IMMUNOLOGICAL ANALYSIS OF ALLERGNICITY OF SALT-SOLUBLE PROTEIN EXTRACTS FROM DIPLOID, TETRAPLOID, AND HEXAPLOID WHEATS IN AN ADJUVANT-FREE MOUSE MODEL OF FOOD ALLERGY

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ABSTRACT

Hypersensitivity reactions to major allergenic foods such as wheat can be potentially deadly due to high risk of systemic anaphylaxis. Wheats belong to four distinct genotypes (AA, AABB, AABBDD, and DD) that exist as thousands of lines and varieties. It is unknown whether there is naturally occurring variation in the intrinsic allergenic potential among genetically distinct wheats. A validated adjuvant-free mouse model to study intrinsic allergenicity of salt-soluble protein extracts (SSPE) from wheat is unavailable. Therefore, a transdermal sensitization/oral elicitation (TS/OE) mouse model of food allergy established by Dr. Gangur, and colleagues was validated for wheat in this work.

The central hypothesis guiding this research was that the salt-soluble protein extracts (SSPEs) obtained from four genetically distinct wheat species (*Triticum monococcum*, *Triticum durum*, *Triticum aestivum*, and *Aegilops tauschii*) will show significant natural variation in their intrinsic allergenicity *in vivo*. The central hypothesis was tested with two specific aims: Aim 1. Validate the TS/OE model for transdermal sensitization with salt-soluble protein extracts (SSPEs) from four wheats: Determine the natural variation in the intrinsic sensitization potencies from transdermal application of SSPEs from four wheats: *T. monococcum* (genome AA), *T. durum* (genomes AABB), *T. aestivum* (genomes AABBDD), and *Ae. tauschii* (genome DD). Aim 2. Validate the TS/OE model for oral allergic reaction to SSPEs from four wheats: Determine the natural variation in the oral allergic reaction elicitation potencies of SSPEs from the four wheats: *T. monococcum* (genome AA), *T. durum* (genomes AABB), *T. aestivum* (genomes AABBDD), and *Ae. tauschii* (genome DD).

Results from this work show that: (i) repeated skin exposures to SSPEs from all four wheats elicited robust increases in the specific (s)IgE levels; (ii) skin exposures to SSPEs from all four

wheats were sufficient to sensitize mice for oral anaphylaxis as measured by hypothermic shock response (HSR) and mucosal mast cell response (MMCR) to respective SSPEs from the four wheats; (iii) for durum wheat (*T. durum*) validation, both HSR and MMCR showed a strong correlation with each other, as well as with sIgE, and a modest correlation with total (t)IgE levels; (iv) in *T. durum* SSPE sensitized mice, selected Th2/Th17/Th1 cytokines were elevated; (v) in durum wheat allergy model, oral allergen-challenged mice showed selective elevation of IL-6 and a panel of chemokines compared to saline-challenged mice; (vi) among all four species tested, *T. monococcum* elicited the lowest sensitization, and the other three wheats were comparable in their sensitization potentials; (vii) among all four species tested, *Ae. tauschii* elicited the least HSR, followed by *T. monococcum*, *T. durum*, and *T. aestivum*; and (viii) among all four species tested, *Ae. tauschii* elicited the least MMCR, followed by *T. durum* and *T. monococcum*; however, all these three wheats were significantly less potent than the *T. aestivum* in eliciting MMCR. In summary, this study validates TS/OE mouse model, and reports a comparative map of intrinsic allergenic potential of four genetically distinct wheats using a novel adjuvant-free mouse model for the first time.

The validated TS/OE mouse model of wheat allergenicity reported here would be a cost-effective pre-clinical testing tool for evaluating the intrinsic allergenicity of novel wheat proteins including GM wheats, and differently processed wheats. This model could facilitate development of hypo/non-allergenic wheat products and advance mechanisms of wheat allergenicity (e.g., role of genetics and environmental factors) leading to the development of novel preventive and therapeutic methods for life-threatening wheat allergies.

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A. oryzae/sojae: *Aspergillus oryzae/sojae*

Ab: Antibody

Ae. tauschii: *Aegilops tauschii*

A/G: Albumins/globulins

AHG: Acid hydrolyzed gluten

ANOVA: Analysis of variance

B. breve/longum/infantis: *Bifidobacterium breve/longum/infantis*

CFIA: Canadian Food Inspection Agency

CFU: Colony forming units

CV: Co-efficient of variation

Δ°C: Absolute change in rectal temperature

DG: Deamidated gliadin

EDCs: Endocrine disruption compounds

EFSA: European Food Safety Authority

EHG: Enzymatic hydrolyzed gluten

ELISA: Enzyme-linked immunosorbent assay

EPIT: Epicutaneous immunotherapy

FALCPA: Food Allergen Labeling and Consumer Protection Act

FAO: Food and Agriculture Organization of the United Nations

FcεRI: High affinity IgE receptor

FcγR: Low-affinity IgG receptor

FDEIA: Food-dependent exercise induced anaphylaxis

FSA: Food Standards Agency (United Kingdom)

FSANZ: Food Standards Australia and New Zealand

GM: Genetically modified

HG: Alcalase hydrolyzed gliadin

HGP: Hydrolyzed gluten protein

HLA: Human leukocyte antigen

HMW-GS: High molecular weight glutenin subunits

HSR: Hypothermic shock response

HWP: Hydrolyzed wheat protein

IC_{50}: Inhibitory concentration 50%

IC_{75}: Inhibitory concentration 75%

II-ELISA: IgE-inhibition ELISA

IL: Interleukin

IP: Intraperitoneal

LAB: Lactic acid bacteria

L. brevis/higardii/plantarum: *Lactobacillus brevis/higardii/plantarum*

LC-MS/MS: Liquid Chromatography with tandem mass spectrometry

LMW-GS: Low molecular weight glutenin subunits

LOAEL: Lowest-observed-adverse effect level

LOD: Limit of detection

LTP: Lipid transfer protein

MAFF: Ministry of Agriculture, Forestry and Fisheries (Japan)

MFDS: Ministry of Food and Drug Safety (South Korea)

MHC: Major histocompatibility complex

MMCP-1: Murine mucosal mast cell protease-1

MMCR: Mucosal mast cell response

MPS: Model pasta sample

NG: Native gluten

NIH: National Institutes of Health (United States)

NOAEL: No-observed-adverse-effect level

OC: Oral challenge

OD: Optical density

OIT: Oral immunotherapy

P. pentosaceus: *Pediococcus pentosaceus*

PAF: Platelet-activating factor

RBL: Rat basophilic leukemia

RP-HPLC: Reverse phase high performance liquid chromatography

S. cerevisiae: *Saccharomyces cerevisiae*

SD: Standard deviation

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

sIgE: Specific immunoglobulin E

SLIT: Sublingual immunotherapy

SNP: Single nucleotide polymorphisms

SPT: Skin prick test

SSPE: Salt-soluble protein extract

SSWPs: Salt-soluble wheat proteins

S. thermophilus: *Streptococcus thermophilus*

tIgE: Total immunoglobulin E

T. aestivum: *Triticum aestivum*

T. durum: *Triticum durum*

T. monococcum: *Triticum monococcum*

Th (1/2/17): T helper 1/2/17 cell

TLR4: Toll-like receptor 4

TS/OE: transdermal sensitization and oral elicitation

UG: Unmodified gluten

USDA: United States Department of Agriculture

US FDA: U.S. Food and Drug Administration

W. cibaria: *Weissella cibaria*

WDEIA: Wheat-dependent exercise induced anaphylaxis

WHO: World Health Organization

Z. rouxii: *Zygosaccharomyces rouxii*

ω-3 LCPUFA: Omega-3 long chain polyunsaturated fatty acid

CHAPTER 1 INTRODUCTION

1.1. Significance of food allergy

Food allergy is one of the major health issues worldwide. It affects 10.8% of adults and 8% of children in the United States of America (Gupta et al., 2019; Sampson et al., 2018; Warren, Jiang, & Gupta, 2020). Moreover, food allergy is increasing at an alarming rate for reasons that are still incompletely understood (Seth et al., 2020). It is reported that the estimated annual economic burden caused by food allergy in the US was $24.8 billion in 2013 (Gupta et al., 2013); updated information regarding its current economic impact is unavailable, but presumably has risen in conjunction with increases in food allergy prevalence. In general, food allergy refers to the IgE antibody-mediated adverse immune reactions triggered by the ingestion of a specific food. Typical clinical symptoms associated with food allergy can vary from mild reactions such as hives, rashes, vomiting, diarrhea, and rhino-conjunctivitis, to serious life-threatening reactions such as systemic anaphylaxis, and baker's asthma (Sicherer & Sampson, 2018; Warren et al., 2020). Incidences of emergency department visits due to food-induced anaphylaxis are also on the rise (Gupta et al., 2019; Warren et al., 2020). There is no cure for food allergies at present; strict food avoidance is the only method to prevent reactions. However, constant vigilance to avoid the allergenic food can impair the quality of life for not only food-allergic individuals but also their families and health providers (Foong & Santos, 2021; Seth et al., 2020).

1.2. Significance of wheat allergy

Wheat has been identified by the United States Food and Drug Administration (US FDA) as one of the nine major allergenic foods that account for over 90 percent of allergic reactions, the others being milk, eggs, fish, shellfish, tree nuts, peanuts, soybeans, and sesame (Gangur &

Acharya, 2021; US FDA, 2022). Wheat is also regulated as a major allergenic food in Canada, the United Kingdom, Japan, Australia, New Zealand, and in all 28 European Union countries. (EFSA, 2004; FAO, 2020; Health Canada, 2022; Japan, 2019; UK, 2021; US FDA, 2022; Australia & New Zealand, 2021). Wheat allergy affects approximately 0.9-3.6% adults and 0.2-1.3% children in the US (Poole et al., 2006; Venter et al., 2008; Venter et al., 2006; Verrill et al., 2015; Vierk et al., 2007). Similar trends were reported in Europe and Australia (Pereira et al., 2005; Rancé, Grandmottet, & Grandjean, 2005; Woods et al., 2002). However, wheat allergens are under-studied compared to other major food allergens (e.g., peanut, tree nut, milk, and egg) even though clinical studies have demonstrated that over half of the wheat-allergic children who participated in oral food challenges exhibited anaphylactic reactions, which can be potentially fatal (Cianferoni et al., 2013; Pourpak, Mansouri, Mesdaghi, Kazemnejad, & Farhoudi, 2004).

There are at least 8 human clinical conditions that have been associated with exposure to wheat: 1. celiac disease; 2. non-celiac gluten/wheat sensitivity; 3. food protein-induced enterocolitis syndrome; 4. eosinophilic esophagitis. 5. wheat food allergy (including vomiting, diarrhea, hives, systemic anaphylaxis, atopic dermatitis, rhino-conjunctivitis, and oral allergy syndrome); 6. wheat-dependent exercise-induced anaphylaxis (WDEIA); 7. contact urticaria; and 8. baker's asthma (Cabanillas, 2020; Gao et al., 2021; Juhász et al., 2018; Patel & Samant, 2020; Quirce, Boyano-Martínez, & Díaz-Perales, 2016; Ricci et al., 2019). All these conditions are due to the abnormal activation of the immune system by wheat. Celiac disease is an autoimmune reaction triggered by gluten, whereas non-celiac gluten sensitivity is thought to be due to the activation of innate immune system by unknown components of wheat (Cabanillas, 2020). Mechanisms of food-protein-induced enterocolitis syndrome and eosinophilic esophagitis are

incompletely understood. Only the last four diseases (#5 to #8) are mediated by wheat-specific IgE antibodies.

Allergic/anaphylactic reactions to wheat can occur through ingestion of wheat (i.e., wheat food allergy), allergic airway reactions can occur through respiratory exposure to wheat (e.g., baker's asthma and baker's rhinitis), and allergic skin reactions can occur through dermal exposure to wheat (e.g., contact urticaria). All IgE-mediated reactions can be triggered by gluten (gliadins and glutenins) or non-gluten (albumins and globulins) protein fractions of wheat. Systemic anaphylaxis to wheat and baker's asthma can be potentially deadly, and therefore require emergency medical treatment (Cabanillas, 2020; Cianferoni et al., 2013; Jin et al., 2019; Quirce et al., 2016; Ricci et al., 2019). The foci of this dissertation research were: i) sensitization (i.e., IgE response) to non-gluten wheat protein extracts upon skin exposure; and ii) IgE-mediated anaphylactic reactions in response to oral exposure of non-gluten wheat protein extracts in skin-sensitized mice.

1.3. Statement of problems

At the beginning of this project, a validated mouse model to evaluate intrinsic wheat allergenicity was unavailable. Consequently, this research was undertaken to address this problem. This dissertation presents the work focused on the development and validation of a novel adjuvant-free mouse model of intrinsic wheat allergenicity using four genetically distinct wheats. This model can be further applied to address three distinct problems facing the general scientific area of wheat allergy as discussed below.

1.3.1 Genetic diversity of wheats vs. wheat allergenicity

There are three distinct wheat genomes (A, B, and D) that are known to contribute to the genetic diversity of the wheat crop (Shewry, 2018). Currently existing wheats are classified genetically as diploid (AA, DD), tetraploid (AABB), and hexaploid (AABBDD) (Shewry, 2018). Einkorn (*Triticum monococcum*) and emmer (*Triticum dicoccum*) are two ancestor wheats, which are diploid (genome AA) and tetraploid (genomes AABB), respectively. The tetraploid pasta wheat (*Triticum durum,* genomes AABB) was derived from emmer wheat for modern use. In addition, the hexaploid common wheat (*Triticum aestivum*, genomes AABBDD) was developed by hybridization of emmer wheat with an ancient diploid wheat known as wild goat grass (*Aegilops tauschii,* genome DD) (Shewry, 2018). The genetic diversity of wheats has been further increased by the development of thousands of varieties and lines within the tetraploid and the hexaploid wheats via conventional cross-breeding and backcrossing (Pilolli et al., 2019). While common wheat and pasta wheat are the two most frequently used wheats, *T. monococcum* (einkorn) is also available commercially. However, *Ae. tauschii* wheat is not commercially available.

Currently, food allergen regulation by the US FDA assumes that all wheats, independent of their genetics, are alike in their intrinsic allergenicity (US FDA, 2022). It is unknown whether wheats in various genotypes differ in their intrinsic allergenic potential. However, as discussed above, the commonly used wheats belong to various species, varieties, lines, and accessions which may potentially contribute to differences in their relative allergenicity. In this regard, there is emerging but limited evidence from the published literature which suggests that all wheats may not be alike in their allergenicity (Gao et al., 2019; Nakamura, Tanabe, Watanabe, & Makino, 2005; Shewry & Tatham, 2016). Therefore, this problem needs to be clarified as it can

inform identification of potentially beneficial hypo-/non-allergenic wheats as well as potentially dangerous hyper-allergenic wheats that must be prevented from entering the food chain. In this dissertation research, a novel mouse model of intrinsic allergenicity was developed, and validated for the following wheats: diploid ancestor wheat *T. monococcum* (genome AA), diploid ancient wheat progenitor *Ae. tauschii* (genome DD), tetraploid *T. durum* (durum wheat, variety Carpio, genomes AABB), and hexaploid *T. aestivum* (common wheat, variety Ambassador, genomes AABBDD).

1.3.2. Novel wheats vs. wheat allergenicity

Practices of conventional crossbreeding of wheat to develop novel varieties with desired traits have brought into existence thousands of modern wheat varieties and lines (Shewry, 2009). Genetic engineering can also be used to create novel genetically modified (GM) wheats with desired qualities (US FDA, 2022). It is noteworthy that GM wheats are not commercially available at present. However, some field trials and studies have been conducted in the USA and Europe in the past using GM wheats, even though their allergenic potential remains unknown (Beale, Ward, & Baker, 2009; Lupi et al., 2013; Shewry et al., 2006). In addition, a few recent incidents of the escape of some experimental GM wheats in the USA have raised safety concerns among wheat consumers and manufacturers (USDA, 2022). Thus, it is important to identify and prevent potentially hyper-allergenic GM wheats from entering the food chain. To assist with safety assessment of GM foods, international regulatory and health agencies (FAO/WHO) have developed the 'substantial equivalence' concept as a general guideline (FAO/WHO, 2001; Ladics et al., 2014; Selgrade, Bowman, Ladics, Privalle, & Laessig, 2009). This concept can be used to assess the allergenicity of GM foods including GM wheats by comparing them with their

conventional non-GM counterparts (Domingo, 2016; Hollingworth et al., 2003; Selgrade et al., 2009). Use of animal models in testing GM foods for *in vivo* allergenicity has been suggested by FAO/WHO. However, no validated method is available at present (FAO/WHO, 2001). A validated *in vivo* model, such as the one presented in this dissertation, would be useful to evaluate the intrinsic allergenicity of GM wheats, so that potentially unsafe GM wheats can be identified during development and prevented from entering the food chain.

1.3.3. Processed wheat products and allergenicity

Wheat is commonly consumed after processing: baking, boiling, frying, roasting, fermentation, and extrusion (Di Cagno et al., 2002; Poutanen, Flander, & Katina, 2009). These procedures may modify the structures of wheat proteins and thus further affect how proteins are released and broken down during digestion and presented to the immune system (Gao et al., 2021; Jin et al., 2019). There is growing *in vitro* evidence in the literature that common methods used for processing of wheat food products can increase or reduce wheat allergenicity (Gao et al., 2021). Furthermore, industrially processed hydrolyzed wheat protein (HWP) that is widely used in cosmetics has been linked to sensitization as well as life-threatening allergic reactions in wheat-sensitive individuals (Burnett et al., 2018; Chinuki et al., 2013). Therefore, it is critical to decipher how food processing may affect wheat allergenicity as it is a prerequisite to exploring the potential of manufacturing hypo- and/or non-allergenic wheat foods as well as preventing the inadvertent introduction of hyper-allergenic wheat products (Gao et al., 2021). However, validated pre-clinical *in vivo* models such as a mouse model of intrinsic wheat allergenicity was unavailable (Denery-Papini et al., 2012; Jin et al., 2019; Pastorello et al., 2007; Petitot et al.,

2009; Shinoda, Inomata, Chinuki, Morita, & Ikezawa, 2012). Therefore, the focus of this dissertation research was to develop and validate a mouse model for this future application.

1.4. The transdermal sensitization/oral elicitation (TS/OE) mouse model of food allergy

Dr. Gangur and colleagues have developed a groundbreaking adjuvant-free transdermal sensitization and oral elicitation (TS/OE) mouse model of food allergy (Gonipeta, Kim, & Gangur, 2015). This model is based upon their discovery that food ingestion is not the only way to induce food allergy: they have shown that food allergies can develop upon dermal exposure to food allergens. In addition, the TS/OE mouse model is capable of simulating many key aspects of human food allergies (Gonipeta et al., 2015). US EPA regulators viewed this model as highly promising for evaluating allergenic potential of novel foods (Selgrade et al., 2009). This model does not involve adjuvant—a common practice used in food allergy animal model development that artificially enhances food allergenicity (Gonipeta, Kim, & Gangur, 2015; Ladics & Selgrade, 2009). Because the TS/OE model does not use adjuvant, it can be used to evaluate the intrinsic food allergenicity of a specific substance. This model has been previously validated for a number of allergenic foods including hazelnut, cashew nut, sesame, shellfish, egg, and milk (Birmingham, Gangur, Samineni, Navuluri, & Kelly, 2005; Birmingham et al., 2007; Gonipeta, Parvataneni, Paruchuri, & Gangur, 2010; Jin, Boss, Bursley, Gangur, & Rockwell, 2021; Navuluri et al., 2006; Ortiz et al., 2016; Parvataneni, Gonipeta, Acharya, & Gangur, 2016; Parvataneni, Gonipeta, Tempelman, & Gangur, 2009). In this dissertation research, the TS/OE mouse model was validated for four genetically distinct wheats.

1.5. Hypothesis and aims

The central hypothesis guiding this research was that the salt-soluble protein extracts (SSPEs) obtained from four genetically distinct wheats (*T. monococcum*, *T. durum*, *T. aestivum*, and *Ae. tauschii*) will show significant natural variation in their intrinsic allergenicity *in vivo*.

The central hypothesis was tested with two specific aims:

Aim 1. Validate the TS/OE model for transdermal sensitization with salt-soluble protein extracts (SSPEs) from four wheats: Determine the natural variation in the intrinsic sensitization potencies from transdermal application of SSPEs from four wheats: *T. monococcum* (genome AA), *T. durum* (genomes AABB), *T. aestivum* (genomes AABBDD), and *Ae. tauschii* (genome DD).

Aim 2. Validate the TS/OE model for oral allergic reaction to SSPEs from four wheats: Determine the natural variation in the oral allergic reaction elicitation potencies of SSPEs from the four wheats: *T. monococcum* (genome AA), *T. durum* (genomes AABB), *T. aestivum* (genomes AABBDD), and *Ae. tauschii* (genome DD).

The operational definitions used for wheat allergen sensitization and anaphylaxis elicitation are provided in **Table 1.1**.

Table 1.1. Operational definitions and quantification of sensitization and disease elicitation potencies of salt-soluble protein extract (SSPE).

Allergenic Potencies	Definition and Experimental Quantification
Sensitization potency	***Definition:*** ability of SSPE to induce specific (s)IgE antibody production in naïve mice upon transdermal application without adjuvant. ***Quantification:*** blood tests were done to measure specific IgE antibody levels.
Disease elicitation potency	***Definition:*** ability of SSPE to elicit oral allergic reaction in pre-sensitized mice after oral challenge. ***Quantification:*** blood tests were done to measure mucosal mast cell protease-1 levels after oral allergen challenge to determine mucosal mast cell degranulation response (MMCR). Rectal thermometry was used to measure the severity of anaphylactic reaction after oral challenge, as measured by hypothermic shock response (HSR).

Findings from this work are organized and presented in this dissertation as follows:

- Review of literature is presented in Chapter 2 (partly published in Jin, Gao et al., 2019).
- Review of the effect of food processing on wheat allergenicity is presented in Chapter 3 (Gao et al., 2021)
- Preliminary published research leading to the dissertation research is presented in Chapter 4 (Gao et al., 2019)
- Published work on the development and validation of TS/OE mouse model for durum wheat (*T. durum*) is described in Chapter 5 (Gao et al., 2022).
- Comparative intrinsic allergenicity potential of diploid, tetraploid, and hexaploid wheats is presented in Chapter 6 (Gao et al., in preparation).
- Future directions are presented in Chapter 7.

1.6. Scope of the work

This research focused on skin sensitization and oral IgE-mediated anaphylactic reactions to salt-soluble wheat protein extracts in a mouse model. This research involved testing *T. monococcum* (einkorn, genome AA), *T. durum* (durum wheat, Carpio variety, genomes AABB), *T. aestivum* (common wheat, Ambassador variety, genomes AABBDD) wheats, and an ancient wheat progenitor *Ae. tauschii* (genome DD). While the *Ae. tauschii* was grown at Michigan State University (MSU) with the help of Dr. Eric Olson, the *T. aestivum* (Ambassador variety) and *T. durm* (Carpio variety) were obtained from the MSU Wheat Breeding Program and North Dakota State University, respectively, and the *T. monococcum* (einkorn wheat) was purchased from a commercial source (einkorn.com). Balb/c breeding pairs were obtained from The Jackson Laboratory (Bar Harbor, ME), and animals used in the experiments were produced in-house and maintained on a plant-protein-free diet (AIN-93G). Adult female mice (6-10 weeks) were used in animal experiments.

1.7. Impact

A validated TS/OE mouse model of wheat allergy would be a cost-effective pre-clinical testing tool for evaluating the intrinsic allergenicity of wheat proteins. Furthermore, a validated TS/OE model could be used to examine the allergenic potencies of novel wheat lines/varieties and GM wheats when they are developed. In addition, this model can also be used to develop potentially hypo/non-allergenic wheat products. Lastly, a validated TS/OE model could facilitate advancing mechanisms of wheat allergenicity leading to the development of novel preventive and therapeutic methods for life-threatening wheat allergies.

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CHAPTER 2: LITERATURE REVIEW

2.1. Wheats and health

2.1.1. Wheat is a nutrient-rich food commodity as well as an ingredient in cosmetic and skin-care products

Wheat is a staple food worldwide (Shewry & Hey, 2015). As the world's second most produced cereal (after corn), its global production has been steadily increasing during the last decade (Kearney, 2010; USDA, 2016). The supply and demand of wheat have fluctuated around 750 million tonnes/year, especially in the last five years (FAO, 2020).

Wheat is a good source of essential nutrients including carbohydrate, protein, B vitamins and minerals (e.g., calcium, magnesium, phosphorus, etc.) (Sabença et al., 2021). The wheat grain is composed of germ (2-3%), bran (13-17%), and the endosperm (80-85%). Due to the high content of starch (~60–70% of the whole grain and 65-75% of the flour) in the endosperm, or the flour, wheat is a main source of energy especially for people in nations where bread, noodles and other products (e.g., bulgar, couscous) account for the most of their diet (Shewry, 2009). For instance, Du et al (2014) reported a major dietary shift in China from 1952 to 1992 that the consumption of cereals, including wheat, increased in rural areas whereas decreased in urban communities. Besides, wheat plays a major role in producing animal feeds due to its high content of starch (Shewry, 2009). Despite its relatively low protein content (8-15%) compared to soybeans (36-56%) wheat still provides comparable amount of protein to both human and livestock (Shewry, 2009). In addition, the consumption of whole grain (with bran and germ) that is rich in dietary fiber, B-vitamins and minerals is associated with reduced incidence of chronic diseases such as type-II diabetes and cardiovascular disease (de Munter, Hu, Spiegelman, Franz, & van Dam, 2007; Hu et al., 2020; Mellen, Walsh, & Herrington, 2008; Nettleton et al., 2010).

The Industrial Revolution brought mechanization to the bakeries and flour mills which facilitated wheat production substantially (Wrigley, 2009). Wheat is prevalently used as a major ingredient in various foods including pasta, bread, porridge, cookies, crackers, pizza, cakes, doughnuts, breakfast cereals, beer, and vodka (Food Allergy Canada, 2022). In addition to human foods, its by-products (e.g., bran and germ) contribute greatly to the diets of livestock (Shewry & Hey, 2015). Besides, wheat protein derivates (e.g., hydrolyzed wheat proteins or gluten) have been extensively applied in the cosmetic industry for manufacturing skin care products such as soap, shampoo, and conditioner (Burnett et al., 2018; Chinuki et al., 2013; Gao et al., 2021).

2.1.2. Wheat as a cause of human immune-mediated diseases

There are at least 8 human clinical conditions that have been associated with human exposure to wheat and wheat products (**Table 2.1**): 1. celiac disease; 2. non-celiac gluten/wheat sensitivity; 3. food protein-induced enterocolitis syndrome; 4. eosinophilic esophagitis; 5. wheat food allergy (including vomiting, diarrhea, hives, systemic anaphylaxis, atopic dermatitis, rhino-conjunctivitis, and oral allergy syndrome); 6. wheat-dependent exercise-induced anaphylaxis (WDEIA); 7. contact urticaria; and 8. baker's asthma (Cabanillas, 2020; Juhász et al., 2018; Quirce, Boyano-Martínez, & Díaz-Perales, 2016; Ricci et al., 2019). All these conditions are mediated by the inappropriate activation of the immune system. Whereas the celiac disease is an autoimmune condition triggered by the ingestion of gluten in food, the non-celiac gluten sensitivity is thought to be due to the activation of innate immune system by unknown components of wheat present in food (Cabanillas, 2020). Mechanisms of food-protein induced enterocolitis syndrome and eosinophilic esophagitis are incompletely understood. Only the last four diseases are mediated by the IgE antibodies. Both systemic anaphylaxis and baker's asthma can be potentially deadly,

and therefore require emergency medical treatment (Cabanillas, 2020; Cianferoni et al., 2013; Jin et al., 2019; Quirce et al., 2016; Ricci et al., 2019).

Table 2.1. Human clinical conditions that are associated with exposure to wheat and wheat products.

Immune-mediated diseases linked to wheat	Prevalence	Symptoms	Diagnosis	Potential Death	Prevention/Treatment	Mechanisms	References
Celiac Disease	1%	Intestinal inflammation, enteropathy, and villi atrophy; dermatitis herpetiformis, brain white-matter lesions	Serological tests and duodenal biopsies	No	Strict adherence to a gluten-free diet	IgA anti-TG2 Ab, IgG	Al-Toma et al 2019; Cabanillas 2020; Croall et al 2020; Leonard et al 2017
Non-Celiac Gluten/Wheat Sensitivity	Unknown	Delayed intestinal and extraintestinal symptoms: abdominal pain/ distension, diarrhea; fatigue headache, pain in muscles and joints, eczema, etc.	Based on exclusion of celiac disease, wheat allergy, and improvement after excluding gluten from the diet, and confirmed by OFC	No	Strict adherence to a gluten-free, gluten-reduced, or FODMAPs-reduced diet; consumption of ancient diploid wheat or novel wheat with reduced content of gliadin	Unknown, innate immunity seem to play a major role	Cabanillas 2020; Ricci et al 2019; Skypala et al 2019
Eosinophilic Esophagitis	0.05%	Esophageal dysfunction: vomiting, abdominal pain, dysphagia, and food impaction	Esophageal dysfunction, non-responsive to proton pump inhibitor therapy, and >15 eosinophil per high power filed in an esophageal biopsy	No	Allergen avoidance and corticosteroid use	Th2, Non-IgE-mediated	Cianferoni et al 2016; Wilson et al 2020
Eosinophilic Gastritis	0.006%	Tissue eosinophilic inflammation, peripheral eosinophilia, coexisting allergic diseases, and sensitization to multiple foods	Based on exclusion of other causes of eosinophilia: parasitic infections, inflammatory diseases related to gastrointestinal eosinophilia, and drug allergy	No	Allergen avoidance and corticosteroid use	Th2, Non-IgE-mediated	Cianferoni et al 2016; Wilson et al 2020
Baker's Asthma/Rhinitis	1%-10% and 18%-29% of bakers	Wheezing, shortness of breath, runny nose, nasal congestion, and chest tightness	Clinical history, positive sIgE and nasal/bronchial response to provocation	Yes	Strict avoidance of wheat flours and wheat foods	Th2, IgE, basophils, mast cells	Cianferoni et al 2016; Jeebhay and Baatjies 2020
Wheat Food Allergy	0.2-3.6%	Skin lesions, gastrointestinal discomfort, vomiting, urticaria, angioedema, respiratory symptoms, anaphylaxis	Clinical history, positive sIgE/SPT, and confirmed by OFC	Yes	Strict avoidance of wheat; Immunotherapies: oral immunotherapy, sublingual immunotherapy, and epicutaneous immunotherapy	Th2, IgE, basophils, mast cells	Babaie et al 2022; Cianferoni et al 2016; Nwaru et al 2014; Ricci et al 2019
Wheat-Dependent Exercise-Induced Anaphylaxis	<0.1%	Urticaria, angioedema, dyspnea, upper airway obstruction, vascular collapse, and anaphylaxis	Clinical history and positive sIgE/SPT; or OFC followed by exercise at 30 min after intake	Yes	Restriction of exercise up to 6 hours after wheat consumption; use of antihistamine, corticosteroids, and epinephrine are for accidental exposure	Th2, IgE, basophils, mast cells	Cianferoni et al 2016; Scherf et al 2016
Contact Urticaria	Unknown	Wheal and flare reaction	Clinical history and positive sIgE/SPT	No	Strict avoidance of products containing hydrolyzed wheat proteins and wheat foods	Th2, IgE, basophils, mast cells	Kobayashi et al 2015; Ricci et al 2019

Abbreviations: FODMAPS: fermentable oligosaccharides, disaccharides, monosaccharides, and polyols; OFC: oral food challenge; SPT: skin prick test

The *per capita* consumption of wheat flour has dropped in the US by ~7.7% during the last two decades (from 143 lbs./person in 1999 to 131 lbs./person in 2019) (USDA, 2019). Reasons for this reduction are unknown. Potential reasons include avoidance of wheat products due to physician diagnosed health conditions (e.g., food allergy, celiac disease, non-celiac gluten sensitivity etc.) or perceived health concerns without a formal diagnosis (Leonard et al., 2017; Pilolli et al., 2019). In Australia, 11% adults consciously avoid wheat in an attempt to alleviate fatigue and gastrointestinal symptoms (Golley, Corsini, Topping, Morell, & Mohr, 2015). In the United Kingdom, motivating factors for wheat avoidance by people (without celiac disease and without wheat allergy) were: management of symptoms similar to inflammatory bowel disease, infertility, low mood, low energy, immune dysfunction, weight gain, and visual and auditory hallucinations (Harper & Bold, 2018). Thus, real, or perceived health issues linked to wheat pose a serious threat to the growth of global wheat food industry. Furthermore, it is a major public health issue since severe symptoms such as systemic anaphylaxis or baker's asthma could be potentially fatal.

2.2. Wheat allergy

2.2.1. Significance of wheat allergy

Wheat allergy is an adverse immune system mediated reaction to specific wheat proteins (Cabanillas, 2020). It is generally mediated by IgE antibodies produced against wheat proteins known as allergens. Wheat allergies can be induced upon repeated exposures to wheat allergens via food ingestion, skin exposures, airways, and conjunctival exposures (e.g., baker's asthma/rhinitis). The diagnosis of wheat allergies is based on clinical history of symptoms that are specific to IgE-mediated wheat allergy, positive wheat-specific (s)IgE levels and/or positive results

of skin prick test (SPT) (Cabanillas, 2020; Cianferoni, 2016). It is worthy to note that due to shared IgE epitopes, cross-reactivity exists between wheat allergens and allergens from other cereals (e.g., rye, barley, etc.) or from grass pollen (Cabanillas, 2020). Therefore, positive sIgE or SPT result without clinical history to wheat is not diagnostic as demonstrated by individuals who were sensitized to grass pollen (Jones, Magnolfi, Cooke, & Sampson, 1995; Sander et al., 1997). And whether these individuals could tolerate wheat is eventually confirmed by performing an oral food challenge with wheat-containing diet, which is the gold standard in the diagnosis of wheat food allergy (Cianferoni, 2016).

The seriousness of wheat allergy is illustrated by the reports that more than half of affected children have had experienced anaphylactic reactions, which can be potentially fatal (Cianferoni et al., 2013; Pourpak, Mansouri, Mesdaghi, Kazemnejad, & Farhoudi, 2004). Even so, wheat allergens are under-researched relative to other allergenic foods such as peanuts, tree nuts, milk, and egg.

2.2.1.1. Prevalence of wheat allergy

The prevalence of wheat allergy is commonly estimated using various methods including: self-reporting, SPT, food specific IgE antibody testing using the blood, and oral food challenge testing (Cianferoni, 2016). In the US, based on self-reported data there are approximately 0.5% of children (less than 17 years) and 0.5-1.3% of adults are afflicted with wheat allergies (Gupta et al., 2018; Verrill, Bruns, & Luccioli, 2015; Vierk, Koehler, Fein, & Street, 2007). However, data from SPT suggests that there are 0.2-1.3% of US children (2-3 years old) suffer from wheat allergy (Venter et al., 2008). Wheat-specific IgE test results indicate that approximately 3.6% of US adults

are allergic to wheat (Biagini et al., 2004). The prevalence of IgE-mediated food allergy to wheat confirmed by oral food challenge is unknown (Cianferoni, 2016).

Based on self-reported data it is estimated that 1% of children (<15 years) and 1.5% of adults in the Europe suffer from wheat allergy (Nwaru et al., 2014; Zuidmeer et al., 2008). IgE-testing results indicate that the general prevalence of wheat allergy is 3.9% in Europe (Nwaru et al., 2014). The result from oral food challenge testing suggests that 0.2-0.5% of European children (6-14 years old) suffer from wheat allergy. The general prevalence across all ages based on oral food challenge data is 0.1% in Europe (Nwaru et al., 2014; Zuidmeer et al., 2008). In the UK, wheat allergy affects 0.9% of general population based on self-reported data. The SPT testing results indicate that about 0.4%-1.2% of children (<15 years old) in the UK are allergic to wheat (Zuidmeer et al., 2008).

In Japan, wheat is the third most common allergenic food in children after milk and egg (Ebisawa et al., 2020). Approximately 0.37% Japanese children (0-6 years) suffer from wheat allergy (Noda, 2010). The SPT-confirmed wheat allergy is prevalent in 0.15% of Australian children (all ages) (Hill et al., 1997). In addition, a study conducted in Central America suggests that the prevalence of self-reported wheat allergy in Salvadoran adults is 0.75% (Ontiveros et al., 2018). Overall, wheat allergy affects approximately 0.43% of children and 0.4%-2.08% of adults at the global level (Zuidmeer et al., 2008).

2.2.1.2. Natural history of wheat allergy

Most food allergies start early during the childhood, while some can be outgrown by adulthood, others persist for life (Foong & Santos, 2021). Wheat allergy can develop as early as infancy (~2 months) whereas the median age of wheat allergy diagnosis is 13 months (Czaja-Bulsa

et al., 2014). As to the natural resolution, it is estimated that 85% of children who are allergic to milk, egg, soy, or wheat can outgrow, whereas only 15-20% of children can outgrow their allergies to peanuts, tree nuts, and seafood after their teenage (Bettcher, Rockwell, & Ravikumar, 2020).

Studies on sex disparity in food allergy in general has shown that male children appear to be more afflicted than females; however, this trend reverses after adolescence (Kelly & Gangur , 2009; Pali-Schöll & Jensen-Jarolim, 2019). Although sex-disparity in wheat allergy has not been well studied, two US studies show that when randomly selected for research study, there were significantly more male children (64-66%) than females (34-36%) in their cohorts (Czaja-Bulsa & Bulsa, 2014; Keet et al., 2009,).

Although most (85%) wheat allergies are outgrown by the time a child becomes an adult, the prediction of this phenomenon using sIgE levels has met with limited success. For example, wheat-allergic patients with highest sIgE levels can outgrow their clinical disease (Keet et al., 2009). The median age of resolution of wheat allergy is approximately 6.5 years (Keet et al., 2009). In this study, one third of the wheat-sensitive children who participated in a study had their wheat allergies resolved by 4 years of age, and 62% restored their tolerance to wheat by 10 years. The wheat allergies can remain persistent in ~15% of wheat-allergic children throughout their lives – a significant problem as they continue to be vulnerable to life-threatening anaphylaxis (Keet et al., 2009).

2.2.1.3. Prevention and management of wheat allergy

Wheat allergy is a critical public health issue as both systemic anaphylaxis and baker's asthma can be potentially deadly (Cabanillas, 2020; Cianferoni et al., 2013; Jin et al., 2019; Quirce et al., 2016; Ricci et al., 2019). Systemic anaphylaxis triggered by food allergies, including wheat

allergies is a leading cause of American emergency room visits (Du et al. 2016; Scherf et al. 2016). Pourpak and coworkers conducted an oral food challenge on wheat-allergic children, and reported that 54% of the clinical manifestations among these children appeared as anaphylaxis (Pourpak et al., 2004). Similarly in another study, more than half of children allergic to wheat experienced anaphylactic reactions upon wheat ingestion (Cianferoni et al., 2013). These studies illustrate the importance of advancing mechanisms of anaphylaxis to wheat upon food ingestion so that more effective methods can be developed to prevent/treat this condition.

Wheat-dependent exercise-induced anaphylaxis (WDEIA), is a particular type of anaphylaxis that occurs in atopic individuals who performs intense exercise after 1-4 hours of wheat ingestion, it accounts for the majority (56%) of food-dependent exercise induced anaphylaxis (FDEIA) (Scherf et al., 2016) (Harada et al., 2001). Nonetheless, Brockow et al (2015) reported that exercise is not an essential trigger for the onset of allergic symptoms in patients when they were challenged with high dose of wheat-containing diet (Brockow et al., 2015). In their study, a quarter of patients exhibited anaphylaxis after ingesting 10 to 80 g of gluten without exercise or other co-factors (i.e., alcohol or acetylsalicylic acid). Furthermore, the incidence of anaphylaxis among these patients was positively related to their dose of intake in wheat gluten. Therefore, anaphylaxis can occur upon ingestion of high dose of wheat protein independent of exercise or other co-factors.

There is no cure for wheat allergies at present (Seth, Poowutikul, Pansare, & Kamat, 2020) Depending on the route of exposures (i.e., skin, food ingestion, or airway), prevention measures include strict adherence to wheat-/ cereal-free diet or even total restriction of exposure to wheat flours or wheat containing food and skin care products (Cabanillas, 2020). Individuals with wheat food allergy are advised to stick to a wheat elimination diet as well as learn to interpret the food

allergen labels (Cianferoni, 2016; Quirce et al., 2016). Subjects with baker's asthma/rhinitis in occupational settings have to avoid exposure to wheat completely in all circumstances (Cianferoni, 2016). In the case of accidental exposure to wheat, while using antihistamines are helpful in reducing minor symptoms of wheat allergy, prompt treatment with epinephrine can be lifesaving for anaphylactic attacks (**Table 2.1**). In the absence of emergency medical treatment, wheat allergies can be potentially fatal (Cianferoni, 2016).

At present, millions of wheat-sensitive consumers around the world must avoid wheat for their entire life if they do not outgrow it (Leonard & Vasagar, 2014; Shewry & Tatham, 2016). For one thing, inadvertent contaminations with food allergens are all around, as witnessed by the 75-80 class I food recalls every year (US FDA 2021). For another, avoidance requires unstinting vigilance, the strain of which is for some individuals the cause of anxiety attacks (Warren et al. 2016). Therefore, improved methods are needed for prevention/therapy of wheat allergies.

The current immunotherapies for wheat allergies include oral immunotherapy (OIT), sublingual immunotherapy (SLIT), and epicutaneous immunotherapy (EPIT) (Ramsey & Berin, 2021). While the OIT has been recognized as the most effective, but risks life-threatening anaphylaxis (Cianferoni, 2016; Ricci et al., 2019). In contrast, SLIT and EPIT are relatively safe but are less effective (Ramsey & Berin, 2021) (Babaie et al., 2022; Nagakura et al., 2022; Tomsitz, Biedermann, & Brockow, 2021). Thus, future investigations are needed to improve both the safety and efficacy of current immunotherapies. Validated wheat allergy mouse model would be very useful for preclinical investigation of improved immunotherapies.

2.2.1.5. Regulation of wheat as a major allergenic food at the global level

Wheat is among the 9-14 major allergenic foods identified by food regulatory bodies of many nations including the United States (**Table 2.2**; EFSA, 2014; FAO, 2021; US FDA, 2020; Food Standards Australia & New Zealand, 2020; Gupta et al., 2011; Health Canada, 2021; Japan, 2019; Renz et al., 2018; UK Food Standards Agency, 2021). Besides, the following countries have also listed wheat or cereals with gluten as a major allergenic food: Argentina, Belarus, Bolivia, Brazil, Caricom Std, Central America, Chile, China, Colombia, Cuba, Egypt, Fiji, India, Kazakhstan, Malawi, Malaysia, Mexico, Morocco, Phillippines, Russia, Thailand, Turkey, Ukraine, Venezuela, and Vietnam (University of Nebrasaka-Lincoln, 2022).

Table 2.2. Regulation of allergenic foods at the global level.

Food Regulatory Agencies (Country)	Major Allergenic Foods	References
US FDA	Milk, egg, fish, wheat, tree-nut, peanut, soybean, crustacean shellfish, sesame, and sulfites (≥ 10 ppm)	US FDA, 2022
CFIA (Canada)	All foods listed by the US FDA plus cereals with gluten, molluscan shellfish, and mustard	CFIA, 2018
EFSA (EU) and FSA (UK)	All foods listed by the US FDA plus cereals with gluten, molluscan shellfish, mustard, celery, and lupin	EFSA, 2014; FSA 2021
FSANZ (Australia and New Zealand)	All foods listed by the US FDA plus cereals with gluten, lupin, and bee pollen/propolis	FSANZ, 2021
MFDS (South Korea)	All foods listed by the US FDA plus buckwheat, and molluscan shellfish	MFDS, 2022
MAFF (Japan)	Milk, egg, wheat, tree-nut, peanut, shellfish, and buckwheat	MAFF, 2019

Abbreviations: US FDA: U.S. Food and Drug Administration; CFIA: Canadian Food Inspection Agency; EFSA: European Food Safety Authority; FSA: Food Standards Agency; FSANZ: Food Standards Australia and New Zealand; MFDS: Ministry of Food and Drug Safety; MAFF: Ministry of Agriculture, Forestry and Fisheries

Wheat-allergic individuals are typically advised to interpret food allergen labels, which are mandated by the Food Allergen Labeling and Consumer Protection Act (FALCPA) in the USA

(US FDA, 2005). These Acts are deemed to help food allergic consumers to identify and thus avoid exposure to the nine major food allergens (i.e., milk, egg, fish, wheat, tree-nut, peanut, soybean, shellfish, and sesame) identified by the US FDA (US FDA, 2022). Specifically, if a food product contains proteins from one of these nine allergenic foods, the manufacturers are required to include them in the ingredient list. In addition, the US FDA may take regulatory actions such as working with manufacturers to recall a food product when it contains an undeclared allergen (US FDA, 2022).

It is important to note that foods labelled as "gluten-free" should not be confused with those labelled as "wheat-free": individuals with celiac disease or non-gluten celiac sensitivity are advised to avoid consuming wheat, barley, and rye. Whereas wheat allergic individuals are often only need to avoid their exposure only to wheat (Cabanillas, 2020; Ricci et al., 2019).

2.2.2. Wheat proteins and allergens

Wheat contains 10-18% total protein on a dry weight basis. Based on their solubility, the proteins are classified into four families as follows (**Table 2.3**): albumins (water/saline-soluble, 10-12% of the total protein), globulins (saline-soluble, 5-8% of the total protein), gliadins (alcohol-soluble, 30-40% of the total protein), and glutenins (acid-soluble, 45-50% of the total protein) (Osborne, 1907; Uthayakumaran et al., 2017). The non-gluten proteins (albumins and globulins) are the structural and metabolic proteins. They serve as nutrient reserves for germinating the embryo and protecting it from insects and pathogens before germination (Dupont & Altenbach, 2003). The gluten proteins (gliadins and glutenins, also known as prolamins) are the seed storage proteins. Gliadins are monomeric proteins that interact through hydrogen bonds and contain mostly intramolecular disulfide bonds (Karsada, 1989; Johansson et al., 2013; Markgren et al., 2020).

Glutenins are polymeric proteins linked by inter and intramolecular disulfide bonds. Gliadins may be cross-linked to the glutenin network through intermolecular disulfide bonds as well (Karsada, 1989; Johansson et al., 2013; Markgren et al., 2020).

