone compared to doughs fermented by a combination of yeast and bacteria.[49] Generally, free amino acids have been shown to stimulate further bacterial growth even as they are liberated by bacteria,[49] which could contribute to explaining the positive correlation between free amino acid concentration and bacterial biodiversity indicators. Yeasts take up free amino acids in a system but largely lack the peptidases required to liberate them,[50] which explains why levels measured lowest in the control dough. Our data contradicts some earlier work which found significant differences in free amino acid concentration in sourdoughs inoculated with different bacterial organisms;[49] however, that work was performed in model fermentations inoculated with pure cultures of distinct species of LAB (in some cases combined with commercial baker's yeast). In contrast, our study involved mixed cultures with overlapping combinations of bacteria, including non-LAB, and wild yeast. It is therefore not unexpected to find fewer distinctions in amino acid concentration in these wild, mixed systems since the effect of each individual organism could not be isolated as in the

previous study.

3.5 Conclusion

Sourdough microbiomes produce dough and bread that are physically and chemically distinct from yeast-fermented bread. In the present study, there were significant differences between all of the sourdough samples and the yeast control for most of the physical and chemical analyses conducted.

The sourdough samples in this study showed more diversity of yeast populations than bacterial populations. Previous research has reported that yeasts are usually present at a 1:100 ratio with bacteria in terms of cell numbers in sourdough[53] but the makeup of the fungal community is less well understood. Some analyses also do show more diversity in fungal than bacterial microbiomes and associate fungal diversity with variables in bread-making practices.[41] In the present study, starters were propagated and fermented to dough (and subsequently converted to bread) under the controlled similar conditions. Starters for this study were donated from the Global Sourdough Project, which received starter submissions by community scientists[1] Therefore we are unable to account for the causes of species diversity and instead can only focus on its outcomes.

Overall, this study demonstrated that the sourdough microbiome drives differences in dough and bread quality attributes. Results suggest that the fungal population contributes more to outcomes in bread, while the bacterial population is more significant for outcomes in dough.

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CHAPTER 4: CONSUMERS WITH DIAGNOSED GLUTEN SENSITIVITIES REPORT NO REDUCTION IN SYMPTOMS UPON CONSUMING SOURDOUGH-FERMENTED BREAD PRODUCTS: RESULTS OF A CONSUMER SURVEY

4.1 Summary

Public consensus holds that sourdough can mediate the symptoms of gluten-stimulated pathologies relative to yeast-fermented products. This link is frequently reported on websites, blogs, social media, and even academic articles despite a lack of data supporting the claim. This chapter presents the results of a survey aimed at understanding, for the first time, the real experiences and perspectives of gluten-sensitive consumers who consume sourdough bread. Three treatment groups were investigated: consumers with celiac disease (CD), those with non-celiac gluten sensitivity (NCGS), and those who are undiagnosed (UD) by a medical practitioner but identify as gluten sensitive. The survey was designed to gather information about the factors that influence the decision to consume sourdough, sources of knowledge about gluten and dietary guidelines, and the perceived benefits and drawbacks of sourdough consumption. We hypothesized that all three groups of gluten-sensitive consumers would report a reduction in symptoms when consuming sourdough relative to yeast-fermented bread and that perceived health benefits would be the principal motivator to eat sourdough among gluten-sensitive consumers. Results indicated that UD consumers were more likely to be older, female, poorly educated, and have the highest levels of gluten knowledge and the lowest rates of social support in comparison to consumers with diagnosed CD or NCGS. We also found that taste is the main motivator for eating sourdough among all groups of gluten-sensitive consumers, eclipsing the perceived health benefits as a motivating factor. Regression models were built to investigate the

factors contributing to three outcomes: high scores on a gluten knowledge test, eating sourdough (vs. not) and having symptoms upon eating sourdough (vs. not) and it was found that both demographic and behavioral factors contribute to all three outcomes. However, no differences were found between treatment groups in terms of frequency of sourdough consumption or rate of gluten avoidance. The findings of this study may improve how medical professionals and nutritionists communicate with gluten-sensitive patients, offering a greater awareness of their needs, habits, and motivations. This study highlights the need for further research into the potential benefits and risks of sourdough consumption for individuals with gluten sensitivity.

4.2 Introduction

Sourdough refers to a symbiotic mixture of yeast and bacteria used to leaven bread products.[1] Although it was the only technology to achieve leavening for millennia,[2] it fell out of favor in the 19th century due to the emergence of the faster and more convenient packaged baker's yeast.[3] Yet today, interest in sourdough has grown as consumers value sourdough for its flavor, [4] natural ingredients, and perceived health benefits, [5] and they are willing to pay more for these qualities.[6] Even some gluten-sensitive individuals, spurred on by suggestions on websites or social media, may seek out sourdough in the hope that it fails to stimulate gastrointestinal symptoms.

Gluten-sensitivity is a pathology stimulated by the consumption of gluten-containing foods, such as wheat, barley or rye. There are a number of specific pathologies that are considered "gluten-sensitivities", each with their own specific mechanisms and pathogenic cascade. The most well-known of these, celiac disease (CD) has an incidence rate of approximately 1% in the U.S. and Europe[7] but has been found at slightly higher rates in some geographically specific populations.[8–10] Non-celiac gluten sensitivity (NCGS) is a disorder

encompassing patients whose symptoms improve upon eliminating gluten from the diet but who do not meet diagnostic criteria for CD. NCGS is thought to affect up to 5% of the population.[11] General gluten sensitivity is also noted in some cases of irritable bowel syndrome (IBS) and irritable bowel disease (IBD). Individuals with gluten sensitivities do not always seek medical care, and it can be difficult to adhere to a diet of strict gluten abstinence required for a formal diagnosis. While these details make it difficult to determine the precise number of individuals affected by gluten sensitivity worldwide, current estimates predict a prevalence of about 10% of the population.[12]

Currently, CD and NCGS can only be controlled by strict gluten avoidance.[13] Unfortunately, it is difficult to avoid gluten entirely for most individuals. Gluten is a common food additive in the Western diet and is found in unlikely sources, such as meat and fruit preserves.[14] Moreover, a gluten-free diet lacks both sensory and nutritional quality relative to conventional diets.[15,16] Adherence to a gluten-free diet also incurs social and economic costs. There are fewer gluten-free options in supermarkets and restaurants, and they tend to cost more than conventional foods. Individuals avoiding gluten may also be excluded from social or religious interactions including bread or wheat-based products, potentially separating these individuals from social support networks and excluding them from social engagements.[17,18] For all these reasons, even gluten-sensitive individuals who have been advised to adhere to a gluten-free diet may be unwilling or unable to do so all or part of the time.

Individuals struggling to adhere to a gluten-free diet may find solace in the popularly circulated belief that sourdough bread causes fewer gluten-mediated symptoms than conventional bread. Indeed, home bakers, bread consumers, professional bakers, and even academic articles frequently refer to this widely held notion. Academic articles rarely go so far as

to make the claim that sourdough is tolerated by patients with celiac disease, although there is often the suggestion, without citation, that patients with other types of gluten sensitivities experience alleviated symptoms in response to sourdough in comparison to conventionally produced yeast breads.[19] Despite the phenomenon being referenced in peer-reviewed articles, we found no source that provided data from sourdough consumers. Rather, articles cited possible mechanisms explaining the phenomenon[20] or even refer to it as "anecdotal evidence".[21] Furthermore, in background research for the broader work for this study, we encountered numerous professional sourdough bakers who claimed to sell their wares to gluten-sensitive customers on the basis of this unfounded claim (**Chapter 5**).

The objective of this study was to investigate consumer perceptions of the healthfulness of sourdough as it relates to gluten sensitivities. We developed a survey which was distributed to individuals who are diagnosed with CD or NCGS, or have a self-diagnosed sensitivity to gluten (n=1015). This survey asked a variety of questions aimed to understand consumer eating habits, demographic information and knowledge about gluten and gluten-containing foods. We hypothesized that all three groups of gluten-sensitive consumers would report a reduction in symptoms when consuming sourdough relative to yeast-fermented bread and that perceived health benefits would be the principal motivator to eat sourdough among gluten-sensitive consumers. Furthermore, we sought to identify trends in demographic information which may be associated with the eating habits of gluten-sensitive consumers.

4.3 Methods

4.3.1 Survey Distribution and Data Collection

The survey (**Appendix A**) was created with the platform Qualtrics (Provo, Utah, USA) and is covered under IRB protocol #3606. The distribution service Centiment recruited and

screened participants such that the demographics of survey respondents roughly reflected the demographics of United States residents. Respondents were compensated for their participation. The survey contained 20 questions which pertained to participants' diagnosis, their symptoms' response to eating sourdough, their knowledge of gluten-containing foods, the sources of information that pertain to a gluten free diet, impact statements about how their condition has affected their lives, and demographic information.

The number of survey participants was determined by a power calculation based on available data. Clinical trials indicated that showing that when gluten-sensitive individuals consume sourdough, approximately 34% of them experience some kind of reduction in their gluten-mediated symptoms relative to when they consume non-sourdough bread products. Given the absence of prior survey data on the subject, a combination of *ex vivo* and *in vivo* clinical trial data was used instead.[22–33] Sample size estimation was performed according to the formula:

$$n = \frac{(z^2) * p * (1 - p)}{ME^2}$$

Where parameters are assigned as p = 0.34, ME = 0.03, and z = 1.96 (representing a two-sided 95% confidence level). This calculation yielded n= 958, leading us to target 1000 survey responses. This approach ensured a robust foundation for this pioneering survey of gluten-sensitive sourdough consumers.

Recruitment and data collection were carried out by Centiment (Centiment, Denver, CO, USA). Panelists were recruited, validated, and compensated through this service. Participants were targeted for the survey based on a profile that is re-validated every 30 days. Participants were unaware of the survey contents or topic, but instead were told only an

estimated completion time and compensation value.

4.3.2 Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics Version 29.0.0.0 (IBM Corp., Armonk, NY) and GraphPad 10.0 (GraphPad Software, San Diego, CA USA). Respondents were categorized into three treatment groups: those diagnosed with CD (CD), those diagnosed with NCGS (NCGS), and those who self-identify as gluten-sensitive but who remain undiagnosed by a medical practitioner (UD). Continuous variables, such as level of gluten knowledge, quantity of symptoms upon eating sourdough, and demographic variables were manually categorized and counted for analysis. To determine level of gluten knowledge, a score from 0-5 was found by allowing 1 point for every correct answer and subtracting 1 point for every incorrect answer. Scores from 0-1 were assigned as Low, 2-3 was Medium, while 4-5 was High level of knowledge. Categorical variables, such as gluten avoidance, were counted within each treatment group. Associations between categorical variables were explored using chi-squared Fisher's test. One-way ANOVA was used to compare treatment groups within categorical variables.

Regression analysis was used to determine the impact of continuous variables on a categorical outcome variable. Binomial logistic regression models were built to ascertain the effects of demographic and lifestyle factors on three binomial independent outcomes: achieving a high score (of 4-5) on gluten knowledge, choosing to consume sourdough (vs. avoiding it), and experiencing symptoms upon exposure to sourdough (vs. not). A forward stepwise model, using diagnosis category as the base independent variable, systematically tested other variables one by one. All demographic and behavior variables were included in the analysis for all three outcome variables. Only those variables that lowered Akaike's information criterion (AIC) by at least two

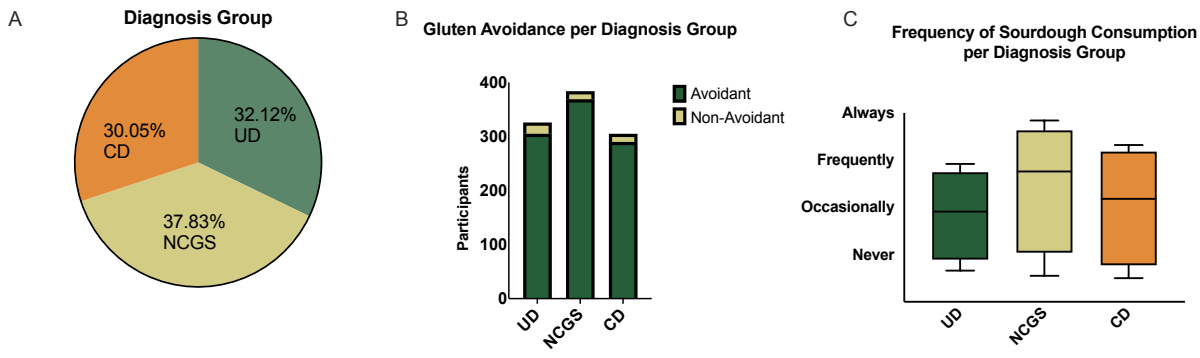

Figure 4.1: Breakdown of participants per (A) diagnosis category, (B) percent of gluten avoidance by diagnosis group, and (C) frequency of sourdough consumption per diagnosis group.

units were retained, although in some cases the coefficient did not achieve a significant p-value. In these cases the variable was retained in the model as contributing to overall fit but not discussed as explanatory. Model fit and variable significance were confirmed using the likelihood ratio test.

4.4 Results

4.4.1 Consumer Characteristics

Participants were distributed approximately evenly among the three diagnosis categories, with 305 individuals in the CD group, 384 in the NCGS group, and 326 in the UD group, constituting a total of 1015 participants (**Figure 4.1 A**). Nearly all surveyed participants (94.98%, or 964 respondents) reported being conscious of gluten avoidance in their diets to some degree, while 51 participants (5.02%) reported no attempt to avoid gluten (**Figure 4.1 B**). A chi-squared Fisher's exact test revealed no significant association ($p = 0.303$) between diagnosis category and rates of gluten avoidance. When queried about the frequency of choosing to eat sourdough products when they are available, 198 participants (19.51%) claimed to consistently choose sourdough when given the option, while 294 respondents (28.97%) indicated that they choose sourdough "frequently". Another 352 people (34.68%) answered that they "occasionally"

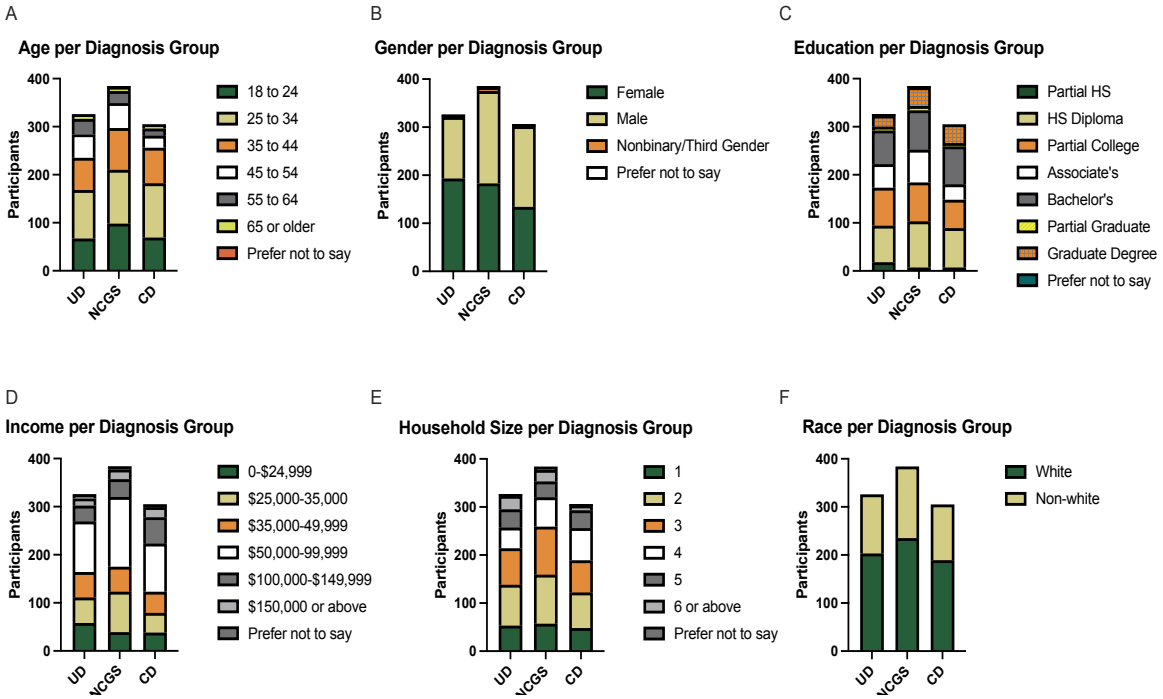

Figure 4.2: Breakdown of participant demographics per diagnosis category. Stacked bar charts indicate the number of participants in each group per diagnosis category for (A) age range, (B) gender, (C) education level, (D) income range, (E) household size, and (F) race (white vs. non-white only).

opt for sourdough over conventional bread products, and 171 participants (16.85%) said that they "never" choose sourdough (**Figure 4.1 C**). No significant associations emerged between frequency of sourdough consumption and diagnosis category (overall p-value = 0.799). Regarding participant demographics, Respondents were also primarily young, with 77.6% of falling into an age category below 44 years old and with the mode age range (32.1% of participants) being 25-35 years old (**Figure 4.2 A**). The distribution of gender was fairly balanced, with males making up 48.1% of the data set and females 50.2%. Nonbinary individuals accounted for only 1.5% of participants (**Figure 4.2 B**). The mode level of education across all three diagnosis categories was "high school diploma" although in the UD category this was very slightly edged out by "partial college" which was selected by an additional three participants (**Figure 4.2 C**). Households in this data set predominantly consisted of two people, and the income category most frequently selected was $50,000 to $99,999 (**Figure 4.2 D-E**). The

majority of participants (61.77%) identified as white, as opposed to non-white (38.23%) (**Figure 4.2 F**).

4.4.2 Consumer Impact and Experiences

Consumers were asked to report on their experiences with symptoms of their gluten sensitivity upon consumption of sourdough in comparison to non-sourdough breads (**Figure 4.3**). Out of a total of 1015 participants, 356 (35.07%) claimed to experience "fewer symptoms" with sourdough. Meanwhile, 292 people (28.79%) reported experiencing the same number of symptoms from sourdough relative to non-sourdough bread products, and 153 participants (15.07%) selected the option indicating that they experience more symptoms upon eating sourdough compared to conventional bread. 44 respondents or 4.34%, claimed to have no

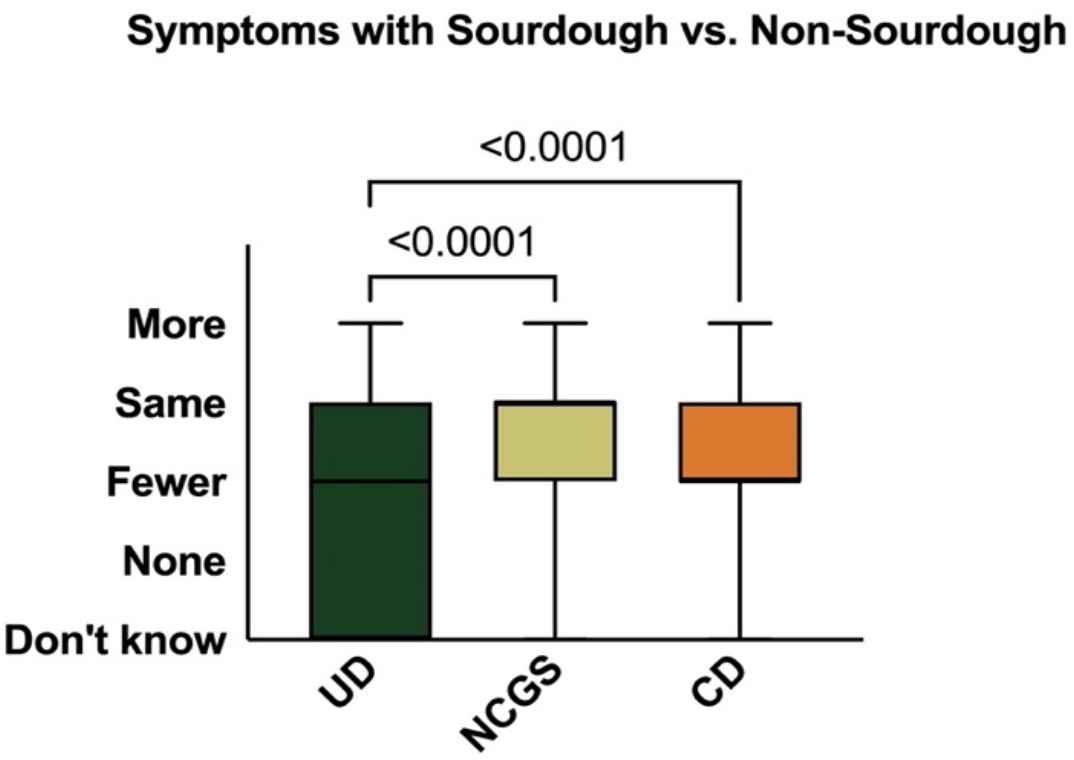

Figure 4.3: One-way ANOVA analysis on number of symptoms experienced upon eating sourdough products compared to non-sourdough products per diagnosis category

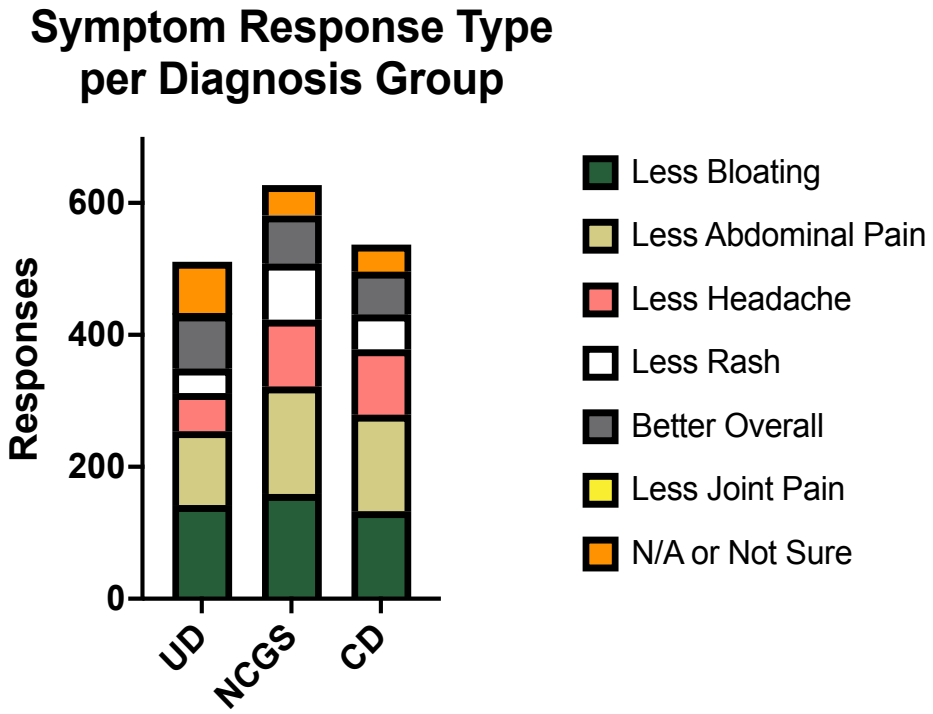

Figure 4.4 Breakdown of participants' impressions of how their symptoms improve in response to eating sourdough products relative to yeast-leavened products.

symptoms regardless of the type of bread product consumed. Finally, 170 participants (16.75%) responded "This does not apply to me/I am not aware of how sourdough affects my symptoms." Notably, respondents in the UD group exhibited a significantly higher tendency to select the responses "This does not apply to me" or "no symptoms regardless of the type of bread I eat" in comparison to the CD and NCGS groups ($p < 0.0001$). No differences in responses were observed between respondents in the CD and NCGS groups (**Figure 4.3**). In terms of specific effects on symptoms, 166 people (16.35%), responded, "This does not apply to me/I do not eat sourdough products" which is roughly similar to the number of people who selected "I am not aware of how sourdough affects my symptoms" when asked whether they have more, fewer, or the same number of symptoms upon eating sourdough relative to conventional bread (16.75% of respondents). The most common response overall was "less bloating", which 434 participants

Table 4.1: Response frequencies for options to the question, "If you tolerate sourdough better than other bread products, which of these statements apply to your experience? Select all that apply."

Symptom Reaction Type	Selection Frequency	Percent of Respondents
Less bloating	434	42.75%
Less abdominal pain	421	41.48%
Less headache	258	25.42%
Feel better overall	220	21.67%
Less rash	175	17.24%
Does not apply	166	16.35%
Less joint pain	1	<0.001%

(42.76%) included in their answer. This was followed closely by "less abdominal pain" which w as selected by 421 participants (41.48%). The other choices were selected less frequently: "less headache" (258 respondents; 25.42%), "feel better overall" (220 respondents; 21.67%), "less rash" (175 respondents; 17.24%), and "less joint pain" from 1 person (<0.001%). "Less bloating" and "less abdominal pain" were the most common co-selections, with 229 respondents

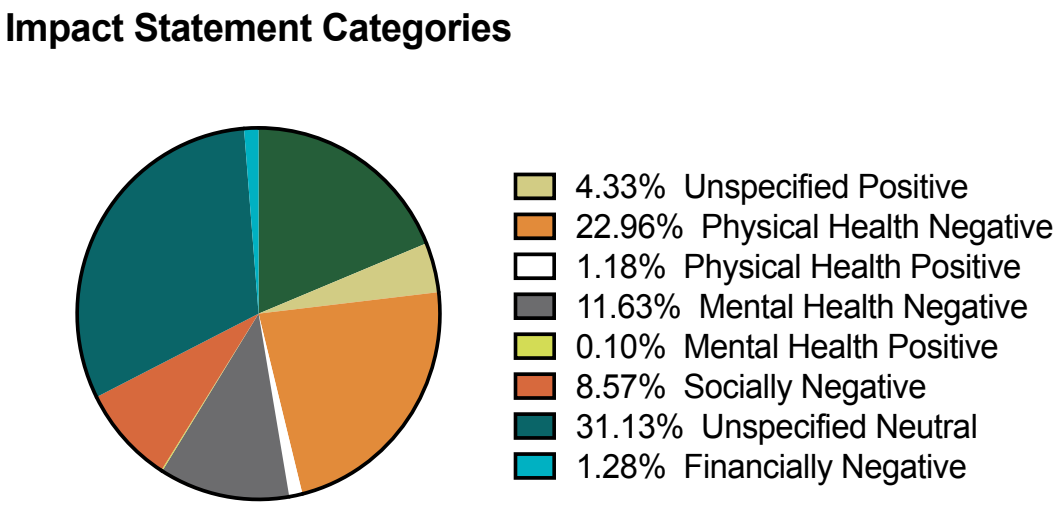

Figure 4.5: Impact statements from survey respondents divided into categories by topic.

choosing them concurrently either alone or in conjunction with other options. The frequency of individual responses is shown in **Table 4.1** and is represented graphically in **Figure 4.4.**

Survey respondents were asked to offer an impact statement, explaining in their own words how their gluten sensitivity has impacted their lives. Statements given in response to this question were categorized according to thematic and topical classes, shown in **Figure 4.5**. These categories provide insight into respondents' experiences and the impact their diagnoses have on various aspects of their lives. Categories were defined as: Unspecified Negative (191 respondents), Unspecified Positive (44 respondents), Physical Health Negative (233 respondents), Physical Health Positive (12 respondents), Mental Health Negative (118 respondents), Mental Health Positive (1 respondent), Socially Negative (87 respondents), Unspecified Neutral (316 respondents), and Financially Negative (13 respondents). Respondents report a wide range of experiences within these categories. Some representative response include:

"It makes it hard to live spontaneously."

"More expensive, but a huge positive impact on health."

"I feel like I'm chained up, I can't live my own life."

"I had to change everything, but I feel more healthy [sic]."

"I tend to stay inside and not socialize much because people don't believe it's a real issue."

"I need to be aware of bathroom locations."

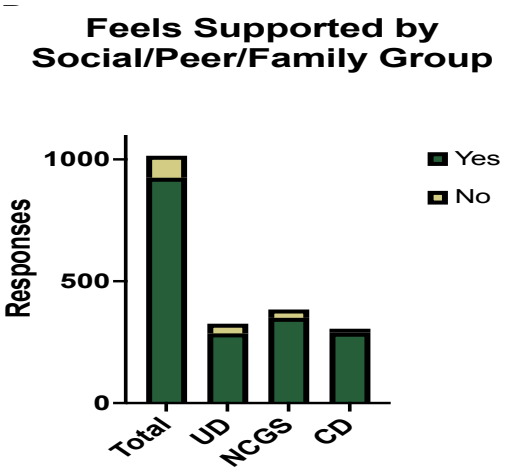

Figure 4.6: Participants' perceived levels of social support for their dietary restrictions and gluten

Lastly, participants were asked whether or not they feel supported in their dietary restrictions by friends, family, and/or partners. Survey participants overwhelmingly claimed to feel satisfied with their level of social support, with 91.13% of respondents answering "yes". UD participants experienced the lowest rates of social support at 87.73% compared to NCGS at 91.14% and CD at 94.75% (**Figure 4.6**). A chi-squared Fisher's test shows a significant association between social support and diagnosis category ($p = 0.0008$)

4.4.3 Consumer Beliefs and Knowledge

Participants were asked several questions that directly or indirectly assessed their knowledge about sourdough and bread products and also identified the sources of knowledge that consumers use to guide their eating habits. When asked why they choose to eat sourdough products, 120 participants (11.82%) responded "This does not apply to me/I do not eat sourdough products" which is slightly fewer than those who chose the comparable option on other questions. The most common reason for choosing to eat sourdough products was "I like the taste" (515 participants, 50.74%). This motivation was followed by "I believe [sourdough

products] contain less gluten" (334 respondents; 32.90%) and "I think [sourdough products] are healthier" (306 respondents; 30.15%). "I tolerate sourdough better than other bread products" was selected by 300 respondents (29.56%) and "I believe [sourdough has] probiotic properties" was chosen by 245 respondents (24.14%). "I think [sourdough is] better for my blood sugar" was the least commonly selected motivation with 170 respondents (16.75%) indicated this motive. Response frequencies are shown in **Table 4.2**. To assess their knowledge of gluten-containing foods, participants were shown pictures of ten foods (five which contain gluten and five which do not) and asked to identify the gluten-containing foods. Responses were scored from 0-5, with one point given for each correct answer and one point removed for each incorrect answer. The UD group had the highest mean score of 2.120, followed by the NCGS group with a mean of 1.763 and CD with a mean of 1.754 (**Figure 4.7**). While there was no significant difference between the means of the CD and NCGS groups, the UD group's mean was significantly different from both NCGS ($p = 0.0101$) and CD ($p = 0.0133$). The survey also investigated where participants sourced their knowledge about which foods are safe for them to eat. The most

Table 4.2: Response frequencies to the question, "If applicable, why do you choose to eat sourdough products? Select all that apply."

Reason for Choosing Sourdough	Selection Frequency	Percent of Respondents
Taste	515	50.74%
Less gluten	334	32.90%
Healthier	306	30.15%
Tolerate better	300	29.56%
Probiotic	245	24.14%
Blood sugar	170	16.75%
Does not apply	120	11.82%

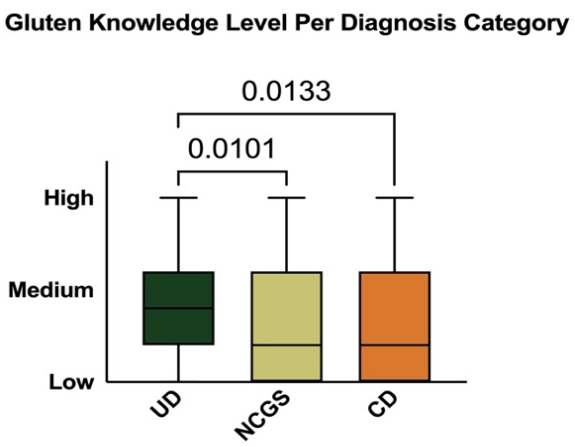

Figure 4.7: One-way ANOVA analysis of scores on gluten knowledge question between diagnosis groups.

commonly identified source of information for dietary decision-making was "product labels/ingredient lists" with 529 respondents choosing this option. This was followed by "gluten-free apps", chosen by 489 participants, and "Google/ internet/ websites" selected by 453 respondents. Other responses were selected less frequently; only 30.84% of survey participants indicated that they turn to a doctor or medical practitioner for information. Total counts for each response option can be seen in **Table 4.3.**

Table 4.3: Response frequencies to the question, "How/from where are you getting information about products you can or cannot eat?"

Sources of Dietary Information	Selection Frequency	Percent of Respondents
Product labels/ingredient lists	529	52.12%
Gluten-free apps	489	48.18%
Google/internet/websites	453	44.63%
Other gluten-free people	340	33.50%
Social media	338	33.30%
Books/cookbooks/recipes	326	32.12%
Doctor/medical practitioner	313	30.84%
My own knowledge/training	206	20.30%
Trial and error	186	18.33%

4.4.4 Factors Contributing to Gluten Knowledge, Symptoms from Sourdough, and Avoiding

Sourdough

Three binomial response variables were considered through regression models. Regression models are used to investigate the relationship between dependent (outcome) variables and independent (predictive or explanatory) variables). In this case, a forward stepwise model was tested to probe the explanatory effect of all demographic and behavior variables on three binomial outcome variables. Variables were fit one at a time to the model, seeking variables that improved the explanatory fit for the outcome variable. Variables failing to lower Akaike's information criterion (AIC) by at least two units were dismissed from the model, and those lowering the AIC by two or more points were maintained and model fit was confirmed using the likelihood ratio test. All variables fitting this criterion were considered to contribute to the model even when calculated p-values were not significant; such variables are noted here and bear 95% confidence intervals that span 1 (**Figure 4.8 A-C**). This model was chosen for its explanatory power over a specific data set. This method does not imply that the variables found to be explanatory for the outcome in this surveyed population would be predictive of the same outcomes in another population. In the case of all three outcome variables, we hypothesized that diagnosis category would be the variable with the most important explanatory power over the outcome (meaning it would contribute to the model fit and have a significant odds ratio).

First, we asked what factors led to participants achieving a high score (4-5) on the gluten knowledge question. In addition to diagnosis category, we hypothesized that sources of knowledge such as medical providers and social media would be most explanatory to this outcome. Odd ratios for contributing independent variables appear in **Figure 4.8 A** with their 95% confidence intervals. Factors associated with greater odds of having high gluten knowledge are being female (93.5% greater), reading ingredient labels (4.183x greater), and having some training or background knowledge of the subject (80.3% greater). Using gluten-free apps, being

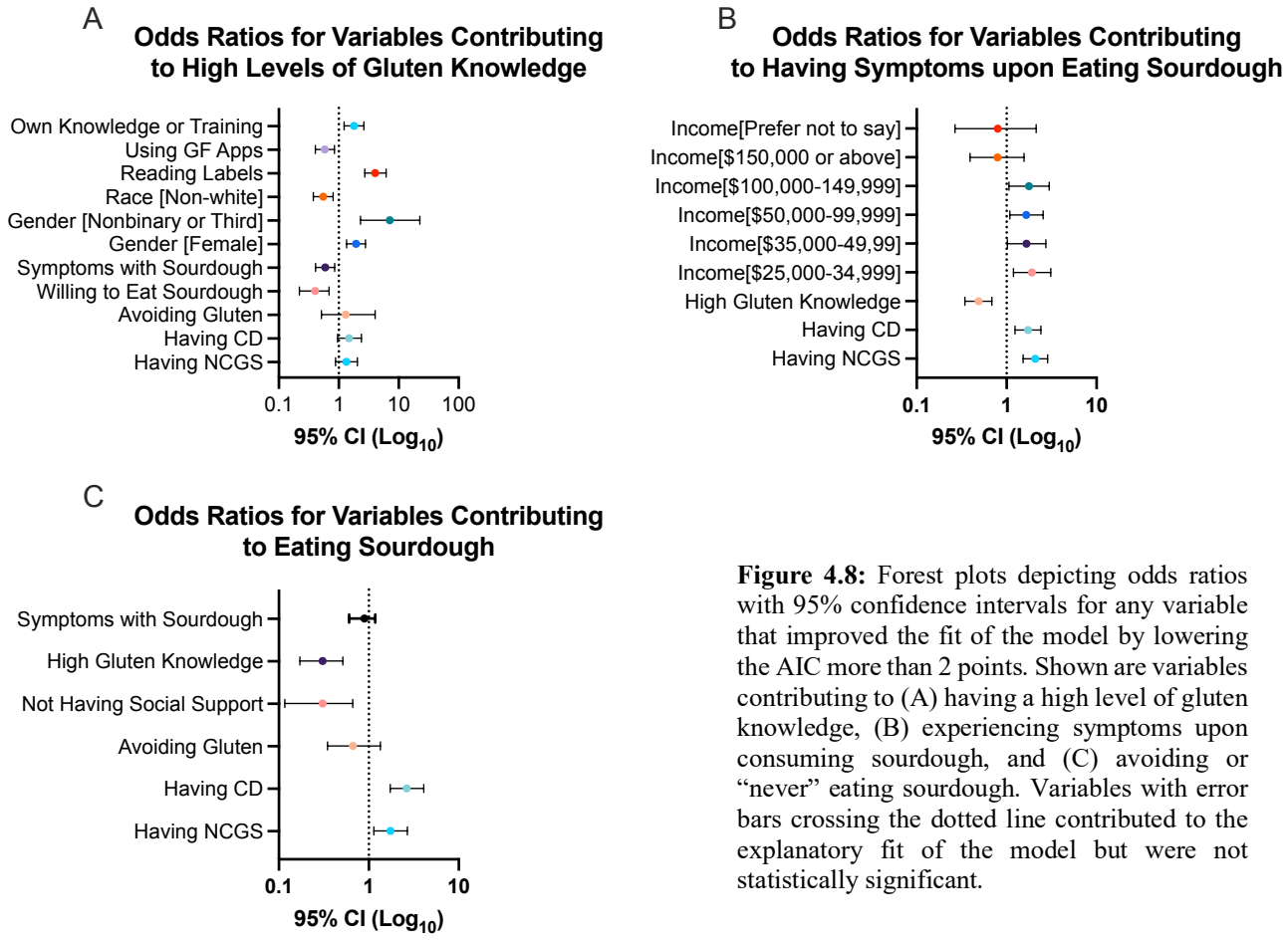

Figure 4.8: Forest plots depicting odds ratios with 95% confidence intervals for any variable that improved the fit of the model by lowering the AIC more than 2 points. Shown are variables contributing to (A) having a high level of gluten knowledge, (B) experiencing symptoms upon consuming sourdough, and (C) avoiding or "never" eating sourdough. Variables with error bars crossing the dotted line contributed to the explanatory fit of the model but were not statistically significant.

non-white, being willing to eat sourdough, and having symptoms upon eating sourdough are associated with lower odds of high performance on the gluten knowledge question (41.30%, 44.74%, 59.83%, and 40.55% lower respectively). Gluten avoidance improved the model fit and the portion of explained data but the variable on its own was not significant.

Next, we examined which variables were associated with participants' having symptoms upon eating sourdough; these odds ratios appear in **Figure 4.8 B**. We hypothesized that diagnosis category would be a major explanatory variable, along with gluten avoidance. Indeed, the analysis does show that diagnosis is a major factor here. Having a diagnosis of CD leads to 1.729x greater odds of symptoms compared to the UD population, and a diagnosis of NCGS is associated with 2.089x greater odds. Increasing income levels also contribute to greater odds of

symptoms upon eating sourdough, except for the very highest income bracket, which was not a significant factor. Having a high level of gluten knowledge is associated with 51.2% lower odds of having symptoms upon eating sourdough.

Finally, we examined the factors associated with what leads participants to eat sourdough (vs. avoiding it entirely); these coefficients and confidence intervals are displayed in **Figure 4.8 C**. We hypothesized that relevant explanatory variables for this outcome would include diagnosis category, social support, and reporting symptoms upon eating sourdough. Once again, diagnosis is a relevant explanatory variable for this outcome as expected; a diagnosis of CD is associated with 1.739x greater odds of being willing to eat sourdough compared to the UD group, while a diagnosis of NCGS is associated with 2.652x greater odds. Claiming to follow a gluten-avoidant diet corresponds with 33.48% lower odds of being willing to eat sourdough, while claiming to have symptoms upon eating sourdough is associated with 13.72% lower odds. Achieving a high score on the gluten knowledge test and having a lack of social support are both associated with 69.42% lower odds of eating sourdough. While the odds ratios for gluten avoidance and symptom manifestation were not significant, both variables contributed to improved model fit.

Upon regression analysis, both NCGS groups and CD groups were found to be associated with approximately two times greater odds of experiencing symptoms upon eating sourdough compared to the UD group. These groups are both more likely to eat sourdough and more likely to experience sourdough-mediated symptoms compared to the UD group. While it is unclear if these two variables bear a causal relationship, a Fisher's exact test on the proportions of respondents who are willing to eat sourdough (vs. not) and who have symptoms after eating sourdough (vs. not) returns a p-value < 0.0001. Indeed, the relationship can be visualized in **Figure 4.9**, in which it is clear that the proportions of affirmative responses for the questions are

nearly reversed.

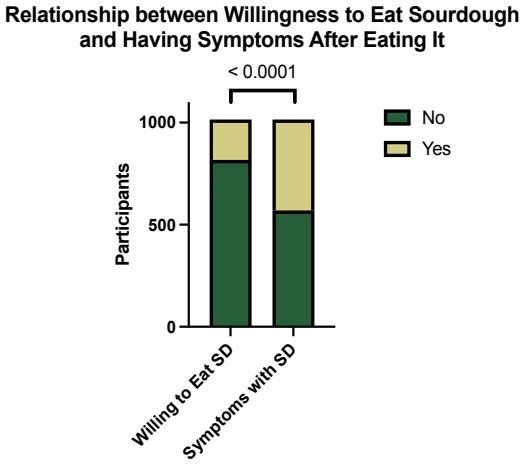

Figure 4.9: Proportions of participants indicating whether or not they are willing to eat sourdough (vs. never willing) and whether or not they experience symptoms upon eating sourdough (vs. experiencing no symptoms).

4.5 Discussion

This is the first study to probe the anecdotally accepted notion that sourdough reduces gluten-mediated symptoms in gluten-intolerant populations and to investigate the sourdough consumption habits and beliefs of these populations. The findings reveal that, contrary to popular belief, most gluten-sensitive individuals do not appear to benefit from the consumption of sourdough bread. Instead, only the UD group reports a significant reduction in sourdough-mediated symptoms compared to the other two groups (**Figure 4.3**) and a diagnosis of both CD and NCGS is associated with greater odds of experiencing symptoms from eating sourdough compared to the UD group (**Figure 4.8 A**). Therefore, we can conclude that for individuals diagnosed with a gluten sensitivity, sourdough is not likely to mitigate their gluten-stimulated symptoms relative to conventional bread. However, for people with undiagnosed or self-

diagnosed gluten sensitivities, sourdough may offer some relief.

4.5.1 The Undiagnosed Group is Statistically Distinct from the Other Diagnosis Categories

Results appear to indicate that sourdough only mediates symptoms of gluten intolerance in the UD group, while the NCGS and CD groups' symptoms do not improve upon sourdough consumption relative to non-sourdough bread products. The UD group is unique in other ways as well; this group stands out with the lowest rates of social support (87.73%, $p = 0.008$), the highest levels of gluten knowledge ($p = 0.0099$), and fewer symptoms when they eat sourdough ($p < 0.0001$). They are more likely to be female ($p = 0.003$) and have the oldest mean age and lowest mean education level ($p < 0.05$). The higher levels of gluten knowledge amount the UD group could suggest that individuals who self-diagnosed or who remain undiagnosed are motivated to actively seek out information or educate themselves about gluten-related issues. This could affect not only their knowledge of gluten but their willingness to engage with "alternative" treatment approaches, such as sourdough, and their perception of how sourdough consumption is likely to affect their symptoms. The fact that the UD group experiences fewer symptoms in response to sourdough (compared to the CD or NCGS groups) may also be partially explained by the possibility that their symptoms are not solely attributable to gluten, but rather to other dietary elements that are distinct in sourdough bread compared to non-sourdough bread products, such as FODMAPs.