All four families of wheat proteins together contain at least 107 allergenic proteins (www. allergome.org). All the 3 wheat genomes (A, B, and D) encode for the wheat allergens. Recently chromosomal locations of 41 allergen encoding genes have been mapped, and IgE epitopes are well characterized for many, but not all of these allergens (Juhász et al., 2018). Major wheat allergens that are well characterized include: ω-1, 2, 5 gliadin, α/β/γ -gliadins, the high and low molecular weight glutenin subunits (HMW-GS and LMW-GS), β-amylase, α-amylase/trypsin/subtilisin inhibitor proteins, lipid transfer protein, chitinase, glyceraldehyde-3 phosphate dehydrogenase, triosephosphate isomerase, peroxidase, glutathione S-transferase, globulin-3, serpins, and α-purothionin (Cianferoni, 2016; Juhász et al., 2018). For detailed information on wheat allergens, and their IgE epitopes, readers are referred to these excellent articles (Denery-Papini et al., 2011; Matsuo, Yokooji, & Taogoshi, 2015; Monaco et al., 2021; Pahr et al., 2013).

Table 2.3. Classification of wheat proteins.

Wheat Protein Fractions	Protein	Solubility	Percentage of Total Wheat Proteins	References
Gluten	Gliadins (Monomers)	70% aqueous ethanol-soluble	30-40%	
	Glutenins (Polymers)	Dilute acetic acid- or alkali-soluble	40-50%	Osborne 1907; Uthayakumaran et al 2017
Non-gluten	Albumins	Water-soluble	10-12%	
	Globulins	Salt-soluble	5-8%	

2.2.3. Mechanisms underlying the genesis of IgE-mediated wheat allergy

2.2.3.1. Genetic factors related to wheat allergy

Several studies investigated the genetic mechanisms underlying wheat allergy and found evidence for the following factors: loss-of-function mutation in the filaggrin gene, HLA-variants, cytokines, and immune receptors. These studies are reviewed below:

2.2.3.1.1. Loss-of-function mutation in the filaggrin gene and wheat allergy

Filaggrin is an epidermal protein that plays an important role in maintaining the skin barrier function. Approximately half of Americans who suffer from severe eczema have at least one mutated filaggrin allele (van den Oord & Sheikh, 2009). Deficiency of filaggrin due to gene mutation is associated with the development of allergic eczema as well as higher incidence of food allergy (Benedé, Blázquez, Chiang, Tordesillas, & Berin, 2016; Pipinić & Macan, 2015). Due to this, atopic individuals are susceptible to the invasion of allergens via the skin. It was reported that infants with eczema are 11 times and 6 times more likely to develop peanut allergy and egg allergy, respectively, suggesting that defects in skin barrier function may facilitate the absorption of food

allergens (Brown et al., 2011). Indeed, loss-of-function mutations in the filaggrin gene was thought to strongly associated with multiple food allergies and alcohol sensitivity (Linneberg et al., 2013). Two studies examined the role of genetics in WDEIA development with a focus on a specific mutation in the filaggrin (Iga et al., 2013; Mizuno et al., 2015). Mizuno et al (2015) reported two individuals with WDEIA carried at least one loss-of-function mutation in their filaggrin, indicating the potential involvement of filaggrin mutations in wheat allergy (Mizuno et al., 2015). Nonetheless, Iga et al (2013) documented a patient with WDEIA who did not have filaggrin mutations (Iga et al., 2013). It is worthy to note, however, that the patient developed symptoms of WDEIA after using facial soap that contained hydrolyzed wheat proteins, the WDEIA-causing capacity of which has been illustrated in many other studies (Hiragun et al., 2011; Nakamura et al., 2014; Yokooji et al., 2013).

2.2.3.1.2. HLA, RBFOX1 variants and wheat allergy

The human leukocyte antigen (HLA) region encodes loci for several key immune response genes (Horton et al., 2004). Fukunaga et al (2021) conducted a genome-wide study to identify the genetic variants associated with WDEIA in 77 adult patients. They reported that HLA-DPB1*02:01:02 allele exhibited the most significant association and individuals carrying this allele have significant increased risk of WDEIA. Noguchi et al (2019) found that the genetic variation in the class II HLA region on chromosome 6p21 contribute to the susceptibility of atopic individuals toward hydrolyzed wheat gluten induced allergy.

RBFOX1 is a type of RNA-binding protein that plays major roles in alternative splicing and neurological development (Prashad & Gopal, 2021). Noguchi et al (2019) reported that

RBFOX1 locus on chromosome 16p13 is significantly associated with hydrolyzed wheat gluten allergy.

2.2.3.1.3. Cytokines and wheat allergy

Certain cytokines are known to facilitate or reduce the progression of allergic reactions due to gene polymorphism. For instance, interleukin 4 (IL-4) is a well-known signature Th2 cytokine that is essential in development of allergic diseases (Castan et al., 2020). Cai et al (2013) examined the association between three single nucleotide polymorphisms (SNP) (IL-4-C590T, IL-4RA A1727G and IL-10-A627C) with WDEIA in 51 patients. They reported that IL-4-C590T is associated with WDEIA. Hur and coworkers (2013) examined the role of IL-4 and the IL-4 receptor α (IL-4Rα) in the pathogenesis of baker's asthma. Their analysis of clinical and genetic data from 373 bakery workers suggested that the IL-4Rα lle375Val and Gln576Arg polymorphisms may be involved (Hur, Ye, Koh, Kim, & Park, 2013).

The cytokine IL-18 has been reported playing an important role in facilitating allergic reactions (Sanders et al., 2016). Kim et al (2012) genotyped three polymorphisms of the IL-18 gene (-607A/C, -137G/C, and 8674C/G) from 373 bakers with lower respiratory symptoms induced by wheat flour. Their results suggested that IL-18 could be altered by gene polymorphism and thus contribute to the development of sensitization to wheat flour (Kim, Hur, Jin, Choi, & Park, 2012). Gao et al (2020) investigated the association of IL-18 variation with WDEIA in 130 patients. By performing SNP genotyping, they found that the polymorphism in the IL-18 promoter region may play a major role in WDEIA development (Gao et al., 2020).

2.2.3.1.4. TLR4 and wheat allergy

Toll-like receptor 4 (TLR4) is involved in the susceptibility of food allergy as demonstrated by the augmented sensitization as well as anaphylaxis in TLR4-deficient mice exposed to peanut allergens (Bashir, Louie, Shi, & Nagler-Anderson, 2004). Cho et al (2011) performed a genetic association study on bakers with occupation respiratory symptoms to wheat flour. They found that the carriers of TLR4 variants are less likely to develop such occupational symptoms (Cho et al., 2011).

Overall, the above studies elucidate the underlying genetic mechanisms in the pathogenesis of WDEIA and respiratory allergic reactions induced by wheat. However, future investigations are necessary to identify the genetic factors related to other forms of wheat allergies (e.g., systemic anaphylaxis). A validated mouse model of wheat allergy such as the TS/OE model could be used for such studies.

2.2.3.2. Environmental factors related to wheat allergy

Environmental factors have been shown to account for 50% of the risk of developing allergic diseases, which is similar to the impact of genes. (Harb & Renz, 2015; Saxon & Diaz-Sanchez, 2005). The following environmental factors have been found to be related to the development of food allergies: hygiene hypothesis, air pollution, gut microbiome, use of antacids/antiulcer medications, antibiotics/antimicrobials, endocrine disruption compounds (EDCs), nutritional factors, and the effect of food processing methods. These studies are briefly reviewed below:

2.2.3.2.1. Hygiene hypothesis, air pollution, microbiome, and wheat allergy

The "hygiene hypothesis" proposes that as people moved from rural to urban environment, they become more hygienic and consequently reduced their exposures to microorganisms. Because of this, their immune system became more prone to develop allergic sensitization (or atopy) (von Mutius, 2007).

A recent study indicates that air pollutants can facilitate the penetration of allergens which enhances the risk of atopic sensitization and exacerbate the symptoms of sensitized individuals (Urrutia-Pereira, Guidos-Fogelbach, & Solé, 2022). They also reported that air pollution can modify the host microbiota which affects the barrier function of skin and respiratory epithelium, thus increases allergic rhinosinusitis and atopic dermatitis. Indeed, studies of gut microbiota in food (including wheat) allergic children have shown that dysbiosis is associated with increased allergy risk (Bunyavanich & Berin, 2019). Besides, the change of lifestyle as mentioned in the "hygiene hypothesis" above is also thought to alter the gut microbiome, which is essential in modulating immune functions of the host (Bunyavanich & Berin, 2019; Iweala & Nagler, 2019; Skypala & McKenzie, 2019).

2.2.3.2.2. Use of antacid/antiulcer agents, antibiotics, and wheat allergy

Antacid/antiulcer medications can interfere gastric digestion substantially which increases the risk of food allergy sensitization (Untersmayr & Jensen-Jarolim, 2008). Indeed, a large retrospective study has demonstrated a strong positive correlation between the use of antacids in infants less than 6 months and their subsequent development of allergic diseases (Mitre et al., 2018). The study also suggests that antibiotic use during infancy was implicated as a risk factor of allergy development, which coincided with a retrospective study led by Li and coworkers who

compared over 500,000 antibiotic users with non-users in children which the former was associated with a faster development of food allergy (Li et al 2018, Mitre et al., 2018). However, the specific effects of these factors in wheat food allergy in particular remains to be thoroughly investigated.

2.2.3.2.3. Endocrine-disrupting compounds (EDCs) and wheat allergy

Endocrine-disrupting compounds (EDCs) are a group of chemicals that can modulate hormone signaling while some were reported having immune-modulating effect (Chalubinski & Kowalski, 2006; Schug, Janesick, Blumberg, & Heindel, 2011). For instance, triclosan and parabens are antimicrobial agents that have been commonly used in not only personal care products but also preservatives in foods, drugs, and cosmetics (Savage et al., 2012). Studies in both mice and huamns have shown that urinary levels of these agents are associated with sensitizations to food allergens and aeroallergens, indicating their allergy-promoting effects (Savage et al., 2012; Tobar et al., 2016). However, their specific role in wheat food allergy remains to be established.

2.2.3.2.4. Nutritional/maternal factors and wheat allergy

It was reported that people who have vitamin D deficiency are more susceptible to food sensitization as well as development of IgE-mediated food allergy (Allen et al., 2013). However, it appears that not only the specific type of nutrient but also their route of exposure may affect the outcomes of allergy development: Tukkola et al (2016) reported that while off-springs of women taking vitamin D supplements exhibited increased risk of cow's milk allergy, those off-springs from moms who had their vitamin D intake from food sources during pregnancy had decreased

risk of developing allergy towards cow's milk (Tuokkola et al., 2016) (Skypala & McKenzie, 2019). Besides, maternal factor may intertwine with nutritional factor to affect the outcomes of allergy development in the off-springs. For instance, the quantity and quality of fats in the maternal diet may be crucial to the allergy outcomes in offsprings. Best and colleagues (2016) reported that supplementation of omega-3 long chain fatty acids (n-3 LCPUFA) by pregnant moms may have protective effects on children's allergies toward peanut and cashew (Best et al., 2016). Nevertheless, the effects of these factors on children's outcomes in terms of wheat allergy remains to be assessed.

In addition to the effect of a specific nutrient, the timing of introduction of allergenic foods including wheat and the risk of allergic outcomes has been long debated. For example, Poole et al (2006) reported that early introduction of wheat to infants is associated with lower incidence of wheat allergy, whereas delaying exposure to wheat until after 6 months may result in a higher risk of wheat allergy development (Poole et al., 2006). However, Chmielewska et al (2017) suggested that the effect of breastfeeding and early introduction of wheat gluten on the risk of wheat allergy remains uncertain, and more solid evidence is necessary before formulating any firm recommendations.

2.2.3.2.5. Food processing, genetic modification of wheat, and wheat allergy

Wheat food processing, industrial processing of wheat, and genetic modifications of wheat by crossbreeding and recombination DNA technology are other potential environmental factors that may contribute to the genesis of wheat allergy (Pilolliet et al., 2019; US FDA, 2022). Detailed description of these aspects is available in 2.3. and 2.4. of Chapter 2 and in Chapter 4.

In summary, role of specific environmental factor in wheat allergenicity remains to be established. Moreover, the use of mouse models for investigating these factors have not been done. Therefore, a validated TS/OE mouse model would be very useful for such research.

2.2.3.3. Development of IgE-mediated wheat allergy: mechanisms

Wheat allergy, similar to other types of food allergy, is thought to develop in two phases (**Figure 2.1**): (i) sensitization, where individuals produce IgE antibodies specific to wheat allergens upon initial exposures; and (ii) elicitation of allergic reactions, where sensitized individuals exhibit clinical symptoms upon oral re-exposure to the wheat allergen (Jin et al., 2019; Sicherer & Sampson, 2018). During the first phase, susceptible individuals are exposed to wheat allergens via various routes: eyes, nose, skin, and oral. These allergens are captured by antigen-presenting cells (i.e., macrophages, dendritic cell), processed and presented to T cells. Coupling with several co-factors such as dysregulated host microbiome and other environmental factor (e.g., detergents in allergen-containing cosmetic products), primed T helper 2 (Th2) cells in sensitized subjects activate B cells to produce allergen-specific Immunoglobulin E (IgE) antibodies. These allergen-specific IgE antibodies then bind to the high-affinity IgE receptor (FcεRI) present on the surface of mast cells and basophils. Upon re-exposure of sensitized individuals, the wheat allergens cross-link the IgE on mast cells and basophils and activate them to release histamine and other mediators (Murphy, Weaver, & Janeway, 2017). These mediators cause clinical symptoms of the allergic disease.

Depending on the dose and route of exposure, symptoms of allergic reactions vary from mild (e.g., rashes, hives, GI discomfort, airway problems) to severe (shortness of breath, hypotension), and even life-threatening (e.g., anaphylactic shock) reactions. Increased fluid

secretion as well as peristalsis of gastrointestinal tract leading to vomiting and diarrhea. Individuals who suffer from allergic rhinitis and conjunctivitis exhibit increased mucus secretion, itching and sneezing (Pawankar, Hayashi, Yamanishi, & Igarashi, 2015). Whereas allergens introduced into the bloodstream can cause increased blood flow and permeability, which lead to edema. Systemic dissemination of histamine in this case is capable of causing airway constriction such as difficulty in breathing as well as severe loss of blood pressure that result in shock (Finkelman, Rothenberg, Brandt, Morris, & Strait, 2005; Sicherer & Leung, 2015). Late-phase reactions can occur due to new mediator release several hours after the first reaction by mast cells/basophils and may persist for up to 24 hours after the challenge due to continued release and synthesis of inflammatory mediators by mast cells (Murphy et al., 2017).

2.2.3.4. Mechanisms of systemic anaphylaxis in humans vs. mouse models

Systemic anaphylaxis is a life-threatening allergic reaction elicited by groups of immune mediators released by mast cells and basophils upon activation (LoVerde, Iweala, Eginli, & Krishnaswamy, 2018). As opposed to the generally mild allergic symptoms (e.g., itchy skin, hives, diarrhea, vomiting, swelling of lips/face/tongue), systemic anaphylaxis involves reaction of the vital organs such as lungs and cardiovascular system simultaneously with the gut and the skin; this is life-threatening (Cianferoni & Muraro, 2012; **Figure 2.1**).

The criteria for diagnosing systemic anaphylaxis in humans is based on the presence of one of the following three conditions (LoVerde et al., 2018): 1) Acute onset of illness with skin (hives), mucosal tissue (swollen tongue and lips), and breathing difficulties (wheezing, stridor) or hypotension; 2) Two or more of the following conditions that occur rapidly after exposure to

known or likely allergen: skin issues, respiratory compromise, hypotension, gastrointestinal discomfort; and 3) Reduced blood pressure soon after exposure to a known allergen.

Systemic anaphylaxis can be IgE-mediated or non-IgE (or IgG)-mediated (Finkelman, Khodoun, & Strait, 2016). The IgE-mediated systemic anaphylaxis is also known as the classic pathway, whereas the IgG-mediated systemic anaphylaxis is commonly referred as the alternative pathway. In addition, anaphylaxis can also be activated by complement system derived anaphylatoxins known as C3a and C5a (**Table 2.4**).

There are many similarities but some differences in the mechanisms of food induced systemic anaphylaxis in humans vs. mice that is reviewed below.

2.2.3.4.1. Mechanisms of food-induced systemic anaphylaxis in humans: IgE and non-IgE (IgG) pathways

In IgE-mediated systemic anaphylaxis, atopic individuals can be sensitized to food, venom from insect sting, or drug. Upon re-expose to the same allergen, which can cross-link with the allergen-specific IgE antibodies attached to the high-affinity IgE receptor FcεRI on mast cells and basophils (Reber et al., 2013). Upon activation, these cells will degranulate to release mediators including histamine, platelet-activating factor (PAF), and tryptase (Cianferoni, 2021). Both histamine and PAF contribute to the development of shock (hypothermia). Elevation of blood levels of tryptase is clinically used as a diagnostic indicator of systemic anaphylaxis in humans.

Non-IgE or IgG-mediated systemic anaphylaxis is commonly referred as the alternative pathway, in which a different group of immune factors such as the low-affinity IgG receptor FcγR, macrophages, and neutrophils (Jönsson et al., 2011). However, the solid evidence of IgG-mediated food induced systemic anaphylaxis is lacking and thus needs to be further investigated. Compared

to the IgE-dependent anaphylaxis, the elicitation of IgG-dependent anaphylaxis relies on considerably higher concentrations of antigen and antibody (Cianferoni, 2021). Therefore, anaphylaxis elicited by a small quantity of antigen such as insect sting is highly likely to be IgE-mediated. Similarly, food allergy-induced anaphylaxis is also most likely primarily IgE-mediated since only a very small portion of the ingested food protein allergen is absorbed with all epitopes intact (Strait et al., 2011).

In humans, anaphylatoxin (C3a, C5a) receptors are present on mast cells, basophils, other myeloid cells, and vascular endothelial cells, suggesting the possibility of a role for these mediators in human anaphylaxis (Füreder et al., 1995; Skeie, Fingert, Russell, Stone, & Mullins, 2010; Van Epps, Simpson, & Chenoweth, 1992; Van Epps, Simpson, & Johnson, 1993). However, concrete evidence for their role in food induced anaphylaxis is yet to be obtained (Cianferoni, 2021).

2.2.3.4.2. Mechanisms of food induced anaphylaxis in mice: IgE and non-IgE (IgG) pathways

Like humans, IgE/mast cells are primarily involved in food induced oral systemic anaphylaxis in mice. Role of basophils is not well studied. In addition, mucosal mast cell protease 1 (MMCP-1) has been shown to be a very specific biomarker of IgE-mediated systemic anaphylaxis to food allergen in mouse (Finkelman, 2007). In contrast, monocytes/macrophages, basophils, and neutrophils are shown to be crucial in IgG-mediated anaphylaxis in mice (Finkelman et al., 2016; Jönsson et al., 2011; Tsujimura et al., 2008).

Complement-mediated anaphylaxis is another non-IgE mediated anaphylaxis that can be induced by food proteins in mice (Finkelman et al., 2016). Upon complement activation, anaphylatoxins (e.g., C3a, C5a) are released, which lead to the activation of mast cells, basophils, endothelial cells, smooth muscles, and etc. (Cianferoni, 2021). Their role has been demonstrated

in mouse studies (Finkelman et al., 2016). For instance, peanut and tree nut proteins can activate complement and generate anaphylatoxins that can induce anaphylaxis in mice via mast cell activation; this pathway can also exacerbate IgE-mediated anaphylaxis to nuts; however its role in wheat anaphylaxis is unknown at present (Khodoun et al., 2009).

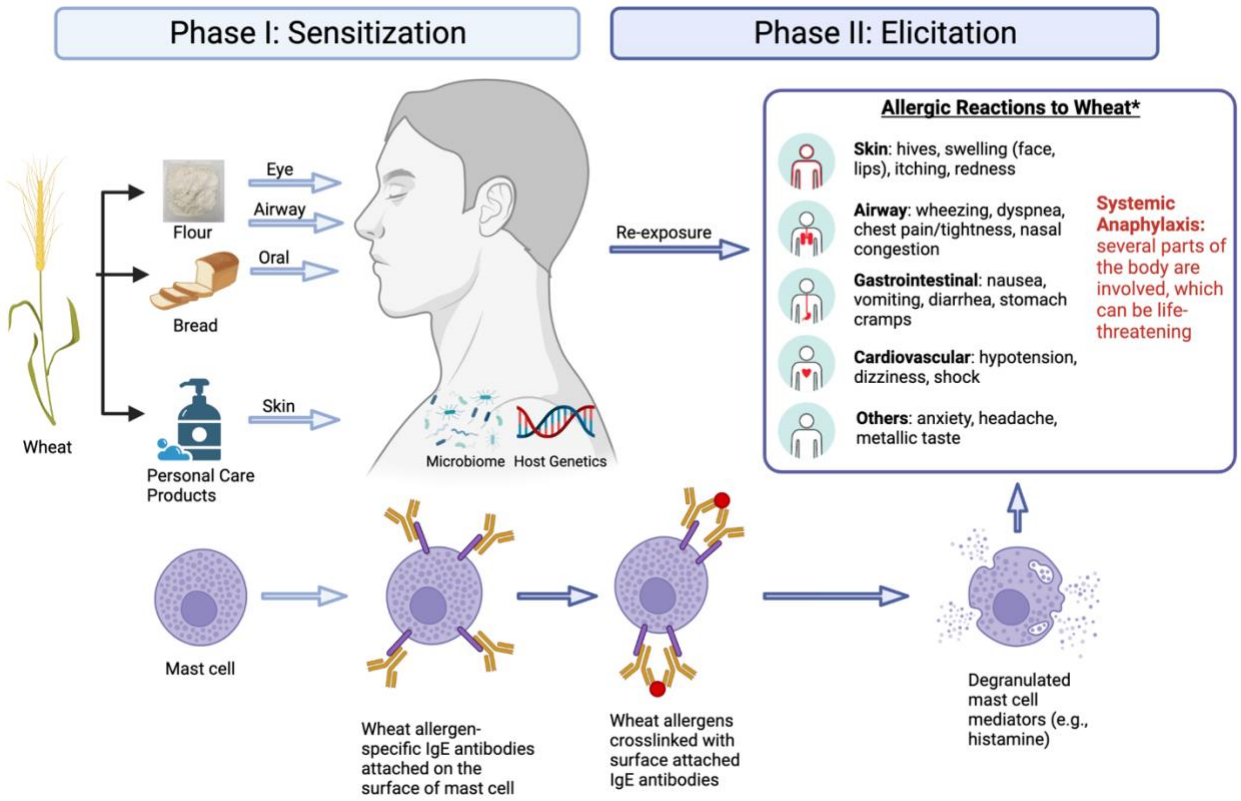

Figure 2.1. The two phases of pathogenesis of wheat allergy: sensitization and elicitation.*Allergic reactions to wheat image source: https://foodallergycanada.ca/food-allergy-basics/preventing-and-treating-allergic-reactions/reaction-signs-and-symptoms/.

Table 2.4. Mechanisms of food-induced anaphylaxis in humans vs. mice.

Type of Anaphylaxis	Human	Mice
IgE-mediated	Individuals become sensitized to food by producing allergen specific IgE antibodies, which attach to the surface of mast cells and basophils via high affinity FcεRI receptor. Upon re-exposure to the same allergens, IgE on these cells gets cross-linked resulting in their activation and release mediators including histamine, PAF, and tryptase (Reber et al., 2013; Cianferoni, 2021).	Similar mechanisms as in humans; murine mast cell protease-1 (MMCP-1) is released upon activation of mast cells which is a specific biomarker of IgE mediated systemic anaphylaxis in mice; role of basophils is not well studied (Finkelman, 2007).
IgG-mediated	Role of macrophages and neutrophils as well as their released mediators have been suggested; solid evidence is lacking (Cianferoni, 2021).	Antigen forms complexes with IgG that cross-link the low affinity IgG receptor FcγRIII and FcγRIV on macrophages, basophils, and neutrophils. Upon activation, these cells release PAF, which is responsible for the development of shock in IP injection mouse models (Finkelman, 2007, 2016). Role in oral food induced anaphylaxis is not well studied.
Complement activation	Anaphylatoxin (C3a, C5a) receptors are found on mast cells and basophils, suggesting the possibility of complement-mediated anaphylaxis. However, the clinical importance of complement-mediated anaphylaxis is unknown (Cianferoni, 2021; Fingert et al., 2010).	Peanut and tree nut proteins can activate complement and generate C3a, C5a in mice and cause anaphylaxis, which can also exacerbate IgE-mediated anaphylaxis (Khodoun et al., 2009); role in oral food induced anaphylaxis in general and wheat induced systemic anaphylaxis is unknown.

2.3. Wheat processing and allergenicity

In general, the inherent factors affecting the allergenic potential of food proteins include the protein's structure, stability to gut digestion process, and the glycosylation patterns (Huby, Dearman, & Kimber, 2000). Wheat foods are typically consumed only after processing as a means of preservation and making foods edible (Mills & Mackie, 2008). Most food processing procedures are able to modify the structure of wheat proteins or introduce new structures, which may affect the way proteins are released, broken down during digestion and presented to the immune system (Ekezie, Cheng, & Sun, 2018; Huby et al., 2000; Jiménez-Saiz, Benedé, Molina, & López-Expósito, 2015; Jin et al., 2019; Lepski & Brockmeyer, 2013; Mills, Sancho, Rigby, Jenkins, & Mackie, 2009). Therefore, deciphering how food processing may affect wheat allergenicity requires elucidation of changes to the structure and properties of wheat proteins at the molecular level. Hence, the impact of food processing on allergenic properties of wheat is a primary challenge

that must be tackled to enable the possibility of creating novel hypo/non-allergenic wheat products. Therefore, we reviewed the effect of processing on wheat allergenicity and published the results (Gao et al., 2021). This published work is presented in Chapter 3.

2.4. Genetic diversity of wheat and wheat allergenicity

2.4.1. Do genetically distinct wheats (i.e., genotypes/lines/varieties) differ in allergenicity?

Wheats are typically classified based on their ploidy levels as follows: 1) diploid species: with genome A (AA, *Triticum monococcum*), D (DD, *Ae. tauschii*) or B (BB, *Ae. speltoides*, extinct now); 2) tetraploid species with genome A and B (AABB, *Triticum durum*); and 3) hexaploid species with genome A, B, and D (AABBDD, *Triticum aestivum*) (Levy & Feldman, 2002) (Jia et al., 2013; Sleper & Poehlman, 2006) (**Table 2.5**, Gao et al., 2021). Polyploidy in wheat has been an evolutionary event that led to the prevalence of tetraploid and hexaploid wheat, which resulted from the spontaneous hybridization using their ancestral species with the genome A, B, and D (Larré et al., 2011).

At present, the world has four distinct natural wheat genotypes: AA, AABB, AABBDD, and DD; BB is extinct now (Shewry, 2018). Breeders have been selecting various wheat varieties with desired traits for crossbreeding to obtain the varieties with technological as well as productivity-related traits. The rapid increase of wheat allergy, celiac disease and non-celiac gluten sensitivity has prompted researchers to evaluate whether or not natural genetic diversity of wheats translates to differences in their toxic, and allergenic potential (Pilolli et al., 2019).

Wheat varieties of AABB and AABBDD genotypes have been used extensively in food industry (e.g., pasta and bread making) as well as cosmetic industry (e.g., hydrolyzed wheat proteins as an ingredient in skin care products). Although the parent species with genome AA and

DD have played a crucial role for the diversity of modern wheats, the allergenicity of these ancient wheats is not well understood currently (Feldman & Levy, 2005). However, there is some suggestion that the ancient wheats may differ in immunotoxicity with respect to celiac disease, an autoimmune disease triggered by wheat gluten (Shewry & Tatham, 2016).

There is some limited but interesting evidence that genetic distinct wheats might differ in their allergenicity as summarized below:

Nakamura et al (2005) screened 324 wheat varieties from many parts of the world for allergenicity based on their abilities in binding to IgE antibodies obtained from wheat-allergic patients. They found allergenicity varies among these wheats and identified several candidates for less allergenic wheat varieties. This human study provided evidence that allergenic potentials of wheats may differ between genotypes for the first time. However, the ELISA-based *in vitro* testing in allergenicity may not predict the *in vivo* intrinsic allergenicity of these wheat varieties.

Larré and co-workers (2011) compared the IgE-binding capacity of salt-soluble wheat proteins (SSWPs) from hexaploid wheat (genomes AABBDD, *Triticum aestivum*) with that of diploid wheat (genome AA, *Triticum monococcum*) (Larré et al., 2011). Using sera from wheat allergic patients as IgE antibody source, they conducted fluorometric ELISA to determine the differences in level of SSWPs-specific IgE. Their results suggested that SSWPs from hexaploid wheat is more reactive in binding with IgE than SSPEs from diploid wheat. This coincided with their results of 2D electrophoresis analysis, which revealed qualitative differences in IgE-binding allergens between the two wheat genotypes. Although this study suggested potential differences in in vitro allergenicity between the two wheat genotypes, there was no *in vivo* method involved to verify and reveal their intrinsic allergenicity.

Lupi et al (2013) compared the albumin/globulins (A/G) fractions from two transgenic wheat varieties (genomes AABB and AABBDD) with their wild-type counterparts. They found that the overall protein content was decreased in genetically modified (GM)-hexaploid (genomes AABBDD) wheat, the proportion of gluten and non-gluten proteins was reversed in GM-tetraploid (genomes AABB) wheat. This observation led to their further investigation on the differences in allergenicities between these two transgenic wheats. Using sera from patients who had wheat food allergy or baker's asthma, they conducted 2D electrophoresis followed by immunoblot to examine the differences in IgE binding capacities between the two wheat varieties. Their results indicated that the A/G fractions of the two transgenic wheat lines exhibited similar IgE-binding profiles compared to their untransformed counterparts. Nevertheless, they found two IgE-binding proteins that were specific to the GM-tetraploid wheat line. One of these two was known to be allergenic and another was not documented in the database for wheat allergens.

Altenbach et al (2015) studied the allergenic potential of two transgenic wheat lines which their ω-5 gliadin genes were silenced by RNA interference. They then used 2D electrophoresis to evaluate the genetic modifications on the wheat flour proteome. Using sera from patients who suffered from wheat-dependent exercise induced anaphylaxis (WDEIA) as IgE antibody source, they tested IgE binding capacities of protein fractions (i.e., ω-5 gliadins, HMW-GS, and albumins/ globulins) from the two GM wheats using 2D immunoblot and ELISA. Their results indicated that while the reduced IgE reactivity of ω-5 gliadin were observed in 7 of 11 WDEIA patients, most patients showed at least a low level of reactivity with other proteins in this GM wheats.

Kohno et al (2016) screened bread wheat varieties to identify those that were deficient in locus of ω-5 gliadin (1BS-18, genomes AABBDD) — a major anaphylaxis causing wheat allergen. They identified one wheat line that lacked ω-5 gliadin locus and confirmed through IgG western

blotting. Guinea pigs were sensitized and challenged via the oral route with gluten from this ω-5 gliadin-deletion line or from a commercially available source. Allergic reactions upon challenge were evaluated using clinical scoring, which indicated that the ω-5 gliadin-deletion line is less allergenic compared to the commercial gluten. The strength of this study was that it evaluated the allergenic potency of an altered wheat *in vivo* for the first time. However, limitations of this study included the following: 1) the IgG western blot used in testing antibody reactivity does not reveal the allergenicity but instead antigenicity of wheat; 2) allergenic potential of non-gluten proteins were not investigated in this study; and 3) Despite that the clinical scoring can be useful in evaluating the severity of allergic shock and difference between allergens, this method can be subjective (Castan et al., 2020).

As preliminary studies leading to this dissertation research, we have investigated the relative differences in the *in vitro* allergenicity of SSPEs from three genetically distinct wheats: *T. durum* (genomes AABB), *T. aestivum* (common wheat, genomes AABBDD), and *Ae. tauschii* (genome DD) (Gao et al., 2019). Using sera from durum SSPE sensitized mice, we conducted wheat-specific IgE inhibition ELISA to test the relative allergenicity of SSPE from common wheat and *Ae. tauschii* compared to durum SSPE. Our results showed that SSPEs from these two wheats were significantly less allergenic than the durum SSPE. Two major limitations of this study were: i) it studied cross-reactivity of IgE antibodies elicited by durum wheat SSPE in mice for their ability to bind to SSPE obtained from the other two wheats; there these data suggested that *T. aestivum* and *Ae. tauschii* SSPE may be less allergenic in durum wheat sensitized host; and ii) mice were sensitized to durum wheat (*T. durum*) SSPE along with the alum adjuvant; therefore, inference on intrinsic allergenicity could not be drawn. Detailed description of this published study is presented in Chapter 3 of this dissertation.

Jorgensen et al (2022) compared and contrasted wheat allergens that can cause wheat allergies in Balb/c mouse model with known wheat allergens reported for humans. Balb/c mice were sensitized to salt-soluble protein extracts (SSPE) from durum wheat. IgE-binding capacity of the wheat allergens were examined using western blot followed by sequencing using LC-MS/MS. In addition, the cross-reacting allergens of the SSPE from an ancient wheat *Ae. tauschii* were identified. Overall, they reported that all but two of the salt-soluble allergens from durum wheat, and all IgE cross-reacting salt-soluble allergens from *Ae. tauschii* identified in this wheat-allergic mouse model were identical to those reported in wheat-sensitive humans. There are few limitations of this study: 1) as opposed to the natural exposures to wheat in humans (e.g., ingestion, airway, skin etc.), mice were sensitized through IP injections with allergen and alum adjuvant; 2) The effect of gastrointestinal digestion on wheat proteins and allergens is an additional factor that may alter the allergic outcomes in humans. Whereas anaphylaxis in mice were induced through IP challenge, which precluded the possibility of investigating the effect of digestion on wheat allergens; and 3) exercise could serve as a necessary co-factor that contributes to the onset of wheat-dependent exercise-induced anaphylaxis in humans. In contrast, IP challenge was sufficient to induce anaphylaxis which exercise was not required in the mouse model used in this study.

The two human studies that used transgenic wheat lines reviewed above provided qualitative and quantitative data that greatly advanced our knowledge in the potential effect of biotechnology on allergenicity of wheat proteins *in vitro* (Altenbach et al., 2015; Lupi et al., 2013). However, further detailed analyses are necessary to evaluate the allergenicities of these transgenic/altered/GM wheat lines *in vivo*. Nonetheless, it is not possible to evaluate the allergenicity of GM wheat *in vivo* without knowing the natural variation in allergenicity of their conventional non-GM wheat counterparts (genomes AA, AABB, AABBDD, and DD).

Overall, there is limited but compelling evidence that genetic distinct wheats may differ in allergenicity based on *in vitro* testing but intrinsic allergenicity *in vivo* has not been evaluated systematically. An adjuvant-free mouse model that is capable of simulating both sensitization and disease elicitation of human food allergy will be highly useful to address this issue.

Table 2.5. Three genetically distinct wheats are commonly used in food products and/or animal feed.

Genome(s)	Common Name/s	Species*	Common Uses
AA	Wild einkorn	*Triticum boeticum*	Cookies, brownies, cakes, bread, pizza, rolls, soup, cereals
AABB	Emmer	*Triticum dicoccon*	Soup, beer, animal feed
	Durum	*Triticum durum*	Pizza, pasta, bread, semolina, upma, couscous, pudding
		Triticum turanicum	Bulgar, pasta, cereals, bread, waffles, beer
	Persian wheat	*Triticum carthlicum*	Bread
	Rivet wheat	*Triticum turgidum*	Feed
AABBDD	Common wheat	*Triticum aestivum*	Bread, cookies, crackers, cereals, pastas, risotto, stews
	Spelt	*Triticum spelta*	Pasta, crackers, bread, cereals, risotto, cookies, stews
	Clubed wheat	*Triticum compactum*	Cake, crackers, cookies, pastries

**Taxonomy based on Integrated Taxonomic Information System (ITIS, 2021). Source: updated version of the table published in Gao et al 2021.*

2.4.2. How to ensure that novel/GM wheat varieties would not be hyper/super-allergenic?

Genetically modified (GM) foods are developed with the use of recombinant DNA technology, where a foreign gene is introduced from one species to another (US FDA, 2022). This agriculture biotechnology has been utilized in developing varieties of crops with desired quality and quantity. For instance, GM crops contain transgenic proteins that enabled them to have enhanced nutritional and organoleptic values. In addition, more and more insects- and fungus-resistant crop species sprung up with the advancement of this biotechnology. Despite the benefits that GM crops have, they could possibly pose threats toward human health, animals, and the environment (Mishra & Arora, 2017). Among all safety concerns about GM foods, allergenicity is the one that has been most frequently prompted by consumers and food manufacturers. For instance, a foreign gene that is transferred to the host species can possibly serve as an adjuvant, create a new allergen *de novo*, or enhance the expression of a minor allergen in the GM crop, and therefore increase the allergenicity of the host species (Joshi et al., 2016).

Currently, the GM foods that are allowed to be distributed on the market have been carefully examined for their safety towards the consumers health (US FDA, 2022). They are commonly assessed for safety per the '*substantial equivalence*' concept developed by international regulatory bodies (Domingo, 2016; Hollingworth et al., 2003; Selgrade, Bowman, Ladics, Privalle, & Laessig, 2009). Codex Alimentarius Commission initiated the guidelines in 1999 to address the increasing safety concerns among the world's population about the GM foods. The finalized safety assessment standards were established in 2001 by Joint FAO/WHO Consultation on Foods Derived from Biotechnology (FAO, 2009). The 'weight of evidence' approach developed by Codex in 2003 also served as an essential protocol for safety assessment of GM foods. Briefly, GM food is compared to the conventional non-GM food for nutritional, toxicological, and

allergenic properties. If the GM food is found substantially equivalent to the non-GM conventional food in all these properties, it is considered safe for human consumption. FAO/WHO international expert panel developed a decision tree in 2001 to assess the allergenic potential of GM food. Overall, they recommended using preliminary testing procedures that analyzing the source of the transgene followed by *in vitro* and *in vivo* assessment of the transgenic protein. In particular, the first step in evaluating the allergenicity of GM foods is to focus on its sequence homology if its non-GM counterpart is considered allergenic. The product is then considered allergenic when sequence homology is confirmed from a known allergen, whereas specific serum screening is necessary when sequence homology cannot be demonstrated. While positive serum screening results indicate the product is most likely allergenic, a negative outcome of serum screening test warrant further analyses including targeted serum screening, pepsin resistance, and *in vivo* testing using animal models (FAO 2009). Although animal models are not able to reveal all aspects of the allergenic potentials of GM foods compare to human studies, there are substantial evidence that they can contribute valuable information in this matter.

As mentioned, wheat is a staple food that consumed by millions of people across the world. Novel dietary protein sources are imperative to be introduced to the food supply chain to the meet the needs of rapid growing world population (Castan et al., 2020). Today's wheat varieties were developed by cross-hybridization and selection. However, these conventional plant breeding are often hamstrung by the available gene pool that limited the increasing production to meet the growing demand. In contrast, GM enabled acquiring desirable attributes of the host species by transferring a single gene or couple of genes in a much more controllable and precise way (Rommens, 2007). Scientists have been dedicating to develop GM wheat that are resistant to herbicides, insects, fungal pathogens, and viruses (Mishra & Arora, 2017). In addition, application

of biotechnology in agriculture has shown promising in conferring wheats with tolerance to drought, high soil salinity, and heat (Mishra & Arora, 2017). Until now, no GM wheat has been approved for commercial use, and therefore they are commercially unavailable. Nonetheless, many transgenic wheat lines have been produced and studied with field trials in the US and the Europe for future introduction (Beale, Ward, & Baker, 2009; Bruce et al., 2015; Lupi et al., 2013; Meyer et al., 2013; Shewry et al., 2006; Yadav et al., 2015). It is unknown whether GM wheat lines will be similar or different in allergenicity compared to their conventional counterparts. This critical question cannot be addressed effectively because there is no validated method to assess the allergenic potential of GM wheat - a critical need for food safety (Domingo, 2016; Hollingworth et al., 2003; Ladics et al., 2014; Selgrade et al., 2009). This critical need is illustrated by the controversy associated with the allergenic potential of a GM corn, the StarLink™ corn that contaminated the US food supply from 2000 to 2005, necessitating a cleanup that costed hundreds of million dollars (Bucchini & Goldman, 2002; Siruguri, Sesikeran, & Bhat, 2004).

More importantly, the five incidences of the escape of experimental GM wheat that occurred in North America have made food processors wary of consumers reaction to products containing GM wheat (Canadian Food Inspection Agency, 2018; USDA Animal and Plant Health Inspection Service, 2022). Few of these were glyphosate-tolerant GM wheat from Monsanto that had been tested in field trials. These cautionary tales demonstrated that without a validated method to assess the allergenic potential of GM wheat products, it will be far more difficult to prevent potentially allergenic altered wheat products from entering the food chain. To address this imperative issue, the FAO/WHO formulated a decision tree in 2001 that recommended including an animal model in safety testing of GM food — but there is none (FAO/WHO, 2001). If an animal model was available, these incidences could have been avoided. Therefore, method validation to

interpret the potential allergenicity of GM wheat and novel wheat varieties is crucial to ensure public health protection.

2.5. Overview of animal models of wheat allergenicity

There are published studies on using dog, rat, and mice to develop wheat allergenicity animal models. An animal model for wheat allergy was first developed using dog, and subsequently using mouse and rat species. Overall, the three animal models (dog, rat, and mouse) have their own strengths and limitations. We published a comprehensive review on this topic (Jin, Gao et al., 2019). Parts of this published study are presented in this section.

2.5.1. Overview of dog model of wheat allergenicity

Buchanan et al (1997) used inbred high IgE responder dogs (spaniel/basenji) that had been genetically selected for over 15 years for showing allergy to pollens and foods. They developed a complex protocol involving adjuvants to study wheat allergenicity. It is worth noting that these wheat-sensitized dogs developed vomiting and/or diarrhea, which is remarkable features that are valuable to human studies. Overall, although dog model can develop allergic symptoms such as vomiting which is crucial in studying human allergic responses to wheat, both its cost of management and the degree of difficulty in protocol development are relatively higher than the other two available species.

2.5.2. Overview of rat model of wheat allergenicity

Kroghsbo and co-workers in 2014 used inbred Brown Norway (BN) rats to produce ‘gluten-free’ rats by breeding three generations on a gluten-free diet. They used ELISA-based

method to compare the differences in allergenicities (IgE) and antigenicities (IgG) between native gluten (NG), acid hydrolyzed gluten (AHG), and enzyme hydrolyzed gluten (EHG) (Kroghsbo, Andersen, Rasmussen, Jacobsen, & Madsen, 2014). Their results suggested that EHG was both more allergenic and immunogenic than NG and AHG. Whereas AHG lead to the formation of novel IgG-binding epitopes which shows higher immunogenic capacity compared to that of NG. They concluded that only enzyme hydrolysis was able to enhance the allergic potentials of gluten via oral sensitization. Ballegaard et al (2019) investigated the sensitization potencies of native gluten vs. AHG in wheat-tolerant rats fed with wheat-containing diet and naïve rats that were maintained on a wheat-free diet (Ballegaard, Madsen, & Bøgh, 2019). Instead of sensitizing orally, they applied the allergens epicutaneously to these rats to study their allergenicities. They reported that both NG and AHG were able to elicit allergic reactions in naïve rats, whereas only AHG could prompt IgE antibody response in wheat-tolerant rats. This phenomenon was explained by their inhibition assay, which revealed that new epitopes were formed during acid hydrolysis. Therefore, these novel epitopes were able to elicit an IgE response even in rats that were tolerant to NG. In summary, these two studies that both used Brown Norway (BN) rats elucidated the mechanisms of allergenic sensitization to hydrolyzed wheat gluten at the molecular level. In addition, using 'gluten-free' rats for the study of oral sensitization without adjuvant is encouraging. However, endpoints parameters (e.g., anaphylaxis, gut reactions, skin reactions etc.) for disease elicitation are crucial in predicting the clinical outcomes of food allergens and they were lacking in these two studies. Besides, sensitization potencies of non-gluten proteins (albumin and globulins) were not investigated.

2.5.3. Overview of mouse model of wheat allergenicity

Mice have been widely used to study human biology as they have some unique characteristics including similar physiology, genome, and immune system to humans (Haley, 2003). They are also cost-efficient in terms of maintenance and breeding to generate a large colony in a relatively short period of time. Furthermore, the ease of performing experiments under strictly controlled conditions, widely available commercial kits reagents for analyses, and abundant sources of gene-modified strains (e.g. forward genetics, reverse genetics, transgenesis, targeted mutations, knock ins/outs) make them highly preferred in model development compare to other animal species (Justice, Siracusa, & Stewart, 2011).

There are several mouse models of wheat allergy reported since 2006 (**Table 2.6)** (Abe et al., 2014; Adachi et al., 2012; Bodinier et al., 2009; Castan et al., 2018; Denery-Papini et al., 2011; Gao et al., 2022; Gourbeyre et al., 2012; Jin et al., 2017, 2020; Jorgensen et al., 2022; Kozai et al., 2006; Tamehiro et al., 2021; Tanaka et al., 2011; Xue et al., 2019). These mouse models of wheat allergenicity are reviewed below

Table 2.6. Wheat food allergy mouse models: a summary.