4.5.2 Diagnosis Group Helps Explain Symptom Manifestation and Willingness to Eat Sourdough

Regression analyses indicates that diagnosis category is not an explanatory factor with regards to participants' level of gluten knowledge, but it does help explain which consumers are willing to eat sourdough and which consumer respond by developing symptoms. There are

several plausible explanations for why individuals in the CD and NCGS groups are more likely to manifest symptoms after sourdough intake. Because individuals in the CD and NCGS groups have a medical diagnosis of gluten sensitivity, while those in the UD group do not, it is possible that some members of the UD group misinterpret their symptoms. Because this group is self-reported as gluten-sensitive, it is conceivable that some of them have not interacted with medical professionals or been taught how to accurately assess symptoms of gluten sensitivity. Symptoms caused by some other dietary element that are misattributed to gluten would not necessarily manifest in response to sourdough, resulting in this group's lower odds of experiencing symptoms in response to sourdough. A higher level of gluten knowledge is also associated with reduced odds of sourdough-mediated symptoms, indicating that having knowledge of gluten, its effects, and which foods contain gluten may help gluten-sensitive individuals manage or strategically mitigate their own symptoms.

Some patterns of responses at first seem surprising but upon further examination appear to be internally consistent. For example, it may seem counterintuitive that the CD and NCGS groups are both more likely to eat sourdough and more likely to experience sourdough-mediated symptoms compared to the UD group. This relationship, shown in **Figure 4.9**, demonstrates that the proportions of affirmative and negative responses to each of these questions are reversed. Perhaps a failure to experience symptoms encourages a willingness to eat sourdough, or maybe the reverse is true, and exposure to sourdough mitigates symptom severity.

4.5.3 Social Factors Affecting Choices and Symptoms

It appears that demographic, social, and lifestyle factors are associated with the consumer's choices regarding sourdough consumption. For example, income is a one factor that explains whether or not participants experience symptoms after consuming sourdough. There is

little difference between income brackets. In fact, participants in all income brackets above $25,000/year have approximately 75% greater odds of experiencing symptoms upon eating sourdough except for the very highest bracket, which is associated with lower odds but does not achieve statistical significance (**Figure 4.7 B**). This may indicate that survey participants managing the dietary needs of household on a severely restricted income are more likely to overlook symptoms associated with cheaper and more accessible dietary options15 It could also be that increasing income provides access to a greater variety of food choices and eating habits,34 such that individuals at higher income levels are more aware of how their bodies respond to a number of different foods including sourdough. Similarly, different income brackets may exhibit incongruencies in lifestyle or access to health care, both of which could affect how their symptoms respond or their awareness of their symptoms.

A lack of peer and family support, which contributes to lower odds of willingness to eat sourdough, is another example of social factors at play. This effect could be attributed to a reluctance to experiment without sufficient sanction from one's peer or social group. Gender and race also play an explanatory role in performance on the gluten knowledge test. Being non-white is associated with lower odds of achieving a high score, while the opposite is true for being female or non-binary (**Figure 4.7 A**). Both of these trends track with clinical data showing that women are diagnosed with both CD35 and NCGS36 at rates much higher than men. Conversely, CD is diagnosed less frequently in non-white populations.[37] It is possible that higher rates of diagnosis within a demographic group lead to greater efforts to self-educate about dietary needs and restrictions. Likely, a feedback loop exists in which higher rates of diagnosis in a group are associated with greater awareness, education, and social support for the condition, which in turn leads to even more diagnoses as patients are exposed to symptom management strategies. The

opposite would also be true; few diagnoses would generate little support or awareness within a community, leading to avoidance or misinterpretation of symptoms and a failure to identify or manage the condition. These results suggest that socioeconomic dynamics play a role in consumer experiences, but more work is needed to understand the degree to which social influences govern dietary choices overall and sourdough consumption specifically.

4.5.4 Sources of Gluten Knowledge and Factors Affecting Gluten Avoidance

The analysis of factors contributing to gluten knowledge illuminated some social and demographic factors, as well as practical ones. Unsurprisingly, reading ingredient labels is associated with greater odds of having high levels of gluten knowledge, as is having some background knowledge or training. What is more surprising is that traditional resources, such as medical practitioners and websites, were not associated with any increase in odds of scoring high in gluten knowledge. This could suggest that information from these resources is ineffectively communicated or perhaps that people who use these resources (as opposed to reading labels or seeking training) are less motivated to retain the information. Gender (being female or non-binary) was also a contributing factor to odds of having high scores for gluten knowledge. Survey bias is an unlikely explanation, since gender demographics in survey respondents (50.24% female, 48.07% male, 1.57% nonbinary) approximately reflect U.S. population demographics.38 Women are more likely than men to say they follow a gluten-free diet39 and American women do more cooking and grocery shopping.40 Gender has also been linked in the past as an explanatory feature of various purchasing decisions, including food purchasing.41 While none of these factors can be directly linked to gluten knowledge, it is not unreasonable that individuals who interact more with food preparation and food purchasing, and who are more likely to be gluten-avoidant, would acquire greater knowledge of this food category. Further

research is needed to explore the explanatory link between women and gluten knowledge and to determine if the same assumptions extend to non-binary individuals, about whom research is currently very limited. Being non-white was also associated with lower odds of achieving a high score on the gluten knowledge test. It is likely that systemic issues account for this, as gluten sensitivities such as celiac disease have long been underdiagnosed or misattributed in non-white populations,42,43 possibly leading to lower availability of information, medical care, or social awareness that may contribute to gluten knowledge. Finally, one unusual feature of this analysis was the fact that being willing to consume sourdough (as opposed to never consuming it) was associated with lower odds of high performance on the gluten knowledge test. This connection might be clarified by the notion that people who possess better knowledge of managing their condition are less likely to eat foods that could provoke symptoms. Indeed, this explanation is further supported by the fact that having a high level of gluten knowledge is associated with lower odds of choosing to eat sourdough in a separate regression analysis.

4.5.5 Factual and Counter-Factual Motivators

Finally, this survey highlights several commonly held beliefs and behaviors regarding sourdough, providing insight as to what consumers value when they make purchasing decisions. For example, these data reveal that when gluten-sensitive consumers choose to eat sourdough, taste is the principal motivating factor (selected by 50.75% of respondents), indicating that the sensory appeal of sourdough plays a major role in consumers' choice to eat it, even among the gluten-sensitive population. This data also reveals that 24.24% of respondents incorrectly believe that sourdough has probiotic properties. This is likely due to a conflation between fermented and probiotic foods, categories between which often overlap. Consumers may be aware of the mounting evidence for the importance of a healthy gut microbiome in managing

symptoms of gluten-mediated intestinal inflammation and the indications that probiotics may be a useful therapy in some cases.[44] If some gluten-sensitive consumers are eating sourdough in the belief that it provides probiotic therapy, they may in fact be aggravating the symptoms they intend to alleviate. This prompts us to consider how we can tackle this widespread misconception.

In short, while demographic factors appear to be associated with sourdough consumption habits, diagnosis category is not a major factor. Although sourdough is associated with perceived health benefits and improvement of symptoms in the minds of many gluten-sensitive individuals, the data from this survey does not support that these benefits reliably manifest for consumers with celiac disease or non-celiac gluten sensitivity.

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CHAPTER 5: PROFESSIONAL SOURDOUGH BAKERS AS BROKERS OF HEALTH ADVICE: RESULTS OF A SURVEY ON BAKERS' BELIEFS AND PRACTICES

5.1 Summary

In response to reports that some commercial sourdough bakers believe that sourdough can alleviate the symptoms of gluten-mediated intestinal disorders, a survey was developed to probe the beliefs and attitudes of professional sourdough bakers. The survey investigated the 25 bakers' starter maintenance practices, their estimated sales in different areas, their willingness to recommend sourdough to gluten-sensitive customers and their motivations for doing so, and their impressions of how their gluten-sensitive customers respond to eating sourdough. The survey also tested the bakers for their knowledge of gluten-free foods and labeling practices.

Results indicate that commercial sourdough bakeries' starter maintenance practices vary widely in terms of type of grain used to feed the starter, hydration percentage and storage temperature. Nearly half of all responding bakeries declared their willingness to recommend sourdough bread to their gluten-sensitive customers. In all cases, their motivations for doing so were health-related, and some motivations included incorrect information, such as the belief that sourdough is probiotic. Sourdough bakers demonstrated an overall high level of gluten knowledge despite apparent ignorance of gluten-free labeling requirements. Little relationship was found between bakery practices, baker knowledge, and behavior towards customers. This study sheds light on how many commercial sourdough bakers view themselves as key touchpoints in the chain of wellness information for gluten-sensitive customers and highlights the importance of making sure bakers are well-equipped to interact with this population.

5.2 Introduction

In recent years, sourdough bread has experienced a resurgence in popularity, capturing the interest of consumers due to its purported health benefits and association with natural, clean-label status. Customers are increasingly taking into account to the health claims and ingredient list of the products they purchase.[1] There is evidence that part of sourdough's uptick in popularity may be connected to the fact that consumers increasingly prefer products with fewer chemical preservatives.[2] Sourdough has also benefited from an association with health since at least Pliny the Elder's writings.[3] Today, sourdough is associated with improved bread quality, flavor, aroma, texture, shelf-life, and nutritional properties compared to yeast-leavened bread.[4] Some demonstrated health benefits include an improved glycemic response,[5] easier digestibility, [6,7] and better nutrient availability.[8] Overall, sourdough has gained a reputation for the ability to improve gastrointestinal health of consumers.[9] All of these factors likely contribute to recent increases in demand for sourdough. Commercial bakers have responded to this surge in enthusiasm by offering more sourdough products. As the demand for sourdough continues to rise and more and more commercial bakers step in to fill the need for sourdough products, it is imperative to understand how bakers exchange information with their customers about sourdough, which is often promoted for health reasons rather than for its organoleptic properties. Are commercial bakers well-informed about the health claims made around sourdough? Are they able to distinguish common misconceptions ("sourdough is probiotic") from claims supported by research? Which, if any, of these beliefs do they pass along to their customers?

Sourdough is also anecdotally reported to reduce the symptoms of gluten-mediated disorders relative to yeast-leavened bread.[10] **Chapter 4** reported on the results of a survey examining this question from the point of view of gluten-sensitive consumers. However, as we engaged in background research and preparation for other aspects of this study, we had the

opportunity to interact with commercial sourdough bakers. Almost every baker claimed to serve a population of gluten-sensitive customers who benefited from the baker's sourdough products. As producers of sourdough products, commercial sourdough bakers are likely seen by customers as authoritative sources of information about the health benefits and risks of consuming sourdough bread, and many bakers seemed to view themselves as arbiters of health and wellness information, especially for gluten-sensitive clients. Thus, we determined to include the perspectives of commercial sourdough bakers as a point of comparison to the survey of gluten-sensitive consumers presented in **Chapter 4**.

The purpose of this survey is to understand the role of bakers in communicating health and safety information about sourdough to their customers—specifically, to gluten-sensitive customers. This survey sheds light on commercial sourdough bakers' starter maintenance practices as well as their beliefs about sourdough, their willingness to share those beliefs with gluten-sensitive customers, and the feedback they receive from gluten-sensitive consumers regarding the effect of sourdough on their gluten-mediated symptoms.

Undertaking this study is crucial for several reasons. First, it allows for an examination of the range of practices that bakers employ to ensure consistency and quality in their sourdough baking. Second, it will provide insights into the strategies that bakers use to manage their gluten-sensitive customers and how they offer their customers a safe and enjoyable experience. Moreover, by investigating the bakers' beliefs about sourdough and their motivations for recommending sourdough to gluten-sensitive consumers, we can gain insight into the perceived benefits of sourdough bread from the perspective of those who craft it daily. This study will also allow for a better understanding of the feedback interaction between gluten-sensitive customers and bakers; it will demonstrate which beliefs about sourdough are at least partially based on

direct interactions with gluten-sensitive customers and are therefore inherently anecdotal. Finally, understanding professional sourdough baker's knowledge of ingredient and labeling practices contributes to an understanding of the industry's awareness and adherence to regulations and guidelines. It is important to gain a picture of how closely industry practices align with said guidelines for the purposes of gauging consumer confidence and safety, especially for consumers following a gluten-restricted diet.

5.3 Methods

5.3.1 Survey Design

A survey (**Appendix B**) was developed under the guidance of Colorado State University faculty and Extension agents and its content was reviewed by faculty and Extension specialists with experience in survey design. They survey consisted of 28 questions and was built through the Qualtrics survey platform (Qualtrics, Provo, UT). The survey was deemed exempt by the International Review Board (IRB) due to minimal likelihood of risk to participants and was approved under protocol number 3963. Survey content addressed professional baker's interactions with their gluten-sensitive clientele including feedback received from gluten-sensitive customers regarding the effects of consuming sourdough bread. Additional questions probed the baker's sourdough starter care and maintenance practices as well as their knowledge of labeling and gluten-containing foods. These questions were largely designed to compare commercial bakers' responses to consumers' responses to similar questions posed in the consumer survey described in Chapter 4. Some questions were also included to assess potential areas of future investigation into commercial bakeries' starter handling practices.

5.3.2 Survey Distribution

Recruitment for the survey was achieved through requests on social media platforms including the researchers personal Instagram and Twitter accounts as well as the Food Science and Human Nutrition Department Instagram account. Survey information and links were also posted on relevant Reddit forums and disseminated through various listservs and through connections through CSU Extension. The survey link was shared with extension colleagues, who distributed the link through their own listservs or to contacts who were candidates for participation. Inclusion criteria required that participants be over 18 years of age and that they own or be employed by a commercial bakery that produces some portion of sourdough products. They survey received a total of 42 participants across multiple U.S. states. Of these, 17 participants were eliminated for not meeting the inclusion criteria or for unintelligible responses, leaving a sample size of n=25.

5.3.3 Statistical Analysis

Statistical analysis was performed first by cleaning and processing the data in Microsoft Excel. Excel formulae were used to calculate the frequencies of each response type. These data were transferred to Graphpad Prism 10.0 where the data were plotted and analyzed further for correlations. Graphpad Prism 10.0 was used to build all figures.

To determine level of gluten knowledge on Question 22 (**Appendix B**), a score from 0-5

Table 5.1: Codes used to convert categorical responses to continuous numerical responses to investigate correlations.

Response	Code/Value	Response	Code/Value
Question 3: Number of Starters		Question 10: Hydration Percentage of Starter	
1 starter	1	Answered	Numerical value of response
2 starters	2	Not answered	0
3 starters	3	Question 11: Starter Contains Other Ingredients?	
4 starters	4	Yes	2
4 or more starters	5	No	1
Question 4: Make Gluten-Free Product?		Not answered	0
Yes	2	Question 12: Time Starter Continuously Maintained	
No	1	Answered	Numerical value of response
Not answered	0	Not answered	0
Question 5: Have Gluten-Sensitive Customers?		Question 13: Starter Feeding Interval	
Yes	2	Answered	Numerical value of response
No	1	Not answered	0
Not answered	0	Question 15: Starter Holding Temperature	
Question 6: Approximate Sales from Sourdough?		Warm	4
75-100%	4	Ambient	3
50-75%	3	Variable	2
25-50%	2	Refrigerated	1
0-25%	1	Not answered	0
Not answered	0	Question 17: Customers have symptoms with SD?	
Question 7: Approximate Sales from Gluten-Free?		Same	4
75-100%	4	Fewer	3
50-75%	3	No symptoms regardless	2
25-50%	2	Don't know/Don't Discuss	1
0-25%	1	Not answered	0
Not answered	0	Question 20: Recommend SD to GS Customers?	
Question 8: Make Gluten-Free Product (21 CFR 101.91)?		Yes	2
Yes	2	No	1
No	1	Not answered	0
Not answered	0	Question 23: Limit of 20ppm for Labeling GF Foods	
Question 9: Base Grain Used to Feed Starter?		Correct	2
Wheat only	4	Incorrect	1
Rye only	3	Not Answered	0
Wheat and rye combo	2	Question 24: Gluten-Containing Foods Test	
GF grains	1	Response	Numerical score on test
Not answered	0	No response	0

was identified by allowing 1 point for every correct answer and subtracting 1 point for every incorrect answer. No points were deducted for missing correct answers. Scores from 0-1 were assigned as Low, a score of 2-3 was Medium, and a score of 4-5 was considered a High level of gluten knowledge.

Responses were places on a continuous scale to allow further analysis of correlations within the data set. The coding schemes used are shown in in **Table 5.1**. Questions 1 and 2 were inclusion criteria questions, and were not coded or included in analysis. Questions 15, 16, 18,

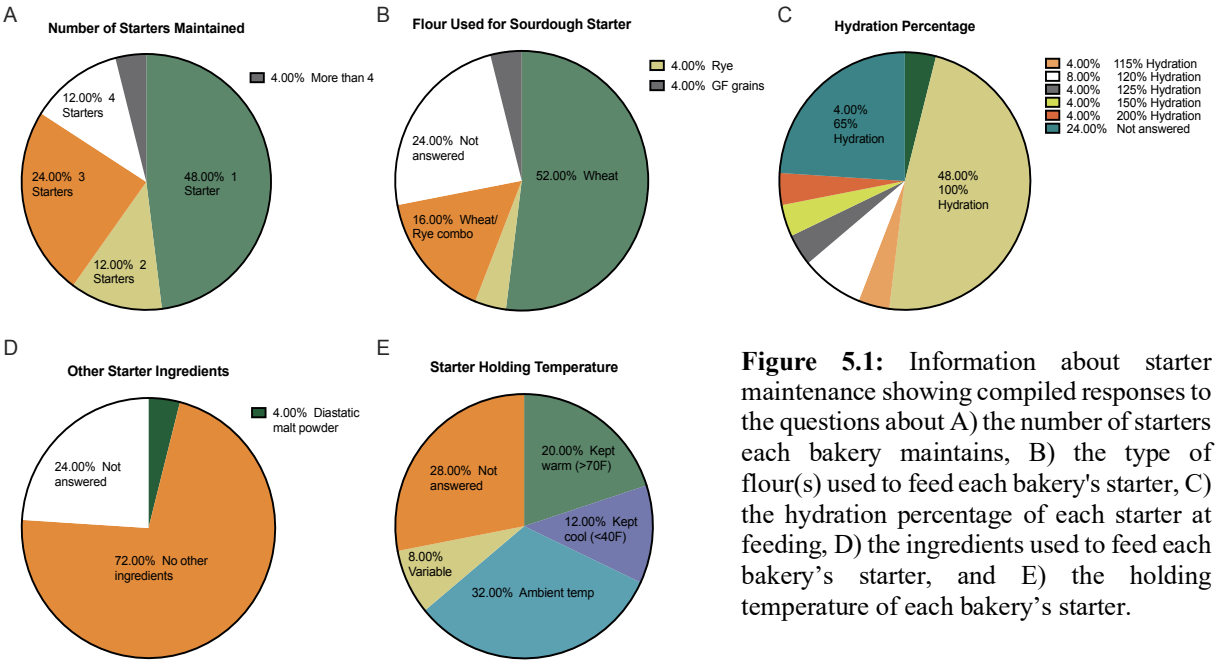

Figure 5.1: Information about starter maintenance showing compiled responses to the questions about A) the number of starters each bakery maintains, B) the type of flour(s) used to feed each bakery's starter, C) the hydration percentage of each starter at feeding, D) the ingredients used to feed each bakery's starter, and E) the holding temperature of each bakery's starter.

19, 21, and 22 were not coded or included in correlation analysis because these responses were check-all-that-apply, open write-in responses, or other types of responses that could not be coded on a continuous scale.

5.4 Results

5.4.1 Starter Ingredients and Maintenance

The survey investigated connections between bakers' sourdough starter maintenance practices and the beliefs about sourdough that constitute the information they communicate with their customer base. Bakers were asked to report how many starters they maintain, the feeding and maintenance conditions of their most frequently used starter, and the ingredients included in their most frequently used starter. All bakers reported on the number of starters they maintain, but six bakeries opted not to provide any further information about their starter ingredients or maintenance practices.

Nearly half of bakers (48.0%, or 12 respondents) reported maintaining only a single

starter, while the other 13 participating bakeries maintain multiple starters (**Figure 5.1 A**). Subsequent questions specified that the bakers should respond with information pertaining only to their "most commonly used starter." Bakers were asked about the flour used to feed and maintain their starters. 52.0% of bakeries reported using only wheat flour to maintain their starters, while one bakery (4.0%) uses only rye. 16.0% of bakeries reported using a combination of wheat and rye, and one bakery (4.0%) uses various gluten-free grains (**Figure 5.1 B**). Six bakeries withheld responses to this question.

Next, bakers were asked to report the hydration percentage of their starter, as defined by:

$$\frac{\text{Grams Flour Fed}}{\text{Grams Water Fed}} \text{X } 100 = \text{Hydration Percentage}$$

Responses ranged from 65% hydration to 200% hydration, with the mode response being 100% hydration (**Figure 5.1 C**). Six bakeries declined to respond. Bakeries were also asked to report on any other ingredients used to feed their starter besides flour and water. One bakery (4.0%) reported including diastatic malt powder in feedings. The remaining respondents claimed to feed their starters no ingredients other than flour and water (**Figure 5.1 D**).

Responses related to starter storage conditions varied widely (**Figure 5.1 E**). While other responses varied widely, 20.0% of bakeries surveyed incubate their starters at constant warm temperatures (above 70°F), while 12.0% incubate the starters at constant cool temperatures (below 40°F). The maximum constant holding temperature recorded was 82°F, while the minimum constant holding temperature was 35.5°F. Another 32.0% (8 bakeries) allow the starters to rest at ambient room temperature, with no effort to control for daily or seasonal temperature shifts. The remaining 8.0% claim that the holding temperature of their starter varies depending how far it has progressed through its feeding cycle, with temperatures in variable

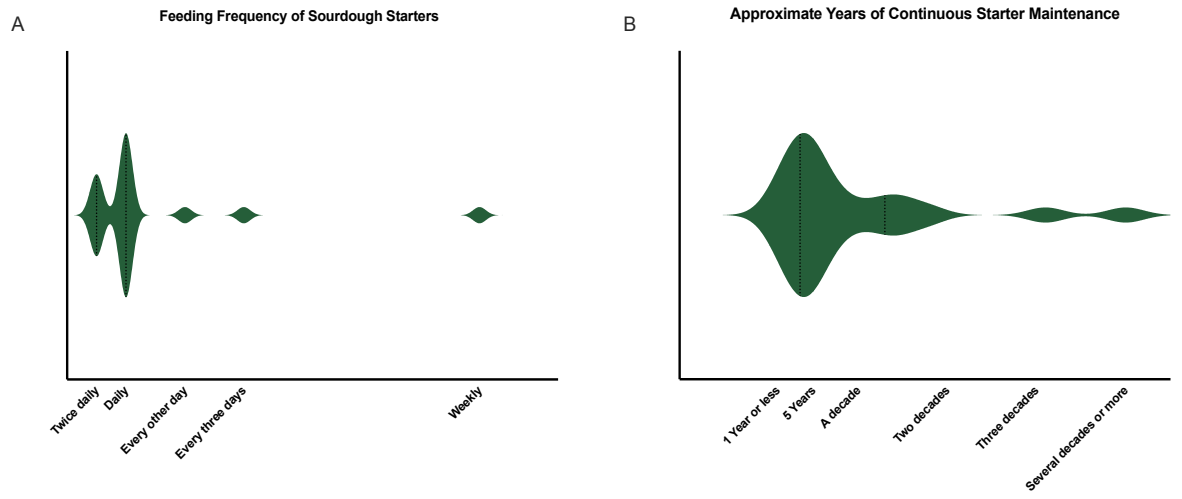

Figure 5.2: Compiled information about starter maintenance, showing A) hours elapsed between feedings and B) estimated number of years that the starter has been continuously maintained by the baker.

cycles recorded from 35°F to 85°F. Seven bakeries declined to share information about their starter holding temperature.

When asked about the feeding frequency of their starters, 40.0% of bakeries shared that they engage in daily feedings, with the second most common response being twice daily feedings (20.0% of bakeries). Other responses varied with the longest feeding interval being weekly feedings, a practice claimed by only one baker (4.0% of responses). The same seven bakeries who did not disclose starter holding temperature also opted to share no information about feeding frequency (**Figure 5.2 A**). Finally, bakeries were asked to share how long their starter had been continuously maintained. Responses ranged from less than one year to several decades. One baker with a decades-old starter claimed that the same starter had been maintained for "generations" while another claimed to have personally maintained the starter for 31 years but alleges that it was originally transported from Belgium 200 years ago. Three bakers neglected to answer this question (**Figure 5.2 B**).

5.4.2 Bakery Customer and Sales Information

The survey collected some general information about each bakery, asking whether each bakery prepared sourdough and/or gluten-free products, what percentage of bakery sales comprised each of these product categories, and whether they served gluten-sensitive customers. Most bakeries offered information for this set of questions, with only one bakery abstaining.

Of the bakeries surveyed, 92.0% (23 respondents) claim to serve some portion of gluten-sensitive customers, while 1 bakery (4.0% of respondents) claimed to not be aware of any gluten-sensitive customers. One bakery declined to answer (**Figure 5.3 A**). Only 24.0% of bakeries surveyed (6 respondents) prepare gluten-free products compliant with the Code of Federal Regulations Title 21, which governs preparation and labeling of gluten-free foods,[11] while the remaining 76.0% do not (**Figure 5.3 B**). When asked if they prepare any products which are sourdough-fermented as well as gluten-free, 12.0% (3 bakeries) replied yes, while

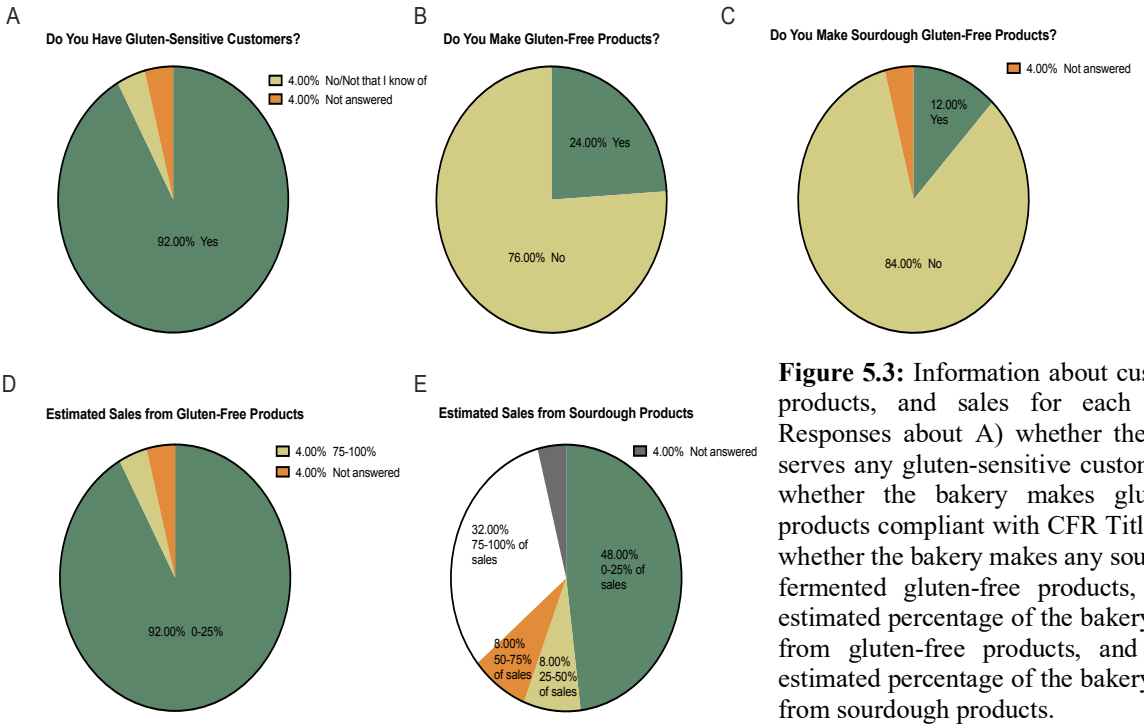

Figure 5.3: Information about customers, products, and sales for each bakery. Responses about A) whether the bakery serves any gluten-sensitive customers, B) whether the bakery makes gluten-free products compliant with CFR Title 21, C) whether the bakery makes any sourdough-fermented gluten-free products, D) the estimated percentage of the bakery's sales from gluten-free products, and E) the estimated percentage of the bakery's sales from sourdough products.

84.0% (21 bakeries) replied no, and one declined to answer (**Figure 5.3 C**).

Bakeries were also asked to estimate what percentage of their sales are represented by both sourdough and gluten-free products. Gluten-free products represent a small share of most bakeries' sales. One bakery replied that gluten-free products account for 75-100% of their sales, while the remaining 96.0% of bakeries (23 responses) indicated that gluten-free products represent from between 0% and 25% of sales, with one bakery choosing not to respond (**Figure 5.3 D**). Sourdough products account for a larger share of most bakeries' sales, with 32.0% (8 respondents) indicating that sourdough accounts for 75-100% of their sales and 48.0% (12 respondents) claiming that sourdough products represent 0-25% of sales (**Figure 5.3 E**).

5.4.3 Interactions with Gluten-Sensitive Customers

Next, bakers were asked to provide information about how they discuss sourdough with their gluten-sensitive customers and about the feedback they receive from their gluten-sensitive clients. When asked if they specifically recommend sourdough products to gluten-sensitive customers, 11 bakers (44.0%) replied "Yes" while 9 bakers (36.0%) replied "No" and 5 bakers abstained from the question (**Figure 5.4**). The same five opted out of the subsequent question,

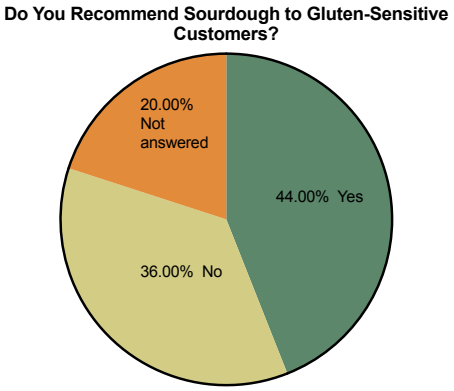

Figure 5.4: Breakdown of responses indicating whether bakers claim to recommend sourdough to their gluten-sensitive customers.

which asked, "If applicable, what are your reasons for recommending sourdough to your gluten-sensitive customers?" Seven of the 9 bakeries who claimed not to recommend sourdough to their gluten-sensitive customers selected the response "I don't know/I don't talk to my customers about this topic" to describe their motivation, showing consistency between responses to the two questions. However, one of these 9 selected the response, "I believe sourdough has probiotic properties" while another selected, "I think they will like the taste" indicating that there may be a disparity in some bakers between perceived and actual behavior, possibly resulting in the transmission of mixed messages to customers. The remaining 11 bakers, who all replied "Yes" to the question "Do you recommend sourdough to your gluten-sensitive customers?" provided a variety of motivations (**Figure 5.5**). "I believe sourdough has lower levels of gluten" was only mentioned as a motivation by two bakers.

Next, bakers were asked to recount how they have heard gluten-sensitive customers describe their experiences with consuming sourdough and its effect on the symptoms of their condition. Nearly half of bakers (48.0%, or 12 respondents) reported that their customers experience fewer gluten-mediated symptoms upon eating sourdough than non-sourdough bread. One baker (4.0% of respondents) asserted that their customers seem to have the same symptoms

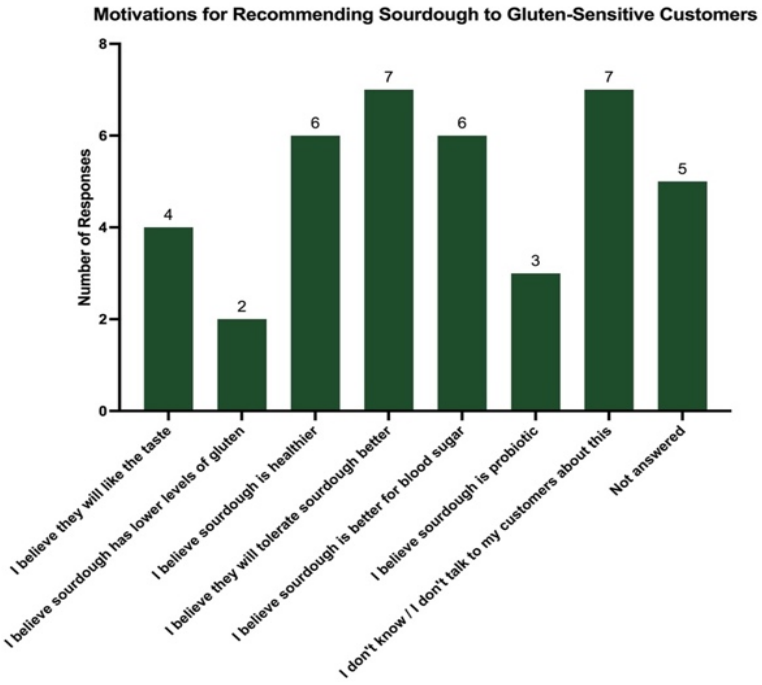

Figure 5.5: Baker's motivations for recommending sourdough to their gluten-sensitive customers. Those in the "not answered" category are the same five who opted out of the question "Do you recommend sourdough to your gluten-sensitive customers?"

with sourdough as with non-sourdough bread, while another baker (4.0% of respondents) replied that their customers do not experience symptoms with any type of bread. Notably, this was not the same baker who claimed to be unaware of serving gluten-sensitive consumers. Six bakers (24.0%) indicated some version of insufficient information to answer, and 5 abstained from the question (**Figure 5.6 A**). When asked how their gluten-sensitive customers' symptoms improved upon consuming sourdough bread, 5 bakeries chose not to respond, and 10 others claimed that they didn't know or didn't discuss this topic with their customers. The remaining bakeries provided feedback in a variety of categories, with "less bloating" and "less abdominal pain" being the dominant responses (**Figure 5.6 B**).

Bakers were also asked to assess their gluten-free customers' motivations for consuming sourdough bread. Five bakeries declined to answer, while 6 replied, "I don't know/I don't discuss this with my customers." "They believe sourdough is healthier", "They like the taste",

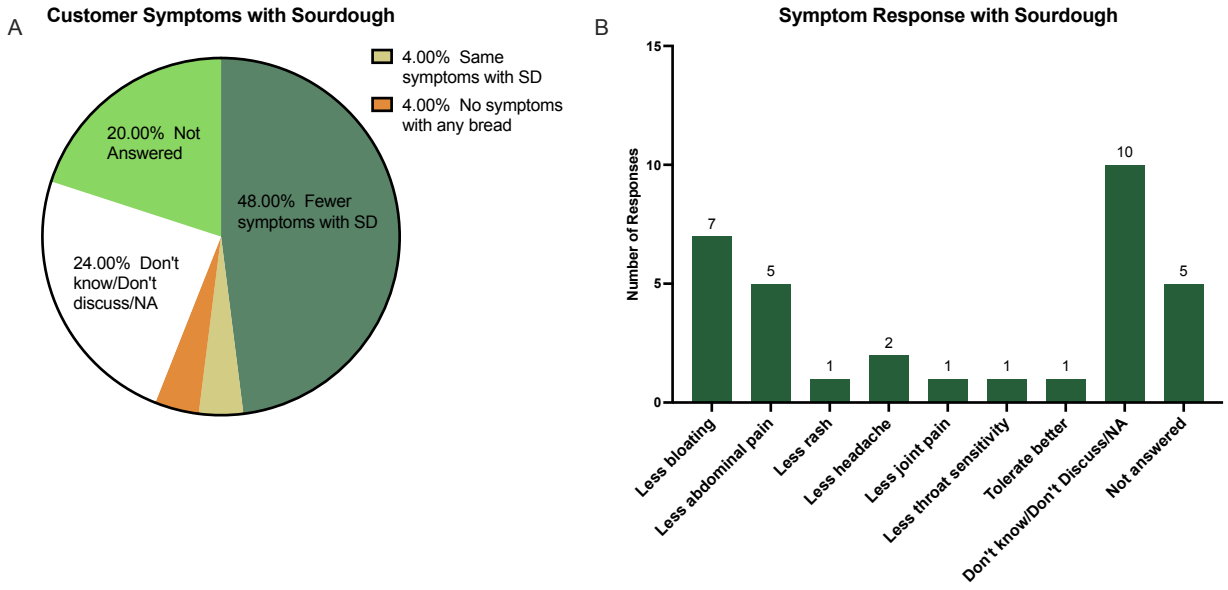

Figure 5.6: Responses showing feedback bakers have received from gluten-free customers about these customers' symptoms in response to consuming sourdough. Bakers reported on A) how they perceive their gluten-sensitive customers' symptoms to manifest upon eating sourdough relative to yeast-leavened bread, based on interactions with these customers, and B) in what way the gluten-sensitive customers symptoms have changed upon eating sourdough relative to yeast-leavened bread, based on interactions with these customers.

and "They tolerate sourdough better" were the dominant motivations cited with 9, 10, and 11 mentions each, respectively. Other motivations such as "They believe sourdough is probiotic", "They believe sourdough contains lower levels of gluten", and "They believe sourdough is good for their blood sugar" each received 5-6 mentions each, while "They believe sourdough is less processed" and "They believe sourdough is easier to digest" were each mentioned only one time each (**Figure 5.7 A**).

Finally, the survey finished with three brief knowledge tests relating to sourdough, gluten, and labeling. Under the hypothesis that bakers might be transmitting information about the management of the gluten-mediated intestinal symptoms, it seemed relevant to determine if bakers are well-informed about the causative agent of this pathology and with their comfort handling and labeling baked products. The first knowledge test pertained to labeling of sourdough products. It asked, "Which of the following should appear on the ingredient label for

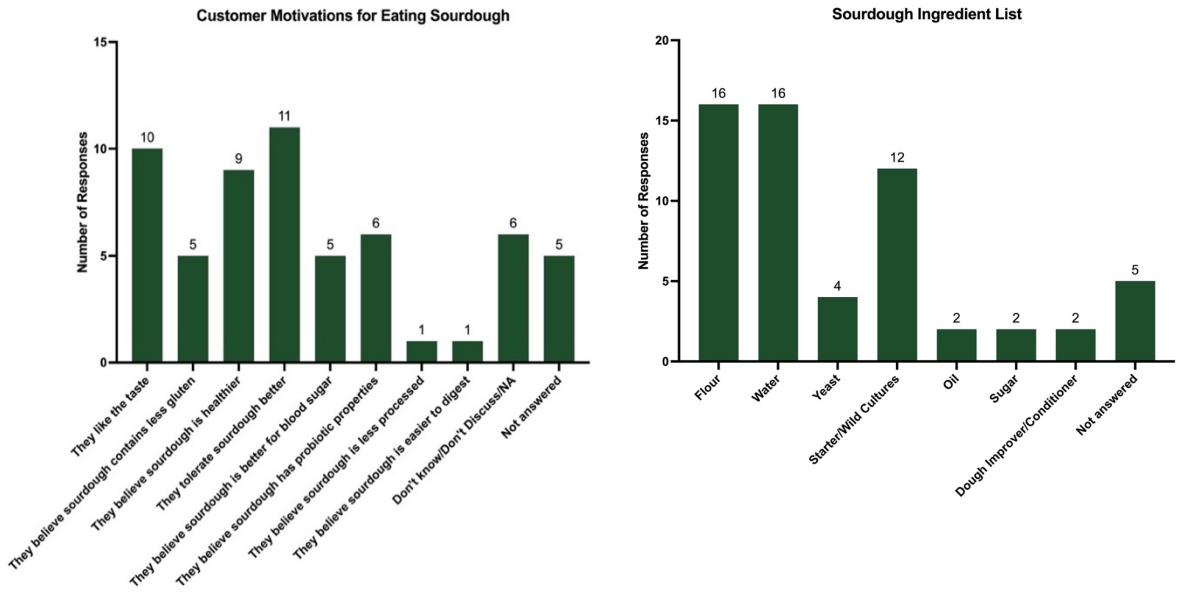

Figure 5.7: (A) Bakers' assessment of why gluten-free consumers might choose to consume sourdough, based on interactions with these customers, and (B) number of times each ingredient was mentioned in response to the question, "Which ingredients should be included on an ingredient label for traditional sourdough?"

traditional sourdough bread?" and asked respondents to choose from a list. Eight bakers (32.0%) abstained from this test. Of the 17 who offered answers, 16 (64.0%) of them included both "Flour" and "Water" as responses; 12 (48.0%) also included "Yeast". Another 4 (16.0%) included "Starter/Wild cultures" instead of "Yeast" in addition to both "Flour" and "Water". "Oil", "Sugar", and "Dough improvers/conditioners" were included by two bakeries each. One respondent listed *only* "Sugar" and "Dough improvers/conditioners" (**Figure 5.7 B**). Several other responses were not chosen by any respondent. When asked the second knowledge test, "What is the upper limit of gluten content for a product to be labeled 'gluten-free'?", nearly half of participants (12 bakeries) failed to provide any answer. Of the 13 bakeries who attempted a response, only 6 (24.0% of total) replied correctly with a response of 20 ppm (**Figure 5.8 A**). This limit, 20 ppm, is the upper limit of gluten that a food may contain in order to be labeled "gluten-free" in compliance with CFR 101.91[12]. On the third and final knowledge test, bakers were shown images and names of ten foods, five of which contained gluten and five which did not. They were asked to list all of the foods which contained gluten, as typically prepared. Seven

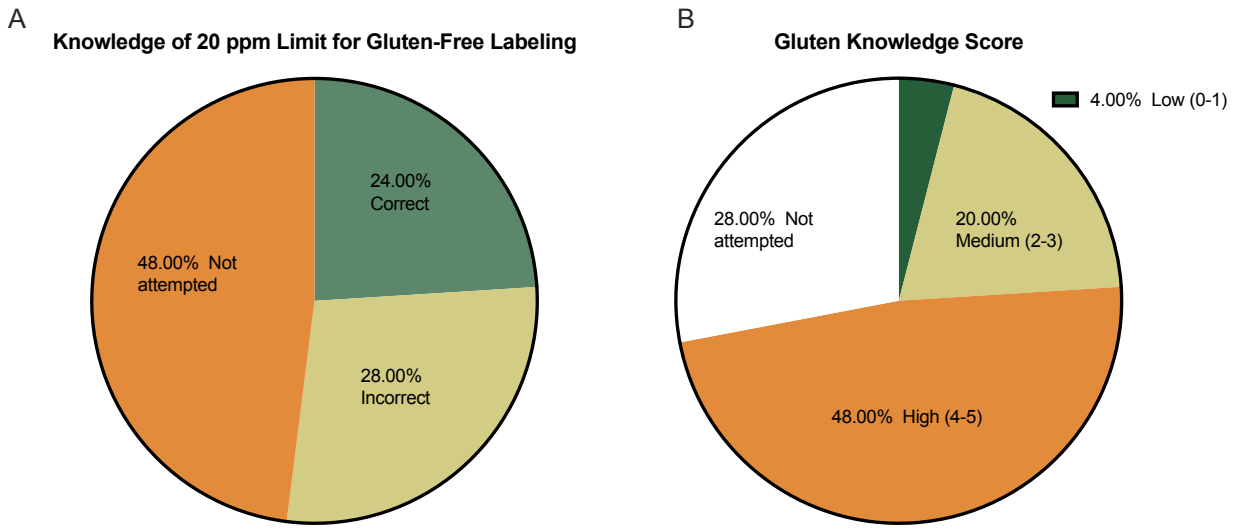

Figure 5.8: Results of two questions testing bakers' knowledge of gluten labeling (A) and of gluten-containing foods (B).

respondents declined to attempt this knowledge test. Twelve participants (48.0% of the total) achieved a High score (of 4 to 5), 5 participants (20.0%) managed a Medium score (of 2-3), and only 1 participant (4.0%) a Low score (of 0 to 1) (**Figure 5.8 B**).