Authors	Wheat proteins/allergens used	Mouse model used	Sensitization	Disease Elicitation
Kozai et al 2006	SSPE, gliadin, and glutenin	B10.A mice (age unspecified, female)	IP route: four times, 14 days apart, 10 ug + 1 mg alum	Oral route: 20 mg followed by moderate exercise
Bodinier et al 2009	Gliadin	Balb/c mice (3 weeks, female), B10.A and C3H/HeJ (4-5 weeks, female)	IP route: four times, 10 days apart, 10/20 ug + 1 mg alum	Nasal route: 10 ug on day 40
Tanaka et al 2011	Gliadin, purified ω-5 gliadin	B10.A mice (5 weeks, female)	IP route: six times, 7 days apart, 100 ug for the first injection and 50 ug for the next fix injections with 1 mg alum	Oral route: gliadin at 0.1 and 0.8 mg; ω-5 gliadin at 0.1 mg followed by acute exercise
Denery-Papini et al 2011	Gliadin, LTP	Balb/c mice (3 weeks, female)	IP route: four times, 10 days apart, 10 ug + 1 mg alum	Nasal route: 10 ug on day 40
Gourbeyre et al 2012	DG	Balb/c mice (6 weeks, female)	IP route: four times, 10 days apart, 10 ug + 1 mg alum	IP route: 1 mg + 1 mg alum on day 38
Adachi et al 2012	NG, AHG, and AHG + detergent	Balb/c mice (7 weeks, female)	Skin: twelve times on days 1-3, 8-10, 15-17, and 22-24; 0.5 mg	IP route: 1 mg on day 18 or 25
Abe et al 2014	UG and DG	Balb/c mice (5 weeks, male)	IP route: twice, 14 days apart, 50 ug + 1 mg alum	Oral route: 10 mg on day 28, 30, 32, 34, 36, 38, and 40
Jin et al 2017, Gao et al 2019, Jorgensen et al 2022	SSPE	Balb/c mice (6-8 weeks, female)	IP route: four times on days 0, 10, 24, and 40; 10 ug + 1 mg alum	IP route: 0.5 mg/mouse
Castan et al 2018	DG, NG, and HG	Balb/c mice (4 weeks, gender unspecified)	IP route: twice, 10 days apart, 10 ug + 1 mg alum	Oral route: 20 mg, 1 week after sensitization
Xue et al 2019	Gluten processed by physical, chemical, and enzymatic treatments	Balb/c mice (6 weeks, male)	IP route: three times, 7 days apart, 50 ug + 50 ug alum	Not done
Jin et al 2020	SSPE	Balb/c mice (4-6 weeks, female)	IP route: four times on days 0, 10, 24, and 40, 10 ug + 1 mg alum; Skin: six times, 7 days apart, 1 mg	IP route: 0.5 mg/mouse
Gao et al 2022 (**This dissertation work; Chapter 5**)	SSPE	Balb/c mice (6-8 weeks, female)	Skin: nine times, 7 days apart, 1 mg	Oral route: 20 mg/mouse

This is a modified and updated version of the table published in Jin, Gao et al 2019; Abbreviations: IP= intraperitoneal; RBL= rat basophilic leukemia; UG=unmodified gluten; EHG= enzymatic hydrolyzed gluten; AHG= acid hydrolyzed gluten; NG= native gliadin; DG= deamidated gliadin; HG= alcalase hydrolyzed gliadin; SSPE: salt-soluble protein extract; LTP: lipid transfer protein.

In 2006, Kozai et al (2006) developed the first mouse model of wheat allergy to investigate the molecular mechanisms underlying WDEIA. Groups of mice (B10.A) were sensitized to SSPE, globulin, and glutenin through IP injections. Sensitized mice were orally challenged with each protein fraction followed by exercise (treadmill) to elicit WDEIA. Their results indicated that while SSPE did not sensitize B10.A mice for WDEIA, gliadins and glutenins were able to cause sensitization and elicit WDEIA. Besides, they reported that exercise induced mucosal lesions upon oral challenge with wheat gliadins and glutenins, which were found to leak into the liver of allergic mice.

Bodinier et al (2009) compared the allergic responses to wheat gliadins between mice and humans. Three mouse strains (Balb/c, C3H/HeJ, and B10.A) were sensitized through IP injections and challenged via the intranasal route to measure allergic responses. Their results suggested that: 1) Balb/c mice appears to be the highest responders among the three strains tested as demonstrated by antibody/cytokine responses and responses in their airways; 2) Similar to children who are allergic to all five fractions of wheat gliadins, which displayed allergenicity in Balb/c mice in descending order as follows: $\alpha/\beta > \gamma > \omega1 > \omega2 > \omega5$. However, ω5 appears to be the most allergenic to wheat allergic adults; and 3) the use of alum adjuvant favored the formation of conformational epitopes against IgE.

In 2011, Tanaka et al (2011) investigated that whether ω5-gliadin can cause anaphylaxis without exercise as a co-factor. They sensitized B10.A mice with total gliadin or ω5-gliadin and alum adjuvant through IP route. Anaphylaxis was induced by oral gavage with total gliadin or ω5-gliadin. Their results suggested that in mice that were sensitized to total gliadins, most IgE antibody responses were specific to ω5-gliadins. In addition, they reported that sensitized mice developed similar extent of anaphylaxes upon oral elicitation with total gliadins or ω5-gliadins.

Denery-Papini et al (2011) compared the IgE epitopes on gliadins and LTP1 in Balb/c mice with those in humans. IgE epitopes were mapped using pepscan technique, and then those continuous IgE epitopes were identified using reduced and alkylated forms of proteins. For IgE epitopes on LTP1, while only one shared linear IgE epitope on LTP1 were found between mice and humans, all others were conformational in nature in both species. However, they found many IgE epitopes on ω5-gliadins in both mice and humans. A similar trend was found as to other gliadins and low molecular weight glutenin subunit. They concluded that the IgE epitopes on gliadins and LTP1 that recognized by mice are like those by humans.

Deamidation of wheat gluten has been prevalently used in both food as well as cosmetic industry as this practice yields desired solubility of gluten to be readily used as an ingredient. Gourbeyre et al (2012) compared the sensitization and disease elicitation capacities of native gliadins (NG) with deamidated gliadins (DG) using IP injections with gliadins and alum adjuvant. There were several findings from this study: 1) While the NG induced higher Th1-type and DG induced higher Th2-type immune responses, the two gliadins elicited comparable extent of anaphylaxis in sensitized mice upon IP challenge. Moreover, NG-specific IgE bound to all five gliadins whereas DG-specific IgE bound not only to the five DGs but also the five NGs. Therefore, they concluded that DG indeed is more allergenic than NG. In 2014, Abe et al (2014) studied the oral elicitation potency of DG. Using carboxylated cation-exchange resins, they made a novel type of DG without bond hydrolysis or polymerization. Balb/c mice were sensitized with NG (via IP route) and were orally gavaged with this DG. Interestingly, they noted that this DG appears to be less allergenic compared to NG in eliciting anaphylaxis. Thus, authors of this study suggested that cation exchange may be promising in producing hypoallergic wheat.

Hydrolyzed wheat has also been widely used for manufacturing cosmetics and personal care products. However, they have raised safety concerns which were investigated by several studies reviewed as follows: Adachi et al (2012) studied whether skin exposures to acid hydrolyzed gluten can elicit anaphylaxis in Balb/c mice. Mice were sensitized to native gluten, acid hydrolyzed gluten, or acid hydrolyzed gluten with detergent (0.5% sodium dodecyl sulfate), and were IP challenged to induce anaphylaxis. They reported that: 1) With the addition of detergent, native gluten but not by itself can sensitize Balb/c mice for anaphylaxis; 2) In contrast, acid hydrolyzed gluten can cause sensitization for anaphylaxis with or without the presence of detergent; and 3) The increased solubility of acid-hydrolyzed gluten led to enhanced permeability, thus making it appears to be more allergenic than native glutens. In 2018, Castan and coworkers conducted a study to investigate the allergenic potential of native gliadins, deamidated gliadins, and acid hydrolyzed gliadins. Balb/c mice were sensitized through IP injections and then orally challenged with these three forms of gliadins to measure their allergic responses. They found that acid hydrolyzed gliadins and deamidated gliadins induced significant stronger allergic reactions compared to that by the native gliadins.

Xue et al (2019) studied the effect of several processing methods including physical (hot water bath/ microwave), chemical (phosphorylation), and enzymatic treatments (hydrolysis with alcalase and papain) on the allergenic potential of wheat gliadins. Balb/c mice were sensitized with modified gliadins via the IP route with adjuvant and then measured for serum gliadin specific IgE levels, index of spleen, histamine, and serum cytokine concentrations. They reported that gliadins that were treated with phosphorylation and hydrolysis with alcalase and papain had their allergenicity significantly reduced as indicated by the parameters measured above.

Jin et al (2017) reported a mouse model of wheat allergy using durum wheat SSPE and alum adjuvant. Balb/c mice were sensitized to wheat SSPE with alum and were challenged with SSPE to elicit allergic diseases. They monitored SSPE-specific and total IgE for antibody responses upon sensitization and measured hypothermic shock responses (HSR) as well as mucosal mast cell protease-1 (MMCP-1) levels in the plasma to determine the extent of allergic reactions. Using the same model, Gao et al (2019) compared the relative *in vitro* allergenicity of SSPE from three genetically distinct wheats (*T. durum*, *T. aestivum*, *T. monococcum*, and *Ae. tauschii*). For this they developed a novel IgE inhibition ELISA method and reported differences in allergenicity among these wheats. Detailed description of this work is presented in the Chapter 3.

Using the model developed by Jin et al (2017), Jorgensen et al (2022) studied the specific salt-soluble wheat allergenic proteins in durum wheat (genome AABB) and *Ae. tauschii* wheat (genome DD). They found that all but 2 of the durum wheat allergens and all *Ae. tauschii* wheat allergens identified in Balb/c mice had been reported in human wheat allergens in the literature, thus validating the extensive similarity of the wheat allergenicity in this mouse model with that of wheat allergic humans.

Jin et al (2020) compared the immune mechanisms in alum-adjuvant based mouse model with a novel adjuvant-free mouse models using durum wheat SSPE. They demonstrated for the first time repeated six skin exposures with SSPE was sufficient to clinically sensitize Balb/c mice for systemic anaphylaxis by the IP route of challenge. They noted comparable levels of allergenicity readouts in both adjuvant-free and adjuvant-based models. However, they found that the immune activation patterns were substantially different between these two models.

As part of this dissertation work, Gao et al (2022) developed a novel adjuvant-free mouse model of durum wheat allergy using oral route for elicitation of disease. This work is presented in the Chapter 5.

Taken together, Balb/c mice has been used extensively in studies of wheat allergies, and immune responses in this model show extensive similarities with wheat allergenicity in humans. Therefore, there is strong rationale in using Balb/c mice for further research on human wheat allergies and wheat allergenicity.

2.5.4. The importance of adjuvant-free skin-exposure models

Mouse models of food allergy can be divided into adjuvant-based and adjuvant-free models (Gonipeta, Kim, & Gangur, 2015; Jin et al., 2019). Adjuvant based models use alum or bacterial toxins (e.g., cholera toxin, or enterotoxin super-antigen) to enhance sensitivity to food allergens (Gonipeta et al., 2015; Jin et al., 2019; Jin et al., 2017). Although adjuvant-based models are based on an artificial situation of co-exposure to both allergen and adjuvants, they are useful to study mechanisms of disease. However, they are not considered suitable to evaluate intrinsic allergenicity of GM foods because the adjuvants are thought to enhance sensitivity and reduce specificity (Kimber et al., 2003). The effect of adjuvant is usually difficult to be differentiated from that of allergens, thus complicates the interpretation of results. Therefore, adjuvant-based models are generally not preferred to evaluate the inherent allergenic potencies of food proteins (Dearman & Kimber, 2009; Dearman & Kimber, 2007; Selgrade et al., 2009). The mouse models published mostly used alum-adjuvant for enhancing allergenicity of wheat proteins. Furthermore, these models, except for one of our previous studies that used salt-soluble protein extract (SSPE) from durum wheat (Jin et al., 2017) have used alcohol-soluble gliadins in testing. As discussed in the

introduction, SSWPs are also important allergens in wheat allergic humans (Cianferoni, 2016; Jin et al., 2019). Notably, an adjuvant-free mouse model is unavailable at present for testing SSPEs.

The transdermal sensitization and oral elicitation (TS/OE) mouse model would be more suitable for safety assessment because it uses food allergens without co-exposure with adjuvant (Gonipeta et al., 2015; Kimber et al., 2003; Ladics et al., 2014; Selgrade et al., 2009). The TS/OE mouse model has these similar features to simulate the two phases of allergy development in humans: (1) mice are sensitized transdermally using wheat SSPEs; (2) sensitized mice are orally challenged with same allergens to elicit allergic reactions. Selgrade et al (2009) described the adjuvant-free TS/OE mouse model as promising for GM food testing, suggesting it should be validated with more allergenic foods. Dr. Gangur and co-workers have since validated this model for hazelnut, cashew nut, egg, shellfish, and milk (Birmingham, Gangur, Samineni, Navuluri, & Kelly, 2005; Birmingham et al., 2007; Gonipeta, Parvataneni, Paruchuri, & Gangur, 2010; Jin, Boss, Bursley, Gangur, & Rockwell, 2021; Navuluri et al., 2006; Ortiz et al., 2016; Parvataneni, Gonipeta, Acharya, & Gangur, 2016; Parvataneni, Gonipeta, Tempelman, & Gangur, 2009). Here we validated it with wheat because wheat allergens are under-studied and GM wheat varieties are on the horizon. For GM wheat or novel wheat varieties to be safe, they should be substantially equivalent to non-GM conventional wheat in eliciting sensitization and oral allergic reactions upon ingestion (Gonipeta et al., 2015; Ladics et al., 2014; Selgrade et al., 2009). Therefore, validating the TS/OE model for predicting intrinsic allergenicity of GM wheat varieties will consider not only intrinsic sensitization potential but also disease elicitation capacity.

Mechanisms of sensitization to food allergens are generally thought to occur via an oral or skin route (Berin & Sampson, 2013; Gonipeta et al., 2015; Ungar et al., 2017). There is extensive evidence that transdermal sensitization in mice was capable of eliciting clinical disease upon oral

challenge, and the features of which in Balb/c mice were very similar to those in humans (Gonipeta et al., 2015). For wheat allergy it is unclear at present that which route, oral or skin, results in human sensitization. Previous alum adjuvant-based Balb/c model studies convincingly show that IgE responses in mice, including IgE epitope structures, are remarkably similar to those of wheat allergic human subjects in a side-by-side comparison study (Denery-Papini et al., 2011). Thus, there is strong justification to improve the Balb/c model by evaluating the adjuvant-free transdermal approach we have developed for wheat allergy studies.

2.5.5. Food allergies in TS/OE mouse model vs. humans

Sensitization to food allergens is thought to mainly occur via oral or skin route in humans especially during infancy and childhood (Ungar et al., 2016, Gonipeta et al., 2015). Plenty of studies have demonstrated that hydrolyzed wheat proteins, an ingredient in cosmetics and health care products can sensitize atopic individuals for systemic anaphylaxis including WDEIA (Chinuki & Morita, 2012; Gao et al., 2021; Shinoda, Inomata, Chinuki, Morita, & Ikezawa, 2012) (**Table 2.7**). The transdermal exposures to food/wheat proteins in the absence of adjuvants used in the TS/OE model thus would closely simulate sensitization in wheat allergic individuals. The onset of food allergies in humans mostly relies on the ingestion of foods, and thus the oral route used for disease elicitation in the TS/OE model resembles this feature (Gonipeta et al., 2015). Using this model, we have developed quantifiable ways to measure: i) sensitization: elevation of wheat-specific IgE antibodies in the blood; and ii) oral allergic reaction: elevation of plasma murine mast cell protease-1 (MMCP-1) upon oral challenge — a specific biomarker of IgE antibody-mediated mucosal mast cell degranulation and anaphylaxis (Gonipeta et al., 2015, Jin et al., 2017, Jin et al., 2019, **Table 2.8**). Indeed, the use of our mouse model in multiple studies including allergies to

hazelnut, cashew nut, and milk have demonstrated that the TS/OE mouse model can display the allergic reactions similar to severe forms including anaphylaxis in humans (Birmingham et al., 2007; Gonipeta et al., 2009; Gonipeta et al., 2010; Parvataneni et al., 2009). Gonipeta et al (2015) reported that hypotension and tachycardia are observed upon oral allergen challenge in TS/OE mouse model of hazelnut allergy (Gonipeta et al., 2015). Besides, respiratory distress upon oral challenge have also been reported in this TS/OE mouse model (Gonipeta et al., 2009; Navuluri et al., 2006; Parvataneniet al., 2009). Furthermore, the persistent nature of certain food allergies such as hazel nut allergy in humans has also been demonstrated using this TS/OE model (Gonipeta et al., 2010).

As discussed earlier, food-induced anaphylaxis appears to be mostly IgE-mediated since only a very small portion of the ingested protein/antigen is absorbed with all epitopes intact (Strait et al., 2011). Therefore, the TS/OE mouse model share the same disease mechanisms underlying food allergies with humans. Previous studies from Dr. Gangur's laboratory have shown that anaphylaxis to hazelnut in TS/OE mouse model is genetically controlled (Parvataneni et al., 2009).

Overall, the allergic disease features using the TS/OE model are remarkably like human food allergies (Gonipeta et al., 2015) (**Table 2.7**). An optimized and well-characterized mouse model of wheat allergy would facilitate the understanding of this disorder in humans despite the differences between the two species.

Table 2.7. Humans and animals are exposed to wheat allergens via the skin: A summary of commercial products containing wheat proteins. (Source: Gao et al 2021).

Product	Wheat content	Type of product	Examples
Human	Hydrolyzed Wheat Protein	Shampoo	The Naked Object, Rituals. TRESemmé, Garnier Fructis, Neutrogena, Cetaphil
		Nail Polish	Esika
		Primer-	IT Cosmetics
		Eyeliner-Mascara	N/A
		Moisturizer	Glow Recipe, Marcelle
		Soap	Jolly Jabón, Canmepris
		Hand Sanitizer	Sally Hansen, Essential Organics, Kosho
		Hand/Nail Care	Botaneco Garden, Kypwell Essentials
		Exfoliating Towelettes	Alba Botanica
		Hair Gel/Mask/Wax	TREsemmé, Briogeo, Watson's
		Face/Neck Care	Erin's Faces, Lancome Paris, Omved Therapies
		Eye Cream	Lancome Paris, Revlon, Erin's Faces
		Hair Conditioner	Miracle 9, L'Oreal Paris
		Antiseptic	Tea Tree Therapy
		Baby diaper rash cream	Mom & World
	Wheat Germ/Bran Extract	Lipstick	Estee Lauder
	Hydrolyzed wheat gluten	Shower Gel	Korres Natural World
Veterinary		Conditioner	Tiger Brands
		Hair Oil	Conatural
	Wheat Protein Extract	Shampoo and Conditioner	Buddy Wash
	Hydrolyzed Wheat Protein	Shampoo	Furminator
		Conditioner	TropiClean

Table 2.8. Comparison of the major features of food allergy in humans and in the transdermal sensitization/oral elicitation (TS/OE) mouse model developed by Dr. Gangur and colleagues at the Michigan State University.

Characteristics		Human Food Allergy [1,2]	TS/OE Mouse Model [3-6]
Sensitization	Markers of sensitization	Specific IgE antibody	Specific IgE antibody
	Route	Oral or skin route	Skin
	Mechanisms	Th2 dependent, IgE-mediated	Th2 dependent, IgE-mediated
	Allergens involved	Proteins from allergenic foods (e.g., Cor a 9 in hazelnut anaphylaxis)	Proteins from allergenic foods (e.g., Cor a 9 in hazelnut anaphylaxis)
Disease Elicitation	Route	Oral	Oral
	Mechanism	IgE/Mast cell/basophil mediated	IgE/Mast cell mediated
Systemic anaphylaxis		Hypothermia, hypotensive shock, convulsions, dizziness, fainting, loss of consciousness, sense of impending doom, tremors, fatal or near fatal	Hypothermia, hypotensive shock, convulsions (tree nut allergy), fatal or near fatal
Skin Reactions		Hives; urticaria	None observed
Gut Reactions		Angioedema of lips; swollen tongue; oral pruritis; nausea, vomiting, diarrhea	Not studied
Airways Reactions		Nasal congestion, sneezing, pruritis, cough, dyspnea	Dyspnea, wheezing, abdominal breathing
Cardiovascular Reactions		Hypotension, tachycardia then bradycardia	Hypotension, tachycardia, cardiac mast cell degranulation
Role of sex		Both affected (more prevalent among males before puberty, more prevalent among females in adults)	Both affected
Role of Genetics		HLA (MHC) and non-HLA genetics	Non-MHC genetics in hazelnut allergy
	Age of onset	As early as infancy or childhood; can also start at adulthood	Adult mice are used for technical reasons
Natural History	Persistent/Transient	Milk, egg, wheat, and soy allergies are transient; peanut, tree nut, fish, shellfish, and sesame allergies are persistent in most cases	Milk allergy is transient, hazelnut allergy is persistent; others yet to be studied

[1] Purington et al., 2018; [2] Beyer et al 2002; [3] Birmingham et al 2007; Gonipeta et al., 2009, 2010, 2015a,b; [4] Navuluri et al., 2006; [5] Parvataneni et al., 2009, 2010; [6] Gonipeta et al., 2015; *Abbreviations: SPT = skin prick test; NA = not applicable; HLA = human leukocyte antigen; MHC: major histocompatibility complex*

2.5.6. Summary of major gaps in knowledge in the field of wheat allergy

Overall, major gaps in knowledge in the field of wheat allergy include but not limited to the following: i) lack of a well characterized adjuvant-free mouse model for evaluating the intrinsic allergenicity potential of wheat allergens; i) lack of adequate information on the differences in allergenicity profiles of genetically distinct wheats (i.e., AA, AABB, AABBDD, and DD); iii) lack of adequate information on the allergenicity of non-gluten wheat proteins in particular as most studies have focused on glutens; and iv) absence of a validated mouse model for assessing the intrinsic allergenicity of alter/novel/GM wheats and differently processed wheat products.

2.5.7. Potential benefits of a validated adjuvant-free mouse model of wheat allergy using the TS/OE approach

Validating the TS/OE mouse model, which closely resembles human allergies, will create a new *in vivo* reference standard for assessing the allergenic potential of GM wheat and other novel/altered wheat varieties without resorting to use adjuvant as an artificial allergenicity stimulant (Gao et al., 2021). Besides, there are numerous studies have shown that food processing can alter the allergenicity of food proteins (Masthoff et al., 2013; Ortiz et al., 2016; Vanga, Singh, & Raghavan, 2017). A validated TS/OE mouse model would be an essential tool to evaluate the allergenic potentials of these allergens under ultra-processing or combinations of novel processing methods including fermentation technology. A validated TS/OE mouse model can also be used to develop novel methods to prevent and treat wheat allergy (e.g., vaccines, immunotherapies). In addition, a validated TS/OE mouse model can be used to study role of environmental and genetic factors in wheat allergy and advance mechanism of disease. In summary, validating this animal

model for all the four existing genotypes of wheats will advance basic and applied research on wheat allergenicity.

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CHAPTER 3 CREATING HYPO/NON-ALLERGENIC WHEAT PRODUCTS USING PROCESSING METHODS: FACT OR FICTION?

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3.1. Abstract

Wheat allergy is a potentially life-threatening disease that affects millions of people around the world. Food processing has been shown to influence the allergenicity of wheat and other major foods. However, a comprehensive review evaluating whether or not food processing can be used to develop hypo/non-allergenic wheat products is unavailable. There were three objectives for this study: 1) to critically evaluate the evidence on the effect of fermentation, thermal processing, and enzyme or acid hydrolysis on wheat allergenicity so as to identify the potential for and challenges of using these methods to produce hypo/non-allergenic wheat products; 2) to identify the molecular effects of food processing needed to create such products; and 3) to map the concept questions for future research and development to produce hypo/non-allergenic wheat products. We performed literature research using PubMed and Google Scholar databases with various combinations of keywords to generate the data to accomplish these objectives. We found that: 1) food processing significantly modulates wheat allergenicity; while some methods can reduce or even abolish the allergenicity, others can create mega allergens; and 2) fermentation and enzymatic hydrolysis hold the most potential to create novel hypo/non-allergenic wheat products; however, pre-clinical validation and human clinical trials are currently lacking. We also identify five specific research

concepts to advance the research to enable the creation of hypo/non-allergenic wheat products for application in food, medical, and cosmetic industries.

3.2. Introduction

Food allergy is one of the major health issues worldwide. It currently affects 10.8% of adults and 8% of children in the United States of America (USA) (Sampson et al., 2018; Gupta et al., 2019). In general, food allergy refers to the IgE antibody mediated adverse immune reactions triggered by the ingestion of a specific food. Typical clinical symptoms associated with food allergy can vary from mild reactions such as hives, rashes, vomiting, diarrhea, and rhino-conjunctivitis, to serious life-threatening reactions such as systemic anaphylaxis, and Baker's asthma (Sicherer & Sampson, 2018; Warren, Jiang, & Gupta 2020). The United States Food and Drug Administration (US FDA) has identified eight major foods that account for over 90 percent of allergic reactions: milk, eggs, fish, shellfish, tree nuts, peanuts, soybeans, and wheat; sesame has been recently added to this list as the 9th major food allergen in the USA (Gangur & Acharya, 2021; US FDA, 2021). In addition, wheat is also regulated as a major allergenic food in Canada, the United Kingdom, Japan, Australia, New Zealand, and in all 28 European Union countries. (EFSA, 2004; FAO, 2020; Health Canada, 2018; Japan, 2019; UK, 2020; US FDA, 2021; Australia & New Zealand, 2020). The overall prevalence of wheat allergy is estimated to be 0.4–3% in the USA and up to 0.9% at the global level (Cianferoni, 2016; Leonard & Vasagar, 2014; Venter et al., 2006a, b). Thus, wheat allergy affects a significant proportion of the global human population.

Wheat is a staple food for most of the world's population. The global wheat production has been steadily increasing during the last decade. In addition, the supply and demand of wheat

have fluctuated around 750 million tonnes/year, especially in the last five years (FAO, 2020). However, the *per capita* consumption of wheat flour has decreased in the US by ~7.7% during the last two decades (from 143 pounds per person to 132 pounds per person) (USDA, 2019). Reasons for this reduction in the US are unknown. Potential reasons include avoidance of wheat products due to physician diagnosed health conditions (e.g., food allergy, celiac disease, non-celiac gluten sensitivity etc.) or perceived health concerns without a formal diagnosis (Leonard, Sapone, Catassi, & Fasano, 2017; Pilolli et al., 2019). In Australia, 11% adults consciously avoid wheat in an attempt to alleviate fatigue and gastrointestinal symptoms (Golley, Corsini, Topping, Morell, & Mohr, 2015). In the United Kingdom, motivating factors for wheat avoidance by people (without celiac disease and without wheat allergy) were: management of symptoms similar to inflammatory bowel disease, infertility, low mood, low energy, immune dysfunction, weight gain, and visual and auditory hallucinations (Harper & Bold, 2018). Thus, real, or perceived health issues linked to wheat pose a serious threat to the growth of global wheat food industry.

There are at least 8 human clinical conditions that have been associated with wheat: 1. celiac disease; 2. non-celiac gluten/wheat sensitivity; 3. food protein-induced enterocolitis syndrome; 4. eosinophilic esophagitis. 5. wheat food allergy (including vomiting, diarrhea, hives, systemic anaphylaxis, atopic dermatitis, rhino-conjunctivitis, and oral allergy syndrome); 6. wheat-dependent exercise-induced anaphylaxis (WDEIA); 7. contact urticaria; and 8. baker's asthma (Cabanillas, 2020; Juhász et al., 2018; Quirce, Boyano-Martínez, & Díaz-Perales, 2016; Ricci et al., 2019). All these conditions are mediated by the overactivation of the immune system. Whereas the celiac disease is an autoimmune reaction triggered by gluten, the non-celiac gluten sensitivity is thought to be due to the activation of innate immune system by unknown

components of wheat (Cabanillas, 2020). Mechanisms of food-protein induced enterocolitis syndrome and eosinophilic esophagitis are incompletely understood. Only the last four diseases are mediated by the IgE antibodies. Both systemic anaphylaxis and baker's asthma can be potentially deadly, and therefore require emergency medical treatment (Cabanillas, 2020; Cianferoni et al., 2013; Jin et al., 2019; Quirce et al., 2016; Ricci et al., 2019). Since the scope of our study was to determine the impact of food processing on IgE antibody mediated adverse reactions to wheat, we excluded the first 4 conditions from our analysis.

Wheat allergies are generally mediated by IgE antibodies produced against wheat proteins. Despite possessing high nutritional value and high palatability, the allergenic proteins present in wheat can trigger serious allergic reactions in genetically susceptible consumers (Cianferoni, 2016; Carter & Frischmeyer-Guerrerio, 2018). Wheat contains 10-18% total protein on a dry weight basis. Based on their solubility, the proteins are classified into four families as follows: albumins (water/saline-soluble, 10-12% of the total protein), globulins (saline-soluble, 5-8% of the total protein), gliadins (alcohol-soluble, 30-40% of the total protein), and glutenins (acid-soluble, 45-50% of the total protein) (Jin et al., 2019). The non-gluten proteins (albumins and globulins) are the structural and metabolic proteins. They serve as nutrient reserves for germinating the embryo and protecting it from insects and pathogens before germination (Dupont & Altenbach, 2003). The gluten proteins (gliadins and glutenins, also known as prolamins) are the seed storage proteins. Gliadins are monomeric proteins that interact through hydrogen bonds and contain mostly intramolecular disulfide bonds. Glutenins are polymeric proteins linked by inter and intramolecular disulfide bonds. Gliadins may be cross-linked to the glutenin network through intermolecular disulfide bonds as well.

All four families of wheat proteins together contain at least 107 allergenic proteins (www. Allergome.org). All the 3 wheat genomes (A, B and D) encode for the wheat allergens. Recently chromosomal locations of 41 allergen encoding genes have been mapped, and IgE epitopes are well characterized for many, but not all of these allergens (Juhász et al., 2018). Major wheat allergens that are well characterized include: ω-1, 2, 5 gliadin, α/β/γ -gliadins, the high and low molecular weight glutenin subunits (HMW-GS and LMW-GS), β-amylase, α-amylase/trypsin/subtilisin inhibitor proteins, lipid transfer protein, chitinase, glyceraldehyde-3 phosphate dehydrogenase, triosephosphate isomerase, peroxidase, glutathione S-transferase, globulin-3, serpins, and α-purothionin (Cianferoni, 2016; Juhász et al., 2018). For detailed information on wheat allergens, and their IgE epitopes, readers are referred to these excellent articles (Denery-Papini et al., 2011; Matsuo et al., 2015; Monaco et al., 2021; Pahr et al., 2013).

In general, the inherent factors affecting the allergenic potential of food proteins include the protein's structure, stability to gut digestion process, and the glycosylation patterns (Huby, Dearman, & Kimber, 2000). Wheat foods are typically consumed only after processing as a means of preservation and making foods edible (Mills & Mackie, 2008). Most food processing procedures are able to modify the structure of wheat proteins or introduce new structures, which may affect the way proteins are released, broken down during digestion and presented to the immune system (Chizoba Ekezie, Cheng, & Sun, 2018; Huby et al., 2000; Jiménez-Saiz, Benedé, Molina, & López-Expósito, 2015; Jin et al., 2019; Lepski & Brockmeyer, 2013; Mills, Sancho, Rigby, Jenkins, & Mackie, 2009). Therefore, deciphering how food processing may affect wheat allergenicity requires elucidation of changes to the structure and properties of wheat proteins at the molecular level. Hence, the impact of food processing on allergenic properties of wheat is a primary challenge

that must be addressed to enable the possibility of creating novel hypo/non-allergenic wheat products.

The common types of wheat processing include fermentation followed by thermal treatment, thermal treatment with or without pressure, and enzyme or acid hydrolysis (**Tables 3.1-3.5**) (EFSA, 2014; Mills & Mackie, 2008; Thomas et al., 2007). Hydrolyzed wheat protein (HWP) and hydrolyzed gluten protein (HGP) are produced at industrial scales for use as ingredients in skin and healthcare products (**Table 3.2)** (Tranquet et al., 2020).

An up-to-date comprehensive review on the effects of processing on wheat allergenicity is unavailable. There were three objectives for this study: 1) to critically evaluate the evidence on the effect of fermentation, thermal processing, and enzyme or acid hydrolysis on wheat allergenicity so as to identify the potential for and challenges of using these methods to produce hypo/non-allergenic wheat products; 2) to identify the molecular effects of food processing needed to create such products; and 3) to map the concept questions for future research and development to produce hypo/non-allergenic wheat products.

To accomplish these objectives, we conducted a literature search without date limits using the PubMed and Google Scholar databases. Various combinations of keywords (IgE, wheat, processing, allergy, allergenicity, immunogenicity, antigenicity, food allergy, anaphylaxis, adverse reaction, and hypersensitivity) were used. Articles in English were retrieved and analyzed to produce summary tables. Since scope of this work was limited to only IgE mediated diseases caused by wheat, articles on non-IgE mediated wheat disorders (celiac disease, non-celiac gluten/wheat sensitivity, eosinophilic enterocolitis, and food protein-induced enterocolitis) were excluded. Our findings and interpretations are presented.

3.3. Impact of food processing on wheat allergenicity: evidence from the literature

Relative to the research reports on the effects of food processing on peanut allergens, the processing effects on wheat allergens have been modestly studied (Blanc et al., 2011; Meng, Li, Chang, & Maleki, 2019; Zhang et al., 2019). Wheat food products are made using two general types of processing methods to create commonly consumed foods (**Table 3.1**): 1) products made by microbial fermentation followed by thermal processing; and 2) products made by thermal processing with or without pressure and with or without drying (boiling, baking, extrusion, and frying). Wheat is also used as an industrial ingredient in a number of skin health and cosmetic products (**Table 3.2**). These products contain HWP or HWG that are produced by hydrolyzing the wheat protein using acids or enzymes (Tranquet et al., 2020). Furthermore, upon consumption, wheat proteins undergo enzymatic digestion in the gut that also can influence their allergenicity. Therefore, wheat allergenicity testing involves not only the effect of wheat processing methods but also the susceptibility of processed products to enzymatic digestion, to simulate gut digestion as discussed below.

Table 3.1. Commonly consumed wheat food products are either thermally processed or fermented plus thermally processed

Wheat Food Product	Processing Method	
	Fermentation	**Thermal Processing**
Cookies	*S. cerevisiae*	Baking (177 °C, 8-12 min)
Crackers	*S. cerevisiae*	Baking (232 °C, 12-15 min)
Pizza	*S. cerevisiae; Lb. sanfranciscensis*	Baking (232 °C, 15-20 min)
Bread	*S. cerevisiae*	Baking (232 °C, 20-25 min)
Sourdough bread	*S. cerevisiae; S. delbrueckii*	Baking (218 °C, 60 min)
	Lb. brevis; Lb. amylophilus	Baking (218 °C, 60 min)
Cornbread	No	Baking (203 °C, 20-34 min)
Breakfast cereal	No	Baking and Extrusion
Naan	*S. cerevisiae*	Baking (260 °C, 4-5 min)
Pretzel	*S. cerevisiae*	Baking (218 °C, 10 min)
Graham cracker	No	Baking (180 °C, 10-12 min)
Pie crust	No	Baking (177 °C, 45-50 min)
Waffle	No	Baking (360 °C, 2-3 min)
Cake	No	Baking (175 °C, 30-40 min)
Cake doughnut	No	Baking (218 °C, 12-14 min)
	No	Frying (190 °C, 3 min)
Yeast doughnut	*S. cerevisiae*	Baking (180 °C, 13 min)
	S. cerevisiae	Frying (191 °C, 2 min)
Roti/chapati	No	Frying (8-9 min)
French toast	No	Frying (4-6 min)
Flour tortilla	No	Frying (1-1.5 min)
Beer	*S. cerevisiae*	Pasteurization (98 °C)
Pasta	No	Extrusion (51 °C)
	No	Boiling (100 °C, 10 min)
	No	Drying (12-13% moisture)
Soy sauce	*A. oryzae/sojae; P. halophilus; Z. rouxii*	Roasted (150 °C, 30-45 sec)

Abbreviations: S = Saccharomyces; Lb = Lactobacillus; A = Apergillus; P = Pediococcus; Z = Zygosaccharomyces

Table 3.2. Both humans and animals are exposed to wheat allergens via skincare and cosmetic products: a summary of commercial products containing wheat proteins

Product	Wheat Content	Type of product	Examples
Human	Hydrolyzed Wheat Protein	Shampoo	The Naked Object, Rituals. TRESemmé, Garnier Fructis, Neutrogena, Cetaphil
		Nail Polish	Esika
		Primer-	IT Cosmetics
		Eyeliner-Mascara	N/A
		Moisturizer	Glow Recipe, Marcelle
		Soap	Jolly Jabón, Canmepris
		Hand Sanitizer	Sally Hansen, Essential Organics, Kosho
		Hand/Nail Care	Botaneco Garden, Kypwell Essentials
		Exfoliating Towelettes	Alba Botanica
		Hair Gel/Mask/Wax	TREsemmé, Briogeo, Watson's
		Face/Neck Care	Erin's Faces, Lancome Paris, Omved Therapies
		Eye Cream	Lancome Paris, Revlon, Erin's Faces
		Hair Conditioner	Miracle 9, L'Oreal Paris
		Antiseptic	Tea Tree Therapy
		Baby diaper rash Cream	Mom & World N/A
	Wheat Germ/Bran Extract	Lipstick	Estee Lauder
	Hydrolyzed wheat gluten	Shower Gel	Korres Natural World
		Conditioner	Tiger Brands
		Hair Oil	Conatural
Veterinary	Wheat Protein Extract	Shampoo and Conditioner	Buddy Wash
	Hydrolyzed Wheat Protein	Shampoo	Furminator
		Conditioner	TropiClean

3.3.1. Wheat fermentation can reduce or even eliminate wheat's allergenicity

Effects of conventional bread fermentation with yeast, sourdough bread fermentation with lactic acid bacteria (LAB), and soy sauce fermentation using mold, yeast, and LAB on the wheat allergenicity have been reported as discussed below (**Table 3.3, Fig. 3.1**).

Di Cagno and colleagues were the first to investigate the effect of fermentation on wheat immunogenicity (Di Cagno et al., 2002). They determined the effects of sourdough fermentation using selected LAB strains. Fifty-five strains of LAB were pre-screened for their proteolytic activity using a digest of albumin and globulin as substrates. The best proteolytic strains were then

used in wheat fermentation. Protein fractions (albumins, globulins, gliadins, and glutenins) were extracted from fermented doughs and subjected to 2D electrophoresis to evaluate proteolysis. Results suggested that albumins, globulins, and gliadins were degraded by 50%, whereas glutenins remained unaffected. Furthermore, gliadins exhibited significantly lower toxicity as measured by an agglutination test. This study showed for the first time that the selected LAB can reduce the total quantity of immunogenic and allergenic proteins. The strength of this study was that they were the first to report the effects of sourdough fermentation on the integrity and toxicity of wheat proteins including allergens. However, they neither directly tested the effect of fermentation on IgE antibody reactivity *in vitro* nor conducted pre-clinical/clinical testing of their products.

De Angelis et al. (2007) investigated the effects of conventional yeast fermentation vs. LAB fermentation on the allergenicity of wheat. They used a cocktail of nine LAB (*Streptococcus thermophilus, L. plantarum, L. acidophilus, L. casei, L. delbrueckii spp. Bulgaricus, Bifidobacterium breve, B. longum and B. infantis*), noted here as VSL#3, for sourdough fermentation. They found that yeast fermentation caused a minor reduction in the IgE reactivity of gliadins, albumins and globulins in the dough compared to the wheat flour. In contrast, fermentation with the VSL#3 caused marked loss of IgE reactivity of albumins, globulins, and gliadins. Further, the allergenicity of the bread prepared from this sourdough was also reduced and subsequent gut enzyme digestion almost completely abolished the allergenicity of the sourdough bread as discussed in later sections (**Table 3.5**). These results were highly encouraging. A major strength of this study is that they provided the first evidence of potential hypo-allergenicity of LAB fermented sourdough bread. Limitations of this study were that they did not test the effect of LAB fermentation on allergenicity of glutenins and they also did not conduct any *in vivo* testing of their product in animal models or in wheat allergic humans.

Leszczyńska et al. (2009) tested the immunogenicity of sourdoughs prepared from yeast plus LAB fermentation. Wheat flour was fermented with six homofermentative (*L. plantarum*) and five heterofermentative (three *L. brevis* and two *L. sanfranciscencis*) strains along with the yeast (*Saccharomyes cerevisiae)*. They used human anti-gliadin antibody (serum) and conducted an indirect IgG ELISA to test the immunoreactivity of gliadins extracted from the fermented sourdough. Lowest immunoreactivity of gliadins was observed when wheat flour was fermented using mixed LAB strains plus yeast at the ratio of 1:1, showing that there was cooperation between the two microbes in reducing the immunogenicity of gliadins. A major strength of this study is that it showed that the combination of yeast plus LAB is better than using LAB alone to reduce the immunogenicity of gliadins. Limitations of their study are that they neither tested the IgE antibody reactivity nor disease elicitation by these products in wheat allergic patients (Table 3).

Stefańska and colleagues studied the effect of LAB fermentation on wheat allergenicity (Stefańska et al., 2016). Commercial wheat flour was fermented using the following mixed strains of LAB that had shown the highest proteolytic activities in a prior experiment: CM4 [*L. curvatus* 750(13), *P. acidilactici* EKO26, *P. pentosaceus* 1850(3) and *L. coryniformis* pA], CM5 [*L. coryniformis* pA, *W. cibaria* EKO31, *P. pentosaceus* EKO23, *L. plantarum* KKP 593/p], and CM10 [*L. helveticus* Lh10, *L. plantarum* W37/54, *P. pentosaceus* 1850(3)]. Albumins, globulins, and gliadins were extracted from sourdough, and tested for their IgE reactivity using a Western blot method. They found that the LAB fermentation significantly reduced the IgE reactivity of albumins, globulins as well as gliadins. However, because LAB did not hydrolyze the glutenins and therefore, they did not study its IgE reactivity. A major strength of this study is that they further confirmed the previous report (De Angelis et al., 2007) that LAB fermentation does reduce the allergenicity of wheat, and extended the concept by using different types of LAB.

Two limitations of their study are that they did not present any data on the allergenicity of glutenins, and they did not test the allergenicity of this product *in vivo* in animal models or in wheat allergic humans.

Soy sauce is a very popular oriental product now consumed worldwide. It has been used as a traditional seasoning in Japan and other East Asian countries for centuries. In general, it is produced using soybean and wheat as food ingredients (at the 1:1 ratio), and mold, yeast and LAB as microbes for fermentation process (**Table 3.1**). Kobayashi et al. (2004) elegantly studied the fate of salt-insoluble (gluten) and salt-soluble (non-gluten) wheat allergens during the various steps involved in the production of a Japanese soy sauce (Higashimaru Shoyu Co. Ltd. Tatsuno, Hyogo, Japan). They used pooled serum from 5 children (who had anaphylactic reactions to wheat) as source of anti-wheat IgE antibodies (with levels of 83 to >100 U/mL) in Western blot, direct ELISA, and inhibitory ELISA to track wheat allergens. The wheat allergic children had IgE antibodies against multiple allergens in both salt-soluble and salt-insoluble wheat protein fractions. They found that during the *koji* stage (mold cultivation and enzyme production), salt-insoluble (gluten) allergens became salt-soluble and during the *moromi* stage (fermentation with yeast and LAB), all salt-soluble allergens were completely degraded by microbial proteolytic enzymes. They then screened 10 commercial soy sauce products of 6 different kinds (Koikuchi, raw-koikuchi, usukuchi, raw-usukuchi, raw-saishikomi, and shiro) for wheat allergens, and found none. Based on these findings, they concluded that *'no wheat allergen is contained in the soy sauce'* products they studied suggesting that these products might be hypo/non-allergenic (**Table 3.3**). It is unclear at present whether or not wheat allergic humans can tolerate these products without allergic reactions as there are no pre-clinical/clinical studies testing them in animal models or in wheat allergic humans. Nevertheless, this study raises the possibility that

soy sauce produced by other countries/companies may also be free from detectable wheat allergens; this remains to be evaluated. These findings suggest that potentially hypo/non-allergenic wheat products, such as this one, may be created using the combination of mold, yeast, and LAB in fermentation.

In summary, these studies together show that: 1) the conventional yeast fermentation of wheat flour does not markedly reduce its allergenicity; 2) LAB fermentation of wheat flour can reduce wheat allergenicity; 3) LABs differ in their ability to reduce wheat allergenicity, and therefore, large-scale screening of LAB strains would be necessary to identity the most suitable ones for producing hypo/non-allergenic wheat proteins and products; 4) the combination of yeast plus LAB offers an improved approach to create hypo-allergenic wheat bread products although allergenicity must be directly tested yet; and 5) Japanese soy sauce may be non-allergenic as it contains no detectable gluten and non-gluten wheat allergens (**Fig. 3.1**; **Table 3.3**). It is noteworthy that although these approaches show potential, *in vivo* validation of such fermented wheat products for hypo/non-allergenicity remains to be done using animal models for pre-clinical testing and finally clinical testing in wheat allergic human

Table 3.3. Effect of fermentation on wheat allergenicity and immunogenicity

Authors	Wheat Variety	Method of Fermentation	Method of Allergenicity Testing	Results	Comments
Di Cagno et al 2002	Unspecified	Fermentation using LAB: (*Lb. alimentarius* 15M, *Lb. brevis* 14G, *Lb. sanfranciscensis* 7A, and *Lb. hilgardii* 51B) at dosage of $5x10^7$ CFU/g of wheat flour	Sourdoughs were used for extracting proteins (albumin, globulins, gliadins, and glutenins); 2D electrophoresis and agglutination test	LAB-fermented dough showed reduced quantity of albumins/globulins (almost all hydrolyzed by ≥ 50%) and to some extent of gliadins; glutenins were not affected; it also reduced agglutination activity of gliadins	Allergen quantity was reduced; gliadin toxicity was reduced; However, IgE reactivity was not investigated
De Angelis et al 2007	Type O wheat flour (*T. aestivum*, AABBDD), variety Svevo, cultivated in the south of Italy	Doughs were fermented with *S. cerevisiae* with or without VSL#3 treatment (*St. thermophilus, Lb. plantarum, Lb. acidophilus, Lb. casei, Lb. delbrueckii subsp. bulgaricus, B. breve, B. longum and B. infantis*)	Sourdoughs were used for extracting albumins, globulins, and gliadins, and proteins tested for allergenicity by SDS-PAGE, 2D electrophoresis, and IgE western blotting	Yeast fermentation caused minor reductions in gliadins, albumins, and globulins (14 kDa reduced mostly; small reductions in 45 to 97 kDa); VSL#3 caused marked loss of IgE reactivity of gliadins, albumins and globulins	Hypoallergenic gliadins, albumins and globulins may be produced using this method; glutenins were not studied
Leszczyńska et al 2009	Wheat flour (Type 500, genome unspecified) from "Kruszynek" mill	Wheat flour was fermented using LAB from six homofermentative (*Lb. plantarum*) and five heterofermentative (three *Lb. brevis* and two *Lb. sanfranciscensis)* strains with *S. cerevisiae*; 2 $x10^7$ CFU/g of dough	Gliadins extracted from fermented dough were tested for immunogenicity in an indirect IgG ELISA	Fermentation using LAB strains mixed with yeast in a 1:1 ratio showed the highest capacity to reduce the IgG reactivity of wheat flour	Allergenicity of gliadins was not investigated
Stefańska et al 2016	Commercial wheat flour of the 550 types (genome unspecified)	Wheat flour was fermented using LAB: CM4 (*Lb. curvatus* 750(13), *P. acidilactici* EKO26, *P. pentosaceus* 1850 (3) and *Lb. coryniformis* pA), CM5 (*L. coryniformis* pA, *W. cibaria* EKO31, *P. pentosaceus* EKO23, *Lb. plantarum* KKP 593/p), and CM10 (*Lb. helveticus* Lh10, *Lb. plantarum* W37/54, *P. pentosaceus* 1850(3))	Albumins/globulins, gliadins and glutenins were extracted from dough and tested for allergenicity by IgE Western blotting	Reduced allergenicity of albumins, globulins (13 proteins: 22-103 kDa), and gliadins (7 proteins: 24-39 kDa)	This method reduced allergenicity of albumins, globulins, and gliadins; glutenins were not studied
Kobayashi et al 2004	Unspecified	Japanese soy sauce fermentation protocol (Higashimaru Shoyu Co. Ltd.) using wheat, soybean, mold, lactic acid bacteria, and yeast	Pooled sera from 5 wheat-allergic children as IgE source; Western blot, ELISA, inhibition ELISA	Progressive and total destruction of gluten and non-gluten allergens to undetectable levels	Fermentation also reduced allergenicity of soybean (Magishi et al 2017); authors suggested that soy sauce maybe tolerated by wheat and soy-allergic patients

Abbreviations: LAB = lactic acid bacteria; CFU= colony forming units; 2D= two dimensional; VSL#3= an experimental preparation of LAB cocktail; B = Bifidobacterium; Lb = Lactobacillus; P = Pediococcus; St = Streptococcus; S = Saccharomyces; W = Weissella; T = Triticum.

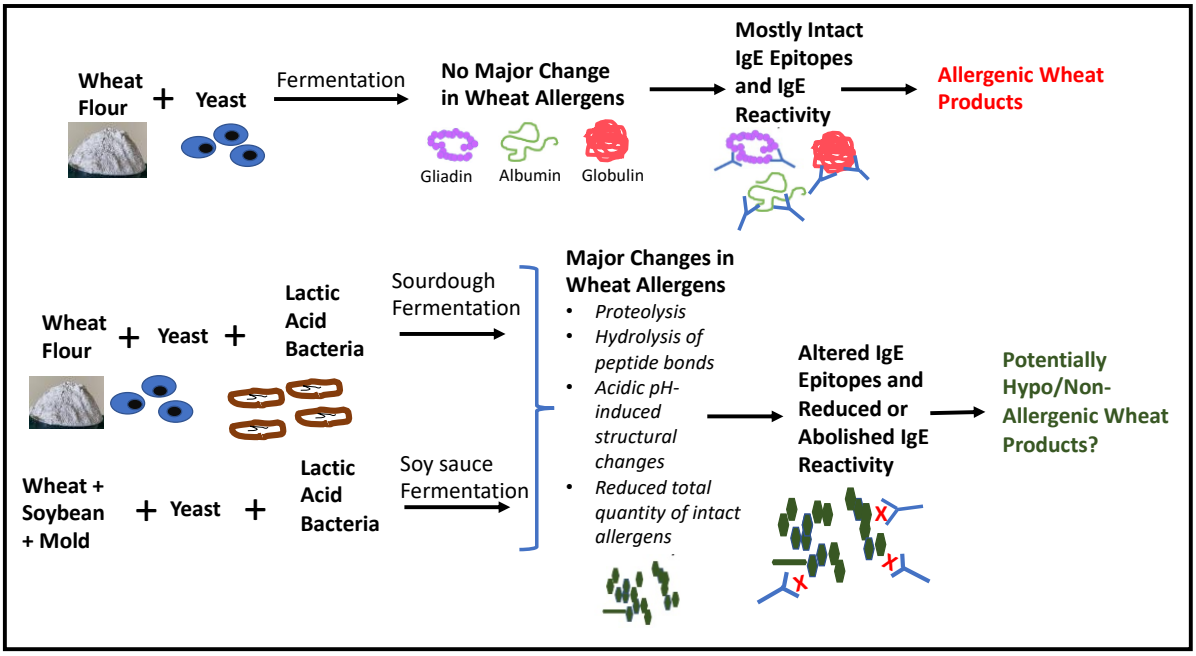

Figure 3.1. Impact of fermentation on allergenicity of wheat. Note: Top of panel: Gliadin allergens present in wheat flour upon traditional fermentation with Baker's yeast (Saccharomyces cerevisiae) do not show major changes in their allergenic structures. Consequently, IgE epitopes remain largely intact, and react with IgE antibodies from wheat-allergic subjects. Baking and gastric digestion can reduce their allergenicity to some extent (see Tables 5 and 6). However, yeast fermented–baked wheat products in general retain substantial allergenicity in vitro and in vivo and therefore must be avoided by wheat-allergic subjects. Bottom of panels: Wheat flour upon sourdough fermentation with a cocktail of selected strains of lactic acid bacteria show major loss of IgE-binding epitopes on albumins, globulins, and gliadins. Baking and simulated gastric digestion further reduce the allergenicity of such products to almost negligible levels in vitro (see **Tables 3.5** and **3.6**). Similarly, during Japanese soy sauce fermentation using microbes (mold plus yeast plus lactic acid bacteria), IgE epitopes on both gluten and nongluten allergens are completely destroyed to undetectable levels. Therefore, such products may be potentially hypoallergenic, which remains to be established by preclinical and clinical testing (see details in the text).

3.3.2. Thermal processing of wheat flour can modulate its allergenicity

Primary goals of thermal processing of wheat food products are to make them edible, safe and shelf stable. However, it can also affect the wheat allergenicity as discussed below (**Table 3.4**).

Simonato et al. (2001) were the first to study the effect of thermal processing (i.e., baking at 220 °C for 30 minutes) on wheat allergenicity. They compared the allergenicity of acid-soluble

proteins extracted from raw wheat dough versus bread (crumb and crust). They used serum from food allergic subjects, who showed reactions upon consuming wheat products, as a source of IgE antibodies and conducted IgE Western blot studies. They found that compared to the raw dough proteins, prolamins in breadcrumb had reduced IgE reactivity. Furthermore, IgE reactivity of α-amylase inhibitor—a major allergen implicated in baker's asthma, was completely lost upon baking. However, they found that large-sized highly reactive mega allergens were present in the bread crust that were not found in the raw dough or the baked crumb. This suggests that direct exposure of the surface of the bread to high temperature during baking causes wheat allergens to form complex Maillard-like aggregates that form highly IgE reactive mega allergens made of mostly gluten proteins. Strengths of this study are that they demonstrated for the first time that baking reduces allergenicity of the bread crumb but increases allergenicity of the crust. These data suggest that avoiding eating bread crusts may be a simple way of reducing the risk of exposure to such mega allergens. A major limitation of this study is that they did not conduct *in vivo* allergenicity testing of their breadcrumbs vs. crusts in animal models or in humans.

Scibilia et al. (2006) were the pioneers in testing the effect of thermal processing on wheat allergenicity *in vivo* by conducting a double-blind placebo-controlled food challenge study. They compared the allergenicity of cooked (conditions unspecified) vs. raw wheat meals with identical contents (wheat flour, water, cocoa, sugar, and lemon aroma syrup). Eleven adult participants were selected based on their clinical reactions upon oral challenge with raw wheat, positive skin prick test results and IgE reactivity to commercial wheat extracts. They found that both cooked wheat meal as well as raw wheat meal elicited food allergic reactions similarly in all subjects. A major strength of this study is that this is the one of the only two reports in the literature in which oral testing was conducted to evaluate the effect of processing on wheat allergenicity. Unfortunately,

specific cooking conditions and the variety of wheats used were not reported. Moreover, due to microbial contamination issues, consuming raw wheat or non-heat-treated wheat is no longer recommended. Furthermore, limited sample size (n=11) and unknown cooking conditions, prevent us from making generalizable conclusions on the effect of cooking on *in vivo* wheat allergenicity from this study.

Pastorello et al. (2007) tested the *in vitro* allergenicity of cooked (boiled) vs. raw wheat flours. They used serum from wheat-allergic subjects and conducted an IgE Western blot study on the albumins, globulins, gliadins and glutenins that had been individually extracted from the boiled vs. raw wheat flours. They found similar IgE antibody reactivities of all four families of wheat allergens extracted from raw vs. boiled wheat flour. A non-specific lipid transfer protein (nsLTP, 9 kDa) was the only exception because IgE from some wheat-allergic subjects did not react with cooked nsLTP. Thus, they showed that wheat allergens in general are resistant to change during the boiling conditions used in cooking wheat flour. A major strength of this study is that this is the first comprehensive study to demonstrate the thermostability of all four families of wheat allergens to boiling conditions *in vitro*. Unfortunately, in this study they did not test the *in vivo* allergenicity of their boiled wheat product in wheat allergic subjects. Furthermore, since they had used a mixture of wheat varieties in their product, interpretations of observed effects to specific wheat variety are not possible (**Table 3.4**).

De Zorzi and others were the pioneers testing the allergenicity of experimental model pasta samples (MPS) (De Zorzi, Curioni, Simonato, Giannattasio, & Pasini, 2007). They prepared various MPS from wheat dough, which were dried at different temperatures (20, 60, 85, 110 or 180°C to reach 10% moisture content). The dried MPS were boiled (10 minutes) and then used in allergenicity testing (**Table 3.4**). Acetic acid-soluble proteins were extracted and used in

testing IgE reactivity by immunoblotting and dot blotting methods. They reported that the MPS prepared at lower drying temperatures (20-110 °C) showed loss of IgE reactivity of LMW albumins; however, LMW-GS and gliadins retained their IgE reactivities. In contrast, MPS dried at 180°C exhibited HMW IgE-reacting protein aggregates suggesting the formation of mega allergens. A major strength of their study is that they controlled all the processing conditions so that accurate interpretations about allergenicity could be made. A limitation of their study is that they did not conduct any *in vivo* testing of their products in animals or in humans.

De Gregorio et al. (2009) conducted a study to evaluate the impact of commercial processing on allergenicity of a large number of food products (French bread, whole meal bread, white tin loaf bread, whole meal tin loaf bread, toasted bread, whole meal toast bread, white pasta, whole meal pasta, fresh pasta, biscuit, pizza crust, baby cereal food, breakfast cereals, wheat flour and bran and raw/cooked pasta). Salt-soluble wheat proteins (SSWPs) were extracted from these products and used in testing. Using IgE-positive pooled serum from a group of wheat-allergic subjects, they conducted Western blot and ELISA to evaluate the relative IgE reactivity of SSWPs. In addition, they also conducted skin prick test (SPT) to evaluate the clinical reactivity of SSWPs. They found that the SSWPs from thermally processed commercial wheat products had a large variation in their allergenicity *in vitro* as well as *in vivo* (SPT), thereby providing the direct *proof-of-concept* evidence that the thermal processing does modulate wheat allergenicity (**Table 3.4**). Overall, commercial bread and cooked pasta exhibited approximately 50% lower IgE reactivities compared to the respective raw flours. Interestingly, pizza crust, baby cereal, and breakfast cereal were least allergenic. Uncooked pasta, and baked biscuits were as allergenic as the raw flour. The major strength of this study is that they assessed *in vitro* as well as *in vivo* (SPT) allergenicity of SSWPs from a large panel of thermally processed wheat food products. It is not possible to deduce

effects of specific conditions used in thermal processing on SSWP allergenicity in this study because they used commercial food products to extract SSWPs. The results suggest that some of the least allergenic food products in their study (e.g., breakfast cereals, pizza etc.,) may be tolerated by people who might be allergic only to SSWPs but not to glutens. However, this remains to be tested in future studies. One limitation of this study is that they tested only the non-gluten proteins and did not test glutens.

Lupi et al. (2019) studied the effect of boiling (100 °C) on the allergenicity of purified gliadins. Wheat flour was used to extract the total gliadins, which were then purified to isolate the α-gliadins by RP-HPLC method. The pure proteins were then boiled and used in testing for IgE reactivity. Pooled serum from a group of allergic subjects was used to perform IgE-based dot blotting. In addition, they also tested the ability of boiled proteins to cause degranulation of mast cells *in vitro* using a cell line assay (RBL-SX38). They found that boiled gliadins completely lost their IgE reactivity as well as their ability to cause mast cell degranulation. Therefore, these data suggest that IgE epitopes on purified gliadins are intrinsically thermolabile and that a simple boiling process may be used to produce a non-allergenic gliadin protein product; however, validating the non-allergenicity in animal models and in humans remains to be done. Furthermore, potential use of such non-allergenic gliadin as a desensitizing allergoid for immunotherapy in wheat allergy also needs to be evaluated. The major strength of this study is that they not only studied the binding of gliadins to the IgE antibodies but also the functional consequence of this binding as measured by *in vitro* mast cell degranulation which occurs *in vivo* during allergic reactions. A limitation of this study is that they did not test the *in vivo* allergenicity of their presumably non-allergenic boiled gliadin in animal models or in humans.

Overall, the studies discussed above suggest that thermal processing has complex, and different effects on non-gluten and gluten wheat allergens as follows (**Fig. 3.2**): 1) non-glutens lose 50% or more of their allergenicity in commercial foodstuffs (bread, pizza, cereals, boiled pasta etc.); they also significantly lose their allergenicity in experimental breadcrumbs and in experimental low temperature dried/boiled pasta; in contrast to non-glutens, glutens (mostly gliadins were studied) in experimental breadcrumbs or boiled pastas largely retain their allergenicity; 2) interestingly, boiling wheat flour does not significantly change allergenicity of non-gluten or gluten proteins; nevertheless, boiling of purified gliadins abolish their allergenicity completely; these results show that IgE epitopes on purified gliadins are intrinsically thermolabile; since boiling of wheat flour does not abolish gliadin allergenicity, it is possible that boiling conditions may promote gliadins to form molecular network with other wheat components (glutenins, non-glutens, carbohydrates) that results in protection of IgE epitopes present on gliadins during the boiling process; 3) pizza crust, baby cereal, and breakfast cereals exhibit least allergenicity of SSWPs—a promising finding towards creating hypo-allergenic wheat proteins that might be tolerated by people who are allergic only to SSWPs; 4) in general, very high temperature treatment (e.g., bread crust, and very high temperature pasta drying) increases wheat allergenicity due to the formation of highly reactive mega allergens of gluten type; and 5) it may be possible to optimize the thermal processing conditions to create novel hypo-allergenic wheat proteins and products; and at the same time, it may also be possible to optimize methods to minimize or prevent inadvertent creation of hyper-allergenic mega allergens (**Table 3.4; Fig. 3.2**).

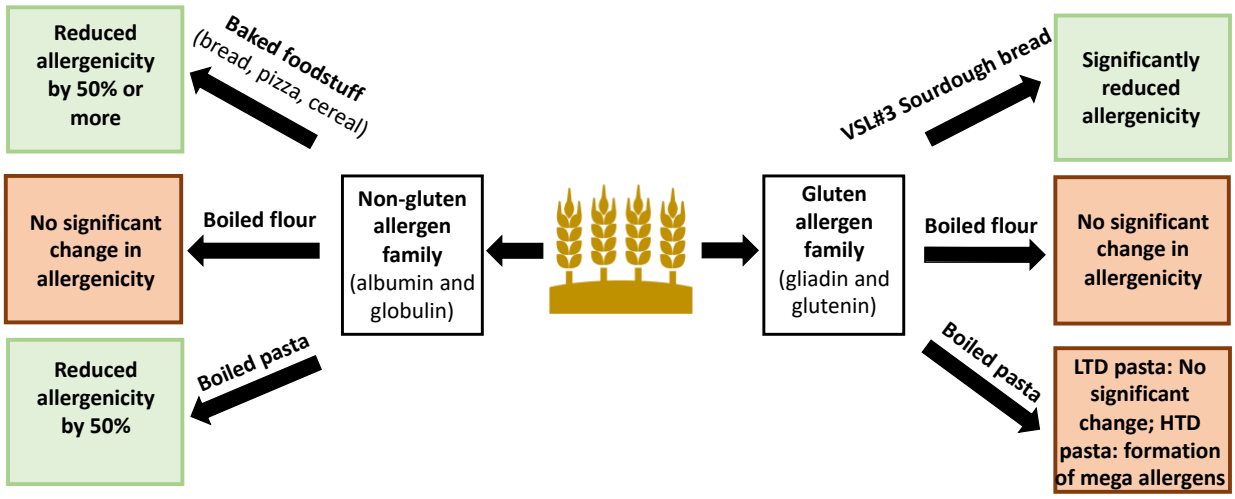

Figure 3.2. Different effects of common thermal processing methods on wheat allergenicity. Note: Left panel: The nongluten allergens (albumins, and globulins) present in baked foodstuffs and boiled pasta lose 50% or more of IgE-binding epitopes compared to those present in the raw wheat flour; in contrast, boiling wheat flour does not markedly reduce the IgE-binding epitopes on nongluten wheat allergens. Right panel: Both gliadins and glutenins retain most of their IgE-binding epitopes in the boiled pasta; gliadins lose significant amount of IgE epitopes in the VSL#3 sourdough bread (glutenins were not studied); boiling wheat flour does not markedly reduce IgE epitopes on gliadins or glutenins. Abbreviations: HTD pasta, high-temperature dried pasta; LTD pasta, low-temperature dried pasta.

Table 3.4. Effect of thermal processing on wheat allergenicity.

Authors	Wheat Variety	Processing Conditions	Method of Allergenicity Testing	Results	Comments
Simonato et al 2001	Common wheat flour (AABBDD) used to prepare round bread loaves in a bakery	Baking at 220 °C for 30 min	HCl-extracted proteins from unheated dough and baked bread crumb and crust were examined using SDS-PAGE and IgE western blot	**In dough**: HMW-, S-poor, and S-rich prolamins (gliadins and glutenins), α-amylase inhibitors, and 16 kDa allergen were IgE reactive; **In bread crumb**: lower amount of all above allergens; 16 kDa allergen disappeared; **In bread crust**: new IgE reactive large protein aggregates formed; 16 kDa allergen was not detectable	Dough contained albumins, globulins (including α-amylase inhibitors and 16 kDa allergen), and prolamins (gliadins and glutenins) allergens; baking reduced all these allergens in the crumb; baking created new large allergens in the crust
Scibilia et al 2006	50% durum (AABB) and 50% tender (genome unspecified)	Cooked wheat (conditions unspecified)	Meals containing raw or cooked wheat were used for double-blind placebo-controlled food challenge testing	Cooked and raw wheat products exhibited no significant differences in allergenicity upon oral challenge	Cooking did not change oral allergenicity of wheat; processing conditions (e.g., time and temperature) of these meals were not specified
Pastorello et al 2007	50% of common (genome AABBDD) and 50% durum (AABB)	Boiled wheat flour in water and cooled immediately	Albumins, globulins, gliadins, and glutenins extracted from raw and cooked wheat flour were tested in IgE western blot	Albumin/globulins (including 12-16 kDa α-amylase/trypsin inhibitor), gliadin and glutenin allergenicity did not change by boiling; 9 kDa LTP lost allergenicity in some cases but not others	Boiling did not change allergenicity of albumins, globulins, gliadins, and glutenins; boiling had inconsistent effect on LTP
De Zorzi et al 2007	Durum wheat (genome AABB)	Flour + water were mixed to form dough, and dried at various temp to reach 10% moisture; Dried samples were then cooked in salted boiling water	Acetic acid-soluble proteins were extracted from dried and boiled MPSs, and subjected to SDS-PAGE, IgE western blot and dot blot	**In 20-110 °C pasta**: HMW-GS, LMW-GS, and α/β/γ-gliadins retained their IgE reactivity while LMW-albumins were abolished; **In 180 °C pasta**: HMW protein aggregates formed	Pasta drying at lower temperatures did not change allergenicity of globulins, gliadins, and glutenins; abolished allergenicity of LMW-albumins; high temperature drying created large-sized allergens
De Gregorio et al 2009	Unspecified commercial products; wheat bran and flour: *T. aestivum*, cultivar Astral (AABBDD)	Baking (commercial products used; conditions unspecified); pastas were boiled for 10 min for dried and 2 min for fresh ones	SSWPs extracted from French bread, whole meal bread, white tin loaf bread, toasted bread, whole meal toast bread, white pasta, whole meal pasta, fresh pasta, biscuit, pizza, baby cereal food, breakfast cereals, wheat flour and bran and raw/cooked pasta were tested for allergenicity using IgE western blot, ELISA, and skin prick test	Breakfast cereals did not provide appreciable IgE reactivity; raw flour and bran, biscuit and non-boiled pasta showed 5-10 major allergens from 5-100 kDa; boiled pastas showed few allergens (2-5), 20-50 kDa; much lower allergens were observed in pizza, baby cereal food, and breakfast cereal; two main allergens: 20 and 32 kDa in breads; no allergens found in toasted breads	SSWPs from commercial breads and cooked pastas exhibited 50% lower IgE reactivities compared to raw flour; uncooked pastas and biscuits had considerable allergenicity; gliadins and glutenins were not studied
Lupi et al 2019	*T. aestivum*, cultivar Recital (AABBDD)	Boiling: 8 mg of purified gliadins with 4 ml of water, heated at 100 °C for 20 min	Total gliadins and α-gliadins were extracted, purified by RP-HPLC, and tested using IgE dot-blot and RBL-SX38 cell degranulation	IgE reactivity of total and α-gliadins were abolished; boiled gliadins did not degranulate RBL-SX38 cells	Boiling abolished allergenicity of purified gliadins

Abbreviations: LTP= lipid transfer protein, HMW-GS= high molecular weight glutenin subunit; LMW=low molecular weight; MPS = model pasta sample; SDS PAGE= sodium dodecyl sulfate poly acrylamide gel electrophoresis; RP-HPLC= reverse phase high performance liquid chromatography, RBL-SX38= rat basophilic leukemia cell line; T = Triticum

Table 3.5. Effect of enzymatic hydrolysis on wheat allergenicity.

Authors	Wheat Variety	Enzymes	Method of Allergenicity Testing	Results	Comments
Simonato et al 2001	Common wheat flour (AABBDD) used to prepare round bread loaves in a bakery	Pepsin and pancreatin	Bread digested with 0.2 N HCl + pepsin followed by pancreatin; supernatant tested; SDS-PAGE and IgE western blot	**In dough**: digestion abolished HMW prolamins and LMW allergens including α-amylase inhibitors; except 16 kDa unknown allergen **In bread crumb**: digestions abolished HMW prolamins; LMW glutenins were resistant **In bread crust**: HMW prolamins/ LMW glutenins formed large aggregates that were resistant; 16 kDa allergen was abolished	Prolamins in dough were more susceptible to digestion; however, allergens in bread crumb and crust were relatively resistant due to formation of large aggregates that retained allergenicity; α-amylase inhibitors were destroyed
Yamamoto et al 2004 & Tanabe et al 2008	Commercial soft flour (genome unspecified)	Actinase treated flour used in making hypoallergenic cupcake	IgE western blot, ELISA, and oral provocation test in nine atopic dermatitis patients allergic to wheat	Degraded IgE epitope (Gln-Gln-Gln-Pro-Pro) on glutenin implicated in atopic dermatitis; seven out of nine patients did not react to eating cupcake made from hypoallergenic wheat flour	Authors also demonstrated that other enzymes (bromelain, cellulase, and collagenase) reduced allergenicity of wheat glutenin epitope as well (Tanabe et al 1996, Watanabe et al 2000). However, only actinase treated wheat product was tested in humans
De Zorzi et al 2007	Durum wheat (AABB)	Pepsin and pancreatin	Digestion of MPSs; acetic acid-soluble proteins extracted and tested; SDS-PAGE, IgE western blot, and dot blot	**MPSs dried at 20-110°C and digested**: no detectable IgE-reactive gliadins, glutenins and albumins/globulins **MPSs dried at 180°C and digested**: high molecular weight protein Maillard-type protein aggregates and one 42 kDa protein retained allergenicity after digestion	Low temperature pasta allergens were destroyed by digestion; high temperature pasta allergens were resistant to digestion; therefore, hypoallergenic pastas can be made using lower drying temperatures; higher drying-temperature pastas were hyper-allergenic
De Gregorio et al 2009	Unspecified commercial products; wheat bran and flour: *T. aestivum*, cultivar Astral (AABBDD)	Pepsin, trypsin, and chymotrypsin	Digestion of commercial bread and wheat flour; SSWPs extracted and tested for allergenicity; SDS-PAGE and IgE inhibition ELISA	Digestion significantly reduced the IgE binding capacity of SSWPs from bread (1.6-fold) and raw flour (3-fold)	Effect of digestion on allergenicity of glutens was not investigated; digestion of commercial breads did not abolish but reduced allergenicity significantly
Li et al. 2016	Commercial wheat flour (genome unspecified)	Papain, flovourzyme, trypsin, α-chymotrypsin, pepsin, and alcalase	Wheat flour was hydrolyzed; SE-HPLC purified gliadins were tested for IgE reactivity using competitive inhibition ELISA	Non-digestive proteases were more effective than digestive proteases in inhibiting IgE-binding capacity of gliadins; pepsin showed the least ability in reducing IgE reactivity; alcalase and papain were the most effective in reducing IgE binding	Sequential use of alcalase-papain treatment yielded the lowest content of gliadin and lowest allergenicity; other protein fractions were not investigated
Lupi et al 2019	*T. aestivum*, cultivar Recital (genome AABBDD)	Pepsin	HPLC-purified gliadins were boiled and digested with pepsin; allergenicity was tested using IgE dot blot and RBL-SX38 cell degranulation	Boiling abolished IgE binding; pepsin digestion of boiled gliadin induced a few new IgE epitopes, which were unable to induce degranulation	Pepsin digestion of boiled gliadins created new non-functional IgE epitopes

Abbreviations: LMW= low molecular weight; HMW= high molecular weight; MPSs= model pasta samples; SSWPs= salt-soluble wheat proteins; VSL#3= an experimental preparation of lactic acid bacteria cocktail; MPS=model pasta samples; T = Triticum

Table 3.6. Three genetically distinct genotypes of wheat are commonly used in food products and/or animal feed.

Genome	Common Name/s	Species*	Common Uses
AA	Wild einkorn	*Triticum boeticum*	Cookies, brownies, cakes, bread, pizza, rolls, soup, cereals
AABB	Emmer	*Triticum dicoccon*	Soup, beer, animal feed
	Durum	*Triticum durum*	Pizza, pasta, bread, semolina, upma, couscous, pudding
	Oriental wheat	*Triticum turanicum*	Bulgar, pasta, cereals, bread, waffles, beer,
	Persian wheat	*Triticum carthlicum*	Bread
	Rivet wheat	*Triticum turgidum*	Feed
AABBDD	Common wheat	*Triticum aestivum*	Bread, cookies, crackers, cereals etc.
	Spelt	*Triticum spelta*	Pasta, crackers, bread, cereals, risotto, cookies, stews
	Clubed wheat	*Triticum compactum*	Cake, crackers, cookies, pastries

*Taxonomy based on Integrated Taxonomic Information System (ITIS, 2021)

3.3.3. Enzyme hydrolysis of wheat can reduce or even abolish its allergenicity

Wheat foods, when consumed, undergo gut digestion processes which are expected to influence their allergenicity. Both the *de novo* allergic immune response to wheat allergens as well as elicitation of allergic reaction in pre-sensitized subjects depends on the availability of adequate amounts of allergenic peptides that would have survived the gut digestion. Consequently, allergenicity of wheat food is a function of its ability to resist gut enzyme-mediated hydrolysis. Therefore, it is critical to evaluate the effects of gut enzymes on the stability of wheat allergens. In addition, non-digestive enzymes have also been used not only for industrial production of HWP and HWG for use as an ingredient in food and skin care products (e.g., papain), but also, in preparing experimental hypo-allergenic wheat food products (e.g., bromelain, actinase) (Tranquet et al., 2020; Tanabe 2008, Yamamoto et al., 2004, Tanabe et al., 1996, Watanabe et al., 1994). The papain enzyme (derived from papaya fruit) is used as a food additive and bromelain (an enzyme extract derived from the papaya stem, juice, and fruit) is used as a food supplement. Therefore, treatment with these non-gut enzymes is a feasible way of developing hypo-allergenic wheat flour. Here we have reviewed the effects of gut digestive enzymes and non-gut enzymes on wheat allergenicity (**Table 3.5**).

Simonato et al. (2001) studied the hydrolytic effects of pepsin and pancreatin on the allergenicity of wheat dough, breadcrumb, and bread crust. They found that almost all IgE-reactive protein allergens, with the exception of a 16 kDa allergen, disappeared after the enzyme digestion of dough. Gut enzyme digestion abolished HMW-prolamins (gliadin and glutenin) and non-glutens but not LMW-glutenins in the baked breadcrumb. Therefore, due to gut digestion, most breadcrumb derived allergens may significantly lose their ability to sensitize and to elicit allergic reactions. Interestingly, large-sized HMW-prolamins/LMW-glutenins that were formed in the breadcrust upon baking, were resistant to gut enzyme digestion. LMW non-glutens were completely hydrolyzed by digestion. These findings suggest that consuming breadcrumb, and avoiding bread crusts, may be a simple way to reduce the relative risk of exposure to wheat allergens. The major strength of this study is that they tested all four families of wheat allergens. A limitation of the study is that they did not test their products for *in vivo* allergenicity in animal models or in wheat allergy patients.

De Angelis et al. (2007) compared the effect of pepsin and pancreatin digestion on allergenicity of conventional yeast bread vs. yeast plus VSL#3 fermented sourdough bread (prepared with a cocktail of selected LAB as described earlier). Albumins, globulins, and gliadins were extracted from the digested bread for allergenicity testing. They found that the digestion of yeast bread reduced IgE-reactivity of 31 to 45 kDa prolamins. It did not affect 14, 60, and 97 kDa proteins. However, more strikingly, digestion of VSL#3 sourdough bread almost completely abolished IgE-reactivity of albumins, globulins, and gliadins. Glutenins were not studied. Therefore, these findings raise the possibility that the VSL#3 sourdough bread may be tolerated by people who are allergic to albumins, globulins, and gliadins. Thus, a major strength of this study is that they provided a novel method to produce potentially hypo/non-allergenic sourdough bread.

However, *in vivo* testing by pre-clinical and clinical studies are yet to be performed for validating this potentially hypo-allergenic wheat food product (**Fig. 3.1**).

De Zorzi et al. (2007) tested the effect of pepsin and pancreatin digestion on allergenicity of experimental model pasta samples dried at different temperatures (20-110 °C and 180 °C). They studied IgE-reactivity of albumins, globulins, gliadins, and glutenins using the Western blot and dot blot methods. They found that digestion of pasta made from lower drying temperature abolished IgE-reactivity of all four wheat allergen families. Thus, lower drying-temperature pasta almost completely lose their allergenicity upon digestion. This suggests that all wheat allergens present in such pasta may be unable to elicit sensitization and allergic reactions because all IgE epitopes are expected to be totally digested in the gut. However, pasta made at high drying-temperature exhibited large-sized Maillard-type protein aggregates and one 42 kDa protein that were each highly IgE-reactive. Therefore, it may be prudent to avoid high temperature for pasta drying to prevent the creation of mega allergens that resist gut enzyme digestion. The major strength of this study is that they demonstrated loss of allergenicity of all four families of wheat allergens upon simulated gut digestion. A limitation of this study is that the *in vivo* allergenicity was not tested.

De Gregorio et al. (2009) studied the effects of pepsin and trypsin enzyme hydrolysis on IgE reactivity of SSWPs from commercial bread and raw flour. They found that enzymatic hydrolysis reduced the IgE binding of SSWP from raw flour by 3-fold and reduced the IgE binding of SSWP from bread by 1.6-fold. Gliadins and glutenins were not studied. These results suggest that such commercial products may have lower sensitization and disease elicitation capacities and may be tolerated by patients who might be allergic only to non-gluten (albumins & globulins) allergens. Notably, in contrast, to Simonato et al. (2001), these authors did not

separate the effects on allergenicity of breadcrumbs vs. breadcrusts. Limitations of the study are that the conditions of commercial bread making were not specified, and the *in vivo* allergenicity was also not tested.

Li and colleagues used six enzymes (alcalase, α-chymotrypsin, flovourzyme, pepsin, and trypsin) to hydrolyze wheat flour to investigate their proteolytic effects on the allergenic potential of gliadins present in hydrolyzed wheat flour (Li, Yu, Goktepe, & Ahmedna, 2016). Gliadins were extracted from hydrolyzed wheat flours, purified by SE-HPLC, and were examined for their allergenicity in an IgE competitive inhibition ELISA. They reported that sequential use of alcalase and papain significantly reduced the gliadin content of hydrolyzed wheat flour, and the IgE reactivity of its gliadin. Similar results of reduced allergenicity of gliadins from alcalase and papain treated flours were reported in a Balb/c mouse model study (Xue et al., 2019). Therefore, two different lines of evidence (human IgE reactivity and mouse model IgE responses) suggest that potentially hypo-allergenic gliadins can be produced using alcalase and papain hydrolysis. Such products may have lower sensitization and disease elicitation potencies, which of course, must be confirmed by clinical studies.

Lupi et al. (2019) studied the effect of pepsin digestion on allergenicity of purified gliadins from wheat flour. They found that IgE reactivity of gliadin was reduced after pepsin digestion. However, when gliadins were heated to 100 °C for 20 minutes, and then digested with pepsin, IgE epitopes reappeared; since these epitopes were not involved in mast cell degranulation, they were therefore considered as non-functional. These results suggest that simple boiling of purified gliadins may be an inexpensive method to create hypo-allergenic gliadins. Whether such gliadins will be unable to cause sensitization and elicitation of allergic reactions is unknown. Once validated, such gliadins might be used in food products as hypo-allergenic proteins. The major

strength of this study is that they tested IgE binding as well as mast cell degranulation *in vitro*. A limitation of the study is that they did not test the *in vivo* allergenicity.

A group of Japanese scientists reported several studies on producing potentially hypo-allergenic wheat flour using non-gut enzymes as follows. Watanabe et al. (1994) reported that treatment of hard wheat flour (Eagle brand, Nihon Flour Milling Co.) and soft wheat flour (Heart brand) with actinase, and collagenase results in hypo-allergenic flours with reduced *in vitro* IgE antibody reactivities of both salt-soluble (non-gluten) and salt-insoluble (gluten) proteins; soft wheat product showed better results than the hard wheat flour. Tanabe et al. (1996) found that bromelain treated soft wheat flour lost IgE reactivity of a major epitope on glutenin (Gln-Gln-Gln-Pro-Pro) implicated in atopic dermatitis among wheat allergic children. Using this flour they prepared a hypo-allergenic bread that resembled English muffins. Subsequently, Yamamoto et al. (2004) prepared a hypo-allergenic cupcake using actinase treated wheat flour that had lost all IgE reactivity *in vitro* and tested its safety and efficacy in a group of atopic dermatitis children (n=9) who had wheat allergy. They reported that upon oral provocation with the hypo-allergenic cupcake, only 2 out of 9 patients showed clinical reactions (eruption, urticaria, and/or wheezing). Furthermore, more than half of the patients who tolerated this cupcake were later able to ingest normal wheat products without reactions suggesting desensitization and induction of immune tolerance to wheat allergens (Tanabe, 2008). Although these studies are exciting, sample size was limited (n=9) in this study. Whether this product may be tolerated by adult wheat allergic patients is also unknown. Furthermore, this study was based on inactivating a major IgE epitope on glutenin involved in atopic dermatitis in this group of children. Therefore, it is unclear whether or not this product may be tolerated by patients allergic to non-glutenin family of wheat

allergens. Overall, this study supports the idea of creating hypo-allergenic wheat products for wheat allergic patients using non-gut enzymes.

In summary, available published studies on *in vitro* enzyme digestion show that: 1) enzymes present in the digestive tract have the capacity to abolish the allergenicity of non-gluten wheat allergens present in conventional yeast breadcrumb and the VSL#3 sourdough bread; further, whereas gliadin allergenicity in yeast bread is slightly reduced and that of the VSL#3 sourdough bread is completely abolished; however, glutenins in both types of breads appear to resist gut enzyme digestion; 2) gut enzymes can completely abolish the non-gluten allergens and gluten allergens present in the boiled pastas that had been prepared at low drying temperatures; however, mega allergens (mostly gliadins and glutenins) created at high temperature processing in yeast breadcrust or in high temperature dried pastas resist gut enzyme digestion; 3) Non-digestive enzymes (alcalase, papain, bromelain, actinase) have the capacity to significantly reduce or eliminate the allergenicity of purified gliadins, as well as, that of glutens (gliadins and glutenins) present in wheat flour; 4) most importantly, hypo-allergenic cupcakes made from actinase treated wheat flour was tolerated by the majority of children (7 out of 9) with atopic dermatitis associated with wheat allergy; subsequently, these children were able to eat regular wheat products without clinical reactions suggesting that hypo-allergenic cupcake was able to cause desensitization; and 5) further, pre-clinical and larger clinical studies are needed to validate such hypo-allergenic wheat products produced by enzyme treatment of wheat flour (**Table 3.5**).

3.3.4. Industrial processing can be used to produce hypo/non-allergenic hydrolyzed wheat products

Hydrolyzed wheat products such as HWP and HWG are produced by industrial processing of wheat protein with acid, alkali, enzymes or steam (Tranquet et al., 2020). These products are used as functional ingredients in food and cosmetics because they can provide emulsifying and foaming attributes (Gabler & Scherf, 2020). Their use in cosmetics include, adding to skin and hair-conditioning products in a large number of personal care products and in some veterinary skin and hair products (**Table 3.2**). These HWP and HWG are mixtures of amino acids and peptides of varying lengths. There is a wide variation in the distributions of the sizes of peptides and polypeptides in such products depending upon the company that produce them (Gabler & Scherf, 2020). However, in general, molecular weights of the hydrolyzed proteins range from 0.5 to 30 kDa (Burnett et al., 2018; Gabler & Scherf, 2020).

If the HWP and HWG products contain peptide sizes larger than 3.5 kDa, then they pose an allergenicity risk in humans *via* both skin and mucosal (eye, oral, respiratory) routes of exposures. In one specific case of a Japanese facial soap, HWP made from partial hydrolysis of gluten with hydrogen chloride (95 °C for 40 min) had been used. This product contained large sized proteins (40-50 kDa). Unfortunately, this product caused anaphylactic sensitization to wheat in a large number (>1000) of Japanese people (Chinuki & Morita, 2012; Chinuki et al., 2013; Fukutomi et al., 2011; Nakamura, Rika, Ryosuke Nakamura, Reiko Adachi, Yasuharu Itagaki, Yuma Fukutomi, 2013; Noguchi et al., 2019; Tranquet et al., 2017; Yokooji et al., 2013). An expert committee that reviewed the allergenicity of HWP and HWG concluded that these products are safe only when they are formulated to minimize peptide lengths greater than 30 amino acids (~3.3 kDa) (Burnett et al., 2018). Furthermore, current advisory is that the cosmetic products containing

HWP and HWG should not be used on damaged skin or in products that may come in contact with the mucosal surfaces such as eyes and airways. Thus, it is possible to produce industrially processed hypo/non-allergenic wheat proteins for use in cosmetics.

3.4. The effect of processing on wheat allergenicity: molecular mechanisms

Wheat allergenicity depends on two phases of immune responses to wheat protein allergens that occur in sequence: *Phase 1*: exposure of non-allergic, but genetically prone, healthy subjects to wheat allergens results in the production of wheat allergen-specific IgE antibodies most of which remain in the body bound to the receptor present on mast cells and basophils; this process is termed sensitization; and *Phase 2*: re-exposure of pre-sensitized subjects to wheat allergens results in physicochemical binding of the cell-bound IgE antibodies to the allergens which initiates the disease; this process is known as allergic reaction elicitation. Without phase 1, disease elicitation is not possible (Jin et al., 2019; Renz et al., 2018).

Food processing can modify the structure of wheat allergens. Therefore, it can potentially affect the capacity of altered allergens to initiate sensitization (i.e., Phase 1) as well as the disease elicitation (i.e., Phase 2). It is noteworthy that all the reports reviewed in this paper focused entirely on the Phase 2 of the process (i.e., interaction of IgE antibody with processed wheat proteins *in vitro* in all studies, and mast cell degranulation and clinical reactions in 3 studies). Therefore, the impact of processing on the Phase 1 of wheat allergenicity is largely unknown at present. Accordingly, the mechanisms discussed here refer only to the Phase 2 of the wheat allergenicity process.

Wheat proteins can function as allergens because of their epitope structures that bind to IgE antibodies through a complementary three-dimensional matching of molecular shapes (Battais

et al., 2005; Battais, Richard, Jacquenet, Denery-Papini, & Moneret-Vautrin, 2008). These IgE epitopes are of two general types: conformational and linear. The quaternary structures including the intra/interchain disulfide bonds and carbohydrates present on the proteins contribute to the integrity of conformational epitope structure. The linear epitopes are determined primarily by the sequence of the amino acids in the polypeptide chain. Consequently, if these epitope structures can be abolished or altered by food processing, then one would expect corresponding changes in IgE antibody binding capacity of wheat allergens, thereby influencing Phase 2 of the wheat allergenicity process.

It is estimated that a functional protein allergen possesses at least two epitopes of 15 amino acids each in length that can bind to one IgE antibody molecule. Such epitopes can be either linear or conformational in nature (Huby et al., 2000). When a person has at least two or more wheat specific IgE molecules bound on the mast cells and basophils in the body, that person is deemed sensitized to the wheat allergen. Subsequently, allergen binding and cross-linking of at least 2 IgE molecules present on mast cells and basophils is required to activate them to cause release of vasoactive chemicals like histamine to trigger an allergic reaction (also known as type I hypersensitivity reaction). Therefore, the primary requirement for a wheat product to function as hypo-allergenic, it must contain markedly fewer IgE binding epitopes compared to the conventional wheat product. If all the IgE binding epitopes are abolished, then the resulting product may be considered non-allergenic. However, both types of products must be confirmed for hypo/non-allergenicity by pre-clinical and clinical testing. These definitions are operationally used in all the studies we have reviewed in this article.

Existing evidence shows that processing conditions can affect the wheat allergen and IgE binding interactions as measured by not only *in vitro* methods such as Western/dot blot, ELISA,

and mast cell degranulation assays, but also *in vivo* methods such as skin prick testing (in 1 study) and oral allergen challenge (in 2 studies) (Verhoeckx et al., 2015). Combined effects of thermal processing plus gut enzyme digestion must be considered to deduce the molecular effects on wheat allergenicity. Thermal processing methods (baking and pasta drying & boiling) alone appear to reduce the IgE binding ability of most non-gluten wheat allergens, suggesting significant destruction of relevant IgE epitope structures (**Table 3.4; Fig. 3.2**) (Cabanillas & Novak, 2019). Gut enzyme digestion completely abolishes the IgE epitopes on non-gluten allergens in bread and boiled pasta. Therefore, almost all IgE epitopes present on non-glutens in baked foodstuffs and in boiled pasta will be expected to be non-functional in the gut when such wheat products are consumed. In contrast, with the exception of breadcrumb, IgE epitopes on gluten allergens generally resist thermal processing such as baking and pasta boiling (**Fig. 3.2**). However, most IgE epitopes on gluten allergens present in VSL#3 sourdough bread and in boiled pasta are easily destroyed by gut enzyme digestion. Therefore, most IgE epitope structures on gluten in such products are expected to be non-functional in the gut when they are consumed. These molecular mechanisms suggest that *in vivo* testing of these products is warranted to confirm the potential clinical benefits of such dramatic molecular changes in wheat allergens induced by the combined effects of thermal processing and gut enzyme digestion.

Boiling of wheat flour, in general, does not induce major changes to IgE epitopes present on gluten or non-gluten wheat allergens (**Fig. 3.2**). In contrast, in the wheat pasta matrix, almost 50% of IgE epitopes on non-gluten allergens are destroyed by boiling but, almost all IgE epitopes on gluten allergens resist boiling. Nevertheless, boiling purified gluten (gliadins) results in complete loss of their IgE epitope structures suggesting intrinsic thermolability of IgE epitopes present on purified gliadins. These observations, together suggest that IgE epitopes of both gluten

and non-gluten allergens, when present in the wheat flour matrix, resist boiling possibly due to their protection conferred by multi-molecular networks formed among gliadins, glutenins, albumins, globulins, and polysaccharides during the boiling process. Similarly, in the pasta matrix also, same mechanism may protect most IgE epitopes on gluten allergens and 50% of IgE epitopes on non-gluten allergens.

Enzymes produced during fermentation reactions can be used to destroy IgE epitopes by hydrolyzing larger proteins into small peptides. Such enzymatic hydrolysis of gluten commonly occurs in the production of fermented foods, in which the acid produced during microbial metabolism activates enzymes that degrade the protein; the net effect on allergenicity would depend on the enzyme specificity and the degree of hydrolysis (Thiele, Grassl, & Gänzle, 2004) (Wang, Zhao, Zhao, Bao, & Jiang, 2007).

Sourdough fermentation of wheat flour, as opposed to the traditional yeast fermentation, appears to markedly reduce wheat allergenicity (**Fig. 3.1, Table 3.3).** It is noteworthy that, the yeast fermentation of wheat flour does not significantly change most of the IgE epitopes structures on glutens or non-glutens (De Angelis et al., 2007); however, use of LABs alone or along with the yeast during the fermentation process appears to consistently reduce the density of IgE epitopes. Furthermore, Japanese soy sauce fermentation method that uses molds, yeasts, and LAB, progressively destroys all IgE epitopes present on both gluten and non-gluten wheat allergens (Kobayashi et al., 2004). These results together demonstrate the extreme vulnerability of the molecular IgE epitope structures of wheat allergens to the effects of microbial fermentation. Therefore, these effects can provide strong molecular basis and rationale on using combination of microbes to reduce or even to eliminate wheat allergenicity.

Several mechanisms are proposed to explain this significant destructive effects of microbial fermentation on wheat allergenicity (**Fig. 3.1**): 1) enzymatic hydrolysis: acidic pH from the fermentation reaction can activate the proteolytic activity of proteases released by LABs; these enzymes can then break the large allergen molecules into smaller sized proteins, polypeptides, oligopeptides and amino acids, causing progressive changes to and loss of the linear as well as conformational epitopes that are required for IgE binding; 2) Non-enzymatic hydrolysis under acidic pH conditions can denature the epitopes by breakage of disulfide bonds and disruption of non-covalent interactions among amino acids. In those cases, where there is complete loss of allergenicity, the mechanism may be due to complete loss of epitope structure present on large-sized protein products of >3.5 kDa, or complete degradation of the protein to peptides less than 3.5 kDa that are incapable of binding to the IgE antibodies (Akiyama et al., 2006; Chahal, 2014).