5.4.4 Weak Correlations Link Bakery Practices and Perception of Customer Symptoms

In order to seek relationships between bakeries' starter management practices and their willingness to act as wellness advisors to gluten-sensitive customers, correlations were investigated between the responses in each section of questions. We hypothesized that because sourdough microbiomes are dependent on their environment and management, if bread quality outcomes are driven by unique sourdough microbiomes, then bakeries with different starter handling practices would produce bread with different quality outcomes. These outcomes would be reflected by the customer response ("Customer Symptoms"). This analysis also helped shed light on the degree and type of responsibility professional bakers take towards their gluten-sensitive customers, reflected in their performance on the gluten knowledge test ("Gluten

Knowledge") and in their willingness to recommend sourdough to glute-sensitive customers ("Recommend SD?"); we investigated correlations for both of these variables with unique bakery practices.

Few strong relationships were identified, but some weak trends emerged. One significant correlation was identified between increasing feeding interval and an increased likelihood that the bakery would offer gluten-free products ($p = 0.001$) and have a higher traffic in gluten-free sales ($p < 0.001$) (**Figure 5.9**). A weaker, but still significant, negative correlation was found

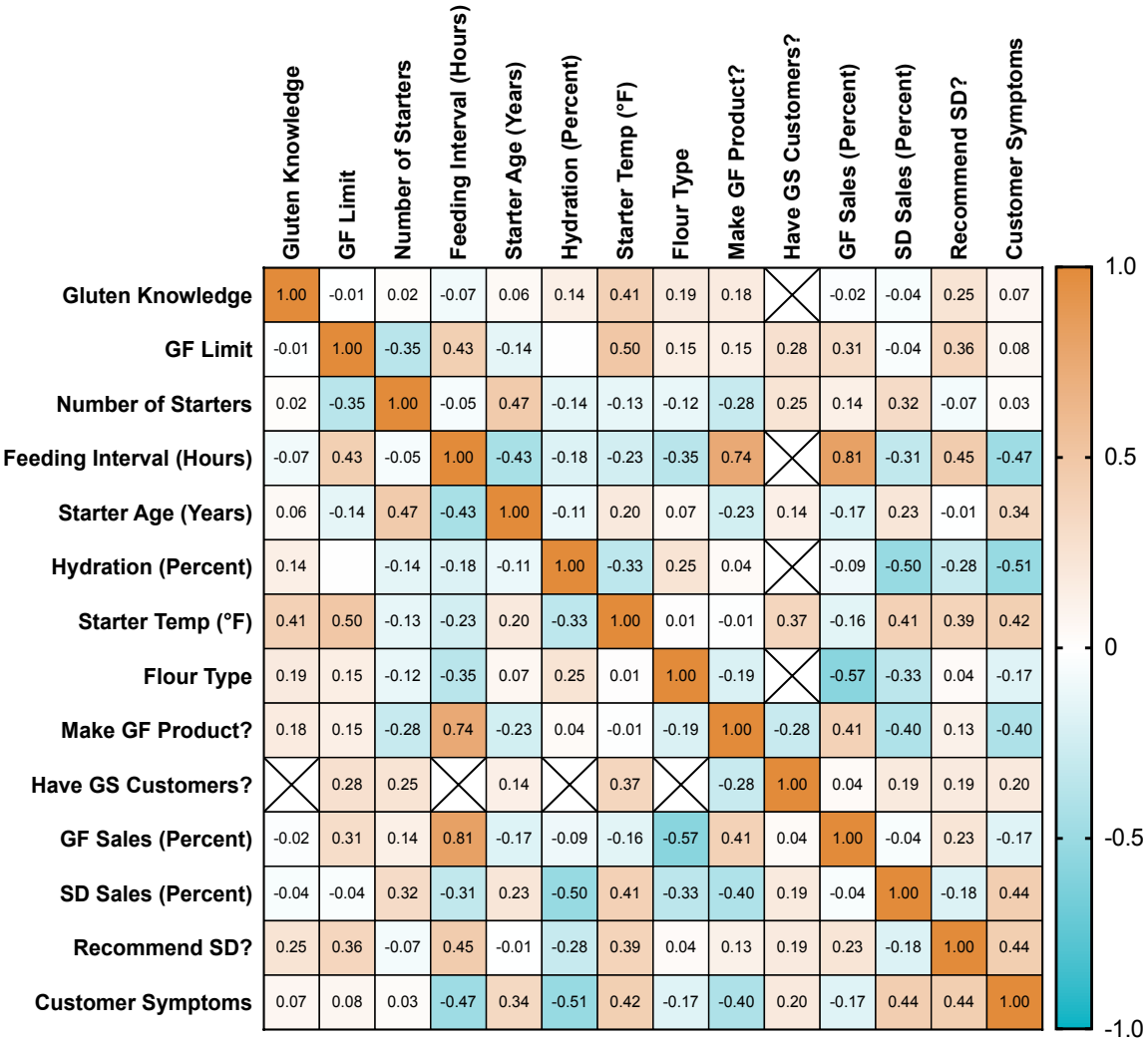

Figure 5.9: Pearson's r correlation matrix showing relationships between bakery knowledge, starter maintenance habits, and customer interaction practices.

between customer symptoms and several bakery practices. Bakeries perceived fewer gluten-mediated symptoms in their customers associated with an increase in time between starter feedings and an increase in starter hydration. Bakeries who perceived fewer customer symptoms from sourdough consumption were also more likely to recommend sourdough to gluten sensitive customers, with a correlation of 0.44 ($p = 0.029$). No significant correlations were found between the bakeries' product and sales information except for the expected relationship between making gluten-free products and claiming that gluten-free products represent a higher percentage of bakery sales. Neither were there any significant correlations between bakers' knowledge of gluten and any individual starter handling practice or other reported data (**Figure 5.9)**.

5.5 Discussion

This study was carried out with the objective of understanding the role of professional sourdough bakers as touchpoints of health and safety information for their gluten-sensitive customers. It investigated their knowledge, starter maintenance practices, beliefs, and willingness to pass along those beliefs, along with their perceptions of how their gluten-sensitive customers are affected by sourdough. Comparing these data allowed us to identify the range of starter maintenance practices in use by commercial sourdough bakeries whose product is accessed by gluten-sensitive consumers such as those in the survey in **Chapter 4**; this is in contrast to many of the lab-produced sourdoughs studied in previous literature. This survey identified a wide variation in starter maintenance practices across temperature, feeding interval, holding temperature, and flour type. No strong correlations were identified between starter handling practices, even where they were expected (for example, between increasing feeding

interval and decreasing holding temperature).

With this survey we were also able to compare these bakery practices to outcomes such as perceiving fewer symptoms in gluten-sensitive customers and being willing to recommend sourdough as a health therapy to gluten-sensitive customers. Results suggest that as feeding interval decreases and hydration percent decreases, bakeries perceive that customers experience more gluten-mediated symptoms upon eating their sourdough. Lower hydration and shorter feeding intervals may affect the bacterial diversity of the starter and its capacity for protein breakdown, with possible downstream effects on gluten toxicity.

Finally, with this survey we were able to assess professional sourdough bakers' knowledge of ingredient and labeling expectations. As distributors of wellness information to a health-compromised population, bakers have a responsibility to be well-informed regarding the product they are recommending and its proper handling practices. Despite nearly half of bakers being to recommend sourdough as a health therapy to gluten-sensitive customers, few bakers were able to produce correct answers when tested on sourdough ingredient standards or gluten-free labeling practices. This disparity indicates a potential breakdown in trust; those who are seen (and who act) as brokers of information for a health-compromised community may be in a position to harm rather than help.

5.5.1 Many Bakers Share Gaps in Understanding of Appropriate Labeling Practices

The findings collected in this survey demonstrate a gap in some bakers' understanding of labeling requirements and conventions. When asked what belongs on an ingredient label for "traditional sourdough bread", most bakers identified flour and water, but nearly half of bakers also listed "yeast" while only a few included "wild cultures". While sourdough contains wild

yeast cultures, the word "yeast" on an ingredient label is often taken to mean baker's yeast (*Saccharomyces cerevisiae*). Some activist groups, such as the Real Bread Campaign,[13] advise consumers to be suspicious of sourdough bread with "yeast" in the ingredient label as they suggest it may indicate compromised authenticity. While there is currently no specific standard of identity for sourdough bread or its labeling,[12] the Real Bread Campaign and similar groups are advocating for identity, labeling, and trade standards for sourdough bread in order to avoid confusion between authentic sourdough and "sourfaux" bread. The results of this survey indicate that perhaps bread producers are also in need of clearer standards and labeling expectations.

Another labeling issue that becomes apparent through these results is bakers' unfamiliarity with gluten-free labeling requirements. 76.0% of respondents were unable to correctly identify 20 ppm gluten as the legal limit for an item to be labeled "gluten-free". Only two bakeries which claim to sell gluten-free product answered correctly. Because this survey did not collect firmographic information on any of the bakeries, it is unknown which bakeries regularly participate in packaging and labeling operations. While bakeries that sell packaged product might engage more closely with FDA regulations, the FDA's gluten-free labeling requirements do apply to all foods regardless of packaging. Although gluten testing of finished food is not a legal requirement, any businesses labeling a product as "gluten-free" are responsible for compliance to the FDA's labeling requirements. Failure to comply with these regulations may result in financial penalties as well as risking the health and safety of their gluten-sensitive consumers.[14]

Despite deficiencies in knowledge of labeling requirements, bakers' performance on the knowledge test of gluten-containing foods indicated familiarity with possible sources of gluten contamination and an understanding of gluten as a functional component of foods. Nearly half of

bakers achieved a high score (4-5 points) on the gluten knowledge test. Conversely, in the survey of consumers reported in **Chapter 4**, only 19.33% of consumers achieved a high score on the same question using the same scoring method. While the sample size of professional bakers was much smaller (n=25 bakers vs. n=1015 consumers), making the two groups difficult to compare, it is a detail worth investigating further in a follow-up study.

5.5.2 Bakers as Arbiters of Health Information

One striking finding from this study was the willingness of bakes to recommend sourdough to their gluten-sensitive consumers. All bakers who indicated a willingness to do this cited health reasons as among their motivations, although taste was also listed by some as an additional consideration. These findings suggest that at least some commercial sourdough bakers may see themselves as distributors of knowledge when it comes to the nutrition and healthfulness of baked goods. One baker described finding articles about the benefits of sourdough and sharing those articles with gluten-sensitive clients as a way to recommend their products. Others shared passionate statements about information they advocate to their customers, such as, "[I]f made with whole grains, [sourdough] helps the body detox due to the bran which acts as nature [sic] scrubby pads, helping to remove old debris from the digestive track. This is why I bake sourdough exclusively!" Conversely, another baker described a policy of refusing to sell sourdough to gluten-sensitive customers, because it is made with a high-protein bread flour.

These examples demonstrate that commercial sourdough bakers hold strong opinions about their product, and they are sometimes willing and even enthusiastic to transmit those opinions to customers. Some of the beliefs expressed by bakers about their sourdough in this survey were a matter of personal opinion ("I believe [my customers] will like the taste"). Other

beliefs ("I believe [sourdough] is more digestible") are accepted as nutritional guidelines, though not necessarily applicable to the gluten-sensitive population.[7] Some responses such as "I believe [sourdough] contains lower levels of gluten" are supported by some evidence, but are not firmly established in dietary recommendations at this time.[15] Notably, one of the beliefs expressed in this survey is unambiguously false: some respondents believe that sourdough has probiotic properties. A requirement for probiotics is the presence of live organisms; because baked sourdough bread is a heat-treated product, it does not contain live organisms and therefore is not probiotic.[16] Three responding bakeries listed a belief that sourdough is probiotic as one of their motivations for recommending it, although only two of these bakeries asserted that they recommend sourdough to their gluten-sensitive clients. Some bakers also think that customers hold this opinion; six responding bakeries claim that a belief in sourdough's supposed probiotic properties is something that motivates their customers to purchase it, although none of these six are the same bakeries who listed probiotics as being among their own motivations for recommending sourdough.

5.5.3 Study Limitations and Response Congruity

The present study is not without limitations. In addition to a small sample size, consistency between responses within the same participant appeared low in some cases, bringing the trustworthiness of some participant responses into question. Some respondents appeared to offer mutually exclusive answers to different questions. For example, when asked, "Do you have customers who claim to be gluten-sensitive, or who are conscious of avoiding gluten in their diets?" one respondent replied, "No." However the same respondent was later asked, "Based on your interactions with them, how do your gluten-sensitive customers claim that they

are affected by the consumption of sourdough products?", the respondent selected the answer, "[My gluten-free customers] have fewer symptoms from sourdough relative to non-sourdough bread products" rather than abstaining to answer. This type of internal inconsistency was observed multiple times from two respondents. While it is unlikely that these inconsistencies invalidate the data entirely, a follow-up survey with improved controls could help ensure consistency. For example, limiting follow-up questions about interactions with gluten-free customers to only those respondents who say "yes" to whether they serve gluten-free customers could improve data reliability.

At first, there appeared to be a principled divide between the two sets of bakers responding to this survey. One set of approximately 9-11 participants appeared cautious or reluctant to recommend, speculate about, or indeed transmit information about sourdough, while a second group of approximately the same size appear to be willing to recommend sourdough as a therapy for gluten-mediated symptoms and feel comfortable in their motivations for doing so.

However, upon further analysis, members of both apparent sets displayed no adherence to a pattern, making them difficult to neatly categorize. For example, one participant claimed not to recommend sourdough to gluten-sensitive customers, but in response to the question "If applicable, what is your motivation for recommending sourdough to gluten-sensitive customers?" the same participant replied, "I believe sourdough has probiotic properties." For all other questions in this group, this participant replied, "I don't know/I don't talk to my customers about this topic." Other bakers appeared reluctant to speculate about the effect of sourdough on their gluten-sensitive customers, replying "I don't know/I don't talk to my customers about this topic" to questions 14, 15, 16, and 18, but indicating "Yes" when asked if they recommend sourdough to gluten-sensitive customers. Instead, it appears that a baker's willingness to consider

sourdough as a potential therapy for gluten-mediated symptoms is situational and may depend on the context around how the question is asked.

5.6 Conclusion

Professional sourdough bakers practice a wide variety of techniques for starter maintenance and mostly share a belief that sourdough confers health benefits, including to the gluten-sensitive population. Based on our findings, many of them are willing to recommend their sourdough products to gluten-sensitive consumers, and many also believe that their sourdough products are better tolerated than yeast-leavened bread by gluten-sensitive consumers. This study provides insight into the extent to which commercial sourdough bakers view themselves as brokers of health and wellness information. It is vital to understand the role of these professionals as important points of contact for gluten-sensitive consumers of baked goods.

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CHAPTER 6: IN VITRO COMPARISON OF GLIADIN TREATED BY FERMENTATION WITH DISTINCT SOURDOUGH MICROBIOMES

6.1 Summary

In this study, the effects sourdough fermentation on the inflammatory properties of gliadin from wheat gluten were evaluated using an *in vitro* model of celiac disease. 20 samples of wheat dough were fermented using unique sourdough starters, with a dough fermented by baker's yeast (*Saccharomyces cerevisiae*) used as a control. Gliadin was extracted from each dough and subjected to *in vitro* digestion. Digested gliadin was then applied to an *in vitro* model of the intestinal epithelial barrier. Cell viability and membrane integrity were assessed after treatment with gliadin from all samples. Both sourdough and yeast samples diminished cell viability significantly more than the untreated control, but no significant differences in viability were noted between treatments. Similarly, no differences in intestinal barrier permeability were observed between treatments. However, the yeast control was observed to lose more barrier function by 4 hours and to recover less barrier function after 24 hours compared to sourdough samples. Several sourdough-fermented gliadin extracts demonstrated improved intestinal barrier permeability in comparison to the yeast-fermented control; each sample also exhibited more extensive proteolysis in the bread dough. A moderate but insignificant relationship between the extent of proteolysis and intestinal barrier integrity was observed. Examination of the taxonomic data for each starter culture suggests that the distinct functionality of the three starters observed may be related to microbial community structure.

6.2 Introduction

Celiac disease is an enteric autoimmune pathology triggered by gluten protein.[1] Gluten is a heterogeneous class of storage proteins found in the endosperm of wheat, barley and rye. Broadly, gluten comprises two substituents- glutenin and gliadin. The gliadin component of gluten is resistant to enzymatic degradation[2] over the course of digestion due to its richness in proline. Incomplete hydrolysis of gliadin results in the formation of peptides[3,4] which trigger both innate and adaptive immune responses in individuals with celiac disease. First, gliadin triggers disruption of tight junction proteins in the intestinal epithelial barrier, causing intestinal permeability which allows the paracellular leakage of gliadin peptides into the lamina propria. This in turn triggers the production of inflammatory cytokines by intestinal epithelial cells, which signal for infiltration of intestinal epithelial lymphocytes (IELs) to the intestinal mucosa.[5] The damage to the intestinal mucosa caused by gliadin stimulates the production of enzyme tissue transglutaminase 2 (TG2); typically associated with tissue repair, TG2 binds to gliadin peptides in the lamina propria and increases their affinity for interaction with major histocompatibility complex (MHC) class II receptors on antigen presenting cells (APCs). APCs bind to gliadin as well as transient gliadin-TG2 complexes. As a result, the subsequent adaptive immune response is targeted towards both gliadin and TG2, with the production of anti-gliadin and anti-TG2 antibodies by effector B cells.[6]

Presently, the only available treatment for celiac disease is a gluten-free diet.[6] However, many strategies to prevent the inflammatory and immune responses of celiac disease have been explored, including attempts to “detoxify” gluten. Sourdough fermentation has been considered as one such strategy. The rationale behind this is that sourdough starter cultures have been found to include microorganisms capable of breaking down gluten protein, essentially “pre-digesting” the gliadin peptide fragments that are resistant to digestion by humans.[7,8] However, sourdough

microbiomes are not monolithic; rather, each starter exhibits a unique combination of microbes.[9] Therefore, starters may not be equivalent in terms of their proteolytic abilities and thus their effectiveness in detoxifying gluten for individuals with celiac disease.

The present study examines the differences in gluten breakdown among 20 distinct, intact sourdough microbiomes and assesses the impact of those differences on intestinal barrier integrity and cell viability *in vitro*. We tested the hypothesis that sourdough fermentation decreases the inflammatory capacity of gliadin as measured by cell viability and intestinal barrier permeability. Furthermore, we hypothesized that the efficacy of sourdough fermentation on each of these outcomes would vary based on the microbial ecology of the starter culture used in fermentation. Our results indicate a relationship between the microbial composition of sourdough starters and their capacity for gluten degradation, with downstream effects on the inflammatory properties of the resulting gluten.

6.3 Methods

6.3.1 Sourdough Benchtop Fermentations

Sourdough starters were donated by Ben Wolfe at Tufts University. Donated starters were brought to room temperature, and 2 g of each starter was combined with 10 g King Arthur All-Purpose flour (King Arthur Flour, Norwich, VT) and 10 g autoclaved MilliQ water. The mixture was stirred gently with sanitized tools and then vortexed briefly to mix before being capped loosely and fermented under ambient conditions for 24 hours. This process was repeated for three days, each day using 2 g of the previous day's propagation instead of freshly donated starter. On day 4, a benchtop fermentation was performed using the formulas indicated in **Table 6.1**, each mixed in a 50 mL polypropylene tube and fermented at 35°C and 30% relative

humidity (RH) for 24 hours. Each propagation and fermentation was carried out in triplicate. After the fermentation, samples were frozen at -80°C and lyophilized (LabConco, Kansas City, MO) to prepare them for gliadin extraction. Dough prepared in this way was used only for assessment of cell viability.

6.3.2 Preparation of Sourdough Bread

Sourdough starters were propagated from cryopreservation at 5 g to a mass of 365 g over 4 days with King Arthur All-Purpose flour and autoclaved MilliQ water. Each starter was used to prepare a benchtop dough fermentation (**Table 6.1**) and also to make approximately 1 kg of wheat dough at 65% hydration (**Table 6.2**). A baker's yeast-fermented dough made with Fleischmann's active dry yeast (Fleischmann's Yeast, Fenton, MO) acted as a control dough. See **Tables 6.1** and **6.2** for dough formulas. Doughs were mixed in standing mixers with dough hooks (KitchenAid, Benton Harbor, MI) for 10 minutes, until a cohesive dough mass had formed, then fermented at 35°C at 30% RH for 24 hours. Yeast control dough was fermented under the same conditions but for only 2 hours to account for more rapid production for CO_2 and consistency with conventional use of this leavening method. After fermentation, each dough was divided into three pieces of 150 g each for baking into bread loaves; these loaves were used for other tests (**Chapter 2**). The remaining dough was frozen in 50 mL conical flasks at -80°C and

Table 6.1: Formulas for mixing sourdough and yeast dough in a benchtop fermentation.

Component	Inclusion in sourdough formula (g)	Inclusion in yeast dough formula (g)
Flour	12	20
Water	6.8	13
Salt	0.36	0.36
Sourdough starter	6	0
Yeast	0	0.30

Table 6.2: Formulas for mixing sourdough and yeast dough in a full-scale fermentation.