Evidence in the literature shows that non-gut enzymes such as bromelain, papain, alcalase, collagenase, and actinase also can reduce or eliminate wheat allergenicity (**Table 3.1**). The mechanism includes proteolysis and breakdown of larger protein molecules into smaller sized peptides and amino acids with consequent loss in total quantity of allergenic proteins associated with loss of both linear and conformational IgE epitopes. Dramatic effects of bromelain and actinase in destroying most IgE epitope structure is illustrated by the resulting wheat flour testing negative for any detectable allergenicity in *in vitro* assays (Tanabe, 2008; Yamamoto et al., 2004). Furthermore, actinase treated hypo-allergenic wheat flour was used to produce cupcake that was tolerated by most wheat allergic children. It also induced clinical desensitization enabling patients to eat common wheat products without problems a year later.

This suggests that immune tolerance-inducing non-allergenic wheat proteins can be produced by treating wheat flour with the actinase enzyme.

The mechanism of reduction in allergenicity of industrially produced HWP and HWG involves the hydrolytic breakdown of large-sized wheat allergens into products containing peptides less than 3.5 kDa that simply lack the ability to bind to IgE antibodies. Therefore, industrial production of non-allergenic HWP and HWG is possible when the products contain protein sizes of less than 3.5 kDa (Burnett et al., 2018). It is noteworthy that partial hydrolysis of wheat proteins using acids does not reduce allergenicity. It is thought to be due to the re-organization of IgE epitopes by the interchange of disulfide bonds, entanglements of chains or due to formation of non-covalent bonds. Such protein products are soluble, contain lots of IgE epitopes and therefore, can trigger serious allergic reactions (Bouchez-Mahiout et al., 2010). Therefore, inadvertent production of such epitopes must be avoided.

3.5. Creating hypo/non-allergenic wheat products using food processing methods: potential opportunities, and challenges

The primary objective of this study was to evaluate the published literature on the effect of food and industrial processing of wheat so as to establish the evidence-based potential for creating novel hypo/non-allergenic wheat products. Other objectives included identification of the knowledge-gaps and challenges preventing advancements, to inform the future research agenda.

A professionally developed and widely accepted definition of hypo/non-allergenicity for wheat products does not exist at present. The *in vitro* studies reviewed here used reduced binding or loss of binding to IgE antibodies obtained from wheat allergic subjects as operational definitions in their experiments. The three *in vivo* studies in humans used the frequency and

severity of clinical reactions (1 study, skin reaction to intradermal injection; 2 studies, oral reactions) as evidence for hypo-allergenicity. The US FDA does not have an approved definition for 'hypo-allergenicity' (US FDA, 2020). Nevertheless, for successful development of hypo-allergenic infant milk products, pediatric clinical scientists, along with the infant food industry professionals have developed clear guidelines for pre-clinical and clinical testing of candidate hypo/non-allergenic products, that includes the following definition*: 'These tests should, at a minimum, ensure with 95% confidence that 90% of infants with documented cow's milk allergy will not react with defined symptoms to the product under double-blind, placebo-controlled condition"* (Baker et al., 2000; Høst & Halken, 2004). We suggest that a similar professional guideline and definition of hypo-allergenicity be established for developing hypo/non-allergenic wheat products also.

Genetically, the modern wheats are derived from 3 genomes—A, B and D (Juhász et al., 2018). Commonly consumed wheats are either diploid (AA) (e.g. Einkorn), tetraploid (AABB) (e.g. durum), or hexaploid (AABBDD) (e.g. bread wheat) (**Table 3.6**, Appels et al., 2018; Juhász et al., 2018; Shewry, 2018). There are many species within hexaploid and tetraploid wheat, and there are hundreds and thousands of varieties and accessories within each species. Since allergenic protein content and their structure are genetically encoded, one can expect variation in the allergenicity of wheat at the ploidy level, at the species level as well at the variety/accession levels (Gao et al., 2019; Nakamura, Tanabe, Watanabe, & Makino, 2005). However, not every study we reviewed specifies the wheat variety used in testing. Furthermore, commercial flours most likely contain mixture of several species/varieties/accessions. Therefore, it is critical that future studies on wheat allergenicity consider using standardized material and document genetic

details on the wheat used so as to advance the ultimate goal of consistently creating hypo/non-allergenic wheat products.

Studies show that the wheat proteins (both gluten and non-gluten) present in wheat flour exhibit high levels of intrinsic allergenicity *in vitro* as well as *in vivo* in humans. Wheat flour is not consumed as such but is subjected to hydration and thermal processing (with or without pressure) or to fermentation followed by thermal processing. Wheat-based food further undergoes gut digestion upon consumption. Not all studies have done this. Therefore, ideally future studies on *in vitro* assessment of the effect of food processing on wheat allergenicity should involve not only testing the IgE binding capacity of processed wheat but also the effect of gut digestion (acidic pH plus gut enzymes) on processed wheat products.

Upon fermentation with baker's yeast, allergenicity of wheat proteins in dough is not reduced in a significant way. When fermented dough is baked to make conventional bread, major changes in the allergenicity of wheat proteins occur. Allergenicity of albumins and globulins is significantly reduced. Gluten proteins in the breadcrumb also lose a significant degree of allergenicity. However, newer and large-sized gluten allergens are formed within the bread crust (Simonato et al., 2001). Digestion studies have shown that the albumins, globulins, and gliadins, but not glutenins, present in the breadcrumb are susceptible to human digestive pH and enzymes and therefore lose much of their allergenicity during digestion. In contrast, gluten proteins present in the bread crust resist digestion. Furthermore, non-gluten proteins extracted from commercial bread (crust and crumb combined) also appear to have reduced allergenicity in skin prick testing. These pieces of evidence together suggest that the conventional breadcrumb may have lower potential to cause sensitization and disease elicitation. Therefore, this needs to be tested in pre-clinical and clinical studies.

Sourdough breads prepared using cocktails of selected strains of LAB (alone or along with yeast) show markedly reduced allergenicity *in vitro*; the extent of reductions is much more dramatic than the effects seen in conventional bread studies (**Fig. 3.1, Tables 3.3, 3.5**). Interestingly, all the residual allergens (albumins, globulins, and gliadins) present in such sourdough breads are much more sensitive to gut digestion compared to that of the conventional yeast-based breads (De Angelis et al., 2007). Therefore, such sourdough bread is expected to have dramatically reduced ability to cause sensitization and disease elicitation compared to the conventional bread. Therefore, future pre-clinical and clinical testing of such product is warranted.

Based on *in vitro* ELISA testing, researchers have reported that Japanese soy sauce products contain no detectable gluten nor non-gluten wheat allergens (**Table 3.1**). Therefore, these products may be tolerated by all wheat allergic subjects. Furthermore, these products may also be incapable of causing allergic sensitization. However, pre-clinical and clinical testing of these products in wheat allergic patients have not been reported so far. It is important to conduct such studies urgently as the *in vitro* data is very compelling. Since these products may vary widely based on the producer, it is also important to evaluate whether other types of soy sauce products (prepared by different methods, other companies, other countries) are also hypo/non-allergenic.

It is noteworthy that the fermentation studies (except soy sauce studies, that tested both soluble and insoluble wheat allergens) extracted and tested the soluble proteins in IgE binding. Therefore, the question remaining to be clarified is whether or not the insoluble proteins present, the ones not extractable by the methods used, also show reduced allergenicity. This can be tested by conducting oral feeding of the food slurries (without extracting any specific proteins) in validated animal models and eventually in humans. Thus, fermentation of wheat using LAB plus

yeast (as in sourdough bread), and mold plus LAB plus yeast (as in soy sauce), provide highly promising methods to create potentially hypo/non-allergenic wheat products.

Several commercial baked wheat products in addition to the bread have been tested for allergenicity of non-gluten proteins. Interestingly, reports show that non-gluten protein obtained from toasted bread exhibited no allergenicity and the allergenicity of non-gluten protein from pizza crust, baby cereal, and breakfast cereal were minimal (**Tables 3.4 & 3.5**; De Gregorio et al., 2009). Although these results are encouraging, a key question would be how much of the non-gluten protein was actually extractable from such products since the processing conditions may make the proteins insoluble and non-extractable with saline. Moreover, such non-extractable proteins may still be allergenic or even worse, could be hyper-allergenic due to the creation of high temperature-induced mega allergens. Nevertheless, if all the non-gluten family of allergens had been indeed destroyed, such products might be tolerated by those who are allergic only to non-gluten wheat allergens. Therefore, further *in vivo* testing of these products would be critical to validate the results.

Available evidence shows that commercial raw pasta is intrinsically as allergenic as the raw flour. However, boiled pasta appears to lose up to half of the allergenicity of its extractable non-gluten proteins. Furthermore, digestion using gut enzymes almost completely eliminated allergenicity of the albumins, globulins, gliadins and glutenins present in the cooked pasta made using relatively lower drying temperature. Therefore, available evidence suggests that lower temperature drying conditions of pasta must be precisely defined to favor the creation of potentially hypo-allergenic products.

There is compelling evidence that treatment of soft wheat flour with non-digestive enzymes (bromelain, and actinase) can produce hypo-allergenic wheat flour that can be used to make bread

and cupcake products. Furthermore, such a cupcake is not only tolerated well by most children with atopic dermatitis (7 out of 9 tested), but also subsequently induce immune tolerance in more than half of the patients to enable consumption of regular wheat products. Although this was a small study, it supports a strong rationale to conduct further pre-clinical and clinical testing of such products in the immediate future.

Boiling of wheat is a very commonly used processing method. Evidence shows that both gluten and non-gluten wheat allergens resist degradation upon boiling and retain their allergenicity *in vitro;* however, boiling abolishes allergenicity of purified gliadins *in vitro* (**Table 3.4**). In the real world, consumers are typically exposed to gliadin in a food matrix. These studies together suggest that extrapolating data from pure protein studies to the whole foods can be misleading. Therefore, to determine the real effects of food processing on wheat food products, testing of wheat protein allergenicity must be done using the wheat food rather than purified single wheat allergens. Nevertheless, when purified wheat proteins are intended for use as a food ingredient or as an ingredient in cosmetic products, testing the allergenicity of those purified proteins might be appropriate.

3.6. Future directions and suggested research agenda

Overall, we found evidence that: 1) in contrast to the effect of food processing on other allergenic foods such as peanuts and tree nuts, effect on wheat allergenicity has been modestly studied (Ortiz et al., 2016; Palladino & Breiteneder, 2018; Zhang et al., 2018); 2) effects of processing have been studied mostly using *in vitro* methods; 3) food processing has significant but variable effects on wheat allergenicity, with reports showing anywhere from a reduction in allergenicity or even abolishment of allergenicity of specific wheat allergen protein families, all

the way to increasing allergenicity by creating newer and larger mega allergens; 4) among the processing methods, fermentation and enzyme hydrolysis offer the strongest promise to produce novel hypo/non-allergenic wheat proteins and products; however pre-clinical and clinical validations are urgently needed; and 5) several scientific concepts can provide fertile ground for future research and development to create hypo/non-allergenic wheat proteins for applications in food, medical, and cosmetic industries (Kohno et al., 2016).

This review shows that there is significant potential for creating novel hypo/non-allergenic wheat products by optimizing specific food processing methods. These findings also inform the following specific scientific concepts for research and development in this exciting area of food science:

1) Establishing professional guidelines for the pre-clinical and clinical testing of potentially hypo-allergenic wheat products. To advance the goal of creating such products for wheat allergic patients, as well as for the general public, precise professional guidelines for pre-clinical and clinical testing including definitions for hypo/non-allergenicity, similar to the model developed for hypo-allergenic infant milk products, are immediately needed.

2) The in vivo validation of the effects of processing on wheat allergenicity. Currently, the majority of studies have reported *in vitro* data on allergenicity. Although valuable as a first level screening method, such methods must be followed by pre-clinical testing (in appropriate animal models) and human clinical trials (Gonipeta, Kim, & Gangur, 2015; Jin et al., 2019). Previous studies have already identified few potential hypo/non-allergenic wheat products (e.g., Japanese soy sauce, VSL#3 sourdough bread, cupcakes from actinase treated wheat flour etc.). Oral challenge studies can be conducted in both animals and humans to determine the allergenicity of processed wheat foods vs. extracted allergens so that a comprehensive picture on the effect of

processing can be drawn. For testing allergenicity of processed wheat proteins used in skin care products, appropriate dermal exposure allergenicity studies may be conducted using an adjuvant-free model described recently (Jin et al., 2020).

3) Does food processing differently affect the allergenicity of wheat products prepared from different wheat species, varieties, and accessions? Commonly consumed wheats belong to different species, varieties, and accessions that are expected to be different in the allergenic protein contents and their susceptibility to the effects of food processing (Gao et al., 2019; Nakamura, Tanabe, Watanabe, & Makino, 2005). Published studies do not always mention the genetic details of the wheat used. To advance the goal of consistently producing hypo-allergenic wheat products, it will be important to relate the effects of food processing at each of these genetic (i.e., species, varieties, and accessories) level (Gao et al., 2019; Nakamura, Tanabe, Watanabe, & Makino, 2005).

4) Does processing alter the sensitization capacity of wheat allergens and convert them to immune tolerance-inducing wheat proteins? The immune process of wheat allergy involves the first phase of sensitization (i.e., production of IgE antibodies) to wheat allergens followed by the second phase of IgE binding with consequent clinical reactions upon re-exposures (Renz et al., 2018). Currently, wheat allergenicity studies have mostly used the IgE binding methods to assess allergenicity, which relates to second phase. It will be important to determine whether hypo/non-allergenic wheat products will be inferior in eliciting sensitization in healthy subjects, and whether they will be able to desensitize wheat allergic subjects by inducing immune tolerance as suggested by the hypo-allergenic cupcake study (Tanabe, 2008; Yamamoto et al., 2004). Future studies should evaluate the immunotherapy-relevant effects of processing on wheat allergens.

5) Determining the effects of additional methods of processing on wheat allergenicity. All reported studies reviewed in this paper examined the effects of the commonly used methods of

thermal processing, fermentation, and enzyme/acid hydrolysis on wheat allergenicity. There is ample opportunity to test the effects of several other methods of processing, including extrusion processing, high pressure processing, plasma treatment, ultraviolet treatment, etc., in future studies (Boreddy, Rose, & Subbiah, 2019; Kim, 2017).

3.7. Conclusions

There is significant evidence in the literature to suggest that wheat allergenicity can be altered (increased, decreased, or abolished) using common food and industrial processing methods. There is significant potential for creating novel hypo/non-allergenic wheat products using optimal fermentation and enzyme hydrolysis methods. Almost all of the studies (with the exception of 3) have evaluated the allergenicity of processed wheat products using *in vitro* IgE binding methods. Furthermore, the effect of processing methods on the sensitization capacities of wheat allergens has not been widely studied. Current focus has been largely to study the effects on binding of IgE antibodies from wheat allergic subjects, and in just three cases allergic reaction elicitation. Therefore, future work is required to validate the effects of processing on wheat allergenicity, both sensitization (i.e., *de novo* IgE production) as well as disease elicitation, by conducting pre-clinical testing using appropriate animal models and finally human clinical trials. These concepts provide fertile grounds for future research and development to create hypo/non-allergenic wheat proteins for applications in the food, medical, and cosmetic industries. Availability of hypo-/non-allergenic wheat products is expected to increase the consumer-base for the wheat industry which is seriously challenged currently by the ongoing increases in adverse health effects of wheat products on a global scale (Yang & Kulis, 2019). Furthermore,

such efforts will be consistent with the emerging concepts of precision food, nutrition, and health as envisioned by the US National Institutes of Health (NIH, 2021)

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CHAPTER 4 DEVELOPMENT AND VALIDATION OF A MOUSE-BASED PRIMARY SCREENING METHOD FOR TESTING RELATIVE ALLERGENICITY OF WHEAT PROTEINS FORM DIFFERENT GENOTYPES

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4.1. Abstract

Wheat allergy is one of the major food allergies that have reached significant levels of global public health concern for reasons that are not completely understood. There are many genetically diverse types of wheat that have been cultivated over the human history. However, potential variation in allergenicity among genetically different wheat is not well studied due to the unavailability of validated methods at present. Here, we developed and validated a novel mouse-based primary screening method for testing relative allergenicity of wheat proteins from three different genotypes. Groups of Balb/c mice weaned on to a plant free diet were sensitized with salt-soluble protein (SSP) extracted from AABB genotype of wheat (durum, carpio variety). Clinical sensitization for anaphylaxis was confirmed by intraperitoneal challenge. Mice were then boosted 7 times over a 6-month period. Bi-weekly blood samples were collected to create an IgE-enriched mini-plasma bank. Using this plasma, wheat specific IgE-inhibition (II)-ELISA was optimized. The optimized II-ELISA was used to determine the relative allergenicity of tetraploid (AABB), hexaploid (AABBDD) and diploid (DD) wheat genotypes. The IgE-inhibition curves

were established to estimate the IC_{50} and IC_{75} values for the SSPs from three wheat genotypes. The sensitized mice showed robust IgE response and marked anaphylaxis upon challenge. The IgE antibody titer of the mini-plasma bank was 2560. The plasma dilution of 1/80 was identified as suitable for II-ELISA development. An II-ELISA was optimized with an inhibition time of 2.5 hours and a co-efficient of variation of <2%. Primary screening for relative allergenicity demonstrated that IgE binding to AABB-SSP was significantly abolished by the other two wheat genotypes as follows: AABBDD-SSP by 91-94% and DD-SSP by 83-85%. On an average, compared to AABB, the relative allergenicity of AABBDD and DD were significantly lower by ~7% and ~17% respectively ($p < 0.01$). Furthermore, IgE inhibition curves showed significant differences in IC_{50} and IC_{75} values for the three wheat genotypes. We report a novel mouse-based primary screening method of testing relative allergenicity of wheat proteins from three different wheat genotypes for the first time. As part of a broader *comprehensive tool kit* for allergenicity testing of wheat proteins, this method will be useful as a primary screening tool to monitor the changes in allergenicity of novel types of wheat produced by genetic modification and differently processed wheat products.

4.2. Introduction

Wheat allergies are a major type of food allergy that affects both children and adults in many countries around the world (Sicherer and Sampson, 2018; Renz et al. 2018; Cianferoni 2016). They are increasingly recognized as a growing public health problem of global significance because they not only affect millions of wheat consumers but also adversely affect the wheat industry and the global economy (Scherf et al. 2016; Cianferoni 2016; Shewry 2018). The overall

prevalence of wheat allergy is estimated to be up to 1-3% in the USA and up to 0.9% at the global level (Cianferoni 2016; Leonard et al. 2014; Venter et al. 2006a, 2006b).

Wheat allergy, similar to other food allergies, is thought to develop in genetically susceptible individuals in two phases: sensitization phase and disease elicitation phase (Sicherer and Sampson, 2018). During the sensitization phase, IgE antibodies are produced against wheat allergens and these IgE antibodies bind to the mast cells *via* the high affinity IgE receptor (Sicherer and Sampson, 2018; Renz et al. 2018). Such subjects are deemed sensitized to wheat. In the second phase, exposure of sensitized subjects to wheat results in binding of allergens to the IgE on mast cells and basophils resulting in activation and release of mediators from these immune cells causing clinical disease (Sicherer and Sampson, 2018; Renz et al. 2018).

Wheat allergy disease can manifest at least in three ways: 1) classical wheat food allergy with symptoms of vomiting, diarrhea, atopic dermatitis, or life-threatening anaphylaxis after consumption of wheat; 2) airways allergies (allergic rhinitis, baker's asthma) and eye allergy (allergic conjunctivitis) among wheat industry workers (e.g., bakery, pizzeria etc.,); and 3) wheat-induced exercise-dependent anaphylaxis, when sensitized subjects develop disease doing exercise immediately after eating wheat products (Cianferoni 2016; Leonard et al. 2014). The binding of mast cell surface attached IgE antibody to the wheat allergenic proteins is central to eliciting allergic disease (Cianferoni 2016). Consequently, allergenicity of proteins is typically measured by their ability to bind to the IgE antibody in ELISA or Western blot methods (Pastorello et al. 2007; Nakamura et al. 2005; Mohan Kumar et al. 2017).

There are five distinct wheat genotypes known to contribute to the genetic diversity of the wheat crop (Shewry 2018). They are AA, BB (extinct today, SS is the closest relative available), DD, AABB, and AABBDD (Shewry 2018). Among them, the last two genotypes are most

commonly used to produce wheat-based food and animal feed. In addition, using these genotypes, wheat breeders have successfully developed thousands of wheat varieties and wheat lines (Shewry 2018; Mishra and Arora, 2017). Furthermore, currently efforts are also underway to genetically modify and produce engineered wheat lines (Mishra and Arora, 2017; Hellemans et al. 2018; Rey et al. 2015; Kohno et al. 2016). Thus, there is tremendous genetic diversity in the wheat crop currently cultivated for human and animal consumption.

It is noteworthy that despite this genetic diversity of wheat, the plausibility of differences in allergenicity of among genetically distinct wheat is not well studied at present (Larre et al. 2011, Nakamura et al. 2005; Mohan Kumar et al. 2017). The major reason being the unavailability of a validated primary screening method to compare the relative allergenicity of wheat proteins obtained from different genotypes of wheat. Such a method is urgently needed because it will help to identify not only the historical changes in the allergenicity of different wheat genotypes--if at all that has had happened, but also to monitor potential future changes in the wheat allergenicity due to ongoing breeding/selection and genetic engineering of wheat (Larre et al. 2011; Mishra and Arora, 2017; Kohno et al. 2016). Furthermore, food-processing methods have been shown to alter food allergenicity including wheat allergenicity (Phromraksa et al. 2008; Vanga et al. 2017; Verhoeckx et al. 2015; Maleki and Hurlburt, 2004). A validated method will also be useful to assess the changes in allergenicity of differently processed wheat products.

In order to address this critical need in this area of cereal science, here, we developed and validated a novel mouse-based primary screening method to determine relative allergenicity of wheat proteins obtained from three wheat genotypes—AABB, AABBDD, and DD.

4.3. Materials and methods

4.3.1. Chemicals and reagents

Biotin conjugated rat anti-mouse IgE paired antibodies and isotype standards (BD BioSciences, San Jose, CA); para-Nitrophenylphosphate (Sigma, St Louis, MO); streptavidin alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA); protein estimation reagents: bovine serum albumin standard and reagents A and B (Sigma, St Louis, MO).

4.3.2. Mice

Balb/cJ mice (female) weaned on to a plant protein-free diet (AIN-93M) were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were housed in the animal facility of the Trout Food Science and Human Nutrition Building at the Michigan State University. Mice were maintained on the plant protein-free diet (AIN-93M). All mice used in this study were 4-6 weeks old. All animal procedures used were in accordance with the Michigan State University policies.

4.3.3. Preparation of salt-soluble protein extract

The following wheat genotypes were used in this study: AABB (Durum, Carpio variety), AABBDD (bread wheat) and, DD (*Aegilops tauschii)*. Protein extraction was conducted following the standard published method (Jin et al. 2017; Tatham et al. 2000). Briefly, ten grams of flour in 100 mL of 0.5 M NaCl was stirred continuously for 2 hours at 20°C followed by centrifugation (5000 x g, 10 min) at 20°C. The supernatant was frozen overnight at -16°C and then freeze-dried. The protein contents of the reconstituted samples were quantified according to Bradford dye-binding method (Bradford 1976). All proteins were stored in aliquots at -70°C.

4.3.4. Sensitization and quantitation of systemic anaphylaxis and mucosal mast cell degranulation responses

Mice were sensitized with the salt-soluble protein (SSP) extract obtained from AABB wheat (durum wheat, Carpio) as described before (Jin et al. 2017). Briefly, groups of mice (n=5/group, total 4 groups, 20 mice) were injected by intraperitoneal (IP) route four times (days 0, 10, 24, 40) with the SSP (0.01 mg/mouse/injection) plus alum (1 mg/mouse). Blood was collected from the saphenous vein on days 26 and 46 after the first injection and used to measure wheat specific IgE antibody. Clinical sensitization was confirmed by IP challenge with SSP (0.5 mg/mouse) and hypothermia shock responses were quantified. Mucosal mast cell degranulation responses were quantified by measuring MMCP-1 protein elevation in the plasma after the challenge as described earlier (Jin et al. 2017).

4.3.5. Generation and characterization of a wheat-specific IgE antibody enriched mini-plasma bank

The overall approach used to generate the mini-plasma bank is shown in Figure 4.1. After confirmation of the clinical sensitization for anaphylaxis, mice were boosted 7 times over a 6-month period. Blood was collected at bi-weekly intervals and pooled to create the AABB mini plasma bank. Aliquots of plasma were stored at -70°C.

The wheat-specific IgE antibody titer of the plasma bank was determined using an optimized wheat-specific IgE ELISA (Jin et al. 2017, Birmingham et al. 2003). Briefly, ELISA plates were coated with SSP (5 mg/mL) in coating buffer (sodium bicarbonate buffer, pH 9.6) at 4^{o}C for 18 hours followed by blocking with 5% gelatin in phosphate buffered saline (PBS), pH 7.4 at 37^{o}C for 3 hours. After 3 times washing (0.05% Tween 20 in PBS), plasma samples were

added in triplicates and a two-fold dilution was conducted in dilution buffer (0.085% BSA in 0.05% Tween 20 in PBS, pH 7.4) and incubated at 4^{o}C for 18 hours. After washing the plates for 3 times, a biotin-labelled anti-mouse IgE antibody (BD Biosciences) was added and incubated at 4^{o}C for 1.5 hours. Plates were washed three times and then streptavidin alkaline phosphatase conjugate was added and incubated at 37^{o}C for 0.5 hour. After three times washing, the para-nitro phenyl phosphate substrate was added and the plates were allowed to develop for 6 and 21 hours. The Optical Density (OD) was read at 405-690 nm using a spectrophotometer (BioTek, Synergy HT). The IgE antibody titer was defined as the reciprocal of the highest plasma dilution that shows positive signal at or above the background ± 3 SD (Birmingham et al 2003).

The intra-assay variation of the wheat specific IgE ELISA was determined by conducting the ELISA with an identical sample in triplicates. The entire experiment was repeated thrice. Variation in the OD was measured and used in estimation of intra-assay coefficient of variation. The inter-assay variation of the wheat specific IgE ELISA was determined by conducting three identical experiments on three different days be the same individual. Variation in the OD between the two independent experiments were measured and used in estimation of inter-assay coefficient of variation.

4.3.6. Optimization and validation of an IgE inhibition (II)-ELISA for wheat protein

ELISA plates were coated with 5 mg/mL of AABB salt-soluble protein (SSP) in coating buffer (sodium bicarbonate buffer, pH 9.6) at 4oC for 18 hours. Blocking was done at 37oC for 3 hours with 5% gelatin. After 3 times washing (0.05% Tween 20 in PBS), plasma sample was added in various dilutions from 1/80 for obtaining the titration curve. Parallel plasma samples were mixed with AABB protein (at 0.5 mg/mL) and incubated for 0.5, 1 and 2.5 hours for IgE binding.

At the end of incubation time, samples were centrifuged at 500x g for 20 seconds. Supernatant was collected and added to the already blocked ELISA wells at the final dilution of 1/80. Control plasma samples without pre-incubation with the AABB protein were added to the control wells. The ELISA plate was incubated at 4^{o}C for 18 hours. After washing, anti-mouse IgE antibody labelled with biotin was added and incubated at 37^{o}C for 1.5 hours. After washing streptavidin alkaline phosphatase conjugate was added and incubated at 37^{o}C for 0.5 hour. After washing, the para-nitro phenyl phosphate substrate was added and the plates were allowed to develop for 6 and 21 hours. The optical density (OD) was read at 405-690 nm (Bio-Tek Synergy HT).

The IgE inhibition results were expressed as % B/B_0, where B corresponds to the specific IgE-binding to immobilized AABB-derived salt-soluble protein when a known concentration of the inhibitor protein is present, and B_0 corresponds to the binding in the absence of inhibitor.

For determination of intra-assay variation of IgE inhibition (II)-ELISA, plasma was used in various dilutions in triplicates and II-ELISA was conducted. Entire experiment was repeated thrice. Variations in OD (405- 690 nm) at 6 and 21 hour reading were measured and used in calculation of intra-assay coefficient of variation. To determine inter-assay variation two experiments were conducted by the same person on two different days. The variation in OD (405-690 nm) at 6 and 21 hours readings were measured and used in calculation of inter-assay coefficient of variation.

4.3.7. Determination of relative allergenicity among the three wheat genotypes

The percent IgE inhibition obtained using AABB as the inhibitor was used as used as 100% allergenicity control and allergenicity of other genotypes relative to this control were determined.

The SSP extracted from Ambassador common wheat was used as a model AABBDD hexaploid genotype for allergenicity determination in the II-ELISA. The SSP extracted from *Ae. tauschii* was used as a model DD diploid genotype for allergenicity determination in the II-ELISA. In these experiments, SSP from these genotypes were pre-incubated at 5 and 1 mg/mL with the AABB-specific plasma (at 1/80) and the IgE inhibition was determined. The relative IgE inhibition percent were calculated using the AABB data as the 100% allergenicity control.

4.3.8. Determination of inhibition concentrations of three wheat genotypes using IgE inhibition curves

Using various concentrations of AABB, AABBDD and DD derived SSP as IgE inhibitors, II-ELISA were conducted. The % B/B_0 values were used to construct the IgE inhibition curves. The concentration of inhibitor that inhibits 50% (IC_{50}) and that inhibits 75% (IC_{75}) of the IgE binding to the immobilized AABB-SSP was determined. In this analysis, an increase in IC_{50} and IC_{75} values correlate with reduced allergenicity of the protein used as inhibitor.

4.3.9. Statistical analysis

Comparison of two groups for significance was done using Student's t-test. For multiple group comparisons, Tukey's test was used. Online software service was used in these analyses (http://www.socscistatistics.com/tests/pearson/). The statistical significance level was set at 0.05.

4.4. Results

4.4.1. Development of a wheat specific IgE antibody enriched mini-plasma bank.

We used a previously published mouse model of wheat allergy to generate a colony of wheat allergic mice (n=20). All mice were tested and confirmed for elevation of wheat specific IgE antibodies. Clinical sensitization for systemic anaphylaxis was confirmed by intraperitoneal challenge with SSP (0.5 mg/mL) and by measuring plasma mMCP-1 protein as described (Jin et al. 2017). The overall method used is shown in **Fig. 4.1A**. These mice then received repeated booster injections (7) without alum adjuvant. By repeated bi-weekly bleedings and pooling the harvested plasma, a genotype specific mini-plasma bank consisting of ~12 mL of hyper IgE plasma was established.

4.4.2. Characterization of the wheat specific IgE antibody enriched mini-plasma bank.

Using a previously published wheat specific IgE ELISA method, the IgE antibody titer of the plasma was determined (Birmingham et al. 2003). As evident, the repeated analysis demonstrated IgE antibody titer of the mini plasma bank to be 2560 (**Fig. 4.1B**). We then determined the inter and intra-assay variation of this analysis. The coefficient of variation (CV) for intra-assay and inter-assay analyses were 2.27% ±0.76% and 8.75% respectively (**Fig. 4.1C, D**).

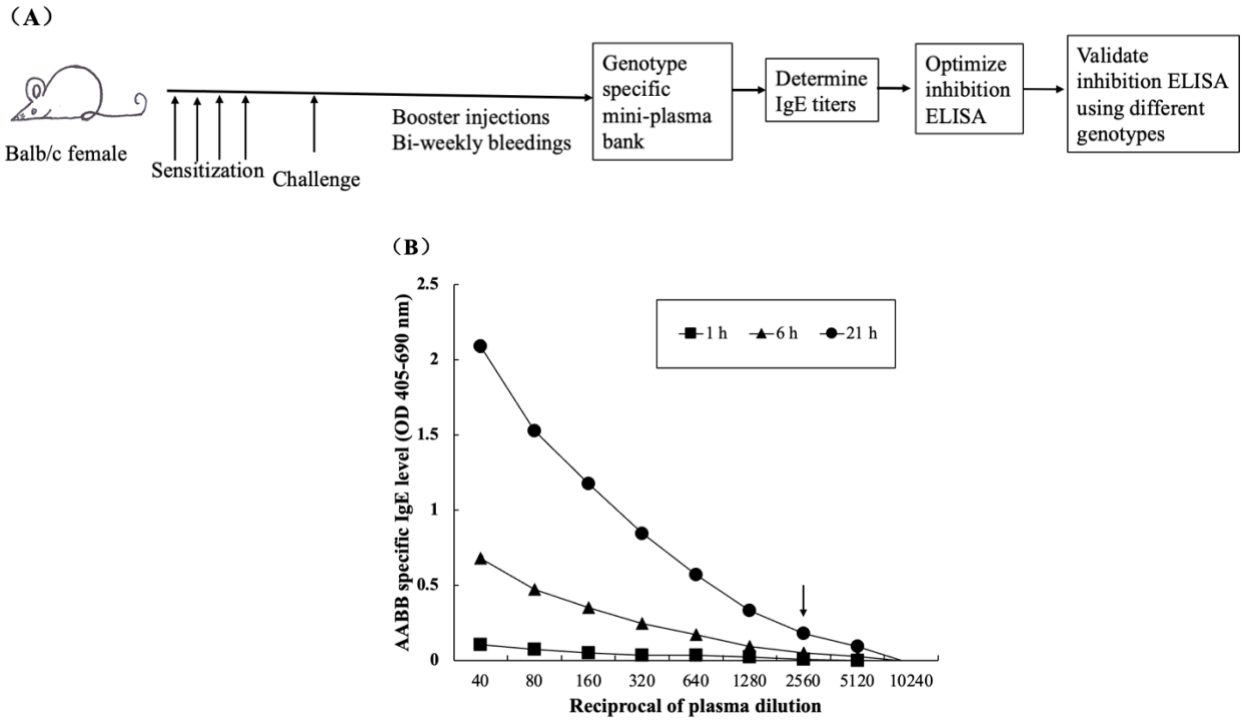

Figure 4.1. Construction and characterization of AABB wheat specific IgE antibody-enriched mini-plasma bank. Fig. 4.1A Groups of Balb/c mice (n=5/group, 4 experiments) were sensitized with purified salt-soluble wheat protein extract (10 ug) from AABB wheat (durum, Carpio) plus alum (1 mg) using the protocol as described in Jin et al (2017). Animals received 7 booster injections with the protein extract over a 6-month period. Blood collected at bi-weekly intervals was used to separate plasma and pooled to prepare a mini-plasma bank and IgE antibody titer determined. Aliquots of plasma was stored at -70°C. **Fig. 4.1B** shows determination of wheat specific IgE antibody titer in the mini-plasma bank. Various dilutions of plasma (as shown) in quadruplicate were used to determine specific IgE antibody titers using a ELISA based method described earlier (Jin et al 2017, Birmingham et al 2003). Data is shown as mean ± SE. Each line shows readings at indicated time point. Antibody titer (down arrow) was determined as highest dilution of the plasma showing OD of more than mean + 3 standard deviation of the background activity as described earlier (Jin et al 2017, Birmingham et al 2003). Determination of intra-assay and inter-assay variation of wheat specific IgE ELISA.

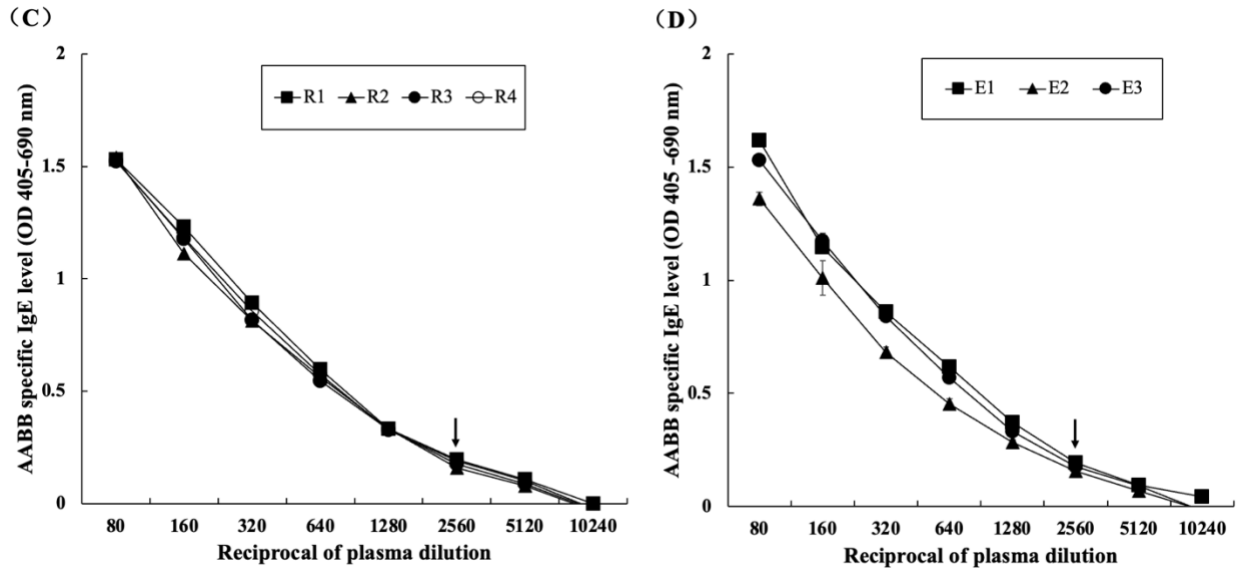

Figure 4.1. (cont'd) Fig. 4.1C shows determination of intra-assay variation; plasma was used in various dilutions as shown in quadruplicates. Figures show variation in the OD at 21 hour reading. **Fig. 4.1D** shows determination of inter-assay variation; ELISA was conducted on different days using the plasma sample at indicated dilutions. Figures show variation in the OD at 21 hour reading from 3 independent experiments (E1, E2, and E3). The vertical downward arrow corresponds to the titer of IgE antibody in the plasma.

4.4.3. Optimization of an IgE inhibition ELISA for wheat protein

Based on the wheat specific IgE ELISA, the plasma dilution of 1/80 that provided a robust signal within the linear portion of the titration curve, was identified as the appropriate plasma dilution to use in the optimization of an IgE inhibition (II)-ELISA (**Fig. 4.1B-D**). First, we determined the optimal pre-incubation inhibition time by pre-treatment of plasma with AABB-SSP at two difference concentrations (0.5 and 1 mg/mL) for 0.5, 1 and 2.5 hour. Results of residual IgE binding are shown at both 6 hour reading time (**Figs. 4.2A, B**) and at 21 hour reading (**Fig. 4.2C, D**). We then calculated the % B/B_0 using these data (**Fig. 4.2E-H**). As shown, the residual IgE binding significantly decreased ($p < 0.05$) as inhibition time increased from 0.5 to 1 hour; there was no significant change between 1 and 2.5 hours although the latter

time was a little better. The same conclusions were drawn based on the % B/B_0 analysis (**Fig. 4.2E-H**).

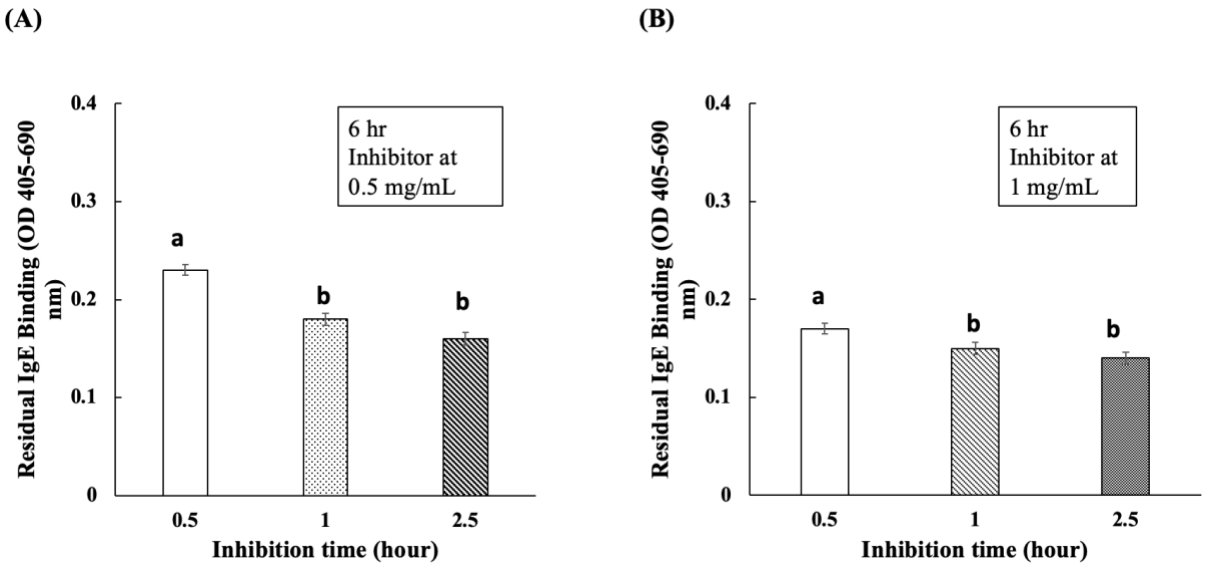

Figure 4.2. Optimization of wheat specific IgE inhibition ELISA. To determine optimal inhibition time, plasma was mixed with 0.5 mg/ml or 1 mg/mL of AABB-derived SSP and incubated for indicated time, and then centrifuged. Supernatant was collected and used in ELISA coated with AABB-derived SSP. (**Figs 4.2A-B)** show residual IgE binding after inhibition time (0.5, 1, and 2 hour) at 6-hour time point of plate reading;

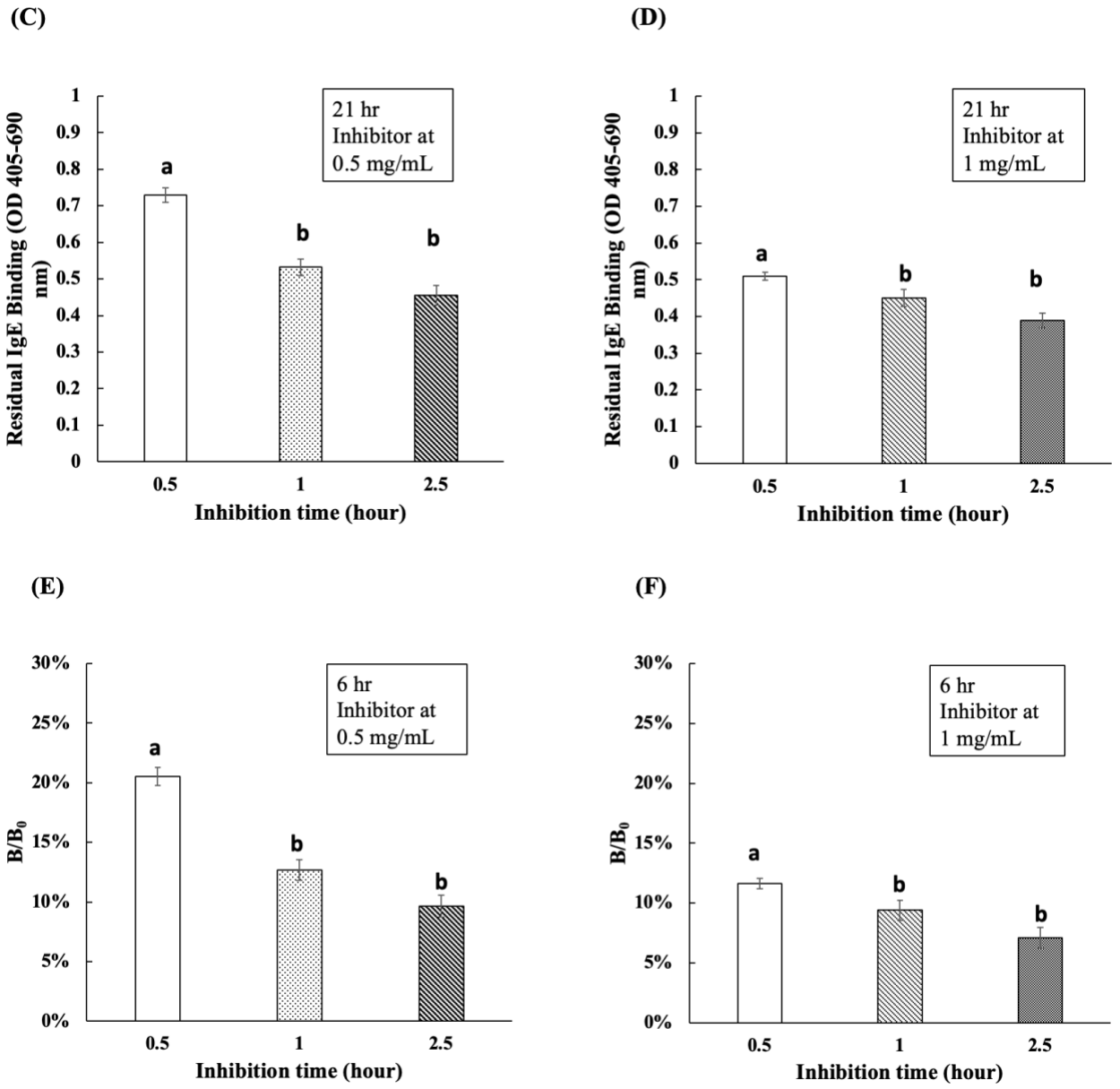

Figure 4.2. (cont'd) (**Figs 4.2C-D)** show residual IgE binding after inhibition time (0.5, 1, and 2 hour) at 21-hour time point of reading as detailed in the method section. Y-axis in figures A-D shows residual IgE binding as optical density (OD) at 405-690 nm. (**Figs 4.2E-F)** show %B/B_0 after inhibition time (0.5, 1, and 2 hour) at 6-hour time point of plate reading;

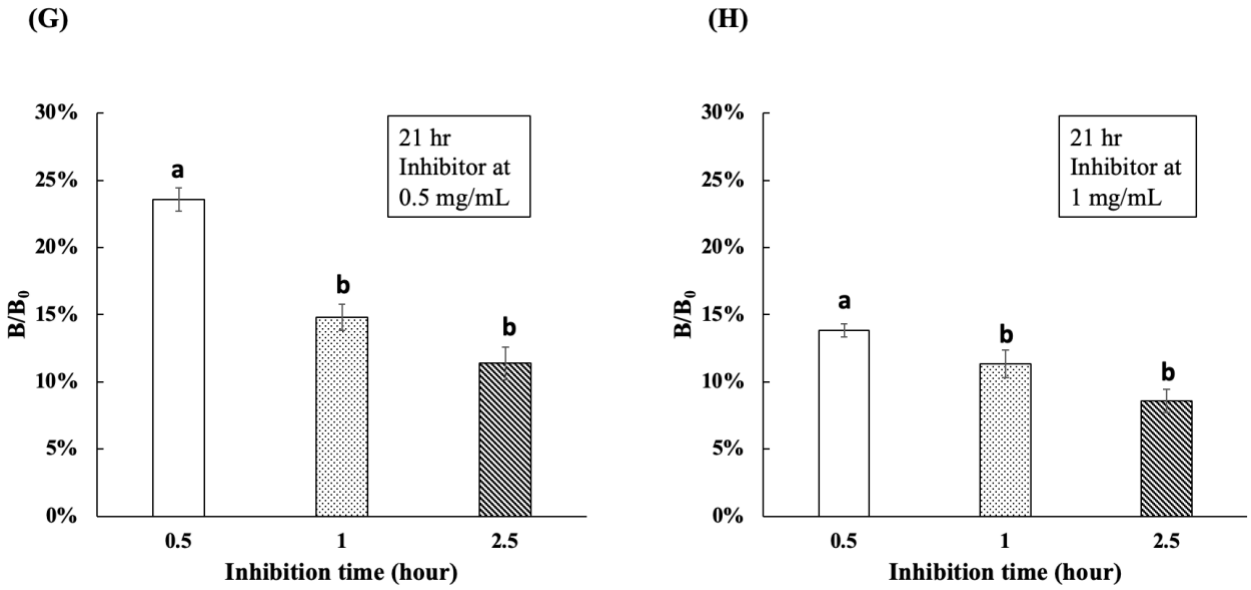

Figure 4.2. (cont'd) (**Figs 4.2G-H)** show %B/B_0 after inhibition time (0.5, 1, and 2 hour) at 21-hour time point of reading as detailed in the method section.

4.4.4. Inter-assay and intra-assay variation of the IgE inhibition ELISA for wheat protein

Then, we determined the intra-assay and inter-assay co-efficient of variation (CV) for the II-ELSIA method (**Fig. 4.3A, B)**. Variations observed among the replicates within an experiment conducted on a single day by an individual (intra-assay variation) for the optimized II-ELISA was acceptable with a CV of 1.77%. Variations observed between the experiments conducted on different days by an individual (inter-assay variation) also was acceptable with an inter-assay CV of 1.5%.

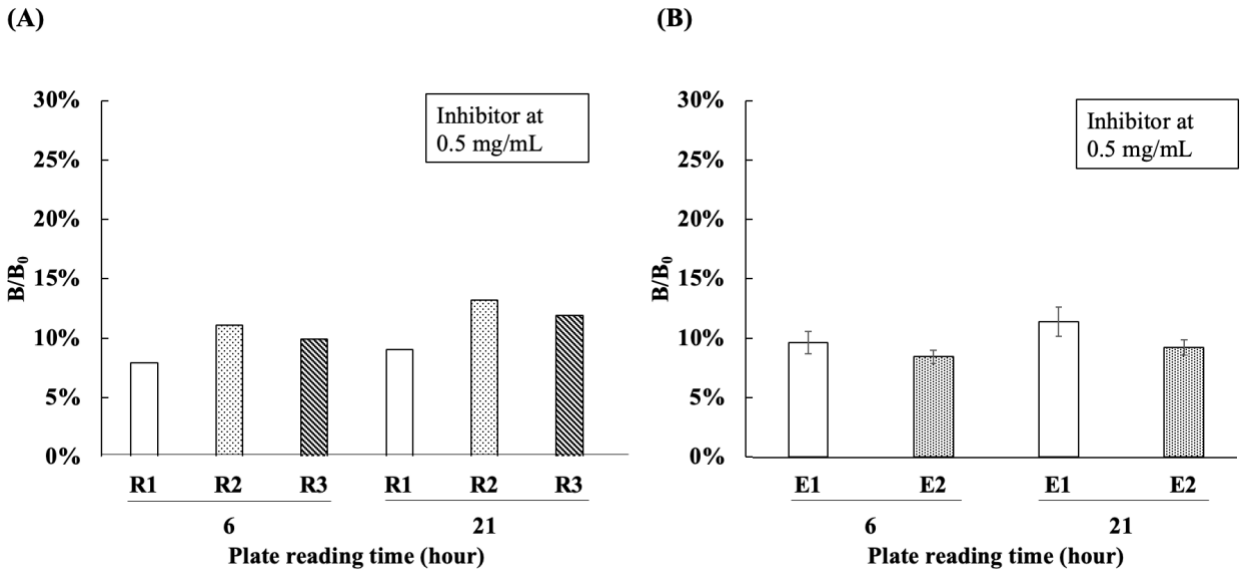

Figure 4.3. Determination of intra-assay and inter-assay variation of IgE inhibition ELISA. To determine intra-assay variation, plasma was used in various dilutions as shown in triplicates. **Fig. 4.3A** shows variation among 3 replicates in the percent B/B_0 at 6 and 21 hours reading. To determine inter-assay variation, ELISA was conducted on different days using the plasma sample at indicated dilutions. **Fig. 4.3B** shows variation among 3 replicates in the percent B/B_0 at 6 and 21 hours reading from two independent experiments (E1 and E2).

4.4.5. Determination of % B/B_0 for AABB, AABBDD and DD wheat genotypes.

Using the II-ELISA, the relative ability of the SSPs obtained from three wheat genotypes to inhibit IgE binding to AABB was quantified as described above. Using the method described earlier in the methods section, the $\%B/B_0$ values were determined. As evident, $\%B/B_0$ values were significantly different among the three wheat genotypes at both inhibitor concentrations (5 and 1 mg/mL) and at both reading time points (6 and 21 hours) ($p<0.01$, all comparisons) (**Fig. 4.4A-D**).

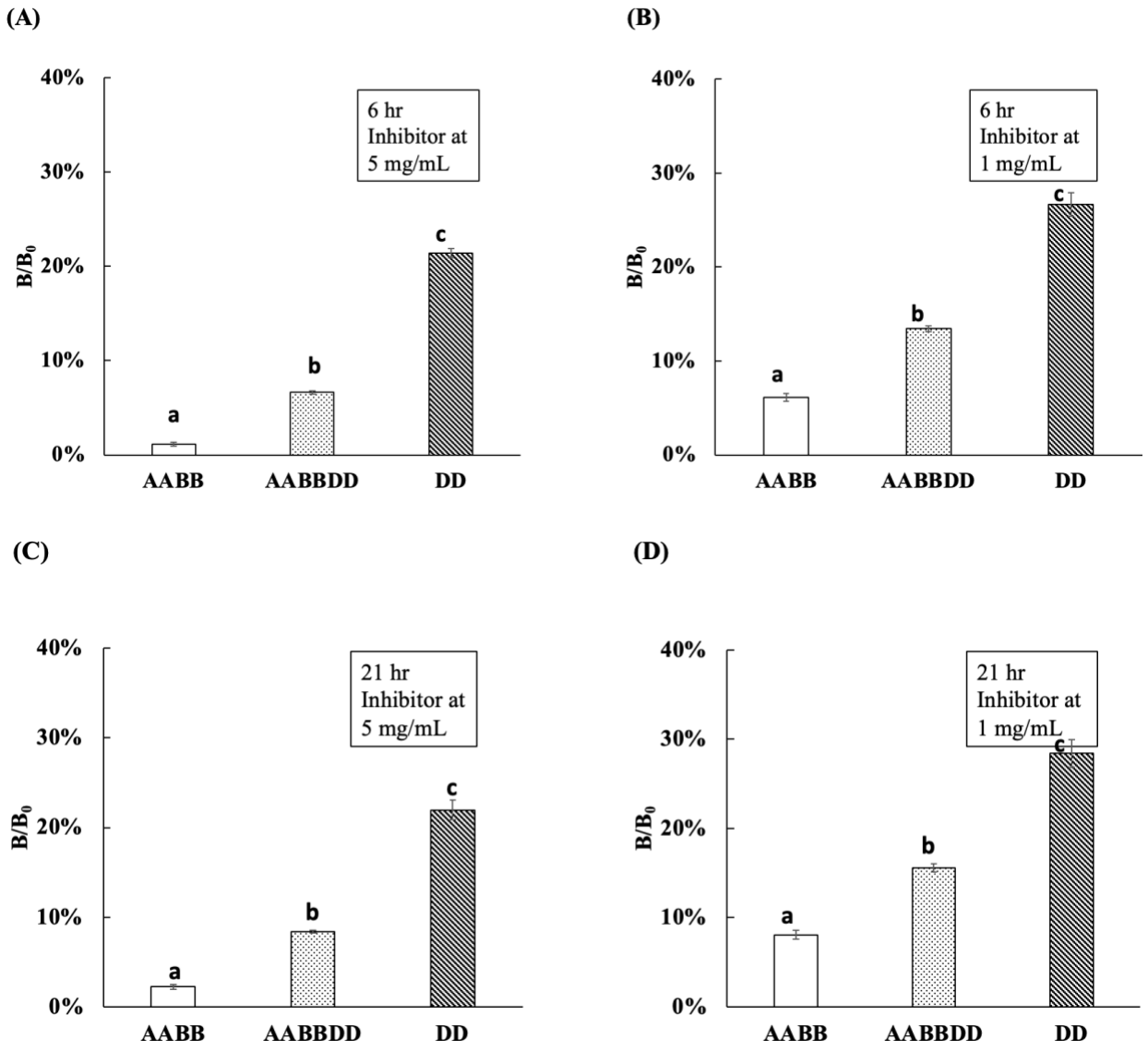

Figure 4.4. Determination of %B/B0 values for AABB, AABBDD, and DD genotypes based on II-ELISA. The IgE inhibition ELISA was conducted using AABB-SSP as coating antigen. The SSPs from AABB, AABBDD and DD wheat genotypes were used as IgE inhibitors by pre-incubating with AABB-plasma bank. **Figs 4.4A, B** show percent B/B_0 at inhibitor concentrations of 5 mg/mL and 1 mg/mL respectively using 6 hour reading. **Figs 4.4C, D** show percent B/B_0 at inhibitor concentrations of 5 mg/mL and 1 mg/mL respectively using 21 hour reading.

4.4.6. Determination of relative allergenicity of AABB vs. AABBDD and DD genotypes using the optimized II-ELISA.

Using the homologous IgE inhibition by AABB as 100% reference level for allergenicity, the relative allergenicity of AABBDD and DD genotypes were determined. As evident, relative to homologous IgE inhibition by AABB-SSP at 100%, the AABBDD-SSP abolished IgE binding by 92.21% ± 0.72% at 6 hour and by 91.85% ± 0.97% at 21 hour reading time with inhibitor concentration of 1 mg/mL and by 94.43% ± 0.06% at 6 hour and by 93.72% ± 0.15% at 21 hour reading time with inhibitor concentration of 5 mg/mL; overall, the average IgE inhibition was by ~93.05% (**Fig. 4.5A-D)**. Similarly, compared to the homologous IgE inhibition by AABB-SSP at 100%, the DD-SSP abolished IgE binding by only 82.66% ± 1.02% at 6 hour and by 79.86% ± 1.19% at 21 hour reading time with inhibitor concentration of 1 mg/mL and by 85.27% ± 0.83% at 6 hour and by 83.39% ± 0.71% at 21 hour reading time with inhibitor concentration of 5 mg/mL; overall, the average IgE inhibition was by ~82.8% (**Fig. 4.5A-D)**. Thus, on an average, compared to AABB, the relative allergenicity of AABBDD was lower by ~7% and the relative allergenicity of DD was lower by ~17%.

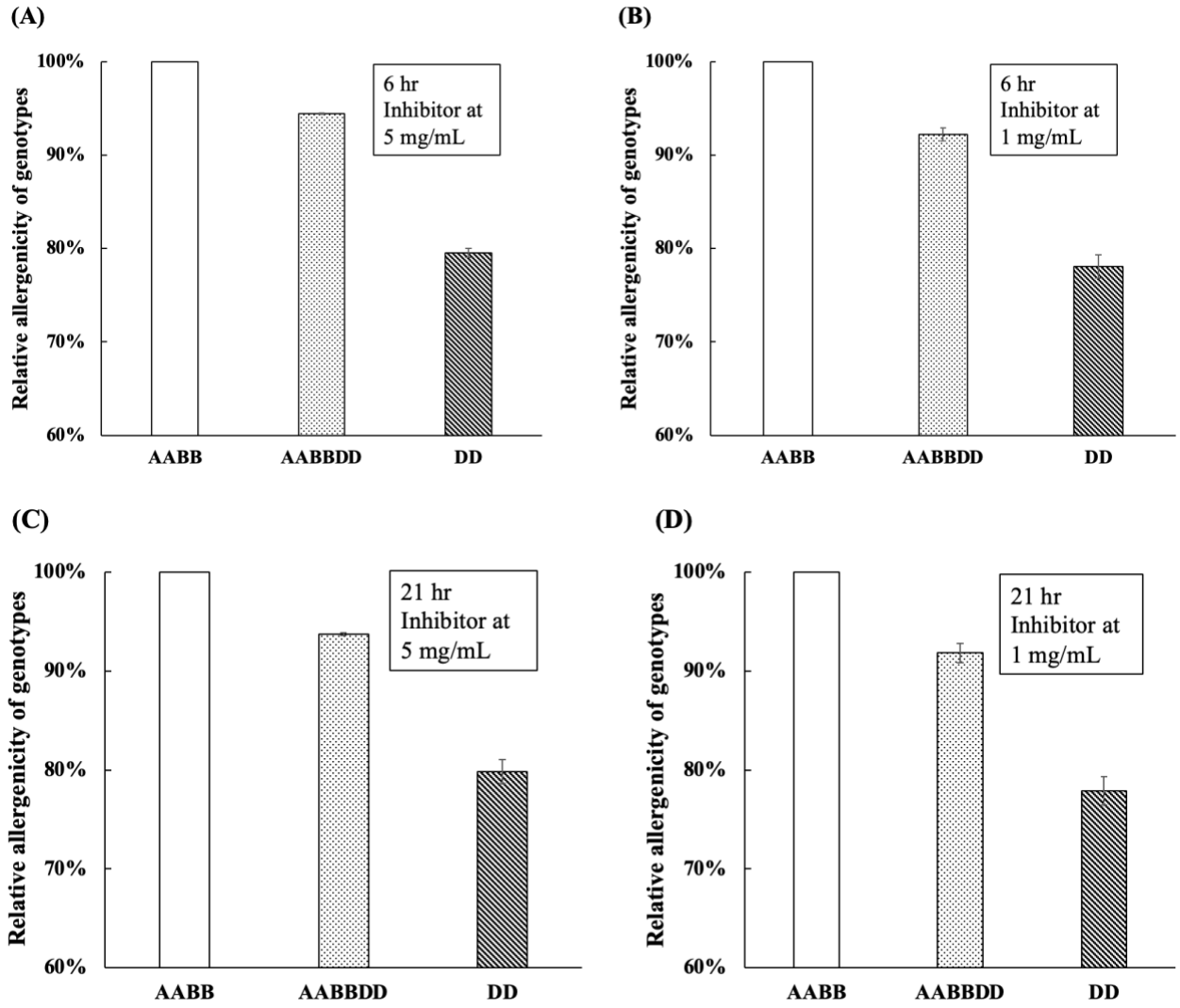

Figure 4.5. Determination of allergenicity AABBDD, and DD genotypes relative to AABB genotype. The IgE inhibition ELISA were conducted using AABB-SSP as coating antigen. The SSPs from AABB, AABBDD and DD wheat genotypes were used as IgE inhibitors by pre-incubating with AABB-plasma bank. Using allergenicity measurements obtained for AABB genotype as 100 % reference allergenicity values, the relative allergenicity of AABB and DD genotypes were calculated. **Figs. 4.5A, B** show relative allergenicity at inhibitor concentrations of 5 mg/mL and 1 mg/mL respectively using 6 hour reading. **Figs. 4.5C, D** show relative allergenicity at inhibitor concentrations of 5 mg/mL and 1 mg/mL respectively using 21 hour reading.

4.4.7. Establishment of IgE inhibition curves and estimation of IC50 and IC75 values for the three wheat genotypes.

We established dose-response IgE inhibition curves using the II-ELISA method for all three wheat genotypes (**Fig. 4.6 A-C**). Using these curves, IC50 and IC75 values were estimated and compared. As evident (**Table 4.1**), there was a significant difference in both IC_{50} and IC_{75} values among the three wheat genotypes ($p < 0.01$ all comparisons).

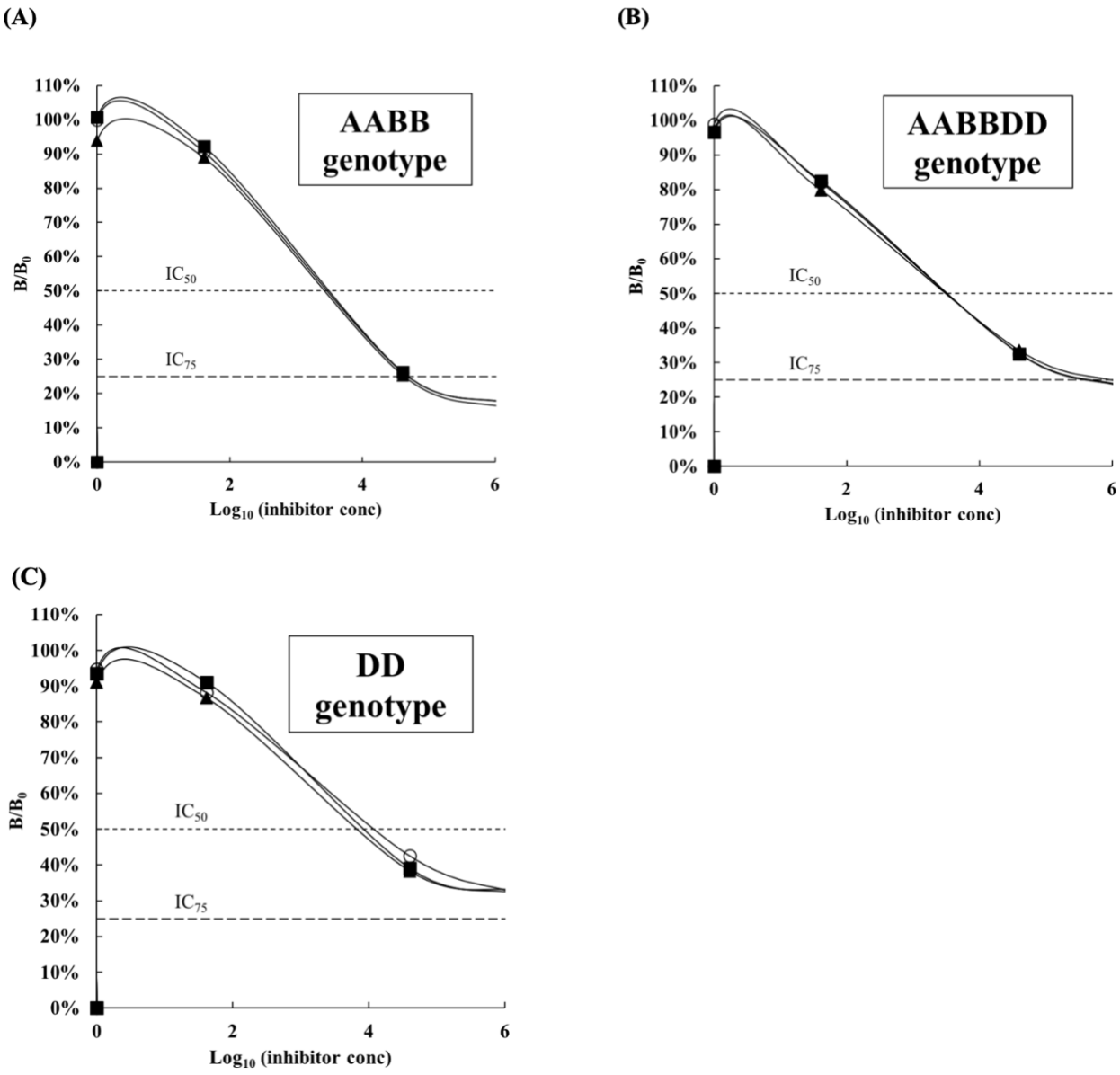

Figure 4.6. Determination of IC50 and IC75 values using IgE inhibition curves for three wheat genotypes. The IgE inhibition ELISA was conducted using AABB-SSP as coating antigen. The SSPs from AABB, AABBDD, and DD were used as IgE inhibitors by pre-incubating with plasma from the mini-plasma bank. Figs. 4.6A, B and C show inhibition curves for AABB, AABBDD, and DD genotypes respectively. Figures show percent B/B0 on y-axis at various concentrations of the inhibitors on the x-axis. The broken horizontal lines show the IC50 and IC75 values.

Table 4.1. Comparison of estimated IC50 and IC75 values of three wheat genotypes using the optimized IgE inhibition ELISA method.

Wheat Genotype	IC_{50} Value (µg/mL)	IC_{75} Value (µg/mL)
AABB	45.05 ± 0.65	87.80 ± 0.87
AABBDD	54.50 ± 0.16	177.21 ± 2.15
DD	62.12 ± 2.33	> 500

IgE Inhibition ELISAs were conducted using different wheat genotype derived salt-soluble proteins at various inhibitor concentrations as detailed in the text. Data shows estimated inhibition concentration values from triplicate analyses. ANOVA with post-hoc Tukey honestly significant difference (HSD) test results: AABB vs AABBDD, AABB vs DD, AABBDD vs DD all $p < 0.01$ for both IC_{50} and IC_{75} comparisons.

4.5. Discussion

In this study, we sought to develop and validate a novel mouse-based primary screening method for testing relative allergenicity of wheat proteins from different genotypes. Our data collectively demonstrate for the first time that it is possible to use the mouse system to accomplish this challenge. There are several novel and significant findings from this study: (i) mouse model can be used to establish a wheat genotype specific IgE antibody enriched mini-plasma bank; ii) the mouse-based mini-plasma bank can be used to optimize an IgE inhibition (II)-ELISA for wheat SSP with acceptable co-efficient of variation; iii) IgE binding analysis using II-ELISA can be used to determine the relative differences in allergenicity among the wheat genotypes; (iv) using II-ELISA one can establish IgE inhibition curves and estimate IC_{50} and IC_{75} values; v) the results demonstrated that compared to the allergenicity of SSP from AABB, SSP from AABBD and DD are relatively less allergenic; vi) the data shows that genetic differences among these three genotypes (tetraploid vs. hexaploid, vs. diploid) indeed translates to measurable variations in allergenicity by this method; data also show that AABBDD, which is a hybrid between AABB and DD genotypes, is intermediate in allergenicity as measured by this method.

We envision developing a '*comprehensive tool kit'* for allergenicity assessment of novel wheat proteins whether they are derived by differential processing methods or by genetic engineering method or by any other method. This can be accomplished in four phases as follows: 1) Primary screening using a genotype specific mini-plasma bank (using the method described here). 2) Secondary screening to test the ability of wheat proteins to elicit degranulation of mast cells *in vitro* using cell based assay system such as rat basophilic leukemia cell based assay and *in vivo* using sensitized mice by oral or intraperitoneal challenges and measuring plasma mMCP-1 levels as a marker of IgE-mediated mucosal mast cell degranulation; 3) Tertiary screening, where threshold doses for both sensitization as well as for elicitation of allergic reactions can be established using mice; and 4) Finally, clinical trial of novel wheat proteins identified by the above three pre-clinical testing phases, using human volunteers. In this framework and scope, the novel primary screening method described here should serve as a pre-clinical testing tool for primary screening of novel wheat proteins for allergenicity potential.

Wheat is a major allergenic food per food regulators in the US, Canada, Europe, Australia and Japan and New Zealand (US-FDA 2018; Sicherer and Sampson, 2018; Gonipeta et al. 2015). For regulation purpose all types of wheat are regarded as a single regulatory entity (US-FDA 2018). However, there are five different wheat genotypes that are genetically distinct (Shewry 2018). Therefore, it is reasonable to hypothesize that genetically distinct wheat might differ in their allergenicity potential. However, this hypothesis has not been thoroughly investigated so far.

There are two studies that used serum from wheat allergic patients and examined the binding of IgE to proteins obtained from various wheat varieties (Nakamura et al. 2005; Mohan Kumar et al. 2017). These studies used a direct IgE ELISA to study IgE binding to wheat

proteins. They reported that there is variation in IgE binding among wheat varieties suggesting that there may be natural variation in the allergenicity among wheat varieties. However, interpretation of the data from these studies is difficult because of two reasons: i) the observed differences in IgE binding to various wheat varieties could be simply due to differences in exposure of allergic subjects to different wheat varieties or different exposures to cross-reacting non-wheat plant proteins; and ii) the use of direct ELISA to study IgE binding to different proteins assumes that the proteins obtained from various wheat types will bind to ELISA plates to the same extent. If there were to be differences in the ability of wheat proteins to bind to ELISA plates, then that would translate into different level of reactivity to IgE antibodies. Therefore, to overcome this technical problem, instead of direct ELISA, inhibition ELISA is better suited to compare the relative binding to different types of proteins to the same target antibody. Nevertheless, these papers provide the first preliminary data and scientific premise to further test the hypothesis that genetically different types of wheat might differ in their relative allergenicity

Here we developed and validated the approach of using an animal based system for evaluating potential differences in allergenicity of genetically different wheat genotypes. We made sure that the IgE antibodies elicited in the mice cause disease (i.e., are functional) by challenge studies for systemic anaphylaxis and mast cell degranulation. This approach has the major advantage over using wheat allergic subjects for this type of analysis because exposure to specific wheat genotypes for sensitization can be completely controlled in animal based system such as mouse models. Furthermore, exposure to cross-reacting plant proteins can also be completely controlled in mouse models. Thus, in this study, mice were maintained on a plant-protein free diet and they were exclusively sensitized to one wheat genotype (e.g., AABB) only. This type of

exposure control is not possible with humans. Consequently, interpretation of the data is straightforward when using the animal based method to assess the relative allergenicity of different wheat genotypes. As a proof-concept, we used AABB exposed mice in this study. Essentially, similar approach could be used to generate other genotype specific mini-plasma banks by exposing mice to other genotypes of wheat also.

Wheat allergens belong to the three classical Osborne's wheat protein fractions: i) water/saline-soluble (albumins, globulins); 2) alcohol-soluble (gliadins) and 3) acid-soluble (glutenins) (Shewry 2009). As a proof-of-concept, here we used saline-soluble wheat proteins because we had previously established a mouse model using this type of protein (Jin et al. 2017). Successful development and validation of this mouse-based method here, suggest that a similar approach could be used to develop and validate methods for allergenicity assessment of alcohol-soluble and acid-soluble proteins from wheat or any other allergenic food. For example, there are five useful mouse models that already have been developed using alcohol-soluble gluten proteins (gliadins) earlier (Bodinier et al. 2009; Denery-Panini et al. 2011; Gourbeyre et al. 2012; Abe et al. 2014; Adachi et al. 2012). These animal model protocols may be employed for developing and validating II-ELISA using the same approach that is described here in this paper.

We are not aware of an IgE inhibition ELISA (II-ELISA) method reported for wheat allergenicity testing in any animal models in the literature so far. Use of II-ELISA to determine relative allergenicity of different wheat genotypes overcomes the problem associated with direct ELISA measurement such as potentially different plate binding capacities of proteins from different wheat genotypes. Furthermore, using II-ELISA, IgE inhibition curves can be generated and used to estimate IC_{50} and IC_{75} values for different wheat genotypes. These values provide a

quantitative comparison of differences in allergenicity. Thus, in this study we noted significant differences in these values for the three genotypes studied. These data suggest presumptive natural variation in the allergenic potential of these three genotypes. However, additional studies (e.g., phase 2 through phase 4 studies) are needed for further confirmation as discussed in the earlier section.

One limitation of the approach reported here includes the volume of the mini-plasma bank that one can establish using mice. Although mice provide little volume of blood, here we demonstrate successfully that indeed a mini-plasma bank (total volume of ~12 mL from 20 mice colony) can be created using the approach we developed. Nevertheless, should there be a need for larger size plasma bank, one might consider a similar approach using larger number of mice.

There are numerous studies showing that food processing can affect allergenicity of food proteins including wheat due to the effect of processing on the IgE binding epitope structure on protein allergens (Verhoeckx et al. 2015). Studies so far have typically used SDS-PAGE, and western blot analysis to study these changes (Phromraksa et al. 2008; Vanga et al. 2017; Verhoeckx et al. 2015). The II-ELISA established in this study can be used in the future to study changes in the IgE binding epitope structure of wheat proteins that have been subjected to different types of food processing. For example, it is possible to study how non-thermal vs. thermal processing of wheat products can alter IgE binding epitope structures and consequently allergenicity. No such studies have been reported in the literature so far largely because of the absence of a validated II-ELISA for wheat proteins so far.

Another application of the method described here is in the development of novel hypo/non-allergenic wheat proteins. Hypo/non-allergenic wheat proteins are highly desired ingredients for human and animal consumption (Lombardo et al. 2015; Kohno et al. 2016; Tanabe 2008;

Phromraksa et al. 2008). Therefore, pre-clinical and clinical testing of novel wheat proteins that are being considered for hypo/non-allergenicity applications is vital. The method optimized here can also be used for primary screening of such products as part of a '*comprehensive tool kit*' for assessment of allergenicity of novel wheat proteins discussed above.

Currently, there are no approved genetically engineered (GE) wheat crop for human or animal consumption (Mishra and Arora, 2017; Hellemans et al. 2018; Rey et al. 2015; Kohno K et al. 2016; Shewry 2018). However, there are ongoing efforts to develop GMO wheat (Mishra and Arora, 2017; Shewry and Tatham, 2016). It will be important to demonstrate whether such GE wheat is similar to or different from native wheat in terms of allergenicity potential (Ekmay et al. 2017; Selgrade et al. 2009; Ladics and Selgrade, 2009). Use of the method described here will be a useful as a primary screening tool for such GM wheat products.

4.6. Conclusions

Here we developed and validated a novel mouse-based primary screening method for testing allergenicity of wheat proteins from three different wheat genotypes. As part of a *comprehensive tool kit*, this primary screening method will be a useful tool to conduct first phase assessment of the changes in allergenicity of novel types of wheat products developed by genetic modification, or different food processing techniques or any other newer technology in the future.

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CHAPTER 5 AN ADJUVANT-FREE MOUSE MODEL USING SKIN SENSITIZATION WITHOUT TAPE-STRIPPING FOLLOWED BY ORAL ELICITATION OF ANAPHYLAXIS: A NOVEL PRE-CLINICAL TOOL FOR TESTING INTRINSIC WHEAT ALLERGENICITY

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5.1. Abstract

Wheat is a major food allergen per the regulatory bodies of various nations. Hypersensitivity reactions to wheat have been steadily increasing for reasons that are not completely understood. Wheat-allergy models typically use adjuvants to induce sensitization to wheat proteins followed by an intraperitoneal challenge to elicit anaphylaxis. Although these models are very useful, they lack the ability to reveal the intrinsic allergenicity potential of wheat. To improve the mouse model of wheat allergy, we tested the hypothesis that repeated skin application of salt-soluble protein extract (SSPE) from durum wheat will clinically sensitize the mice to oral anaphylaxis to SSPE. Balb/c mice were bred and maintained on a plant-protein-free diet and used in the experiments. Adult female mice were exposed to SSPE once a week for 9 weeks via a solution on intact skin. Sensitization was measured by SSPE-specific IgE (sIgE) antibody and total IgE (tIgE) levels. Oral anaphylaxis was quantified by hypothermic shock response (HSR), and mucosal mast cell response (MMCR) was quantified by measuring MMCP-1 after oral

challenge. Using single mouse data, correlation analyses were performed to determine the relationship among the allergenicity readouts. Spleen cytokines were quantified using a protein microarray method. Our results show that (i) repeated skin exposures to SSPE elicited robust increases in the sIgE and tIgE levels; (ii) skin exposure to SSPE was sufficient to sensitize mice for oral anaphylaxis and MMCR; (iii) both HSR and MMCR showed a strong correlation with each other, as well as with sIgE, and a modest correlation with tIgE levels; (iv) selected Th2/Th17/Th1 cytokines were elevated in skin-sensitized mice; and (v) oral allergen-challenged mice showed selective elevation of IL-6 and a panel of chemokines compared to saline-challenged mice. Together, we report the development and characterization of a novel adjuvant-free wheat-allergy mouse model that uses skin sensitization without tape-stripping followed by oral elicitation of anaphylaxis. Furthermore, validation of quantifiable wheat allergenicity readouts makes this model particularly suitable as a pre-clinical testing tool to assess the intrinsic sensitization/oral-anaphylaxis elicitation potential of novel wheat proteins (e.g., processed wheat) and to develop hypo/non-allergenic wheat products.

5.2. Introduction

Food allergies are chronic potentially fatal reactions to common food proteins mediated by the immune system (Sampson et al., 2018). They are on the rise for reasons that are not well understood at present (Seth et al., 2020). In the United States, their prevalence is 8% among children and 10.8% among adults (Gupta et al., 2018, 2019). Other countries, such as Canada, EU, Japan, and Australia, have reported a similar trend (Australian Society of Clinical Immunology and Allergy, 2021; Clarke et al. 2020; Ebisawa et al. 2020; Lyons et al. 2020). One decade ago, the estimated annual economic impact of food allergies in the United States was

$24.8 billion; the current updated impact information is unavailable (Gupta et al., 2013). In addition to its increasing prevalence, emergency department visits due to food- induced anaphylaxis are also on the rise (Gupta et al., 2019; Warren, Jiang, & Gupta, 2020). There is no cure for food allergy at present (Seth et al., 2020). It is reported that the quality of life of food-allergic individuals is significantly impaired as constantly paying stringent attention to diet can introduce extra burdens on individuals, as well as on their families, schools, and healthcare takers (Foong & Santos, 2021).

Wheat is the world's second most-produced cereal, after corn (FAO, 2021). However, per capita consumption of wheat flour has been decreasing in the last two decades in the United States by ~ 7.7% (from 144 lbs./person in 1999 to 131 lbs./person in 2019) (USDA Economic Research Services, 2021). Wheat is among the 8–14 major allergenic foods that are regulated by multiple countries, including the United States, Canada, EU, United Kingdom, Australia, and New Zealand (FAO 2021; US FDA 2020; Health Canada 2021; Japan 2019; UK Food Standards Agency, 2021). Besides wheat, other major food allergens are milk, fish, shellfish, peanuts, tree nuts, eggs, soybean, sesame, celery, lupin, mustard, and sulfites (Gupta et al., 2011; Renz et al., 2018; UK Food Standards Agency, 2021).

In general, wheat has been reported to cause two distinct types of immune-mediated adverse reactions: (i) IgE antibody- mediated allergic/anaphylactic reactions, which are potentially deadly; and (ii) non-IgE-mediated reactions that tend to be chronic conditions; these include autoimmune celiac disease, non-celiac gluten sensitivity, and eosinophil-mediated inflammatory gut reactions (i.e., eosinophilic esophagitis and eosinophilic gastritis) (Cianferoni, 2016; Patel & Samant, 2021; Schiepatti et al., 2020). Validated mouse models play a critical role in advancing

the knowledge of the mechanisms underlying these diseases so that novel methods of prevention and treatment can become available.

The prevalence of wheat allergy in the United States among adults is 0.9%−3.6% (Verrill, Bruns, & Luccioli, 2015; Vierk, Koehler, Fein, & Street, 2007). Its prevalence among United States children is 0.2%−1.3% (Poole et al., 2006; C Venter et al., 2008; Carina Venter et al., 2006). In both Europe and Australia, the prevalence of wheat allergies among adults and children is 0.4% and 1%, respectively (Pereira et al., 2005; Rancé, Grandmottet, & Grandjean, 2005; Woods et al., 2002). The seriousness of wheat allergy is illustrated by the reports that more than half of the affected children have experienced anaphylactic reactions, which can be potentially fatal (Cianferoni et al., 2013; Pourpak, Mansouri, Mesdaghi, Kazemnejad, & Farhoudi, 2004). Although 65% of children with wheat allergy outgrow it, a significant proportion (35%) continue to have persistent wheat allergy into adulthood with a continued risk of life-threatening reactions for the rest of their lives (Keet et al., 2009). Even so, wheat allergens are under-researched relative to other allergenic foods, such as peanuts, tree nuts, milk, and egg. For example, an adjuvant-free mouse model to study oral elicitation of anaphylaxis to wheat is unavailable at present–the focus of this study.

Wheat is a highly nutritious staple food, particularly because of its high protein content. On a dry weight basis, wheat contains 10%−14% of protein that includes gluten and non- gluten fractions. The non-gluten fraction (i.e., albumins and globulins; water/salt-soluble proteins) accounts for 15%−20% of the total proteins. The remaining 80%−85% of total protein is comprised of glutens (that include gliadins and glutenins) (Jin et al., 2019). Although wheat proteins are important sources of nutrients for most people, both types of proteins are also equally important sources of allergens for wheat-allergic subjects. Relative to non- glutens, most

published research on wheat proteins has focused on glutens, and therefore, there is a need to advance the knowledge on the allergenicity of non-gluten proteins also—the focus of this study.

Animal models are critical to advancing our knowledge of wheat food allergies (Y. Jin et al., 2019). Consequently, dogs, rats, and mice have been used to develop wheat-allergy models (Abe et al., 2014; Adachi, Nakamura, Sakai, Fukutomi, & Teshima, 2012; Ballegaard, Madsen, & Bøgh, 2019; Bodinier et al., 2009; Buchanan et al., 1997; Castan et al., 2018; Denery-Papini et al., 2011; Gourbeyre et al., 2012; Yining Jin et al., 2017, 2020; Kozai, Yano, Matsuda, & Kato, 2006; Kroghsbo, Andersen, Rasmussen, Jacobsen, & Madsen, 2014; Tanaka et al., 2011; Xue et al., 2019). Mice are very attractive and popular due to relatively lower costs, wide availability of immunological reagents for mouse protein targets, and availability of gene knockout strains (Jin et al., 2019). Among the mouse strains, Balb/c was shown to exhibit wheat allergenicity similar to that of humans (Jin et al., 2019). However, there are two major limitations facing the wheat allergenicity mouse models: (i) adjuvants for inducing systemic sensitization or tape-stripping of stratum corneum of skin for skin sensitization are commonly used that tend to elevate subject sensitivity; this however limits the ability to assess the intrinsic allergenicity potential of any tested wheat proteins, including novel proteins, such as processed wheat proteins and novel wheat varieties/lines; and (ii) intraperitoneal injection to elicit anaphylaxis does not simulate the oral-wheat-induced anaphylaxis noted in humans (Yining Jin et al., 2020). An adjuvant-free mouse model without tape-stripping on the other hand is more desirable as it makes data interpretation of the intrinsic allergenicity of wheat proteins possible, and therefore, is more suitable for allergenicity testing (Denery-Papini et al. 2011). Furthermore, oral elicitation of anaphylaxis using wheat proteins will enable studying mechanisms of oral anaphylaxis and assist in developing novel methods to prevent and treat oral anaphylaxis. Therefore, an adjuvant-free

skin-sensitization/oral-anaphylaxis-elicitation mouse model of wheat allergenicity is urgently needed—the focus of this study.

Here, we have tested the hypothesis that repeated skin application of salt-soluble protein extract (SSPE) from durum wheat will clinically sensitize the mice for oral anaphylaxis to those wheat proteins. There were seven objectives for this study: (i) to establish a colony of plant-protein-free Balb/c mice; (ii) to test for specific (s)IgE and total (t)IgE antibody response to SSPE from durum wheat upon repeated skin exposures to SSPE in adult female mice; (iii) to test for oral anaphylaxis to SSPE as quantified by hypothermic shock response (HSR) in skin-sensitized mice; (iv) to evaluate mucosal mast cell response (MMCR) upon oral challenge with SSPE in skin-sensitized mice; (v) to determine the correlations among four quantifiable readouts of wheat allergenicity (sIgE, tIgE, HSR, and MMCR); (vi) to evaluate spleen cytokine response in skin-sensitized mice; and (vii) to identify the spleen immune markers that are elevated upon oral SSPE vs. saline challenge in SSPE-allergic mice.

Together, we report the development and characterization of a novel wheat-allergy mouse model that uses skin sensitization without tape-stripping, followed by oral elicitation of anaphylaxis. Furthermore, validation of quantifiable wheat allergenicity readouts makes this model particularly suitable as a pre-clinical testing tool to assess the intrinsic sensitization and oral-anaphylaxis-elicitation potential of wheat proteins, including novel wheat proteins (e.g., processed wheat), and in the development of hypo/non-allergenic wheat proteins.

5.3. Materials and methods

5.3.1. Chemicals and reagents

Biotin-conjugated rat anti-mouse IgE-paired antibodies were purchased from BD BioSciences (San Jose, CA, United States). p- Nitro-phenyl phosphate was obtained from Sigma (St Louis, MO, United States). Streptavidin alkaline phosphatase was obtained from Jackson ImmunoResearch (West Grove, PA, United States). BSA standard (at 2 mg/ml) was purchased from Sigma (St. Louis, MO, United States). Alkaline copper tartrate was purchased from BioRad (Hercules, CA, United States). Folin reagent was purchased from BioRad (Hercules, CA). The following reagents were obtained as listed: IgE Mouse Uncoated ELISA Kit with Plates; Streptavidin-HRP, TMB substrate; MCPT-1 (mMCP-1) Mouse Uncoated ELISA Kit with Plates; Avidin-HRP, TMB substrate (all from Invitrogen, MA, United States); Tissue Protein Extraction Reagent (T-PERTM, a proprietary detergent in 25mM bicine, 150mM sodium chloride, pH 7.6; from ThermoFisher Scientific, MA, United States); protease (serine, cysteine, and acid proteases, and aminopeptidases) inhibitor cocktail (Sigma- Aldrich, MO, United States).

5.3.2. Mice breeding and establishment of a plant-protein-free mouse colony

Adult Balb/cJ breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME). Upon arrival, they were placed on a plant-protein-free diet (AIN- 93G, Envigo, Madison, MI). After acclimating for a week, the breeding pairs were set up as one male: two females per cage. Pregnant females were separated and after delivery, pups were weaned at 4 weeks. Adult female mice (6–8 weeks) were used in the experiments. All mice were maintained on the plant-protein-free diet (AIN-93G) throughout the study. All animal procedures were as per the Michigan State University policies.

5.3.3. Preparation of salt-soluble protein extract from durum wheat flour

Durum wheat flour (genomes AABB, variety Carpio) was used for protein extraction. Salt-soluble protein extract (SSPE) was prepared using a method published previously (Tatham, Gilbert, Fido, & Shewry, 2000). Briefly, flour and sterile 0.5M NaCl were mixed in a 1:10 ratio (m/v), and stirred continuously for 2 h followed by centrifugation (5,000×g, 10min) at 20◦C. The supernatant was frozen at −70°C overnight and freeze-dried the next day. Lyophilized SSPE powder was reconstituted with sterile saline. Protein concentration was determined using the Bio-Rad method (Lowry, Rosebrough, Farr, & Randall, 1951).

5.3.4. Skin sensitization, bleeding, and plasma sample preparation

Adult mice were used in the experiments (specific numbers for each experiment are provided in the RESULTS Section). Hair on the rump of the mice was removed using a hair clipper (Philips, Amsterdam, the Netherlands). A total of 50 µl of durum wheat SSPE (10 mg/ml) or vehicle (10% sterile NaCl solution) was applied over both sides of the clipped area on the rump (1mg in 100 µl per mouse per exposure). The mice were then covered with a non-latex bandage (Johnson & Johnson, New Brunswick, New Jersey) for 1 day. The above procedure was repeated weekly for 9 weeks. Bleeding was done 1 week before the 1st exposure and after the 8th exposure via the saphenous vein. Blood was collected in anticoagulant (lithium heparin) coated vials (Sarstedt Inc MicrovetteCB 300 LH, Germany) and centrifuged to harvest the plasma. Individual plasma samples were stored at −70 °C until used in the analysis.

5.3.5. Elicitation of oral anaphylaxis and hypothermic shock responses

Two weeks after the 8th exposure to durum SSPE, the mice were challenged orally with durum wheat SSPE (20 mg per mouse in 300 µl sterile saline) or vehicle (300 µl sterile saline) by using curved feeding needles (22-gauge, length: 1.4 in, Kent Scientific, Torrington, CT, United States). Specific number of mice for each experiment is provided in the RESULT Section. Rectal temperature (°C) was recorded before and after the challenge every 5 min up to 30 min by using a thermometer with a probe (DIGI-SENSE, MA, United States). Actual temperatures and changes in rectal temperature (Δ°C) every 5min compared to the pre-temperatures for each mouse were used in the analyses.

5.3.6. Measurement of wheat SSPE-specific IgE antibody levels

Wheat SSPE-specific(s) IgE antibody levels in the plasma were measured using an ultrasensitive ELISA method as we have reported before (Gao et al., 2019; Jin et al., 2017, 2020). This method was a modified version of the published method we have reported previously for food-specific IgE antibody measurement in the mouse system (Birmingham et al., 2003). Briefly, 96-well plates (Corning 3369) were coated with durum SSPE, followed by blocking with 5% gelatin, washing, plasma sample addition, washing, addition of biotin-conjugated anti-mouse IgE antibody, washing, and the addition of Streptavidin Alkaline Phosphatase and PNPP detection system as described previously (Gao et al., 2019; Jin et al., 2017). Individual mouse samples were tested in quadruplicate.

5.3.7. Measurement of total plasma IgE concentration

Total(t) IgE concentrations were determined using a commercial ELISA kit (Invitrogen, Waltham, MA) that contained antibody pairs (i.e., anti-mouse IgE as capture antibody and biotin- conjugated anti-mouse IgE as detection antibody) and a recombinant mouse IgE standard as described before (Jin et al., 2017, 2020). Briefly, 96-well plates (Corning Costar 9018) were coated with capture antibody (anti-mouse IgE), followed by adding samples and standards (recombinant mouse IgE). A secondary antibody (biotin-conjugated anti-mouse IgE) was then added. Detection was based on Streptavidin-HRP and TMB substrate system. Assay sensitivity: 4 ng/ml. The standard range used for quantification: 250 – 4 ng/ml. Individual mouse samples were tested in quadruplicate.