Component	Inclusion in Sourdough Formula (g)	Inclusion in Yeast Formula (g)
Flour	400	665
Water	225	430
Salt	12	12
Sourdough starter	200	0
Yeast	0	10

lyophilized. The dough prepared in this way was used for permeability and free amino acid assays.

6.3.3 Extraction of Gliadin from Fermented Dough

10 g of lyophilized dough powder were combined with 30 mL 1 M NaCl and shaken for 1 h, then centrifuged at 3500 x g for 20 minutes at ambient temperature before discarding the supernatant. The pellet was resuspended in 30 mL 70% ethanol and shaken at 60°C for 1 h before centrifuging again on the same settings. The resulting supernatant was collected and cooled to -18°C to encourage sedimentation of gliadins. Next, ethanol was decanted off the solids in the bottom of the tube into a 100 mL round bottom flask. Remaining ethanol was evaporated from the solids by shaking samples in a water bath set to 65°C while the decanted ethanol was evaporated in a rotary evaporator (Buchi, Saint Gallen, Switzerland) until any remaining dissolved gliadins precipitated out of solution. 4 mL of acidified water (pH 1.5) were added to the flask to re-dissolve the precipitated gliadin, which was then recombined with the solids remaining in the tube. Gliadin extracts were then frozen to -80°C before being lyophilized and stored at -20°C.

6.3.4 In Vitro Digestion of Gliadin

Pepsin-trypsin digested gliadin (PT-gliadin) was prepared as previously described.[10] Dried gliadin extracts were dissolved in 10 mM sodium phosphate (pH 2.0) at a concentration of 20 mg/mL. Pepsin (Sigma-Aldrich, St. Louis, MO) was added to each sample to reach a final concentration of 0.3 mg/mL pepsin. Samples were then incubated in a shaking water bath at

37°C for 2 h. The pH was then raised to 7.4 with NaOH, and trypsin (Sigma-Aldrich, St. Louis, MO) was added to a final trypsin concentration of 0.3 mg/mL. Samples were incubated in a shaking water bath at 37°C for 4 h. After incubation, samples were heated to 100°C for 30 min to inactivate all enzymes. The digested samples were frozen to -80°C, then lyophilized.

6.3.5 Densitometric Analysis of Protein Degradation

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to quantitatively assess protein degradation in fermented doughs and gliadin extracts collected from each sample. Dough samples were prepared at a concentration of 5 mg/mL and gliadin samples at a concentration of 1 mg/mL in acidified water [pH = 1.5]. Gliadin extracts were compared against a gliadin standard (Sigma-Aldrich, St. Louis, MO). Samples were mixed with Laemmli sample buffer (Sigma-Aldrich, St. Louis, MO) and electrophoresed on polyacrylamide gels at 80 V for 10 minutes followed by 140 V for 70 minutes. Gels were subsequently stained with Coomassie Brilliant Blue dye (Sigma Aldrich, St. Louis, MO) and imaged using an EpiChemi II (UVP Inc., Upland, CA) system. Image Studio 5.2 software (LiCor Biosciences, Lincoln, NE) was used to identify lanes and bands on the gel images for comparison of signal intensity across samples. The intensity of each band was calculated by normalizing the signal strength of each band or lane to its area, then by dividing the normalized signal of each band by the normalized signal of its lane.

6.3.6 Caco-2 Cell Culturing and Maintenance

Caco-2 TC7 cells were purchased from American Type Tissue Collection (Rockville, Maryland) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with

20% FBS, 1% non-essential amino acids, and 1% penicillin/streptomycin. Cells were maintained at 37°C in an incubation chamber under 5% CO_2/95% humidified air. Cells were passaged approximately every 2-3 days at 70% confluence.

6.3.7 Cell Viability

Cell viability after incubation of Caco-2 cells with PT-gliadin from fermented dough was assessed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Caco-2 cells were seeded at a density of 3 x 10^5 cells/mL onto a 96-well plate and incubated at 37°C for 48 h. After 48 h, growth media was aspirated from each well and cells were treated with 1 mg/mL PT-gliadin suspended in serum-free DMEM (SFM). The cells were incubated with the treatment for 2 hours at 37°C and 5% CO_2. SFM was used as a negative control. After 2 h, the treatment was removed, and each well was rinsed with SFM. MTT reagent dissolved in SFM at a concentration of 0.5 mg/mL was applied to each well, and the plate was again incubated for 2 hours under the same conditions. The MTT reagent was aspirated out and replaced with dimethyl sulfoxide (DMSO) before a final incubation period of 15 minutes. Absorbance was measured at 570 nm using a BioTek Synergy H1 plate reader (Agilent Technologies, Santa Clara, CA). Each treatment was applied in quadruplicate, and each replicate of gliadin was plated in duplicate.

6.3.8 Permeability Assays

Membrane permeability was assessed through transepithelial electrical resistance (TEER). Cells were seeded into a transwell monolayer at a density of 3 x 10^5 cells/mL and grown for 22 days to confluence, with media refreshed every 2-3 days. The day before the assay (day 21) media was refreshed and an EVOM2 volt/ohm meter (World Precision Instruments,

Sarasota, FL) was used to confirm that all wells included in the experiment measured above 300 Ω•cm^2. On day 22, all wells were treated with 1 mg/mL PT-gliadin from each sourdough sample suspended in SFM. SFM was used as a negative control and one well absent of cells was used as blank. TEER was measured immediately after as well as after 4 and 24 hours of treatment. This experiment was repeated in triplicate.

6.3.9 Free Amino Acid Concentration of Fermented Dough

Free amino acid concentration was measured in lyophilized dough as a measurement of protein hydrolysis using the ninhydrin assay. First, 1 g lyophilized dough was dissolved in 4 mL 1% trichloroacetic acid and 310 µL ethanol. The solution was shaken for 1 h before centrifugation at 7700 x g for 15 min. The supernatant was reserved and diluted 1:10 with 2% ninhydrin reagent (Santa Crus Biotechnology, Dallas, TX) before heating to 99°C for 15 min and cooling to 4°C. 25 µL of each sample was then mixed with 225 µL carbonate buffer (pH 10) in a 96-well plate. Absorbance was measured at 570 nm. Free amino acid concentration was determined using a standard curve of L-leucine (Thermo Scientific, Waltham, MA).

6.3.10 Statistical Analysis

Data was processed in Microsoft Excel and analyzed using GraphPad Prism v10.0.3 (GraphPad Software, Inc., San Diego, CA, USA). Values are expressed as the mean ± standard deviation. One- or two-way ANOVA were performed when appropriate and paired with Tukey's multiple comparisons to compare samples; values of $p < 0.05$ were considered significant. Deming (Model II) linear regression was used with Pearson's correlation coefficient to evaluate

the relationship between protein hydrolysis as measured by free amino acid concentration and intestinal permeability, testing the hypothesis that greater proteolysis will result in less disruption of the intestinal barrier *in vitro*.

6.4 Results

6.4.1 Cell Viability Was Reduced in All Treatments

Caco-2 cells treated with 1 mg/mL PT-gliadin responded with a significant reduction in viability relative to the untreated negative control ($p < 0.0001$). Average viability of treated cells ranged from 50.78% in sample 120 to 67.29% in sample 542. The yeast treatment demonstrated an average viability of 63.60%. No significant differences were observed between treatment groups (**Figure 6.1**)

6.4.2 In Vitro Intestinal Permeability

Caco-2 cells grown in transwells were treated with 1 mg/mL PT-gliadin from each fermented dough for 24 h. Transepithelial electrical resistance was measured after 4 and 24 h. A

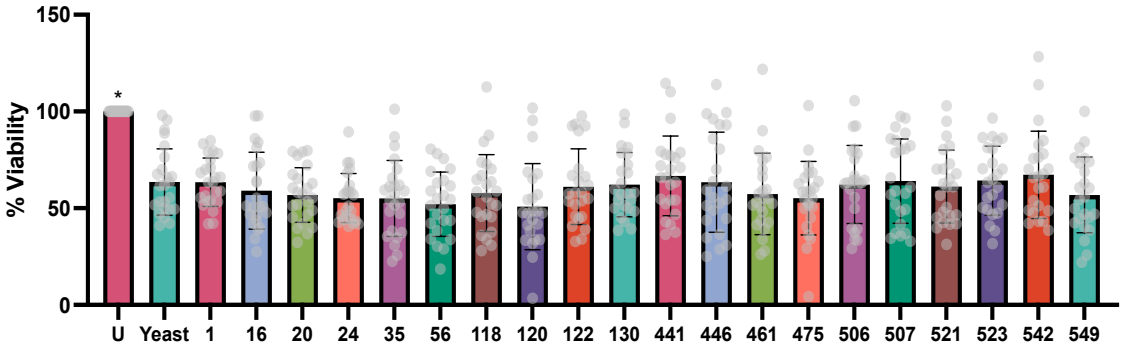

Figure 6.1: Viability of Caco-2 cells after exposure to 1 mg/mL PT-gliadin from each fermentation treatment. U represents the untreated negative control (media only) to which treatments were normalized. Values are expressed as mean ± SD. Differences were detected between groups using one-way ANOVA with a Tukey's multiple comparisons post-test. Asterisks indicate significant differences ($p < 0.05$).

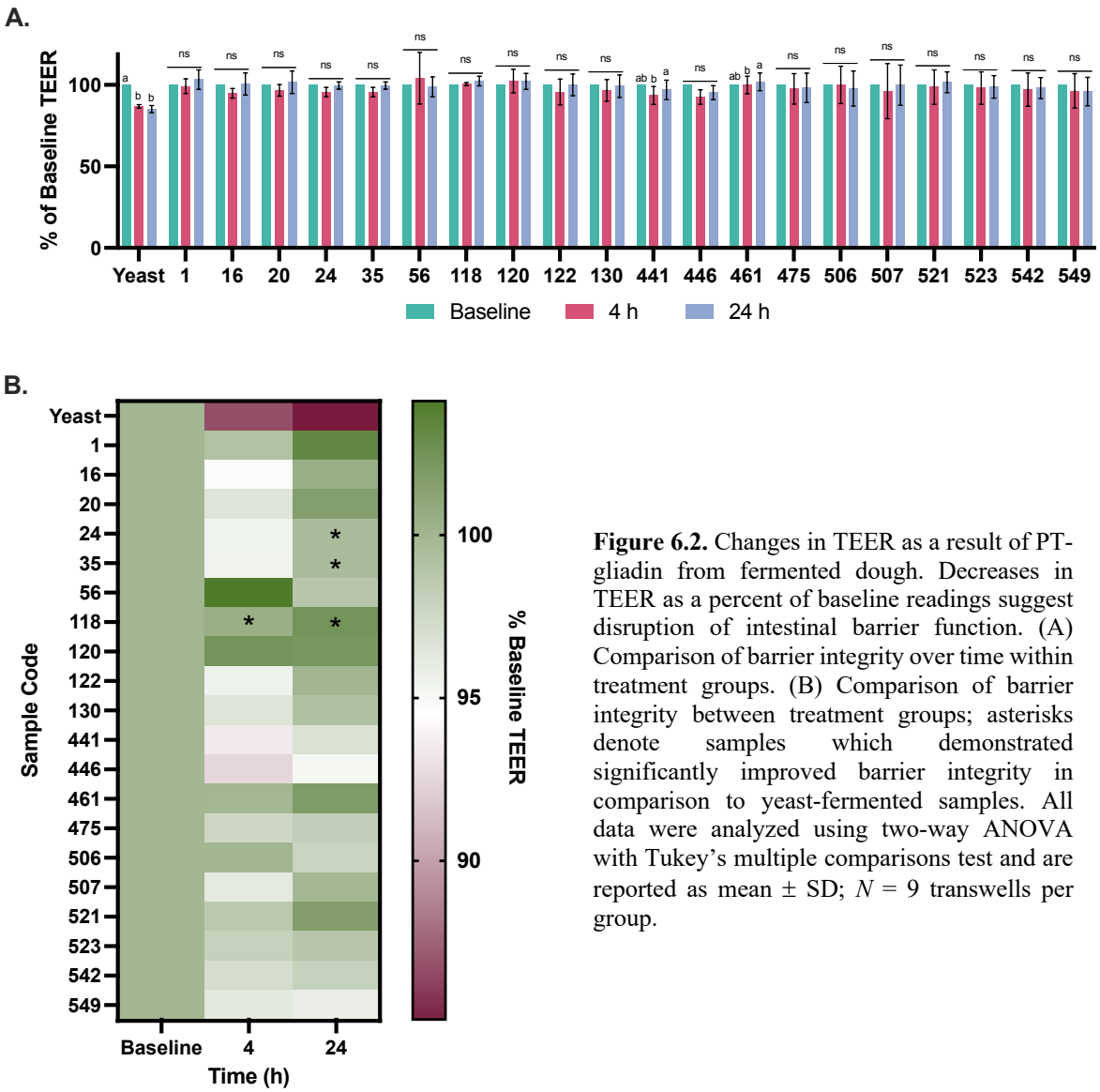

Figure 6.2. Changes in TEER as a result of PT-gliadin from fermented dough. Decreases in TEER as a percent of baseline readings suggest disruption of intestinal barrier function. (A) Comparison of barrier integrity over time within treatment groups. (B) Comparison of barrier integrity between treatment groups; asterisks denote samples which demonstrated significantly improved barrier integrity in comparison to yeast-fermented samples. All data were analyzed using two-way ANOVA with Tukey's multiple comparisons test and are reported as mean ± SD; $N = 9$ transwells per group.

significant decrease in TEER in comparison to baseline was observed only in the yeast-fermented sample after 4 h and 24 h (**Figure 6.2 A**). Comparison between samples at each timepoint revealed that only one sourdough-fermented sample, sample 118, demonstrated improved barrier integrity in comparison to the yeast-fermented sample after 4 h, while three sourdough-fermented samples demonstrated improved barrier integrity in comparison to the yeast-fermented sample after 24 h (samples 24, 25 and 118) (**Figure 6.2 B**). No differences were

detected between individual sourdough samples.

6.4.3 Differences in Gluten Toxicity May Be Influenced by Extent of Proteolysis

The extent of proteolysis of each sample over the course of fermentation was evaluated by measuring the free amino acid content of each dough as well as densitometric analysis of each sample separated by molecular weight using SDS-PAGE. While the takeaways from these analyses are limited in comparison to more precise methods such as targeted and non-targeted proteomics, these inexpensive and relatively simple methods provide preliminary insight towards our overarching hypothesis that fermentation by different starter culture microbiomes may differentially affect gluten proteolysis. This was investigated as a potential explanation for the differences observed in intestinal barrier integrity *in vitro* between gliadin extracted from fermented dough samples.

Differences in free amino acids were observed in the fermented dough samples; the yeast-fermented control contained the lowest average concentration of free amino acids at 12.85 ± 1.47 mg free amino acids per 100 g, which was not significantly different from 2 out of 20

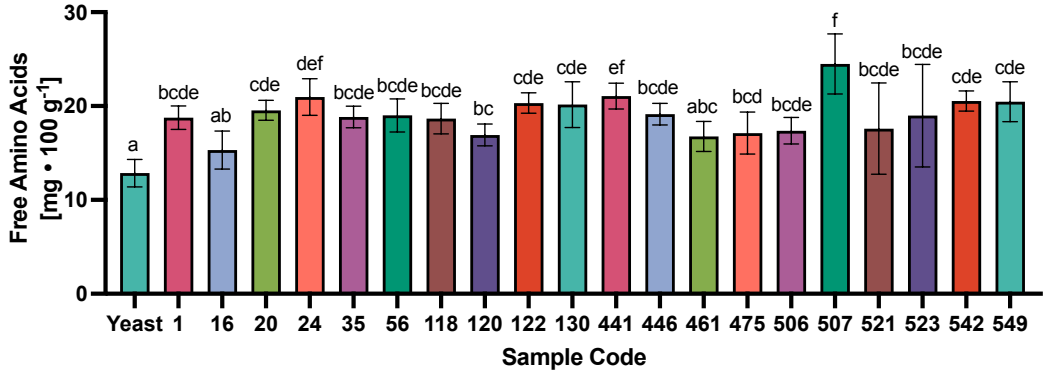

Figure 6.3: Free amino acid content of lyophilized fermented dough samples. Values are expressed as mean ± standard deviation from three independent experiments with three replicates per treatment. Values not sharing common letters are significantly different from one another ($p < 0.05$). Note: This data set was also used in **Chapter 3**.

sourdough-fermented samples (samples 16 and 461). The remaining 18 sourdough-fermented samples contained significantly greater concentrations of free amino acids than the yeast-fermented sample (**Figure 6.3**).

Gliadin extracts from fermented dough samples were analyzed with densitometry to evaluate the relative frequency of gliadins from three distinct molecular weight ranges as a result of fermentation; protein signal greater than 50 kDa was classified as high molecular weight (HMW) gliadins, protein signal between 15 and 50 kDa was classified as medium molecular weight (MMW) gliadins and signal below 15 kDa was classified as low molecular weight (LMW) gliadins. Pairwise comparisons between sourdough-fermented samples were omitted due to the absence of differences between samples in their effects on intestinal barrier permeability *in vitro*. Instead, all fermented gliadins were compared to an unfermented commercial gliadin standard and the yeast-fermented control (**Figure 6.4**). Significant hydrolysis of HMW gliadins was observed in 5 out of 20 sourdough-fermented gliadin extracts, though only one sample (sample 35) demonstrated a lower relative frequency of HMW gliadin than the yeast-fermented control. Gliadin hydrolysis was also considered from the perspective of increased frequency of LMW gliadins in comparison to the gliadin standard and yeast-fermented control; a greater relative proportion of LMW gliadins compared to the gliadin standard was observed in 9 of the 20 sourdough-fermented samples; the relative frequency of LMW gliadins in sample 35 was also greater than levels observed in the yeast-fermented control.

Interestingly, all three sourdough-fermented samples which demonstrated reduced intestinal barrier disruption *in vitro* in comparison to the yeast-fermented control were noted as containing a greater concentration of free amino acids in the complete dough sample in comparison to the yeast-fermented control. Deming (Model II) linear regression was used to

investigate the relationship between the extent of protein hydrolysis as measured by concentration of free amino acids and gluten toxicity as measured by TEER in the Caco-2 cell

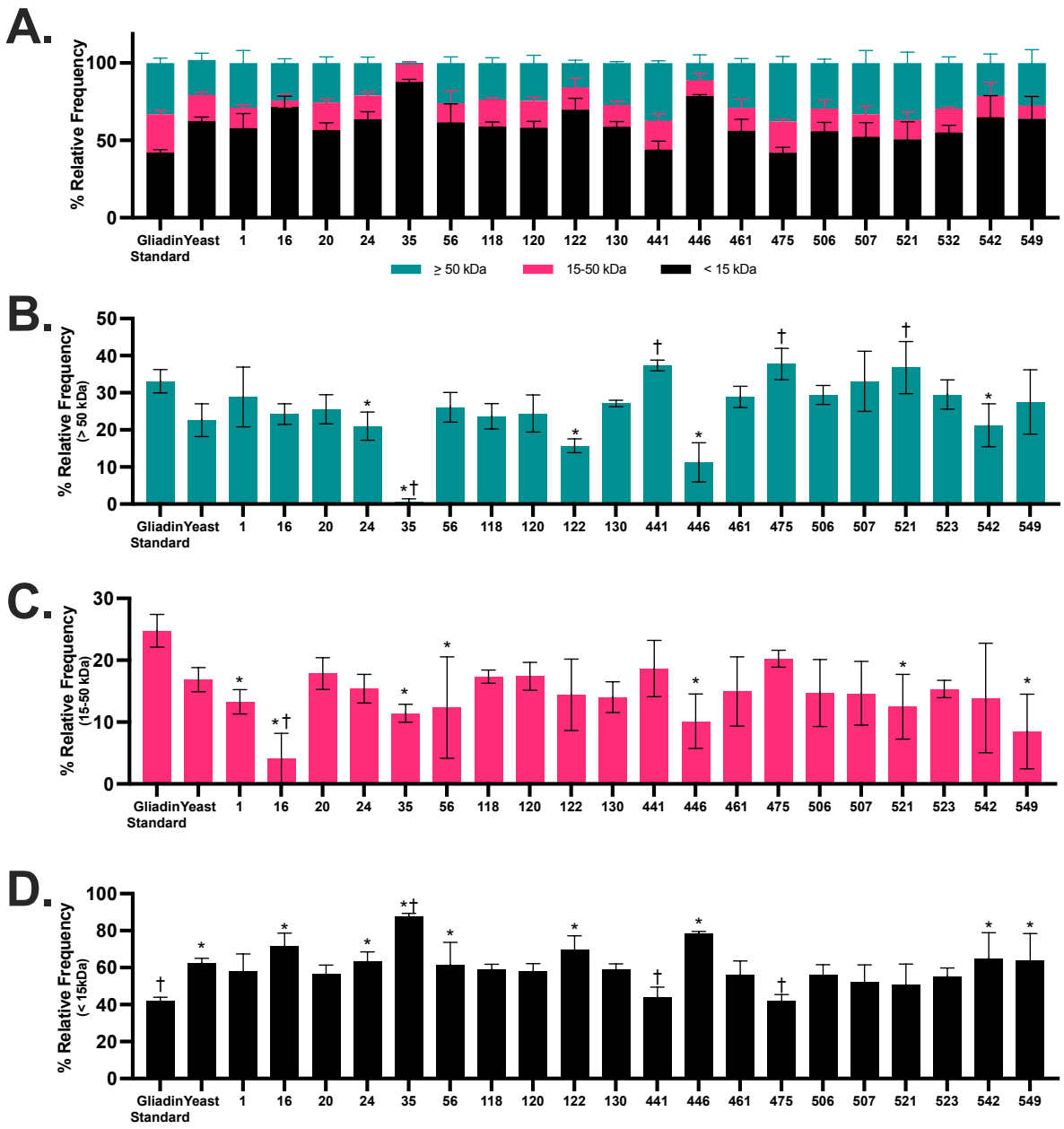

Figure 6.4: (A) Relative frequencies of high, medium, and low molecular weight gliadins extracted from fermented dough samples. (B) Relative frequencies of high molecular weight gliadins, (C) relative frequencies of medium molecular weight gliadins, (D) relative frequencies of low molecular weight gliadins. Values are expressed as mean ± SD. All data were analyzed using one-way ANOVA with Dunnett's post-test to compare each sample to the gliadin standard (differences indicated by asterisk) and to the yeast-fermented control (differences indicated by obelus) ($p < 0.05$).