5.3.8. Measurement of wheat SSPE-specific IgG1 antibody levels

Wheat SSPE-specific(s) IgG1 antibody levels in the plasma were measured using an ELISA method as we have reported before (Gao et al., 2019; Jin et al., 2017, 2020). Briefly, 96-well plates (Corning 3369) were coated with durum SSPE, followed by blocking, sample addition, and secondary antibody (biotin-conjugated anti-mouse IgG1 antibody). The plate was developed by Streptavidin Alkaline Phosphatase and PNPP detection system as described previously (Gao et al., 2019; Jin et al., 2017).

5.3.9. Quantification of mucosal mast cell protease-1 (MMCP-1) level

The blood collected at 1-h post-challenge was used in measuring MMCP-1 levels (ng/ml) in the plasma using an ELISA-based method according to Invitrogen as we described previously (Jin et al., 2017, 2020). Briefly, 96-well plates (Corning Costar 9018) were coated with capture

antibody (anti-mouse MMCP-1), followed by adding samples and standards (recombinant mouse MMCP-1). A sandwich was then formed when a secondary antibody (biotin-conjugated anti-mouse MMCP-1) was added. Detection was based on avidin-HRP and TMB substrate system. Assay sensitivity: 120 pg/ml. The standard range used for quantification: 15,000–120 pg/ml. Individual mouse samples were tested in quadruplicate.

5.3.10. Preparation of spleen extract and analysis of immune markers

The mice were euthanized 1 h after the oral challenge. Their spleens were harvested, snap-frozen, and stored at −70◦C until used for tissue extraction, which was performed using the method we have described before (Jin et al., 2020). Briefly, spleen tissues were immersed in a Tissue Protein Extraction Reagent (T-PER^{TM}) with a protease inhibitor. For each 100mg of tissue, 10 µl of protease inhibitor per 1ml T-PER buffer was used. The spleen tissue was homogenized by ultra-sonication for 30 s twice, with a 5-min interval in between, and then, was rested for 15 min before centrifugation (13,500 × g) for 10min at 4◦C. The supernatants were collected and stored in aliquots at −70◦C until used in the analysis. The Quantibody microarray (RayBiotech, Atlanta, GA) was used to quantify a panel of immune biomarkers. All samples were analyzed in quadruplicate using standards (https://www. raybiotech.com/mouse-cytokine-array-q2000/).

5.3.11. Statistics

An online software service was used in these analyses (https:// www.socscistatistics.com/tests/). The significance level was set at $p < 0.05$. Student's t-test was

used to compare the two groups and ANOVA was used for multiple comparisons. Pearson correlation analysis was used to determine the relationship among the allergenicity readouts.

5.4. Results

5.4.1. Repeated skin exposures to durum wheat SSPE elicits robust specific-IgE and specific-IgG1 antibody responses in Balb/c female mice

Groups of Balb/c female mice were transdermally exposed to durum wheat SSPE or saline by repeated weekly exposures as described in the Methods. The SSPE used in this study had been characterized for protein quantity and quality previously (Supplementary Figure 5.1). Blood collected before vs. after 8 skin exposures was used in measurements of sIgE levels. As can be seen in **Figures 5.1A,B**, a robust induction of sIgE antibody levels after transdermal exposure with SSPE but not vehicle was noted (~17-fold increase in allergic mice vs. vehicle control mice).

5.4.2. Repeated skin exposures to durum wheat SSPE also elevates total IgE levels in Balb/c mice

We measured the tIgE concentrations in the plasma before and after skin sensitization. As evident, a dramatic increase in tIgE levels was observed in SSPE-sensitized but not in vehicle-sensitized mice (~50-fold increase in allergic mice vs. vehicle control mice; **Figures 5.1C,D**).

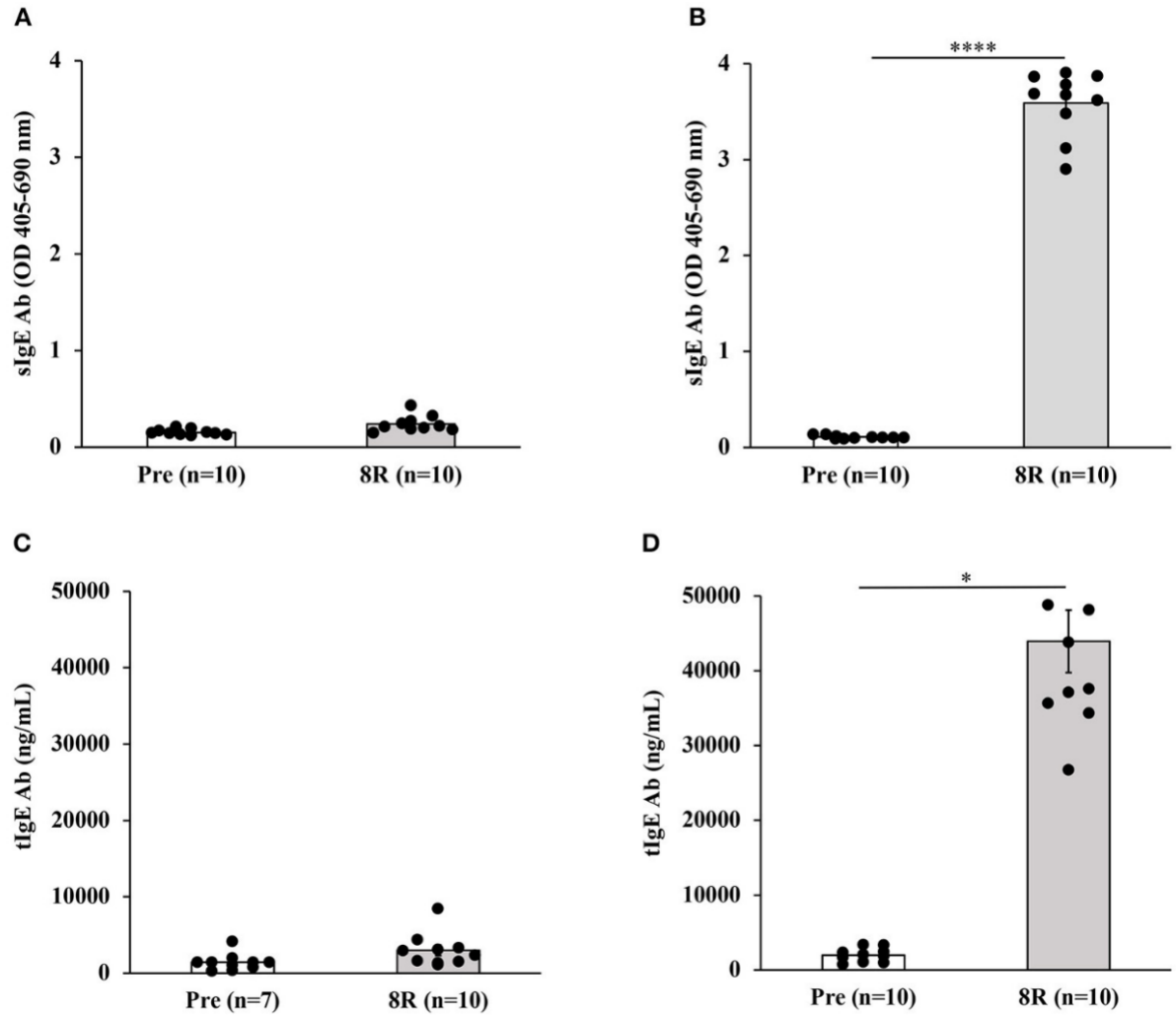

Figure 5.1. Transdermal exposure to durum wheat (genomes AABB) SSPE elicited exposure-dependent SSPE-specific (s) IgE antibody responses and elevation of total (t) IgE in Balb/c mice that correlate with each other. Mice were exposed to SSPE or to saline as described in Materials and Methods. Blood was collected before 1st exposure (Pre) and after 8th exposure (8R). Plasma was used in measurement of SSPE-specific IgE levels (OD 405–690 nm) using an ELISA method described previously. (A) SSPE-specific IgE levels in control mice. (B) SSPE-specific IgE levels in sensitized mice. ****$p < 0.001$; Ab, antibody. (C) Total IgE levels in control mice. (D) Total IgE levels in sensitized mice. *$p < 0.05$.

5.4.3. Repeated skin exposures to durum wheat SSPE is sufficient to clinically sensitize mice for anaphylactic responses after oral allergen challenge

We used parallel groups of skin-sensitized mice to induce anaphylaxis by performing the allergen challenge via the oral route. Anaphylactic reactions were quantified by hypothermic

shock reactions (HSR) using rectal thermometry. The actual temperatures before and after oral challenge with the allergen (15 mg/mouse) or saline at 5-min intervals are shown in **Figure 5.2A**. The change in temperature every 5min post-challenge compared to pre-challenge temperatures is shown in **Figure 5.2B**. There was no HSR upon vehicle (i.e., zero allergen) challenge. Furthermore, oral administration of allergen to non-allergic mice also did not elicit HSRs. On the contrary, acute HSRs were observed upon oral allergen challenge in skin-sensitized mice (**Figures 5.2C,D**). Significant HSRs were noted from 15 to 30 min (ANOVA, $p < 0.05$).

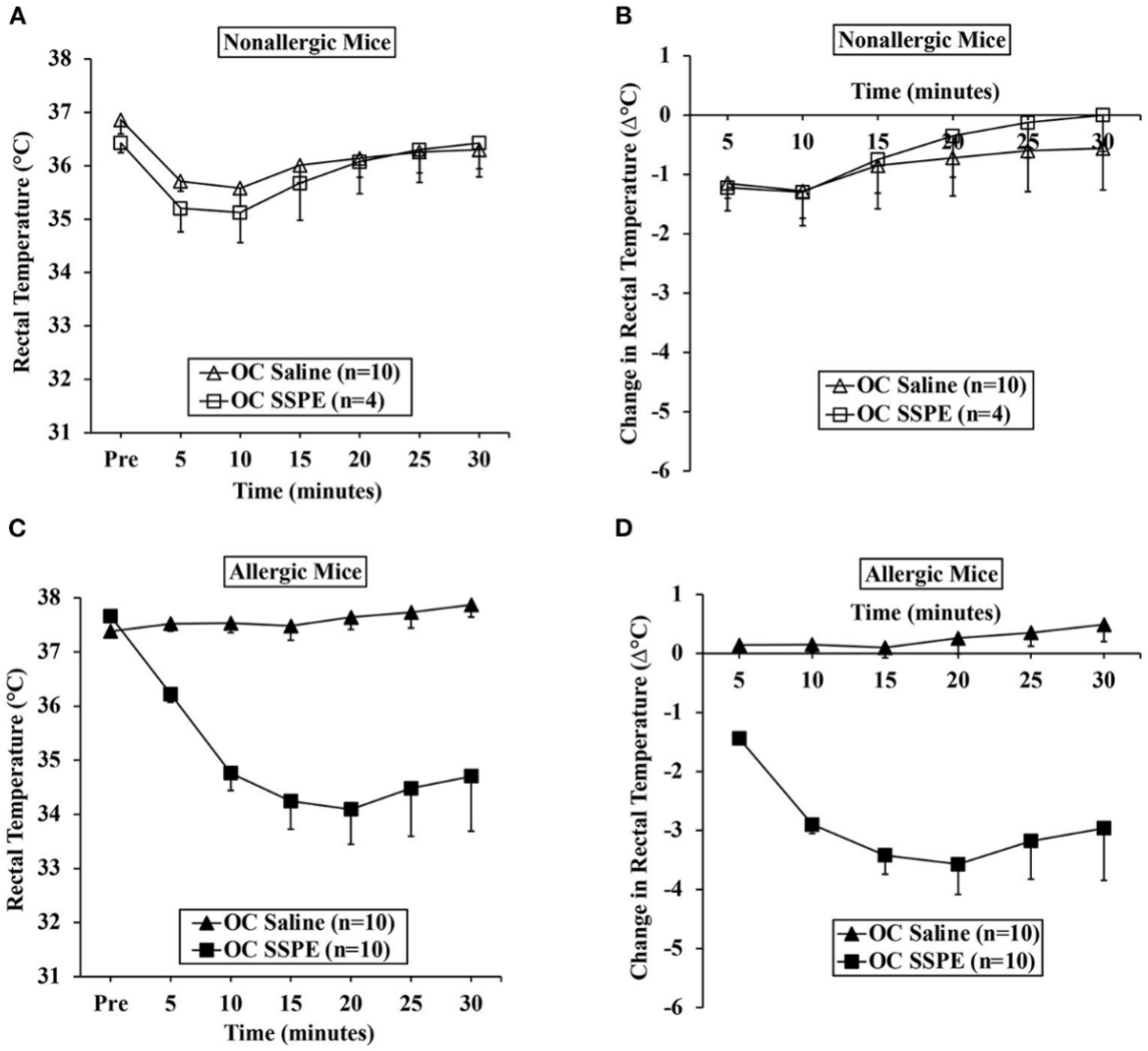

Figure 5.2. Transdermal exposure to durum wheat (genomes AABB) SSPE sensitized Balb/c mice for anaphylaxis upon oral challenge. Mice exposed to SSPE or to saline were orally challenged (OC) as described in MATERIALS AND METHODS. (A) Rectal temperatures (◦C) at indicated time points in non-allergic mice challenged with SSPE or saline. (B) Change in rectal temperature (Δ◦C) at indicated time points in non-allergic mice challenged with SSPE or saline. (C) Rectal temperatures (◦C) at indicated time points in allergic mice challenged with SSPE or saline. (D) Change in rectal temperature (Δ◦C) at indicated time points in allergic mice challenged with SSPE or saline.

5.4.4. Hypothermic shock responses of durum wheat SSPE-sensitized allergic mice correlate more strongly with specific-IgE antibody than the total IgE levels

Using single mouse data, we determined the relationship between HSR and IgE antibody levels (sIgE and tIgE) in oral allergen-challenged mice. The correlation between change in the temperature (Δ°C) at every 5min post-challenge compared to the pre-temperature and sIgE is shown in **Figure 5.3A**. As evident, both sIgE and tIgE showed significant correlations with Δ°C; however, the sIgE levels consistently showed stronger correlations with Δ°C than did the tIgE levels (**Figure 5.3A**). We noted the strong correlations at 15min post-oral allergen challenge for both sIgE and tIgE (**Figures 5.3B,C**).

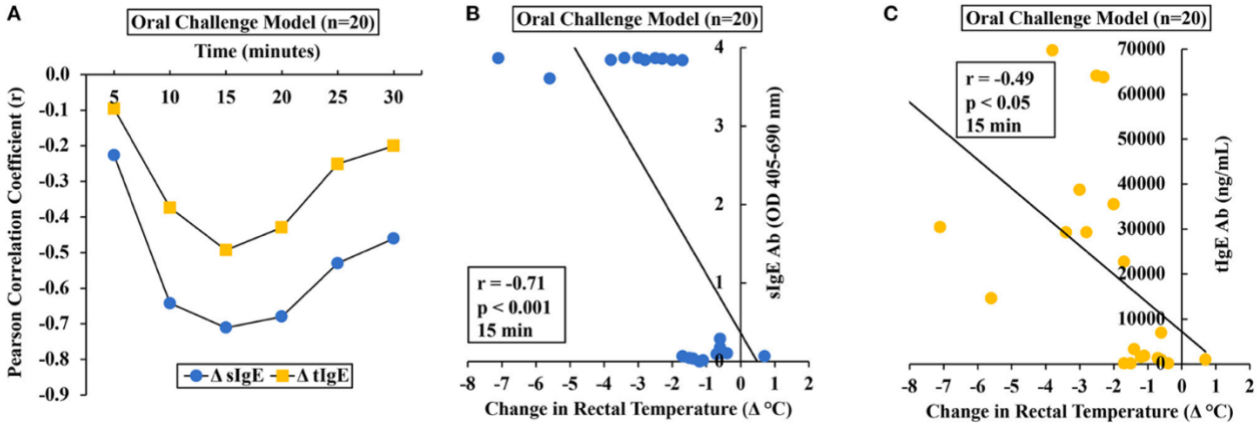

Figure 5.3. Anaphylactic responses in allergic mice upon oral challenge with durum wheat (genomes AABB) SSPE correlated with specific and total IgE levels. Mice were exposed to SSPE or to saline and challenged as described in Materials and Methods. (A–C) Pearson correlation coefficient (r) between antibody responses (sIgE or tIgE levels) and change in rectal temperature (Δ◦C) in mice sensitized and orally challenged with SSPE (20mg). (A) Pearson correlation coefficient (r) at indicated time points. (B) Pearson correlation analysis between sIgE and Δ◦C at 15min post challenge. (C) Pearson correlation analysis between tIgE and Δ◦C at 15min post challenge. sIgE, specific IgE; tIgE, total IgE; Ab, antibody.

5.4.5. Analysis of mucosal mast cell degranulation responses upon allergen challenge in durum wheat SSPE-sensitized mice

It has been shown in a previous study that degranulation of mucosal mast cells resulting in acute elevation of blood levels of murine mucosal cell protease (MMCP)-1 after allergen challenge is a biomarker of IgE-mediated systemic anaphylaxis in mice (Khodoun et al., 2011). Therefore, we determined MMCP-1 responses in allergic mice upon oral allergen challenges.

Results of MMCP-1 responses in oral allergen-challenged mice are shown in **Figure 5.4**. As expected, non-allergic control mice did not show marked elevation of MMCP-1 levels upon vehicle oral challenges (**Figures 5.4A,B**). In contrast, allergic mice exhibited robust MMCP-1 responses upon oral allergen challenge (20 mg dose; **Figures 5.4C,D**).

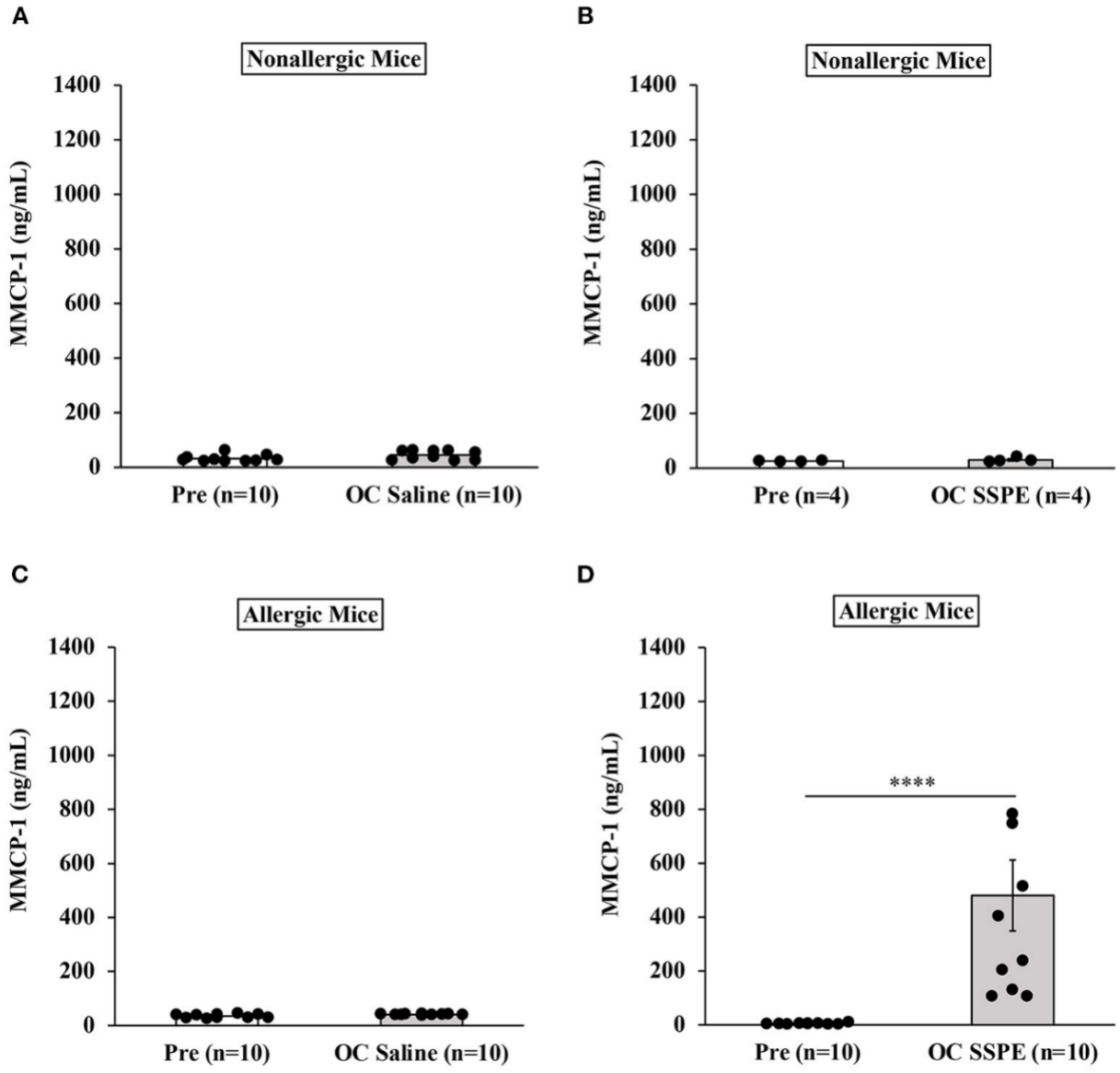

Figure 5.4. Transdermally sensitized allergic mice exhibited degranulation of mucosal mast cells upon oral challenge with durum wheat (genomes AABB) SSPE. Mice were treated as described in MATERIALS AND METHODS. Their serum mucosal mast cell protease-1 levels (ng/mL) were measured using an ELISA-based method described in the texts. (A) MMCP-1 levels in control mice challenged with saline. (B) MMCP-1 levels in control mice challenged with durum SSPE. (C) MMCP-1 levels in allergic mice challenged with saline. (D) MMCP-1 levels in allergic mice challenged with durum SSPE. ****$p < 0.001$. MMCP-1, mucosal mast cell protease-1.

5.4.6. Correlation analysis between mucosal mast cell responses and hypothermic shock responses in this mouse model

To determine the relationship between HSR and mucosal mast cell responses (MMCRs), we conducted Pearson correlation coefficient analysis using single-mouse data oral allergen-challenged mice. Results of correlation analysis between HSR and MMCR in oral-challenged mice are shown in **Figure 5.5**. We observed a strong correlation (r = −0.88) between MMCR and HSR from 15 to 30 min post-challenge period (**Figures 5.5A,B**).

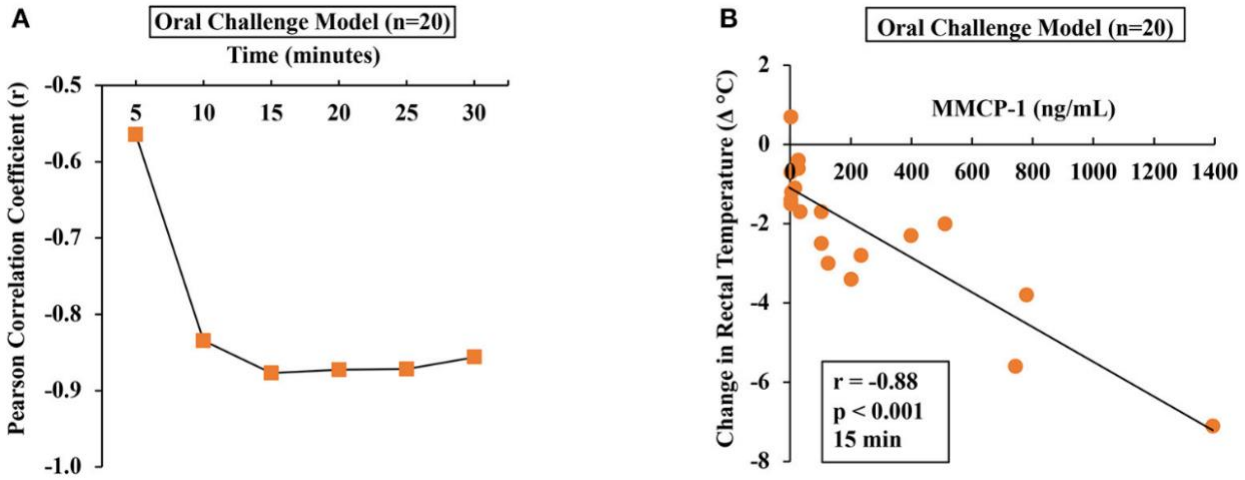

Figure 5.5. Hypothermic shock responses in durum wheat (AABB genome) SSPE-allergic mice correlated with their degranulation of mucosal mast cells. Mice received treatments as described in Materials and Methods. Their serum MMCP-1 levels (ng/ml) were compared with change in rectal temperature (Δ◦C) post-challenge using Pearson correlation analysis. (A) Pearson correlation coefficient (r) at indicated time points in orally challenged allergic mice with SSPE (20 mg). (B) Pearson correlation analysis at 15 min post-challenge in saline-challenged control mice (n = 10) and SSPE-challenged (20 mg) allergic mice (n = 10) via the oral route. MMCP-1, mucosal mast cell protease-1.

5.4.7. Correlation analysis between mucosal mast cell responses and IgE levels in this mouse model

To determine the relationship between MMCRs and IgE levels, we conducted Pearson correlation coefficient analysis using single-mouse data from oral-challenged mice. We found that only sIgE showed a strong correlation with MMCR (**Figures 5.6A,B**).

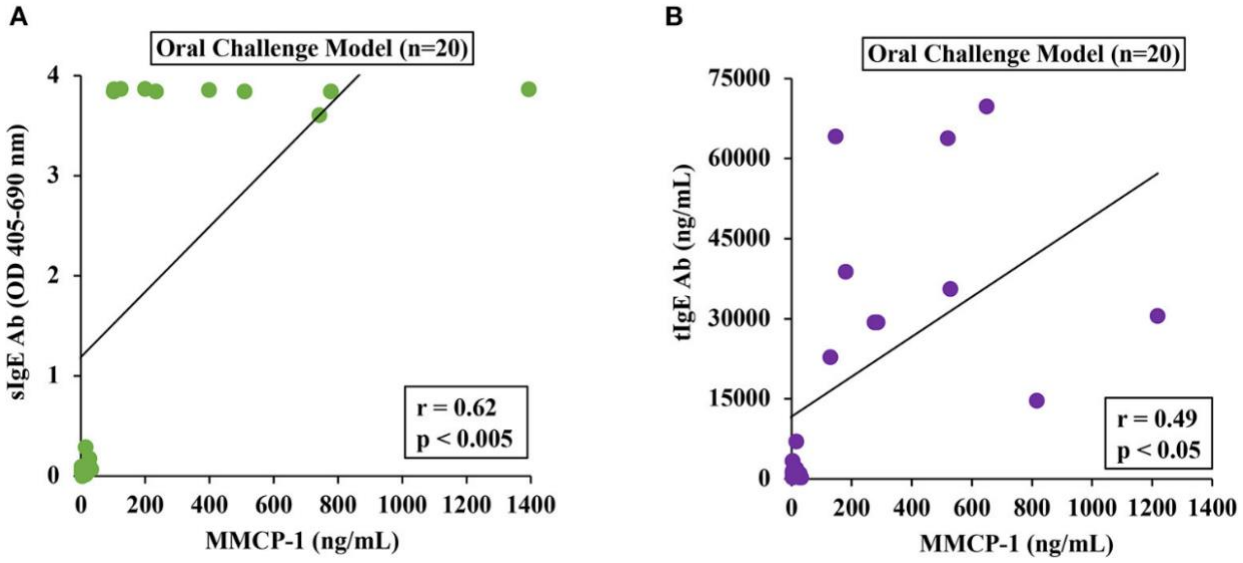

Figure 5.6. Mucosal mast cell responses upon challenge with durum SSPE correlated with specific and total IgE levels. Mice were treated as described in MATERIALS AND METHODS. Pearson correlation analysis was used to test the relationship between MMCP-1 levels (ng/ml) and antibody responses (sIgE or tIgE) in the plasma after 8th transdermal exposure (8R) to SSPE. Data from saline challenged control mice (n = 10) and SSPE-challenged (20mg) allergic mice (n = 10) via the oral route. (A) Pearson correlation analysis between sIgE and MMCP-1 levels in orally challenged (OC) mice. (B) Pearson correlation analysis between tIgE and MMCP-1 levels in orally challenged (OC) mice. sIgE, specific IgE; tIgE, total IgE; Ab, antibody.

5.4.8. Identification of immune biomarkers associated with sensitization vs. oral anaphylaxis to durum wheat SSPE

We screened a selected panel of spleen immune makers to study the cytokine responses upon sensitization. As shown in Table 1, compared to unsensitized mice, durum wheat SSPE-sensitized mice had significantly higher levels of prototypic Th2 cytokines (IL-4, IL-5) and a Th17 (IL-17E) cytokine. Also, Th1 cytokines (IFN-γ and IL-12p70) were elevated. We also studied the spleen biomarkers that increase upon oral allergen vs. saline challenge in skin-sensitized mice. Interestingly, none of the prototypic Th2, Th17, or Th1 were significantly changed after the oral allergen challenge compared to the oral saline challenge (data not shown). However, IL-6 was the only cytokine that was significantly elevated (sensitized mice, orally challenged with saline: 36.41 ± 2.67 ng/ml; sensitized mice, orally challenged with allergen: 85.06 ± 10.57 ng/ml; student's t-test, two-tailed, $p < 0.005$). Furthermore, we identified the following 4 chemokines that were also significantly elevated upon oral allergen but not the saline challenge of skin-sensitized mice: CCL5, CCL20, CCL22, and CXCL1 (**Table 5.2**).

Table 5.1. Spleen cytokine levels in unsensitized mice vs. durum wheat sensitized mice.

Cytokines*	Unsensitized Mice (n=5)	Sensitized Mice (n=5)	Student's t-test, (2-tailed)
IFN-γ	< 8 (LOD)	42.50 ± 9.49	$p < 0.05$
IL-2	182.42 ± 67.46	582.19 ± 110.21	$p < 0.05$
IL-12p40	37.43 ± 6.01	29.90 ± 6.13	ns
IL-12p70	18.79 ± 3.55	118.14 ± 11.81	$p < 0.001$
IL-23	2166.99 ± 304.05	2547.63 ± 275.73	ns
TNF-α	18.23 ± 9.80	33.34 ± 17.62	ns
IL-4	2.37 ± 0.29	6.34 ± 1.24	$p < 0.05$
IL-5	28.87 ± 6.27	98.36 ± 15.26	$p < 0.01$
IL-6	7.29 ± 0.87	35.52 ± 2.60	$p < 0.001$
IL-13	< 12.1 (LOD)	< 12.1 (LOD)	na
IL-17A	< 5.9 (LOD)	< 5.9 (LOD)	na
IL-17E	53.81 ± 10.23	181.70 ± 34.77	$p < 0.05$

** pg/mL of spleen protein extract; LOD: limit of detection. ns: not significant. na: not applicable.*

Table 5.2. Identification of spleen chemokines that are increased upon oral allergen but not saline challenge in durum wheat sensitized mice.

Spleen Immune Markers*	Sensitized Mice Orally Challenged with Saline (n=5)	Sensitized Mice Orally Challenged with Allergen (n=5)	Student's t-test (2-tailed)
CCL1 (TCA3)	99.11 ± 10.16	92.97 ± 10.88	ns
CCL2 (MCP-1)	175.03 ± 48.81	111.25 ± 36.91	ns
CCL3 (MIP-1a)	< 5.5 (LOD)	10.54 ± 5.04	ns
CCL5 (RANTES)	885.30 ± 17.06	951.05 ± 18.65	$p < 0.05$
CCL9 (MIP-1g)	713.60 ± 7.05	740.15 ± 19.16	ns
CCL12 (MCP-5)	< 2 (LOD)	3.65 ± 1.65	ns
CCL19 (MIP-3b)	57.78 ± 5.12	60.22 ± 2.52	ns
CCL20 (MIP-3a)	17.96 ± 6.45	52.75 ± 5.42	$p < 0.01$
CCL22 (MDC)	214.13 ± 12.05	283.63 ± 5.00	$p < 0.005$
CCL24 (Eotaxin-2)	1045.27 ± 35.93	992.89 ± 10.24	ns
CXCL1 (KC)	50.90 ± 0.85	78.65 ± 7.70	$p < 0.05$
CXCL4 (PF-4)	73323.82 ± 1479.44	72812.41 ± 1400.74	ns
CXCL11 (I-TAC)	< 40 (LOD)	< 40 (LOD)	na

** pg/mL of spleen protein extract; LOD: limit of detection. ns: not significant. na: not applicable.*

5.5. Discussion

Here we tested the overall hypothesis that repeated exposure to salt-soluble protein extract (SSPE) from durum wheat via the skin without tape-stripping or the use of an adjuvant

will be sufficient to clinically sensitize mice for oral allergen-induced anaphylaxis. Our data together support this hypothesis.

There are seven novel findings from our studies: (i) repeated skin exposures—once a week for 8 weeks to durum wheat SSPE dramatically elevated the sIgE levels, as well as the tIgE levels in blood; (ii) repeated skin exposures to durum SSPE is sufficient to clinically sensitize mice for oral SSPE-induced anaphylaxis as quantified by hypothermic shock responses (HSR); (iii) strong correlations between sIgE and HSR in individual mice analysis confirm that IgE antibodies contribute to the HSR; (iv) oral SSPE- induced anaphylaxis is associated with significant mucosal mast cell degranulation response (MMCR) confirming that IgE/mast cell pathway is engaged in this model; (v) significant correlations between HSR and MMCR in single mice analysis confirm that MMCR contributes to HSR; (vi) strong correlations between sIgE and MMCR in single mice analysis confirm that MMCR is mediated by the sIgE-oral SSPE interaction resulting in mucosal mast cell response in the gut; and (vii) identification of a panel of spleen immune markers that are significantly elevated upon oral allergen but not saline challenge in wheat-sensitized mice.

Wheat contains two families of allergenic proteins—gluten and non-gluten proteins. More than 100 specific allergens within these two families have been well characterized (Juhász et al., 2018). Although both types of allergens can cause human wheat allergies, most mouse model studies to date have been done using gluten allergens (Abe et al., 2014; Adachi et al., 2012; Bodinier et al., 2009; Castan et al., 2018; Denery-Papini et al., 2011; Gourbeyre et al., 2012; Jin et al., 2020, 2017; Kozai et al., 2006; Tanaka et al., 2011; Xue et al., 2019). To advance the knowledge on the biology of non-gluten allergens, we have been characterizing the immune response to SSPE using durum wheat as a model tetraploid (genomes AABB) wheat.

Using a popular and widely used alum-adjuvant-based model, we have previously demonstrated that durum SSPE when administered by intraperitoneal (IP) injections along with alum induces IgE responses and sensitizes mice for anaphylaxis upon IP injection with SSPE alone (Jin et al., 2017). Furthermore, a long-term study suggested that some of the SSPE- allergic mice developed severe atopic dermatitis (Jin et al., 2017). Later, we demonstrated that the durum SSPE can also elicit IgE responses upon skin exposure (once a week for 6 weeks) and sensitize mice for anaphylaxis upon IP injection with SSPE (Jin et al., 2020). However, it was unknown whether repeated skin exposures to SSPE from durum wheat will be sufficient to clinically sensitize mice for oral SSPE- induced anaphylaxis. Here, we demonstrate that repeated nine skin exposures (once a week for 9 weeks) clinically sensitizes mice for oral SSPE-induced anaphylaxis. Thus, together with this study, we have further advanced the scientific knowledge of allergic immune responses to durum SSPE.

Human wheat allergies develop via unknown mechanisms of allergic sensitization to wheat proteins. For example, skin exposure to wheat proteins, such as SSPE via the skin, can happen when wheat flour/dough is handled with bare hands (e.g., kitchen, baking industries). However, whether such exposures can have any clinical consequences is completely unknown. Recently, there is growing interest in studying immune responses via the skin environment (Akdis, 2021; Leung, Berdyshev, & Goleva, 2020). Therefore, to advance the knowledge of the clinical consequence of skin exposure to food proteins, we have been studying immune responses to various types of food allergenic proteins, including tree nuts, shellfish, and sesame . Here, we further advance the biology of immune responses via skin without tape-stripping of stratus corneum using durum wheat SSPE and demonstrate that it can clinically sensitize Balb/c

mice for oral anaphylaxis via the IgE/mucosal mast cell degranulation responses for the first time.

The model that we have developed and characterized in this study stands out as a significantly improved animal model of wheat allergy because of the following two critical characteristics: (i) as opposed to our model described here that uses oral SSPE challenge to elicit anaphylactic responses, none of the previously described models had reported this method to elicit anaphylaxis to SSPE and (ii) as opposed to our model described here using detailed analysis of single-mouse data, we determined the correlations among the 4 quantitative readouts of wheat allergenicity (sIgE, tIgE, HSR, and MMCR), none of the previous models had reported such detailed analysis. Correlation analyses have established and validated the sIgE as a strong quantitative readout of wheat SSPE-allergic sensitization that leads to anaphylaxis upon oral challenge, and HSR and MMCR as quantitative readouts of sIgE-mediated allergic reactions. Establishing these characteristics was critical in a wheat food allergy animal model for the following reasons: (i) wheat allergies in humans typically occur through food ingestion. Therefore, simulating the mouse model to reflect human oral exposure conditions is vital to validating a model; (ii) validating the quantitative readouts of wheat allergenicity has major future applications. For example, one may use these readouts to determine whether the in vivo allergenicity of various wheat lines developed by breeding and cross-hybridization would be different, thereby, identifying potentially hypoallergenic and hyper-allergenic wheat. Furthermore, if genetically modified (GM) wheat is to be developed in the future, this model could be used to establish whether or not such GM wheat is “substantially equivalent” to non-GM wheat variety in eliciting clinical sensitization that leads to oral allergic reactions; validation of HSR and MMCR as markers of sIgE-mediated wheat allergy upon oral challenge can be used

to establish this critical requirement in the assessment of the allergenic potential of GM foods as recommended by the FAO/WHO in their decision tree method (FAO/WHO, 2001; Ladics & Selgrade, 2009); (iii) one can use these readouts to examine the impact of food processing on wheat allergenicity, especially its effect on intrinsic sensitization and oral elicitation potencies; (iv) in addition, the readouts measured in our study may be useful to establish no-observed-adverse-effect level (NOAEL) and lowest-observed-adverse effect (LOAEL) for wheat. Until now, it has not been possible to do such research simply because validated quantitative markers of intrinsic sensitization and orally elicited wheat allergy were not available; and (v) this model can be used to elucidate the mechanisms of wheat food allergy.

Completion of model characterization in wheat allergy not only could help eliminate the hyper-allergenic wheat lines from the human food chain but also identify and develop hypo/non-allergenic wheat. Moreover, this model may be utilized for developing therapeutics to prevent/treat wheat allergies without the need to use excessive unnatural activation of the immune system using adjuvants.

To understand the immune mechanisms underlying wheat allergies, cytokine response has been studied in a limited number of animal models of wheat allergies. Using an adjuvant-based mouse model of gliadin allergy, Bodinier et al. reported elevated IL-4 and IL-5 in the lung fluid of gliadin- allergic mice challenged by nasal route. Besides these prototypic Th2 cytokines, Jin et al. (2017) reported elevation of Th1 and Th17 cytokines in mice that had developed atopic dermatitis upon chronic intraperitoneal exposures to durum wheat SSPE with alum-adjuvant. Later in 2020, Jin et al. (2020) reported that Th1, Th2, and Th17 cytokines were increased in both an alum-adjuvant mouse model, as well as in an adjuvant- free mouse model upon intraperitoneal injections with durum wheat SSPE. Here, we further extend the knowledge

underlying the cytokine mechanisms of wheat allergy by identification of selected Th1, Th2, and Th17 cytokines elevated in the spleen of skin-sensitized mice after nine skin exposures. Together, these studies demonstrate the critical role of these cytokines in wheat allergenicity irrespective of the specific protocol used and the target site organ used in the study (lungs, skin, and spleen). In all these conditions, wheat allergens consistently appear to activate pathogenic Th1, Th2, as well as Th17 biomarkers in the body. Therefore, these pathways may represent future potential therapeutic targets for wheat allergies. In addition, the selective elevation ofIL-6 and a small panel of chemokines (CCL5, CCL20, CCL22, and CXCL1) in the spleen upon oral allergen but not saline challenge suggests their key role in eliciting anaphylaxis via the oral route. They may also represent therapeutic targets of anaphylaxis triggered upon wheat ingestion. Recent evidence indeed suggested that elevated IL-6 is a diagnostic marker of systemic anaphylaxis in human food allergy in general (Kara et al., 2020; Nguyen et al., 2021). Thus, IL-6 and the chemokines identified in this model may be considered for investigation as potential diagnostic markers of anaphylaxis caused by wheat ingestion in humans.

We were surprised to note that in addition to Th2 cytokine activation, Th1 cytokines were also activated in this model. However, despite the activation of Th1 cytokines, we found robust IgE response, hypothermia shock response, and mucosal mast cell response. Therefore, we do not think that the Th1 activation will interfere with the investigation of allergy in this model.

We conducted a systematic individual mouse analysis to establish correlations among the four wheat allergenicity readouts in this model that has been rarely done before in any animal model of food allergy. The rationale for this was as follows: (i) sIgE measurements require extensive optimization for each food type, require higher volumes of blood samples, and data are difficult to compare across different laboratories (Birmingham et al., 2003; Castan et al., 2020;

Gao et al., 2019). On the contrary, commercial kits are available for tIgE measurements; tIgE measurements are therefore easier and comparable across laboratories. Our data show that in this model, tIgE correlates with sIgE measurements, and therefore, could be used as a surrogate for sensitization analysis in case sIgE measurements are not feasible to do. However, modest to a poor correlation between tIgE with MMCR or HSR caution against using tIgE to predict oral allergic reactions in this model. In contrast, only sIgE responses appear to be strong predictors of oral anaphylaxis; (ii) hypothermic shock response (HSR) has been widely used because it is relatively cost-effective and easy to measure. However, it does not reveal the mechanism underlying the reaction. In contrast, MMCR as measured by MMCP-1 levels reveals that reactions are indeed mediated by the sIgE antibody interaction with the allergen on the mucosal mast cell surface in the gut (Khodoun et al., 2011). Therefore, a strong correlation between the MMCR and HSR in this model demonstrates that sIgE-mediated MMCR contributes to the HSR observed in this model and therefore provides a mechanistic basis for the anaphylaxis observed in this model; and (iii) using correlation analysis we determined the best time points for quantifying HSR that are most likely mediated by sIgE, and mucosal mast cells in this model. The time points that show the strongest correlations between sIgE vs. HSR and MMCR vs. HSR (i.e., 15–20min post-oral challenge) are expected to be the best time points are recommended for future studies on oral anaphylaxis as they reflect HSR that is most likely associated with sIgE/mucosal mast cell degranulation response.

Wheat allergic mice in this study displayed clinical symptoms such as scratching and labored breathing upon oral challenge with SSPE. However, we did not observe diarrhea in these mice upon oral challenge with SSPE. Here, we wanted to develop objectively quantifiable readouts of wheat allergy. Therefore, we focused on hypothermic shock response and MMCP-1

measurements as quantitative readout of oral allergenicity as they are robust objective readouts of food allergenicity in mice in general.

In this study, we report long-term (eight exposures) skin-sensitization data. However, we have studied sensitization after short-term (four exposures) also. For example, significant specific IgE antibody responses appear after 4th skin exposure to wheat protein in this model (**Supplementary Figure 5.2**). Therefore, if the goal of a researcher were to study short-term sensitization, then just 4 exposures would be adequate. We noted that specific IgE levels continue to increase after 4 weeks of exposure and reach much higher levels after the 8th exposure. Our goal in this study was to induce severe life-threatening anaphylaxis in wheat allergic mice upon oral allergen challenge. Therefore, we sought to saturate the system with specific IgE levels that we were able to achieve by longer-term skin exposures as we demonstrate here. Our data show that life-threatening hypothermia shock responses are induced upon oral challenge. Therefore, if the goal of a researcher were to study life-threatening oral anaphylaxis with robust hypothermia shock responses, our data suggest that eight or more skin exposures would be used. Thus, depending on the goal, other researchers might choose the appropriate length of skin sensitization and customize this model to their individual needs. The dose of protein used for skin sensitization in this study was also based on previous optimization studies.

In human studies, it is common to orally challenge patients with whole wheat flour extract. In this mouse model study, we administered salt-soluble protein extract. This is because our goal of this study was to develop a life-threatening oral anaphylaxis mouse model of wheat allergy to salt-soluble wheat proteins. We accomplished this goal with an oral challenge dose of 20mg of salt-soluble wheat protein per mouse. On a dry weight basis, whole wheat contains

~14% total protein (~11.6% would be of gluten and 2.6% would be salt-soluble non-glutens). Thus, to orally challenge mice with whole wheat extract to get optimal reactions to life-threatening anaphylaxis, we would have to challenge each mouse with ~0.84 g of whole wheat flour. The oral dosing volume advised for mice is generally 0.25–0.3ml per mouse (assuming 25 g as the average body weight of mice). Having 0.84 g of wheat flour in 0.3ml volume of saline would be technically not feasible to make, and orally administer in mice. Therefore, this is a technical limitation of the mouse model we have developed.

5.6. Conclusions

In summary, we report the development and characterization of a novel mouse-based in vivo tool for assessment of intrinsic sensitization without tape-stripping of stratum corneum or using adjuvant (that are commonly used in previous methods to create wheat-allergy models), followed by oral anaphylaxis to wheat. We also validate four quantifiable readouts of allergenicity that should be vital in future studies focused on: (i) identifying safer wheat lines; (ii) developing hypoallergenic wheat products using novel processing methods; and (iii) developing effective biologics and immunotherapies to prevent and treat wheat allergies. Finally, the panel of oral anaphylaxis-associated immune markers may represent diagnostic markers and/or therapeutic targets of life-threatening wheat anaphylaxis.