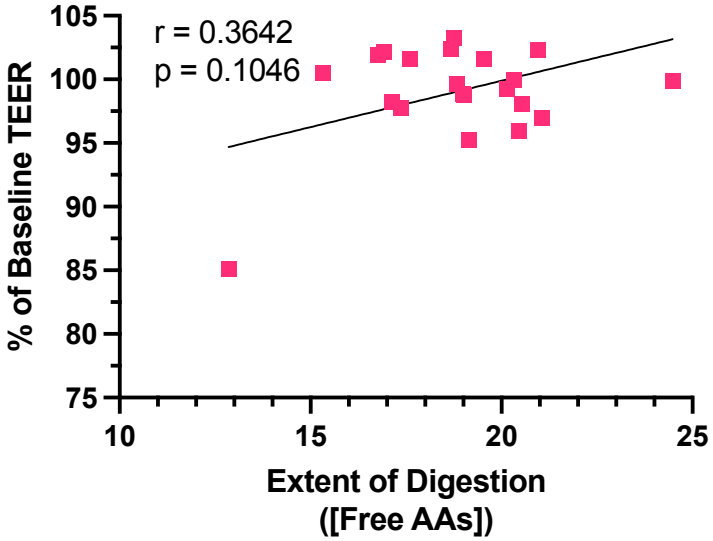

Figure 6.5: Deming (Model II) linear regression demonstrating a moderate positive relationship between protein hydrolysis in fermented dough and reduction of intestinal permeability *in vitro*.

model over 24 h of exposure to gliadins (**Figure 6.5**). The resulting linear regression demonstrated a moderate positive relationship (r = 0.3642) between extent of digestion and TEER, suggesting that increased hydrolysis of protein in dough may play a role in reducing the inflammatory effects of gliadin in fermented bread products. However, this trend was not

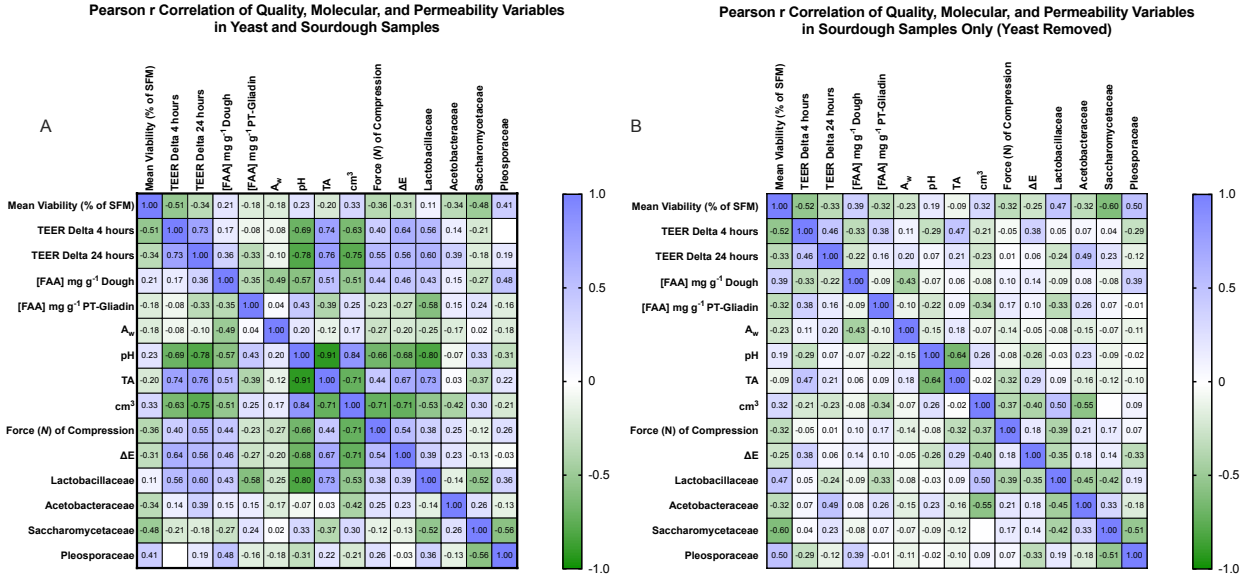

Figure 6.6: Pearson r correlations between quality, molecular, and permeability measurements among all samples (A) and only sourdough samples with the yeast control excluded (B)

statistically significant (p = 0.1046).

6.4.4 Correlations between Quality, Molecular, and Permeability Variables are Strongly Affected by Yeast Characteristics

When Pearson r correlations between quality variables, protein degradation profiles, and FAA concentration are calculated (**Figure 6.6 A**), several strong correlations stand out. TEER change from 0 to 24 hours is negatively correlated to both loaf rise volume (-0.75) and pH (-0.78). TEER change from 0 to 4 hours is also correlated to both rise volume and pH, but less strongly (-0.6 and -0.69, respectively). Correlations between TEER measurements and FAA

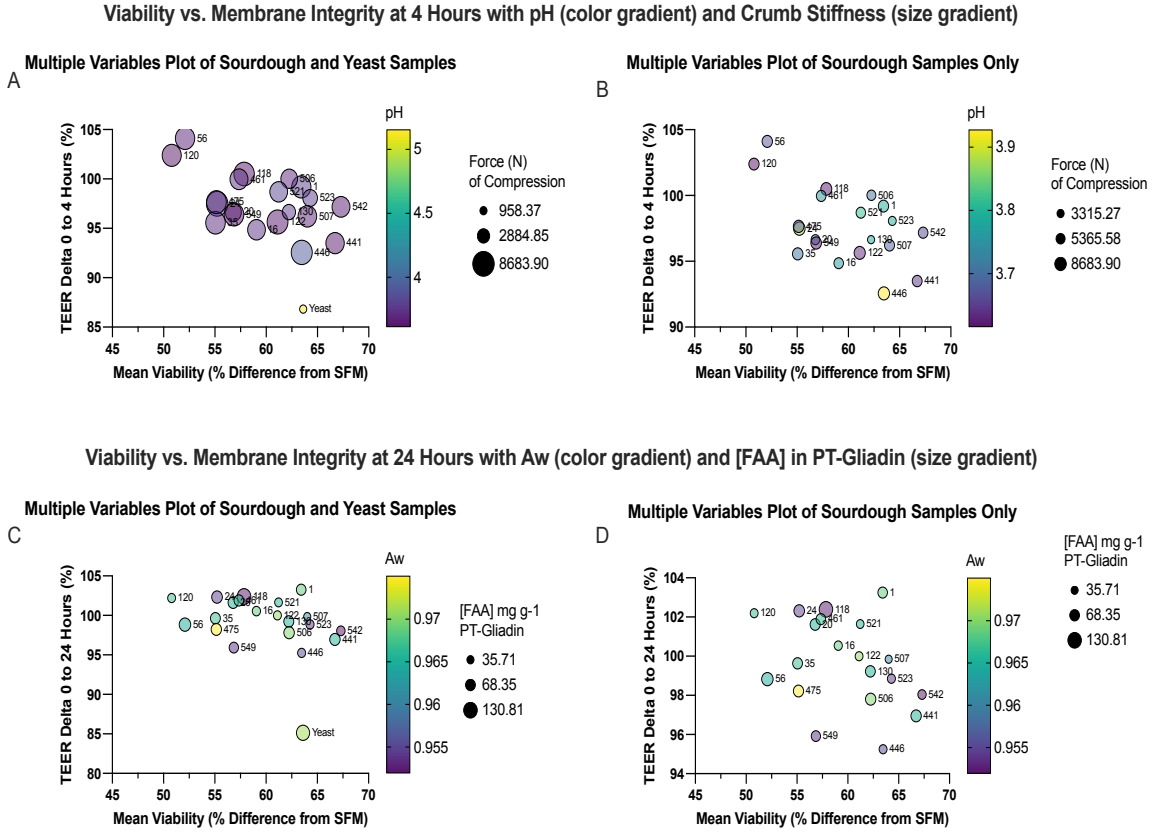

Figure 6.7: Multiple variables plot of all samples (A, C) and sourdough samples only (B, D) showing mean viability on the x-axis and [FAA] membrane integrity at 4 hours (A, B) and 24 hours (C, D) on the y-axis. Other variables are also indicated. The color gradient shows pH (A, B) or Aw (C, D) and the size gradient shows crumb firmness (A, B) or [FAA] in PT-gliadin (C, D). Plots A and C include the yeast control, while plots B and D show only sourdough samples without the yeast control.

levels are weak, as expected based on the linear regression models shown in **Figure 6.5**. Several other correlations, such as those between Lactobacilaceae and pH (-0.80) and between Lactobacilaceae and titratable acidity (0.73) are addressed in **Chapter 3**. However, when the yeast sample is removed from the data set and only sourdough samples are examined (**Figure 6.6 B**), the strong correlations between TEER measurements and pH or loaf volume disappear. Without the yeast sample included, correlations between TEER and FAA measurements strengthen and the direction of those correlations reverse, as also demonstrated in **Figure 6.6**.

Multiple variable plots (**Figure 6.7 A-D**) illustrate the separation of the yeast control from the sourdough samples in more than one measured feature. **Figure 6.7** shows the samples plotted with increasing membrane integrity on the x-axis and increasing barrier integrity at 4 (**Figure 6.7 A and B**) or 24 (**Figure 6.7 C and D**) hours on the y-axis. The color spectrum shows pH or Aw and bubble size shows crumb texture or FAA concentration in PT-gliadin. By all metrics, the yeast sample remains separate while the sourdough samples largely cluster together. For illustration, molecular differences have been compared against quality parameters discussed in **Chapter 3** to demonstrate the extent to which sourdough samples separate from the yeast control.

6.5 Discussion

The overarching objective of this study was to examine the differences in gluten breakdown among 20 distinct, intact sourdough microbiomes and assesses the impact of those differences on intestinal health in vitro. The Caco-2 cell line is an immortalized human cell line derived from human adenocarcinoma which has been used extensively to study the human intestinal epithelium, including many studies focused on gluten-mediated barrier permeability in celiac disease. In the present study, Caco-2 cells were used to measure the influence of

fermented gliadins on cell viability and intestinal permeability when cultured in transwells.

The cytotoxic effects of PT-digested gliadin are well established in Caco-2 cell lines[11] based on disrupted cell proliferation and the inhibition of alkaline phosphatase, which is a critical enzyme in cell differentiation and barrier function. The MTT assay is a colorimetric assay which measures metabolic function of cells through the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which is yellow in color, to formazan, which is purple. Our findings were consistent with those characterizing the cytotoxicity of unfermented PT-gliadin in that viability was reduced by approximately 50% regardless of fermentation treatment.

Differences in the effect of fermentation on the biological effects of gliadin were observed in assessment of intestinal barrier integrity. After 4 h and 24 h of exposure to PT-digested gliadins extracted from fermented bread dough, intestinal barrier permeability increased significantly in the samples exposed to yeast-fermented gliadins in comparison to their baseline permeability. While fluctuations were observed in some sourdough-fermented samples, significant increases in permeability in comparison to each sample's individual baseline were not observed. Interestingly, three samples (#24, 35, 118) of gliadins from sourdough fermentation demonstrated significantly reduced permeability in comparison to the yeast-fermented control.

Though a statistically significant relationship between free amino acid concentration and intestinal barrier integrity was not observed in the present study, it is notable that the three samples demonstrating improved barrier integrity in comparison to the yeast-fermented control also differed from yeast in terms of the extent of proteolysis. Each sample with improved barrier integrity contained a significantly greater concentration of free amino acids in the dough, and two of the three samples (24 and 35) featured a greater relative proportion of LWM gliadins and

lower relative proportion of HMW gliadins than the yeast-fermented control. Enzymatic degradation of gliadins has been studied extensively as a method to "detoxify" gluten; it is possible that the reduced biological response elicited by these samples may be related to the greater extent of protein hydrolysis observed. In the case of sourdough fermentation, protein hydrolysis is driven by the activation of endogenous enzymes in flour through the low pH conditions of the ferment as well as by the production of proteolytic enzymes by microorganisms found in the starter culture. Based on this, we used the taxonomic data for each starter culture

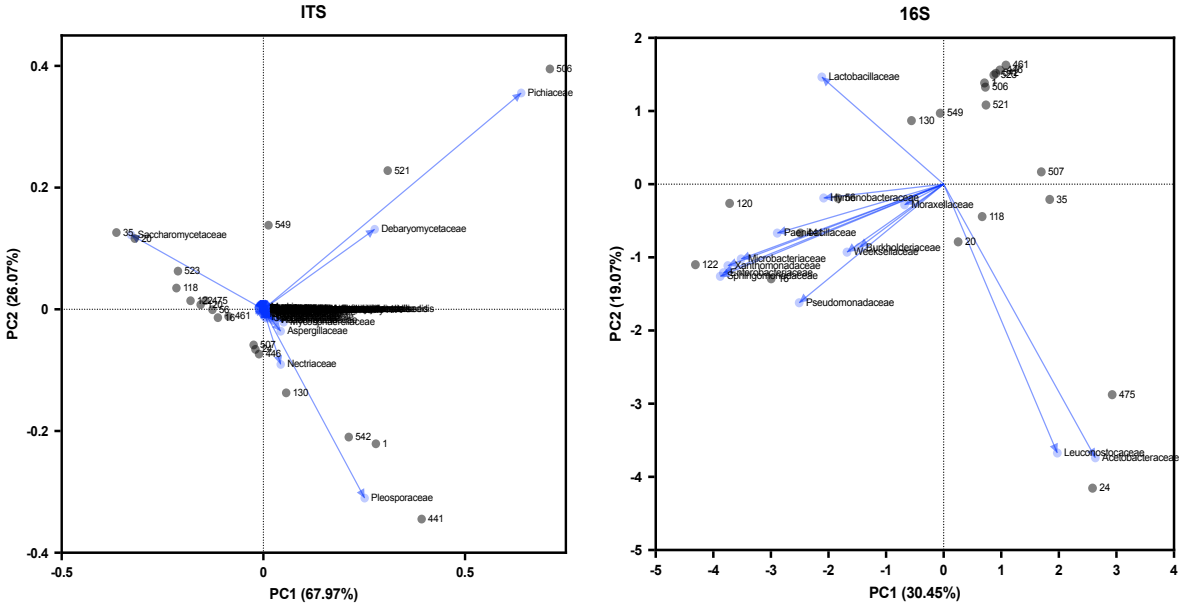

Figure 6.8: PCA biplots of ITS and 16S data for fungal and bacterial communities, respectively. Analyses were performed based on family structure. Each graph shows the loading of each variable (arrows) and each sourdough starter (points).

(presented in **Chapter 3**) to compare the microbial ecology of the starters and identify any commonalities between the starters which produced samples with reduced stimulation of barrier permeability. PCA plots of our taxonomic data reveal that differences in bacterial community structure are driven strongly by Lactobacillaceae, Leuconostocaceae and Acetobacteraceae, while differences in fungal community structure were defined by Saccharomycetaceae, Pichiaceae, Pleosporaceae and Debaryomycetaceae (**Figure 6.8**). In each PCA plot, samples 24,

35 and 118 appear in the same quadrant, suggesting that the functional commonalities of these starters may be related in some part to the commonalities in their overarching microbial community structure.

If microbial community structure does influence function in this way, it is possible that other starter cultures with similar microbial profiles can elicit the same beneficial effect on gluten, but that these effects were not detectable by the analyses used in this study. For example, the analyses used for measuring protein degradation are not as powerful as proteomic approaches. An inherent limitation of the ninhydrin assay is that it only compares the extent of FAA liberation, and it is unable to determine the degradation of proteins into very small peptides, and though we also measured the relative degree of gliadin hydrolysis in each sample, we do not have information about the extent to which the immunostimulatory fragments of gliadin were hydrolyzed, if at all. This information, combined with metagenomic sequencing of our starter cultures, will provide greater insight towards relationships between microbial community structure and their functional abilities in terms of gliadin hydrolysis and protection from celiac-associated inflammation and immunogenicity. An *in vitro* model of celiac disease which features immune cells would also allow more insight towards the specific functional effects of gliadin hydrolysis, as specific amino acid motifs associated with different biological effects have been identified in gliadin peptides including but not limited to cytotoxic activity, immunomodulatory activity, zonulin release/gut-permeating activity and IL-8 release.[12] Though the Caco-2 transwell model is the most popular *in vitro* model for studying celiac disease and gliadin-mediated barrier dysfunction, it limits understanding of the biological implications of processing gliadin to only measuring the effects on cytotoxicity and gut permeability. Additional steps in celiac disease immunopathology could also be targeted, such as a transglutaminase-2

(TG2) binding assay to investigate whether or not processing by unique sourdough microbiomes affect the binding affinity of the resulting gliadin peptides to the TG2 enzyme.[13]

Previous work has shown a relationship between cytotoxicity in wheat proteins and quality outcomes associated with those proteins; that study did not include sourdough treatments and focused on textural quality.[14] The current study has shown that sourdough fermentation affects wheat proteins in such a way it has the potential to affect both bread quality and gluten toxicity. While further tests are needed, this study has shown early results that 1) exposure to gliadin peptides processed by fermentation by 20 distinct sourdough microbiomes have a cytotoxicity similar to that of yeast-fermented peptides, 2) sourdough fermentation positively affects the recovery of membrane integrity after exposure to the resulting gliadin peptides, and 3) also has an impact on quality parameters that distinguishes it from yeast bread. Subsequent testing will seek to quantify the inflammatory response associated with gliadin peptides fermented by each microbiome (and yeast control) and to describe conformational changes the gliadin peptides fermented by each sourdough starter that might affect their immunogenicity or receptor binding affinity, mechanisms by which symptoms of gluten sensitivity could be mediated.

6.6 Conclusion

This study investigated, for the first time, differences in the biological effects of gluten fermented by different sourdough starter cultures within the context of celiac disease. Our data suggest that there may be a relationship between the microbial communities of sourdough starters and their functional abilities to break down gluten, leading to differences in the inflammatory capacity of gluten *in vitro*. Future work is needed to overcome some of the main limitations in this study including more robust analysis of the degradation of cytotoxic gliadin

peptides and the use of a more complex *in vitro* model of celiac disease which would allow understanding of how the immune response to gluten is affected, rather than only the inflammatory response of intestinal epithelial cells.

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CHAPTER 7: CONCLUSIONS AND FUTURE WORK

7.1 Summary of Goals

In this study, we set out to examine the relationship between gluten sensitivity and sourdough bread as it is typically prepared in the traditional style by consumers or professional bakeries. Rather than hypothesizing about how sourdough organisms could be used under ideal conditions or as adjunct ingredients, we were interested in real, fully intact, complex sourdough microbiomes that are intended to ferment dough and leaven bread according to traditional processes without the use of adjunct yeast. This seemed like a relevant pursuit because many consumers already appear to use sourdough this way; eating sourdough is a commonly tip for gluten-sensitive individuals to safely consume gluten as noted in various blogs[1] and websites;[2] references are even made to this in academic work.[3,4] However, the effect of true sourdough fermentation on gluten-mediated diseases is not currently substantiated by any scientific evidence. Sourdough has been studied as a beneficial processing aid to reduce the inflammatory capacity of gluten, but not in this context; instead, flour has been processed by fermentation with lab-curated isolates or co-cultures[5] or by including processing steps that are not part of traditional sourdough making (such as the use of a cytoplasmic extract).[6] Those few commercial sourdoughs which have been studied for their therapeutic usefulness have been proprietary or uncharacterized, and therefore leave us unable to determine whether any effects on gluten are related to specific microorganisms or microbial communities.[7]

In this series of studies, we sought to investigate how sourdough fermentation might mitigate the symptoms of gluten-mediated pathologies when used in a process similar to what an

artisanal bakery or a home consumer could replicate. Using an *in vitro* model of celiac disease, we compared the abilities of gliadin extracts from sourdough-fermented doughs to stimulate inflammation and intestinal barrier permeability. We also compared the quality outcomes of bread made with these different sourdough starter cultures to investigate drivers of quality in sourdough production.

Because the evidence for sourdough's gluten-detoxifying effects has been studied only in laboratory settings, we wanted to involve gluten-sensitive consumers and sourdough bakers in our study. Though communicating with these groups, we aimed to understand people's motivations for eating, or avoiding sourdough (as opposed to yeast bread) and understand where they got their information about what they ought to be eating. We also asked participants about their symptoms in response to sourdough vs. yeast bread, in order to determine the validity of the claim that sourdough relieves symptoms of gluten sensitivity. Finally, we asked similar questions of professional sourdough bakers, searching for a relationship between baker and consumer beliefs and behavior.

7.2 Conclusions

Our findings from this research suggest that sourdough-fermented breads produced under real-world conditions demonstrate clear differences in proteolysis that may be related to observed differences in the inflammatory capacity of gluten. Furthermore, my results have demonstrated that sourdough differs from yeast-fermented bread in terms of quality and consumer perception.

The dough and bread used for both the quality (**Chapter 3**) and permeability (**Chapter 6**) experiments for this dissertation was produced using a process that was within time and temperature parameters that are applied commercially to sourdough ferments (time and

temperature varies widely)[8] but it was both slightly warmer and longer than average[9,8] The objective was to identify one or more microbiomes among the 20 being tested that could produce bread with desirable properties despite a fermentation targeted to drive gluten hydrolysis. Taken together, the data from **Chapter 3** and **Chapter 6** indicate that sourdough samples indeed underwent a greater degree of gluten hydrolysis than the yeast bread. Notably, while all 20 sourdough samples were within expected ranges for pH, TA, and Aw, they all resulted in lower loaf volume and a greater force of compression compared to the yeast control bread. This is likely an indication that the extent of gluten hydrolysis under these conditions compromised the integrity of the gas-trapping network. However, it is worth noting that sample 130 was not statistically different from the yeast control in terms of compressibility, and it had the second greatest rise volume (behind only the yeast control). It was also very similar to the yeast control bread in terms of crust color development (**Figure 3.4 A-C**). In fact sample 130's quality parameters were so unique (and so similar to the yeast control) that it stands alone among sourdough samples, alone in a clade, in the cluster dendrogram shown in **Figure 3.8 C**.

In addition to investigating the differences in quality and gluten breakdown among 20 distinct, intact sourdough microbiomes, this study also sought to correlate those differences with downstream effects on cell membrane permeability in a Caco-2 cell model. A significant decrease in intestinal barrier integrity over the course of 24 h was observed only in the yeast-fermented control. Between samples, three sourdough-fermented samples showed significantly greater intestinal barrier integrity in comparison to the yeast-fermented control, which we attributed to extensive proteolysis as a result of the microbial communities of the starter cultures. However, sample 35 does not perform favorably compared to the control on quality assays; it is pale in color and demonstrates middling volume and compressibility scores (**Figure 3.4 A-C**). It

does not stand out from the other sourdough samples in pH, TA, or Aw (**Figure 3.3 A-C**). While we found that overall, intestinal barrier function was disrupted to a greater extent when treated with baker's yeast-fermented gliadin than with sourdough-fermented gliadin, our present study design does not allow for identification of any single sourdough organism or community producing a unique effect.

Our survey of gluten-sensitive sourdough consumers found that among consumers with different diagnoses (UD, undiagnosed; CD, celiac disease; NCGS, non-celiac gluten-sensitivity) only UD individuals reported a significant reduction in symptoms from sourdough versus conventional bread compared to CD and NCGS groups. We also found that a diagnosis of CD or NCGS was associated with greater odds of experiencing symptoms from eating sourdough compared to the UD group (**Chapter 4**). Based on this survey, it appears that sourdough fermentation, broadly, does not mitigate gluten-stimulated symptoms in people with a diagnosed gluten sensitivity, but it may offer relief for people with unspecified/undiagnosed gluten sensitivities. This survey also used multiple logistic regression to reveal the explanatory factors contributing to consumers' likelihood of manifesting symptoms, willingness to eat sourdough, and knowledge of gluten-containing foods. We found that the diagnosis group helps explain which consumers are willing to eat sourdough and also whether or not they are likely to develop symptoms in response to eating it. A number of demographic and social factors also play into explaining these outcomes. Diagnosis group was not relevant in explaining a participant's knowledge of gluten-containing foods; instead, their gender, race, and habits such as reading labels and eating sourdough more often were more relevant.

Finally, our survey of professional sourdough bakers found that consumers and bakers share some assumptions about sourdough; at least some respondents in both categories indicated

a belief that sourdough is a probiotic food. A majority of both gluten-free consumers and sourdough bakers expressed associations between sourdough and positive health outcomes. Many bakers (more than half of those who responded to the question) affirmed their willingness to recommend sourdough to gluten-sensitive customers on the basis of its healthful properties (**Figure 5.4**), although the assumption that sourdough has lower levels of gluten was not a principal motivator for doing so in this group of respondents (**Figure 5.5**). Slightly over one third of bakers who responded to the survey expressed unwillingness to discuss health recommendations with their gluten-sensitive consumers. This survey also found a gap in professional sourdough bakers' knowledge of gluten labeling requirements, with 76.0% of respondents unable to identify the 20-ppm upper limit for "gluten-free" labeling, despite many of these respondents claiming to sell gluten-free products.

7.3 Future Work

In specific scenarios, sourdough fermentation had already proven effective for decomposing gluten, neutralizing harmful gliadin epitopes, detoxifying wheat-based and contaminated gluten-free flours, as well as mitigating NCGS triggers such as FODMAPs and non-gluten peptides. Here, we have shared a set of preliminary studies which investigate the potential for sourdough fermentation to be used as a processing technology to detoxify gluten in settings more relevant to the food industry.

7.3.1 Future Work Should Test Outcomes Under Multiple Fermentation Parameters

Future tests should attempt to optimize the fermentation parameters for these microbial consortia in order to maximize their usefulness at the intersection of quality and reduced

immunogenicity. In sourdough fermentation, population dynamics, gluten breakdown and quality outcomes have been shown to depend on processing conditions including time[5,10] and temperature.[11] Multiple dough fermentation times and temperatures should be tested, with each set of conditions assessed for its effect on both quality and markers of CD-associated gluten toxicity. A full knowledge of how fermentation and holding conditions affect quality and toxicity outcomes is necessary to make this technique translatable to commercial food processors.

7.3.2 Investigation of Downstream Effects on CD Pathology

Future testing should also look deeper into the relationship between sourdough fermentation and CD pathology. A serum capture assay in transwell plates should be employed, followed by sandwich ELISAs to quantify the pro-inflammatory cytokines such those listed in **Table 7.1** collected from the apical and basolateral serum after treatment with gliadin processed by each sourdough starter. Also, while the present study investigated only the effect of sourdough fermentation on intestinal barrier permeability, the cell inflammatory response should also be considered, as well as potential conformational changes to the gliadin peptides that may affect the availability of TG2 or HLA-DQ binding motifs. These mechanisms could be

Table 7.1: Pro-inflammatory cytokines associated with gliadin exposure in CD.

Marker	Function
IL-6	Stimulation of acute phase of T-cell response, T-cell differentiation[13]
IL-8	Neutrophil recruitment[14]
IL-10	Suppression of gluten-mediated immune cell recruitment[15]
IL-1β	Modulation of intestinal barrier permeability[16]
TNF-α	Modulation of intestinal barrier permeability[16]
MCP-1	Modulation of immune cell regulation, infiltration[17]

investigated through R5 competitive ELISA, which targets hydrolyzed gluten[12] to look at overall gluten quantity remaining in dough after processing by each consortia of organisms. Finally, the binding affinity of the sourdough-processed gliadin peptides should be investigated through transglutaminase (TG2) binding assays.

7.3.3 Quantification of Immunologically Relevant Gliadin Peptides

Fermented doughs should be profiled for metabolites and proteins via reverse-phase liquid chromatography paired with mass spectrometry. Results from the present study showed that fermentation with unique sourdough microbiomes creates a unique protein hydrolysis profile of each resulting doughs. Therefore, it is reasonable to expect that the degree of immunodominant epitope degradation would also differ as a result of fermentation with different sourdough microbiomes, with some microbiomes heavily degrading immunogenic epitopes while others leave them more intact. Microbiomes that result in gluten with a great degree of epitope breakdown would be less likely to stimulate an immune reaction in celiac patients, and future works should attempt to identify these microbiomes.

7.3.4 Confirmation of Sourdough Microbiome Stability

It will also be necessary to confirm stability of the sourdough microbiomes over time. The present study was conducted using sourdough starts propagated directly from cryopreserved, microbially characterized stock, with the expectation that minimal microbial drift would take place over only four days of propagation. To minimize the contribution of external microbial loads, autoclaved water plus sanitized containers and instruments were employed. However,

under non-laboratory conditions starters are propagated continuously and are also sometimes stored for long periods of time without regular feeding. Future work should perform bioinformatics benchmarking assays to assess the degree of drift in each microbial population under typical continual use backslopping and long-term storage conditions. If a microbiome is identified with therapeutic applications, its usefulness under non-laboratory conditions cannot be guaranteed without confirming microbial stability.

7.3.5 Characterization of Relationship Between Sourdough Microbiomes and Shelf-Life

Although this study probed the impact of unique sourdough microbiomes on quality outcome, one aspect of quality that was not investigated was shelf-life. The USDA recommends storing bread for just 2-4 days due to chemical and microbial spoilage.[20] Because of this, hundreds of tons of bread are discarded daily.[21] In commercial settings, preservatives such as calcium propionate, mono- and diglycerides, and potassium sorbate are often added to bread to inhibit microbial spoilage and extend shelf life.[22] However, consumers increasingly seek foods free of these additives.[23] Sourdough fermentation is a biopreservation tool that has been shown to chemically stabilize bread products and inhibit spoilage microbes.[24] Potential avenues of exploration include exopolysaccharides produced by some sourdough-resident LAB, which appear to reduce staling[25,26] by interfering with starch recrystallization.[27,28] Some LAB also produce antimicrobial or antifungal compounds[29,30,31] and organic acids[32] associated with extension of shelf-life.[30,31,33,34]

As with the early work on sourdough and gluten degradation, previous studies that have investigated the relationship between sourdough and shelf-life are not representative of sourdough made or purchased by consumers, because they have been done with fermentation by

isolated organisms not in a consortium[26,31,35,36] or with lab-constructed pairs or groups of organisms.[27] Others have used uncharacterized commercial microbiomes,[37–39] which does not contribute to an understanding of how the microbial community drives quality and functional outcomes. So far, no studies have been conducted on microbially characterized, commercially viable sourdough microbiomes. Therefore, the investigation of shelf-life would be an opportune avenue of research for this project to carry forward, given that the project is designed to engage with intact, commercially viable sourdough starters in forms readily available to consumers. This approach promises immediate translational applicability to commercial settings, but it also offers valuable insights that can be directly beneficial to both consumers and the broader industry.

7.4 Closing Words

Previous work which investigated gluten proteolysis in sourdough systems and found that fermentation with sourdough-associated organisms can reduce intact gluten content overall,[10,40] degrade immunodominant gluten fragments.[40–42] reduce CD toxicity response in *in vitro* or *ex vivo* [18,43,44] and may create flour that is safe for CD patients to consume.[43] These previous studies have taken place with isolated lactic acid bacteria (LAB) cultures or enzymes as opposed to whole food systems with entire sourdough consortia and are not reflective of commercially available sourdough microbiomes.[10,40] Conversely, the study described in the previous chapters was the first study to investigate the relationship between sourdough microbiomes and functional outcomes in using intact native sourdough microbiomes in real dough/bread systems.

I anticipate that further work, building on the foundational concepts in this study, will increase translatability of sourdough fermentation to manipulate quality and therapeutic

outcomes to home and commercial bakeries. This study did not identify a specific consortium of sourdough organisms that is able to make high-quality bread while simultaneously degrading gluten such that it reduces markers of CD pathogenesis in a cell model, but it has succeeded in broadening the understanding of an important and growing field and it has provided a foundation for future researchers to carry these initial findings forward into a large number of potential avenues of investigation.

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APPENDICES

Appendix A: Survey of Gluten Sensitive Sourdough Consumers

A.1 Instructions and Consent

You are being invited to participate in a survey-based research study. In order to participate, you must be 18 years of age and gluten sensitive. Your participation in this is voluntary and you may stop participation at any time without penalty. Any quotes you choose provide as part of this survey are voluntary (not required answers) and may be used in a publication without any information that could identify you. There is no risk or benefit to participating in this study. For participation in this survey, you will receive compensation as outlined in the invitation upon completion. If you have any questions about this opportunity, please contact Charlene Van Buiten at charlene.vanbuiten@ColoState.EDU or Caitlin Clark at caitlin.clark@ColoState.EDU. If you have questions about your rights as a volunteer in this research, contact the CSU IRB at CSU_IRB@mail.colostate.edu or by phone at 970-491-1553. To indicate your consent to participate in this research, please click next to continue to the survey.

A.2 Inclusion Criteria Questions

1. Are you over 18?
    - Yes
    - No

2. Are you gluten intolerant, or have you been diagnosed with a gluten sensitivity or celiac

disease?
- Yes
- No

A.3 Survey Questions

3. Which category best describes your type of sensitivity to wheat/barley/rye-based bread products?
   - I have been diagnosed with celiac disease (1)
   - I have been diagnosed with another type of gluten sensitivity (2)
   - I have a gluten sensitivity but have never received a formal diagnosis (3)

4. Are you conscious of avoiding gluten your diet?
   - Yes, I try to avoid gluten
   - No, I don't worry about avoiding gluten

5. On the occasions that you eat bread products, how often do you choose to eat sourdough products?
   - Frequently
   - Occasionally
   - Never

6. How does the consumption of sourdough products affect the symptoms of your gluten sensitivity?
   - I have fewer symptoms from sourdough relative to non-sourdough bread products
   - I have the same symptoms from sourdough relative to non-sourdough bread products
   - I have more symptoms from sourdough relative to non-sourdough bread products
   - I do not have symptoms from my gluten sensitivity regardless of the type of bread product I eat
   - I am not aware of how sourdough products affect my symptoms

7. If you tolerate sourdough better than other bread products, which statement(s) apply to your experience? Select all that apply.
   - Less bloating
   - Less abdominal pain
   - Less headache
   - Less skin rash
   - I just feel better overall
   - This does not apply to me / I do not tolerate sourdough better than other bread

products
- Other (describe):_____

8. If applicable, why do you choose to eat sourdough products? Select all that apply.
    - I like the taste
    - I believe they have probiotic properties
    - I believe they contain lower levels of gluten
    - I tolerate sourdough better than other bread products
    - I think they are healthier
    - I think they are better for my blood sugar (lower glycemic index)
    - This does not apply to me / I do not eat sourdough products
    - Other (describe)_____

9. Which of the following foods contain gluten, as they are typically prepared? Select all

that apply.

Figure A.1: Options presented as images to consumer survey participants in response to the question about gluten-containing foods.

10. Briefly describe the impact that your diagnosis/gluten sensitivity has had on your life.
    - _____

11. Do you feel supported by your partner/friends/family/etc. in your dietary restrictions?
    - Yes
    - No

12. If you answered "No" to the previous question, please explain.
    - _____

13. How/from where are you getting information about which products you can or cannot

eat?

- Product labels/ingredient lists
- Gluten-free apps
- Google/internet/websites
- Social media
- Cookbooks/recipes
- Other gluten-free people
- Doctor/medical practitioner
- Trial and error
- My own knowledge/training
- Other

You're almost done! We just need a little demographic info about you so we can make sure we know about the populations of people answering our questions. Don't worry, all your answers are anonymous.

14. What is your age range?
    - 18 to 24
    - 25 to 34
    - 35 to 44
    - 45 to 54
    - 55 to 64
    - 65 or over
    - Prefer not to say

15. What is your gender?
    - Male
    - Female
    - Other/Prefer not to say

16. What is the highest grade of school you have completed or the highest degree you have

achieved?

- Less than a high school diploma
- High school diploma or equivalent
- Some college but no degree
- Associate's degree
- Bachelor's Degree
- Master's Degree
- Doctorate or professional degree
- Prefer not to say

17. What field do you work in?
    - Accounting
    - Advertising/Marketing/Sales
    - Agriculture/Fishing/Forestry
    - Architecture
    - Automotive
    - Banking/Brokerage/Financial
    - Carpentry/Electrical/VVS/HVAC/Trades
    - Chemicals/Plastics/Rubber
    - Communications/Information
    - Computer Hardware/Software/IT
    - Construction
    - Education
    - Energy/Utilities/Oil and Gas
    - Engineering
    - Environmental Services
    - Food/Beverage/Hospitality/Tourism
    - Food Science/Nutrition/Dietetics
    - Healthcare/Biotech/Pharmaceuticals
    - Human Resources
    - Insurance
    - Legal/Law
    - Manufacturing
    - Military/Government/Public Sector
    - Non-Profit/Social Services
    - Public Relations
    - Retail/Wholesale Trade
    - Security
    - Shipping/Distribution
    - Telecommunications
    - Transportation
    - Other:_____

18. What is your race?
    - White
    - Black

- Asian
- American Indian
- Hawaiian/Pacific Islander
- Other (please describe): _____
- Prefer not to say

19. Are you Hispanic?
    - Yes
    - No
    - Prefer not to say

20. What is the combined total pre-tax annual income of all the members of your household?
    - $0-25,000
    - $25,000-35,000
    - $35,000-50,000
    - $50,000-100,000
    - $100,000-150,000
    - $150,000 or above
    - Prefer not to say

21. How many people live in your household?
    - 1
    - 2
    - 3
    - 4
    - 5
    - 6 or more
    - Prefer not to say

Thank you for filling out this survey. Your response has been recorded.

Appendix B: Survey of Professional Sourdough Bakers

B.1 Instructions and Consent

You are being invited to participate in a survey-based research study. In order to participate, you must be 18 years of age and an owner or operator of a bakery that produces sourdough products. Your participation in this is voluntary and you may stop participation at any time without penalty. If you have any questions about this opportunity, please contact Charlene Van Buiten at charlene.vanbuiten@ColoState.EDU or Caitlin Clark at caitlin.clark@ColoState.EDU. If you have questions about your rights as a volunteer in this research, contact the CSU IRB at RICRO_IRB@mail.colostate.edu or by phone at 970-491-1553. To indicate your consent to participate in this research, please click next to continue to the survey.

B.2 Inclusion Criteria Questions

1. Are you over 18 years of age?
    - Yes
    - No

2. Do you own or work for a bakery that makes one or more sourdough products?
    - Yes
    - No

B.3 Survey Questions

1. How many different sourdough starters does your bakery maintain (for example, perhaps you use one wheat and one rye starter)?
    - 0
    - 1
    - 2
    - 3
    - More than 3

2. Does your bakery make any gluten-free products (compliant with 21 CFR 101.91)?
    - Yes
    - No

3. Do you have customers who claim to be gluten-sensitive, or who are conscious of

avoiding gluten in their diets?
    - Yes
    - No/Not that I know of

3. Approximately what percentage of your sales is made up of sourdough products?
    - 0-25%
    - 25-50%
    - 50-75%
    - 75-100%

4. Approximately what percentage of your sales consist of gluten-free products (compliant with 21 CFR 101.91)?
    - 0-25%
    - 25-50%
    - 50-75%
    - 75-100%

5. Are any of your gluten-free products (compliant with 21 CFR 101.91) sourdough-fermented?
    - Yes
    - No

The following questions pertain to the composition and care of your sourdough starter. If you prepare more than one starter, answer questions with regards to your bakery's most commonly used starter.

6. What is the base grain of your starter? Please be as specific as possible (Brand name, refined/unrefined, organic/conventional, brominated/unbrominated, bleached/unbleached, protein content, etc.)

7. What is the hydration percentage of your starter (the ratio of flour to water)? For example, if you use 400g of flour for every 500g of water, this is a hydration percentage of 125%, or 400g/500g.

8. Does your starter contain anything other than flour and water? If so, what (please list all

components as a ratio to weight of flour)?

9. How long has this starter been continuously maintained?

10. How often is the starter fed?

11. What percentage of the starter is discarded upon feeding?

12. At what temperature is the starter held?

13. Is there anything else you think I ought to know about your starter?

Now we have just a few questions about how you interact with your gluten-sensitive consumers. We want to know how these consumers talk to YOU about your sourdough products.

14. Based on your interactions with them, how do your gluten-sensitive customers claim that they are affected by the consumption of sourdough products?
    - They have fewer symptoms from sourdough relative to non-sourdough bread products
    - They have the same symptoms from sourdough relative to non-sourdough bread products
    - They have more symptoms from sourdough relative to non-sourdough bread products
    - They do not have symptoms from my gluten sensitivity regardless of the type of bread product I eat
    - I don't know/I don't talk to my customers about this topic

15. If your gluten-sensitive customers claim they tolerate sourdough better than other bread products, how do they claim their symptoms are affected? (Select all that apply)
    - Less bloating
    - Less abdominal pain
    - Less headache
    - Less skin rash
    - Other (describe):_____
    - I don't know/I don't talk to my customers about this topic

16. If applicable, what motivations do your gluten-sensitive customers express for consuming

sourdough products? (Select all that apply)

- They like the taste
- They believe sourdough has probiotic properties
- They believe sourdough contains lower levels of gluten
- They tolerate sourdough better than other bread products
- They believe sourdough is healthier
- They believe sourdough is better for blood sugar (lower glycemic index)
- Other (describe):_____
- I don't know/I don't talk to my consumers about this topic.

17. Do you recommend sourdough products to your gluten-sensitive consumers?
    - No
    - Yes

18. If applicable, what are your reasons for recommending sourdough to your gluten-sensitive customers? (Select all that apply)
    - I think they will like the taste
    - I believe sourdough has probiotic properties
    - I believe sourdough contains lower levels of gluten
    - I believe gluten-sensitive customers tolerate sourdough better than other bread products
    - I believe sourdough is healthier
    - I believe sourdough is better for blood sugar (lower glycemic index)
    - Other (describe):_____
    - I don't know/I don't talk to my consumers about this topic.

19. Which of the following should appear on the ingredient label for traditional sourdough bread? Select all that apply
    - Flour
    - Water
    - Yeast
    - Acetic acid
    - Lactic acid
    - Dough improvers/conditioners
    - Sugar
    - Oil
    - Starter/wild cultures

20. What is the upper limit of gluten content for a product to be labeled "gluten-free"?
    - 5 ppm (parts per million)
    - 10 ppm (parts per million)
    - 20 ppm (parts per million)
    - 50 ppm (parts per million)
    - 100 ppm (parts per million)

21. Which of the following foods contain gluten, <u>as they are typically prepared?</u> (Select all

that apply)

Figure B.1: Options presented as images to bakery survey participants in response to the question about gluten-containing foods.

Thank you for filling out this survey. Your response has been recorded.

LIST OF ABBREVIATIONS

AAB: Acetic Acid Bacteria
CD: Celiac Disease
CE: Cytoplasmic Extract
EPS: Exopolysaccharide
HLA: Human Leukocyte Antigen
HMW: High Molecular Weight
HPLC: High Performance Liquid Chromatography
IBS: Irritable Bowel Syndrome
IFN-γ: Interferon-γ
IL: Interleukin
LAB: Lactic Acid Bacteria
LMW: Low Molecular Weight
MMW: Medium Molecular Weight
PPII: Polyproline II
PT-digest: Pepsin-trypsin digest (*in vitro* human digestion)
NCGS: Non-Celiac Gluten/Wheat Sensitivity
TG2: Human Tissue Transglutaminase II