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CHAPTER 6 INTRINSIC ALLERGENICITY POTENTIAL OF DIPLOID, TETRAPLOID AND HEXAPLOID WHEATS: A PRE-CLINICAL COMPARATIVE MAP FOR IDENTIFYING HYPER/HYPO/NON-ALLERGIC WHEAT VARIETIES

6.1. Abstract

Wheat allergies are potentially life-threatening, and therefore have become a major health concern at the global level. It is largely unknown at present whether genetic variation in allergenicity potential exists among hexaploid, tetraploid and diploid wheat species. Such information is critical in establishing a baseline allergenicity map to inform breeding efforts to develop hypo/non-allergenic varieties. Using a pre-clinical mouse model of intrinsic allergenicity, here we tested the hypothesis that the salt-soluble protein extracts (SSPEs) from wheat species across ploidy levels including hexaploid (*Triticum aestivum* L.) tetraploid *(Triticum turgidum* L.*),* and diploid (*Triticum monococcum*, and *Aegilops tauschii*) will exhibit differences in intrinsic allergenicity potential. Balb/c mice were repeatedly exposed to SSPE via the skin. Allergic sensitization potential was assessed by specific IgE antibody response. Oral anaphylaxis (i.e., disease potential) was quantified by hypothermic shock response (HSR). The mucosal mast cell response (MMCR) (i.e., mechanism potential) was determined by measuring mast cell protease in the blood. Among all species, *T. monococcum* elicited the lowest sensitization and *Ae. tauschii* elicited the least HSR and MMCR. In conclusion, this pre-clinical comparative mapping strategy may be used to identify potentially hyper/hypo/non-allergenic wheat varieties.

6.2. Introduction

Food allergy is a critical public health issue at the global level (Sampson et al., 2018). It currently affects 10.8% adults and 8% children in the US (Gupta et al., 2018, 2019). And the similar trends have been reported by many other countries such as Canada, EU, Japan, and Australia (European Food Safety Authority, 2014; Food Standards Australia New Zealand, 2020; Health Canada, 2021; Japan, 2019; UK Food Standards Agency, 2021; US FDA, 2020). The annual economic impact of food allergy in the US was estimated to be $24.8 billion in 2013 (Gupta et al., 2013). Allergic reactions to offending foods can be potentially life-threatening with manifestation of systemic anaphylaxis (Warren, Jiang, & Gupta, 2020). Individuals affected by allergies must strictly follow elimination diets, which can reduce their quality of life (e.g., need for hypervigilance; increased risk for anxiety attacks) and cause a significant social burden (Gupta et al., 2019).

Wheat has extensive genetic diversity across domesticated and wild species of different ploidy levels. Modern bread wheat, *Triticum aestivum* L. (2n=6x=42) is a hexploid carrying A, B, and D genomes derived from two interspecific hybridization events (Sears, 1952). Durum wheat, *Triticum turgidum* subsp. durum (Desf.) Husnot) (2n=4x=28) is a tetraploid carrying A and B genomes. Einkorn wheat, *Triticum monococcum*, is a cultivated diploid A genome species. *Aegilops tauschii* is the D genome donor that hybridized with a tetraploid wheat species to generate modern bread wheat (McFadden & Sears, 1946).

While, wheat is a global staple food and a source of essential nutrients including protein, B vitamins and minerals, consumption can be associated with various adverse health conditions including: celiac disease, non-celiac gluten/wheat sensitivity, food protein-induced enterocolitis syndrome, eosinophilic esophagitis, wheat food allergy, wheat-dependent exercise-induced

anaphylaxis (WDEIA) and contact urticaria (Shewry & Hey, 2015). Although all these conditions are caused by the overactivation of immune system, the last four are IgE antibody mediated allergic reactions. Approximately 0.2-1.3% children and 0.9-3.6% adults in the US have wheat allergies (Poole et al., 2006; Venter et al., 2008; Venter et al., 2006; Verrill, Bruns, & Luccioli, 2015; Vierk, Koehler, Fein, & Street, 2007). In Europe, wheat allergies affect 1% children and 0.4% adults (Pereira et al., 2005; Rancé, Grandmottet, & Grandjean, 2005; Woods et al., 2002). Wheat is the third most allergenic food after milk and egg among the children in Japan (Ebisawaet al., 2020). Therefore, wheat is regulated in many countries as a major food allergen along with milk, fish, shellfish, peanuts, tree nuts, eggs, soybean, sesame, celery, lupin, mustard, and sulfites (Ebisawa et al., 2020; European Food Safety Authority, 2014; Food Standards Australia New Zealand, 2020; Gupta et al., 2011; Health Canada, 2021; Japan, 2019; Renz et al., 2018; UK Food Standards Agency, 2021; US FDA, 2021).

Wheat proteins include gluten and non-gluten fractions. The gluten fraction accounts for 80-85% of the total wheat proteins, whereas 15-20% is the salt-soluble, non-gluten fraction (Jin et al., 2019). Both types of proteins have been implicated in causing wheat allergies (Cianferoni, 2016). Wheat allergy is often confused with celiac disease, an autoimmune disease triggered by gluten proteins (Gao et al., 2021).

The current food allergen regulation per US FDA assumes that all wheats, independent of their genetics, are alike in their intrinsic allergenicity potential (US FDA, 2021). Pathogenesis of food allergy involves two distinct sequential phases. IgE production first leads to sensitization during initial exposures, followed by disease elicitation upon subsequent exposures. There are several *in vitro* studies suggest that genetically distinct wheats may differ in their IgE binding capacities (Kohno et al., 2016; Larré et al., 2011; Nakamura, Tanabe, Watanabe, & Makino,

2005). These studies examined the IgE antibody binding to proteins extracted from genetically different wheats using immunoassays. However, whether wheat species differ in their *de novo* sensitization potencies and disease elicitation properties is largely unknown. Thus, *in vivo* studies are urgently needed to test whether wheat species differ in their intrinsic allergenicity properties. Although animal models could be used to address this problem, such studies have not been reported so far.

Several animal models have been reported in the literature to study wheat allergenicity (Gao et al., 2021; Jin et al., 2019). We recently reported a novel adjuvant-free transdermal-sensitization followed by oral elicitation (TS/OE) mouse model of wheat allergy using *T. turgidum* (Gao et al., 2022). The TS/OE mouse model is uniquely suited for testing the intrinsic allergenicity potential of genetically distinct wheats because it does not use adjuvant for inducing sensitization to the wheat allergen (Gao et al., 2022). Using this *in vivo* pre-clinical tool, we tested the hypothesis that the salt-soluble protein extracts (SSPEs) from hexaploidy, tetraploid, and two diploid wheats will exhibit differences in their intrinsic allergenicity potential. Objectives for this study are to: i) validate the TS/OE mouse model for allergenic sensitization and oral disease elicitation using SSPE from *Ae. tauschii*, *T. aestivum*, *T. monococcum* and *T. turgidum* and ii) develop comparative maps of intrinsic allergenicity sensitization and intrinsic disease elicitation potential of *T. monococcum* and *T. turgidum*; and vi) develop a comparative map of mucosal mast cell response elicitation potential of these four wheat species. This study further validates the TS/OE mouse model for genetically distinct hexaploid and diploid wheats and provides pre-clinical intrinsic allergenicity potential maps of four genetically distinct wheats that may be useful in the identification of hyper/hypo/non-allergenic wheat varieties in the future.

6.3. Materials and methods

6.3.1. Chemicals and reagents

Biotin-conjugated rat anti-mouse IgE-paired antibodies were obtained from BD BioSciences (San Jose, CA, USA). Streptavidin alkaline phosphatase was obtained from Jackson ImmunoResearch (West Grove, PA, USA). BSA standard (at 2 mg/mL) was purchased from Sigma (St. Louis, MO, USA). p-nitro-phenyl phosphate was obtained from Sigma (St Louis, MO, USA). Alkaline copper tartrate was purchased from BioRad (Hercules, CA, USA). Folin reagent was purchased from BioRad (Hercules, CA). The following reagents were obtained from Invitrogen (MA, USA): IgE Mouse Uncoated ELISA Kit with Plates; Streptavidin-HRP, TMB substrate; MCPT-1 (mMCP-1) Mouse Uncoated ELISA Kit with Plates; Avidin-HRP, TMB substrate. Tissue Protein Extraction Reagent (T-PERTM, a proprietary detergent in 25mM bicine, 150 mM sodium chloride (pH 7.6) was from ThermoFisher Scientific (MA, USA). Protease (serine, cysteine, and acid proteases, and aminopeptidases) inhibitor cocktail was obtained from Sigma-Aldrich (MO, USA).

6.3.2. Generation of a plant-protein-free mouse colony

Adult Balb/cJ breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME) and were acclimated for a week upon arrival. Each male was paired with 2 females. Female pups at 6-8 weeks old were used in the experiments. All mice were maintained on the plant-protein-free diet (AIN-93G, Envigo, IN) throughout the study. Animal procedures were in accordance with Michigan State University policies.

6.3.3. Preparation of salt-soluble protein extract from wheat flours

The following wheats were used in the study: Einkorn (*Triticum monococcum*, genome AA, 2n=2x=14), durum (*Triticum durum*, cv. Carpio, genomes AABB, 2n=4x=28), common wheat (*Triticum aestivum*, cv. Ambassador, genomes AABBDD, 2n=6x=42), and *Aegilops tauschii* (*Ae. tauschii*, genome DD) wheats. *Ae. tauschii* was grown at Michigan State University greenhouses with the help of Dr. Eric Olson. Bread wheat and durum wheat were obtained from the MSU Wheat Breeding Program and North Dakota State University, respectively. Einkorn wheat was purchased from a commercial source (einkorn.com). Salt-soluble protein extracts (SSPE) were prepared from the flours of the four wheats above using a published method (Nagelkirk, 2012; Sisson, 2008). Briefly, wheat flours were mixed with 0.5M NaCl in a 1:10 ratio (m/v), and were stirred continuously for 2 hours followed by centrifugation (5000x g, 10 min) at 20°C. The supernatant was collected and stored at -70°C overnight, followed by freeze-drying the next day. Lyophilized SSPE powder was mixed with sterile saline prior to use for skin sensitization. Protein concentration of the mixture was determined using the Bio-Rad method and was adjusted to 10 mg/mL (Lowry et al., 1951).

6.3.4. Skin sensitization, bleeding, and plasma sample preparation

Adult mice were used in the experiments. Their rump hair was removed using a hair clipper (Philips, Amsterdam, Netherlands). For each mouse, fifty microliters of durum wheat SSPE (10 mg/mL) or vehicle (10% sterile NaCl solution) was applied over both sides of the clipped area (1 mg/100 uL /mouse). Mice were then covered with a non-latex bandage (Johnson & Johnson, New Brunswick, New Jersey) for one day. The same procedure above was repeated once a week for nine weeks. Bleeding was done through the saphenous vein one week before the

1st exposure (Pre) and after the 8th exposure (8R). Blood was collected into anti-coagulant (lithium heparin) coated tubes (Sarstedt Inc MicrovetteCB 300 LH, Germany) and was centrifuged to harvest the plasma. Individual plasma samples were stored at -70°C until used in analysis.

6.3.5. Elicitation of oral anaphylaxis and hypothermic shock responses

Two weeks after the 8th exposure to wheat SSPE, mice were orally gavage with vehicle (300 uL sterile saline) 15 mg or 20 mg wheat SSPE by using curved feeding needles (22-gauge, length: 1.4 inch, Kent Scientific, Torrington, CT, USA). Mice were monitored for rectal temperature before challenge (Pre) and at every 5 min up to 30 min after challenge by using a thermometer with a probe (DIGI-SENSE, MA, USA). Actual temperatures (°C) and change in rectal temperature (Δ°C) at every 5 minutes compare to the pre-temperatures for each mouse were used in analyses.

6.3.6. Measurement of wheat SSPE-specific IgE antibody levels

Wheat SSPE-specific(s) IgE antibody levels were measured using an ELISA-based method we reported previously (Hidalgo & Brandolini, 2014; Gonipeta, Kim, & Gangur, 2015; Shewry, 2009). This method was a modified version of the published method we have reported previously for food-specific IgE antibody measurement in the mouse system (Birmingham et al., 2003). Briefly, 96-well plates (Corning 3369) were coated with wheat SSPE, followed by blocking (5% gelatin), washing, plasma addition, washing, addition of biotin-conjugated anti-mouse IgE antibody, washing, addition of Streptavidin Alkaline Phosphatase and PNPP for

colorimetry as described before (Hidalgo & Brandolini, 2014; Shewry, 2009). Tests were done in quadruplicate for samples from each mouse.

6.3.7. Quantification of mucosal mast cell protease-1 (MMCP-1) level

MMCP-1 levels (ng/mL) in the plasma at 1-hour post-challenge was determined using an ELISA-based method per Invitrogen as described previously (Gonipeta et al., 2015; Shewry, 2009). Briefly, 96-well plates (Corning Costar 9018) were coated with capture antibody (anti-mouse MMCP-1), followed by addition of samples and standards (recombinant mouse MMCP-1). A sandwich was then formed when a secondary antibody (biotin-conjugated anti-mouse MMCP-1) was added. Detection was based on avidin-HRP and TMB substrate system. Assay sensitivity: 120 pg/mL. Standard range used for quantification: 15000-120 pg/mL. Tests were done in quadruplicate for samples from each mouse.

6.3.8. Statistics

An online software service was used in these analyses (https://www.socscistatistics.com/tests/). The statistical significance level was set at 0.05. Student's t-test was used to compare two groups and one-way ANOVA with post-hoc Tukey HSD was used for multiple comparisons. Pearson correlation analysis was used to determine the relationship among the allergenicity readouts.

6.4. Results

6.4.1. Validation of the transdermal sensitization and oral elicitation of disease mouse model for the *T. monococcum* using salt-soluble protein extract*6.4.1.1. Transdermal exposure to salt-soluble protein extract from T. monococcum elicits robust specific IgE antibody response in Balb/c mice*

Groups of Balb/c female mice were exposed via skin to salt-soluble protein extract (SSPE) from diploid *T. monococcum* (Einkorn, genome AA) or to saline by repeated weekly exposures as described in the methods. Blood samples collected before the 1st and after the 8th skin-exposures were used in the measurements of specific (s) IgE levels. A robust induction of sIgE antibody levels after transdermal exposure with SSPE but not vehicle was noted (~29-fold increase in sensitized mice vs. vehicle control mice) (**Figures 6.1A & 6.1B**).

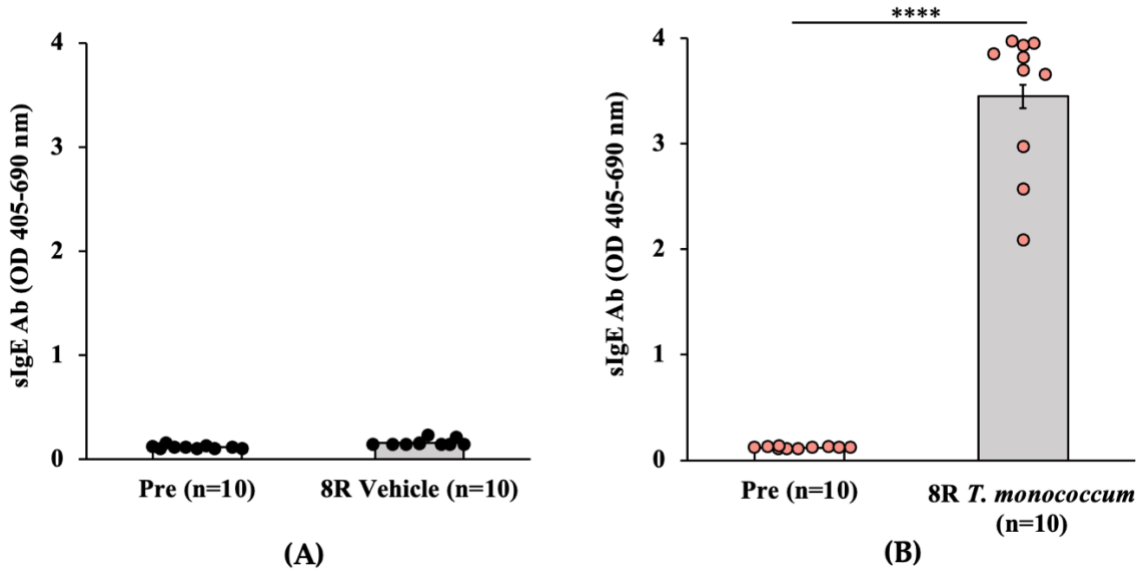

Figure 6.1. Transdermal exposure of Balb/c mice to SSPE from *T. monococcum* (genome AA) elicited robust specific (s)IgE antibody responses. Mice were exposed to *T. monococcum* SSPE or saline as described in Methods. Plasma collected before the 1st exposure (Pre) and after the 8th exposure (8R) were used in the measurement of sIgE levels (OD 405-690 nm). (A) sIgE levels in control mice. (B) sIgE levels in sensitized mice. ****$p < 0.001$, student's t-test. Ab: antibody.

6.4.1.2. Oral challenge with T. monococcum SSPE elicits hypothermia shock responses in skin-sensitized mice

We used parallel groups of skin-sensitized mice to induce anaphylaxis by performing the oral challenge with *T. monococcum* SSPE (20 mg/mouse) or saline. Anaphylactic reactions were quantified by hypothermic shock reactions (HSR) using rectal thermometry as described in methods. There was no HSR upon vehicle (i.e., zero allergen) or SSPE challenge in control mice (**Figure 6.2A, B**). In contrast, acute HSRs were observed upon oral allergen challenge in sensitized mice (**Figure 6.2C, D**). Significant HSRs were noted from 15 to 30 minutes post-oral allergen challenge (ANOVA, $p< 0.05$).

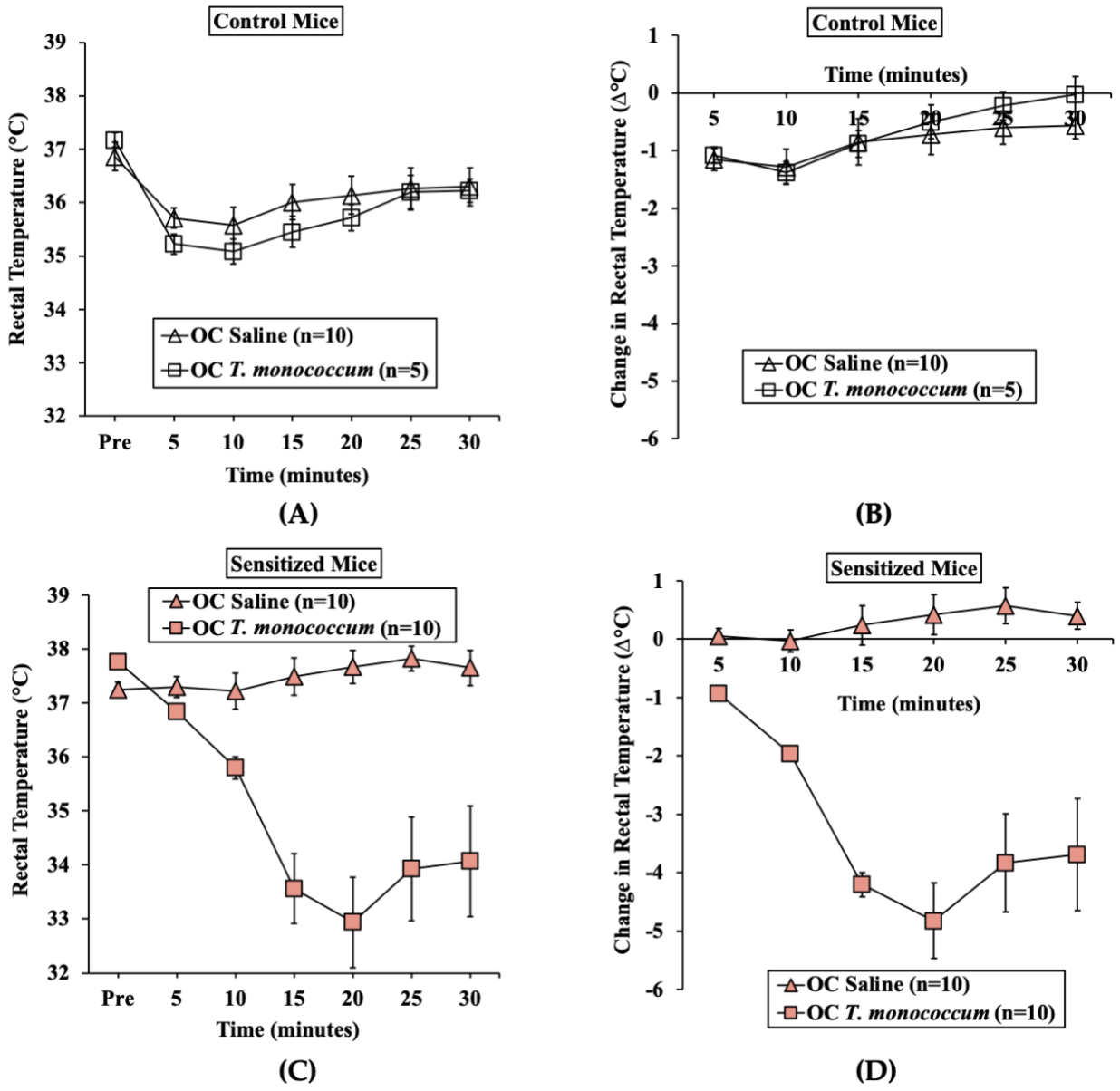

Figure 6.2. Transdermal sensitization with SSPE is sufficient for eliciting oral anaphylaxis using *T. monococcum* (genome AA) SSPE in Balb/c mice. Mice were sensitized and orally challenged with *T. monococcum* SSPE, or saline as described in Materials and Methods. (A) Actual rectal temperature at indicated time points in control mice challenged with *T. monococcum* SSPE or saline. (B) Change in rectal temperature at indicated time points in control mice challenged with *T. monococcum* SSPE or saline. (C) Actual rectal temperature at indicated time points in SSPE-sensitized mice challenged with *T. monococcum* SSPE or saline. (D) Change in rectal temperature at indicated time points in SSPE- sensitized mice challenged with *T. monococcum* SSPE or saline. Ab: antibody, OC: oral challenge, SSPE: salt-soluble protein extract.

6.4.1.3. Oral anaphylaxis elicited by T. monococcum is associated with robust mucosal mast cell response (MMCR) in Balb/c mice

It has been shown in a previous study that IgE-mediated systemic anaphylaxis is induced by degranulation of mucosal mast cells, which is manifested as acute elevation of blood levels of murine mucosal cell protease (MMCP)-1 after allergen challenge (Khodoun, Strait, Armstrong, Yanase, & Finkelman, 2011). Results of MMCP-1 responses in control mice and in sensitized mice are shown in **Figures 6.3A-B**. As evident, oral challenge with *T. monococcum* SSPE (20 mg/mouse) but not saline induces marked elevation of MMCP-1 levels in the blood (**Figure 6.3A, B**).

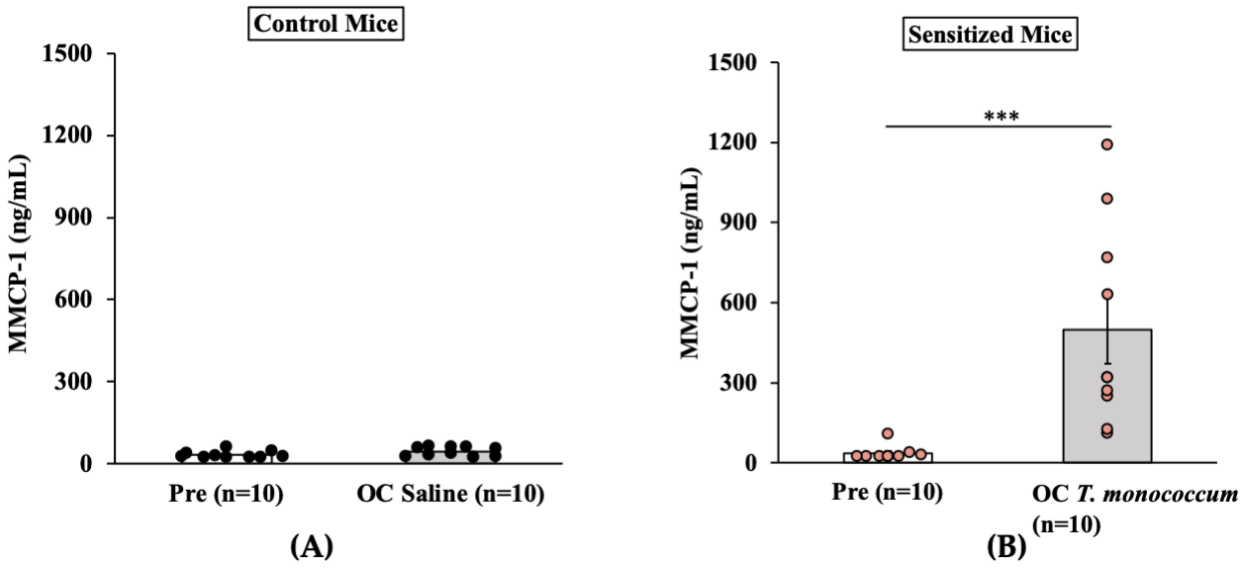

Figure 6.3. Oral challenge with SSPE from *T. monococcum* (genome AA) elicits robust mucosal mast cell response (MMCR) in Balb/c mice. Mice were sensitized and orally challenged with *T. monococcum* SSPE, or saline as described in Materials and Methods. Plasma levels of mucosal mast cell protease (MMCP)-1 levels (ng/mL) in pre and at 1-hour post challenge were measured by ELISA. (A) MMCP-1 levels in control mice challenged with saline. (B) MMCP-1 levels in sensitized mice challenged with *T. monococcum* SSPE. ***$p < 0.005$, student's t-test.

6.4.2. Validation of transdermal sensitization and oral elicitation of disease model using SSPE from *T. aestivum*

6.4.2.1. Transdermal exposure to salt-soluble protein extract from T. aestivum elicits robust specific IgE antibody response in Balb/c mice

Groups of Balb/c female mice were exposed via skin to salt-soluble protein extract (SSPE) from *T. aestivum* (genomes AABBDD) or saline by repeated weekly exposures as described in the methods. Blood collected before and after 8th skin-exposures were used in the measurements of specific (s)IgE levels. As can be seen in **Figures 6.4A**, **B**, a robust induction of

sIgE antibody levels after transdermal exposure with SSPE but not vehicle was noted (~43-fold increase in sensitized mice vs. control mice).

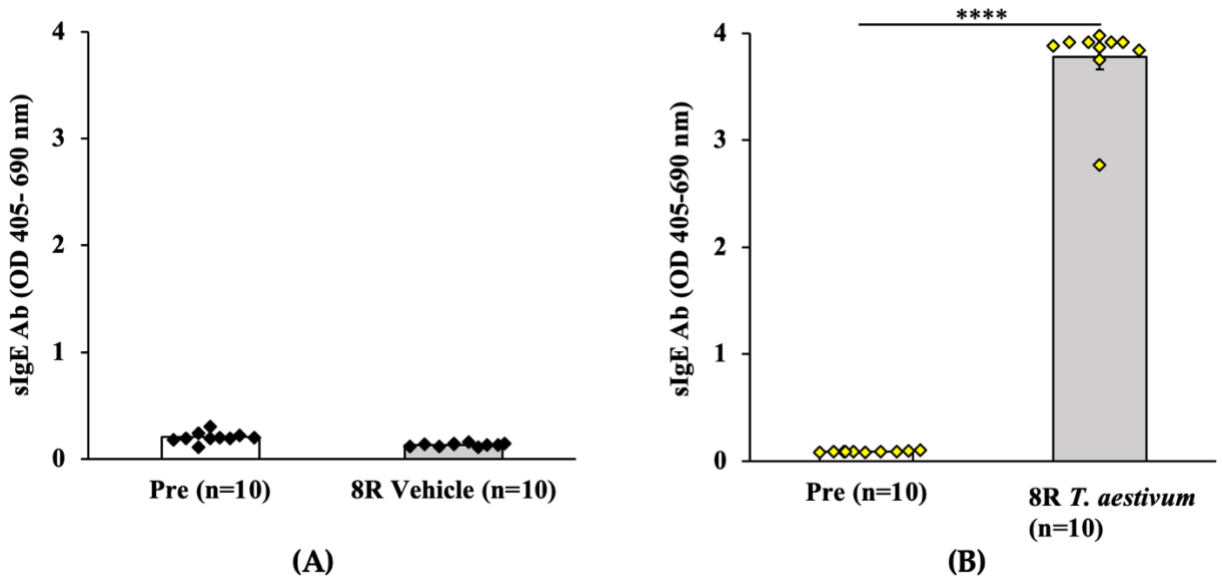

Figure 6.4. Transdermal exposure of Balb/c mice to SSPE from *T. aestivum* (genomes AABBDD) elicited robust specific (s)IgE antibody responses. Mice were exposed to *T. aestivum* SSPE, or saline as described in Methods. Plasma collected before the 1st exposure (Pre) and after the 8th exposure (8R) were used in the measurement of sIgE levels (OD 405-690 nm). (A) sIgE levels in control mice. (B) sIgE levels in sensitized mice. ****$p < 0.001$, student's t-test. Ab: antibody.

6.4.2.2. Oral challenge with T. aestivum SSPE elicits hypothermia shock responses in skin-sensitized mice

We used parallel groups of skin-sensitized mice to induce anaphylaxis by performing the oral challenge with *T. aestivum* SSPE (20 mg/mouse) or saline. Anaphylactic reactions were quantified by hypothermic shock reactions (HSR) using rectal thermometry as described in methods. There was no HSR upon vehicle (i.e., zero allergen) or SSPE challenge in control mice (**Figures 6.5A, B**). In contrast, acute HSRs were observed upon oral allergen challenge in sensitized mice (**Figures 6.5C, D**). Significant HSRs were noted from 15 to 30 minutes post-oral allergen challenge (ANOVA, $p < 0.05$).

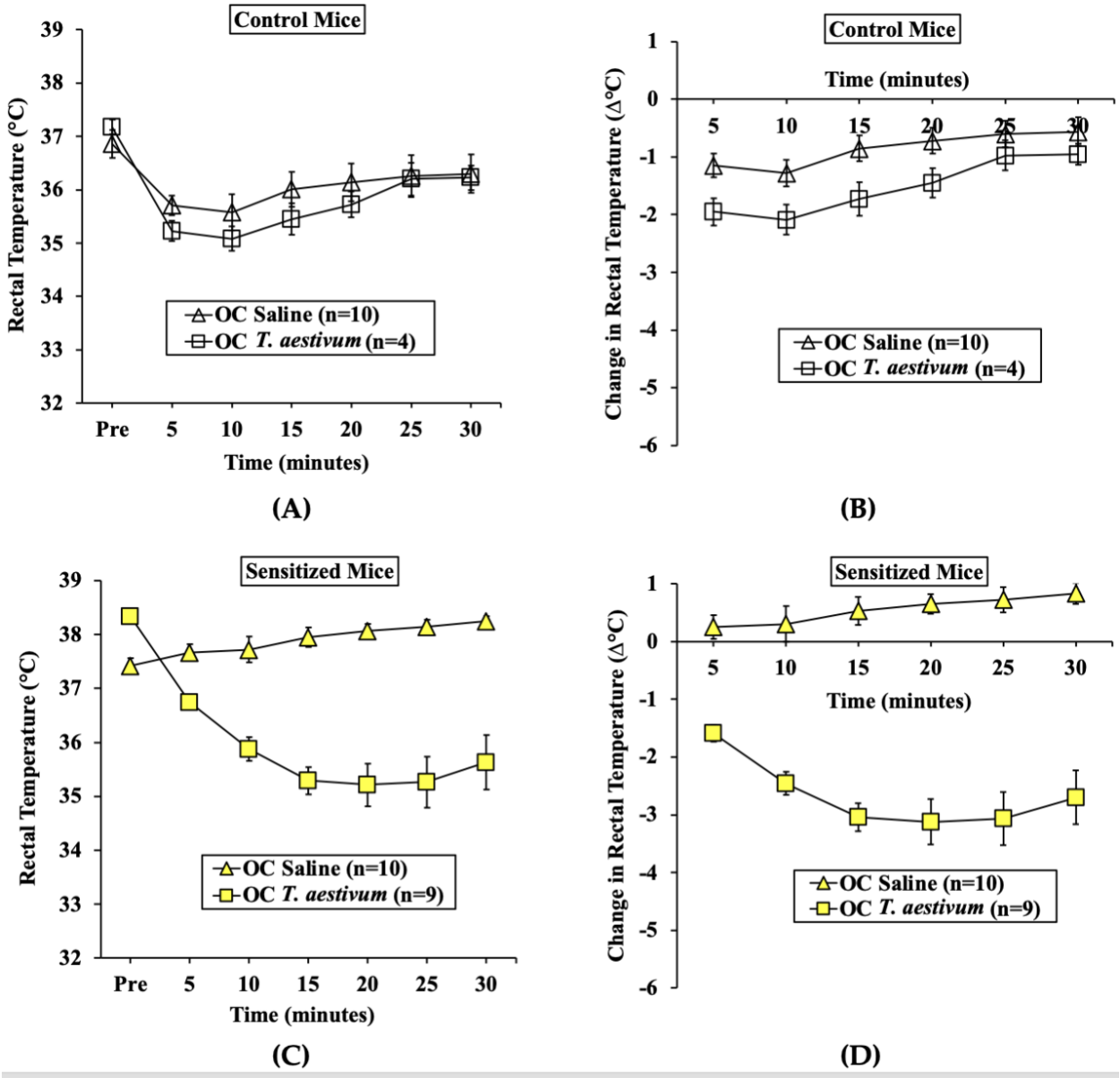

Figure 6.5. Transdermal sensitization with SSPE is sufficient for eliciting oral anaphylaxis using *T. aestivum* SSPE in Balb/c mice. Mice were sensitized and orally challenged with *T. aestivum* SSPE, or saline as described in Materials and Methods. (A) Actual rectal temperature at indicated time points in control mice challenged with common wheat SSPE or saline. (B) Change in rectal temperature at indicated time points in control mice challenged with *T. aestivum* SSPE or saline. (C) Actual rectal temperature at indicated time points in control mice challenged with *T. aestivum* SSPE or saline. (D) Change in rectal temperature at indicated time points in allergic mice challenged with *T. aestivum* SSPE or saline. Ab: antibody, OC: oral challenge, SSPE: salt-soluble protein extract.

6.4.2.3. Oral anaphylaxis elicited by T. aestivum SSPE is associated with robust mucosal mast cell response (MMCR) in Balb/c mice

Results of MMCP-1 responses in control mice and in sensitized mice are shown in **Figures 6.6A, B**. As evident, oral challenge with *T. aestivum* SSPE (20 mg/mouse) but not saline induces marked elevation of MMCP-1 levels in the blood (**Figure 6.6A, B**).

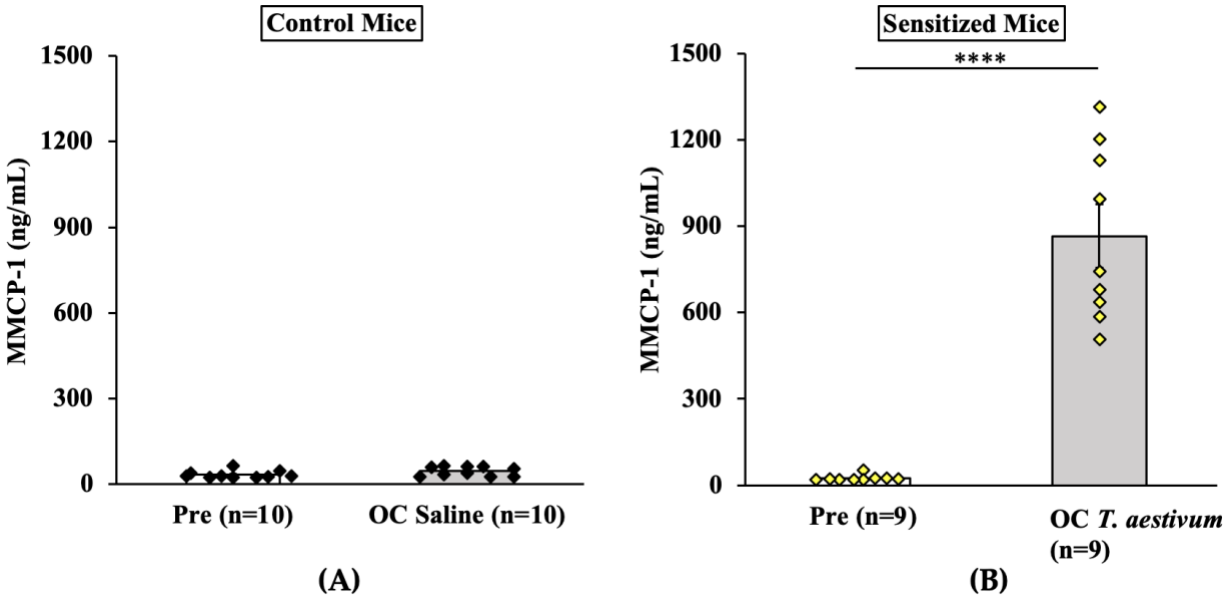

Figure 6.6. Oral challenge with SSPE from *T. aestivum* elicits robust mucosal mast cell response (MMCR) in Balb/c mice. Mice were sensitized and orally challenged with *T. aestivum* SSPE, or saline as described in Materials and Methods. Plasma levels of mucosal mast cell protease (MMCP)-1 levels (ng/mL) in pre and at 1-hour post challenge were measured by ELISA. (A) MMCP-1 levels in control mice challenged with saline. (B) MMCP-1 levels in sensitized mice challenged with *T. aestivum* SSPE. ***$p < 0.005$, student's t-test.

6.4.3. Validation of transdermal sensitization and oral elicitation of disease model using SSPE from *Aegilops tauschii*

6.4.3.1. Transdermal exposure to salt-soluble protein extract from Aegilops tauschii elicits robust specific IgE antibody response in Balb/c mice

Groups of Balb/c female mice were exposed via skin to salt-soluble protein extract (SSPE) from *Ae. tauschii* (genome DD) or saline by repeated weekly exposures as described in the methods. Blood collected before and after 8th skin-exposures were used in the measurements of specific (s)IgE levels. As can be seen in **Figures 6.7A, B**, a robust induction of sIgE antibody levels after transdermal exposure with SSPE but not vehicle was noted (~36-fold increase in sensitized mice vs. control mice).

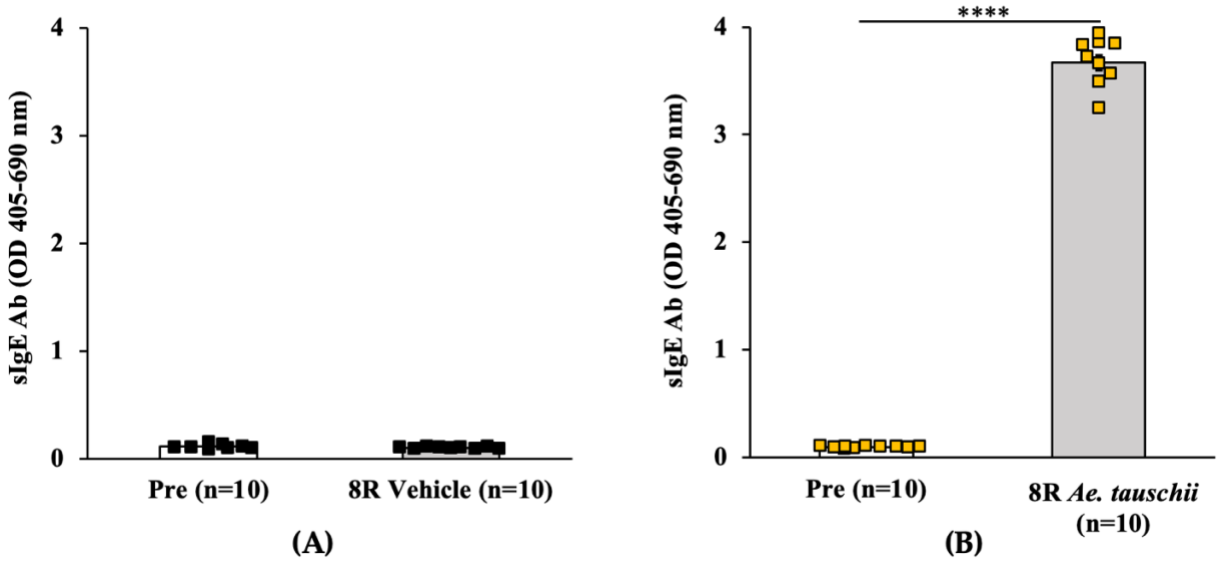

Figure 6.7. Transdermal exposure of Balb/c mice to SSPE from *Ae. tauschii* elicited robust specific (s)IgE antibody responses. Mice were exposed to *Ae. tauschii* SSPE or saline as described in Methods. Plasma collected before the 1st exposure (Pre) and after the 8th exposure (8R) were used in the measurement of sIgE levels (OD 405-690 nm). (A) sIgE levels in control mice. (B) sIgE levels in sensitized mice. ****$p < 0.001$, student's t-test. Ab: antibody.

6.4.3.2. Oral challenge with Ae. tauschii SSPE elicits hypothermia shock responses in skin-sensitized mice

We used parallel groups of skin-sensitized mice to induce anaphylaxis by performing the oral challenge with *Ae. tauschii* SSPE (20 mg/mouse) or saline. Anaphylactic reactions were quantified by hypothermic shock reactions (HSR) using rectal thermometry as described in methods. There was no HSR upon vehicle (i.e., zero allergen) or SSPE challenge in non-allergic control mice (**Figures 6.8 A, B**). In contrast, acute HSRs were observed upon oral allergen challenge in sensitized mice (**Figures 6.8 C, D**). Significant HSRs were noted from 15 to 30 minutes post-oral allergen challenge (ANOVA, $p < 0.05$).

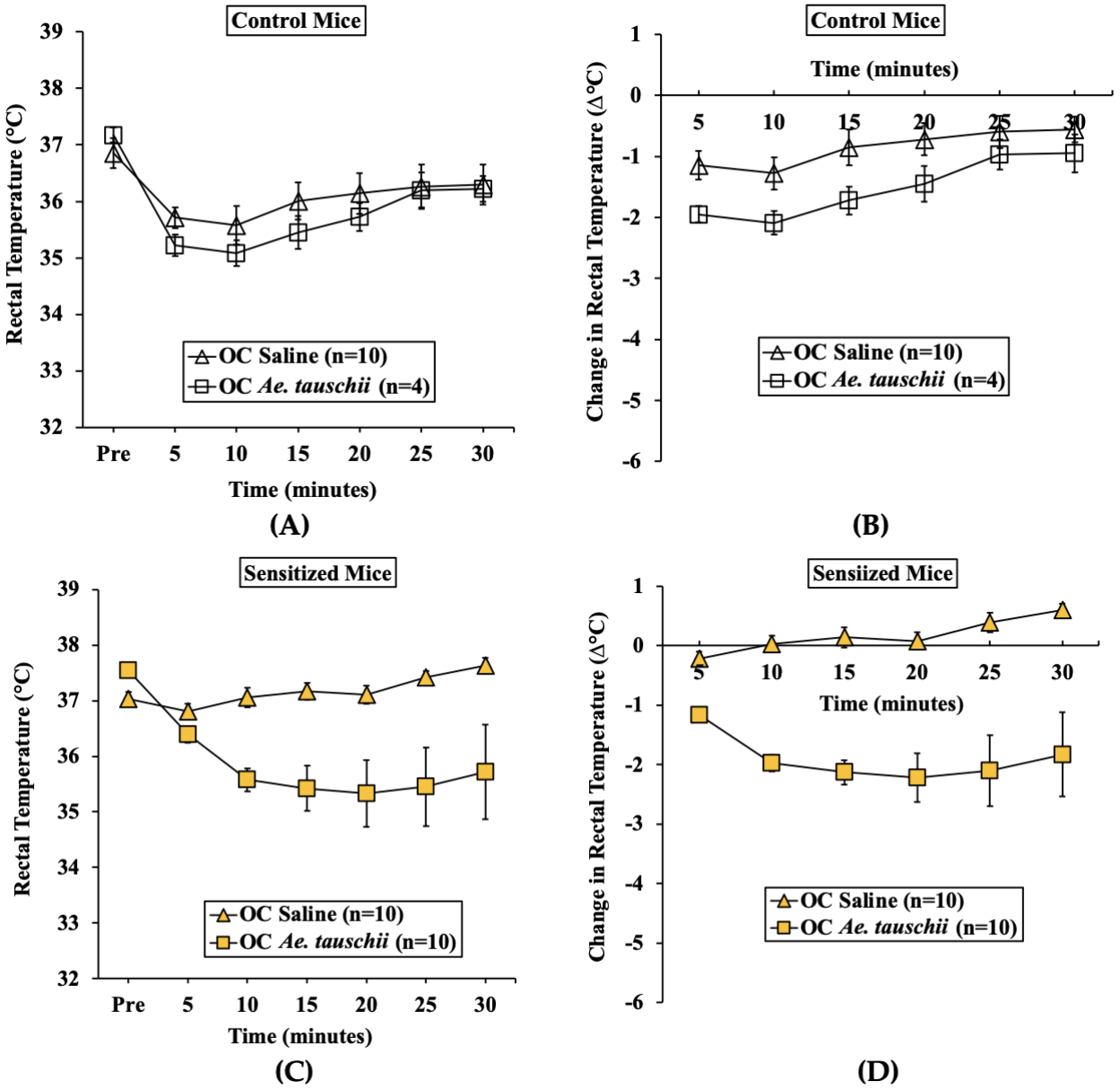

Figure 6.8. Transdermal sensitization with SSPE is sufficient for eliciting oral anaphylaxis using *Ae. tauschii* SSPE in Balb/c mice. Mice were sensitized and orally challenged with *Ae. tauschii* SSPE or with saline as described in Materials and Methods. (A) Actual rectal temperature at indicated time points in control mice challenged with *Ae. tauschii* SSPE or saline. (B) Change in rectal temperature at indicated time points in control mice challenged with *Ae. tauschii* SSPE or saline. (C) Actual rectal temperatures at indicated time points in SSPE-sensitized mice challenged with *Ae. tauschii* SSPE or saline. (D) Change in rectal temperature at indicated time points in SSPE-sensitized mice challenged with *Ae. tauschii* SSPE or saline. Ab: antibody, OC: oral challenge, SSPE: salt-soluble protein extract.

6.4.3.3. Oral anaphylaxis elicited by Ae. tauschii is associated with robust mucosal mast cell response (MMCR) in Balb/c mice

Results of MMCP-1 responses in control mice and in sensitized mice are shown in **Figures 6.9A-B**. As evident, oral challenge with *Ae. tauschii* SSPE (20 mg/mouse) but not saline induces marked elevation of MMCP-1 levels in the blood (**Figure 6.9A, B**).

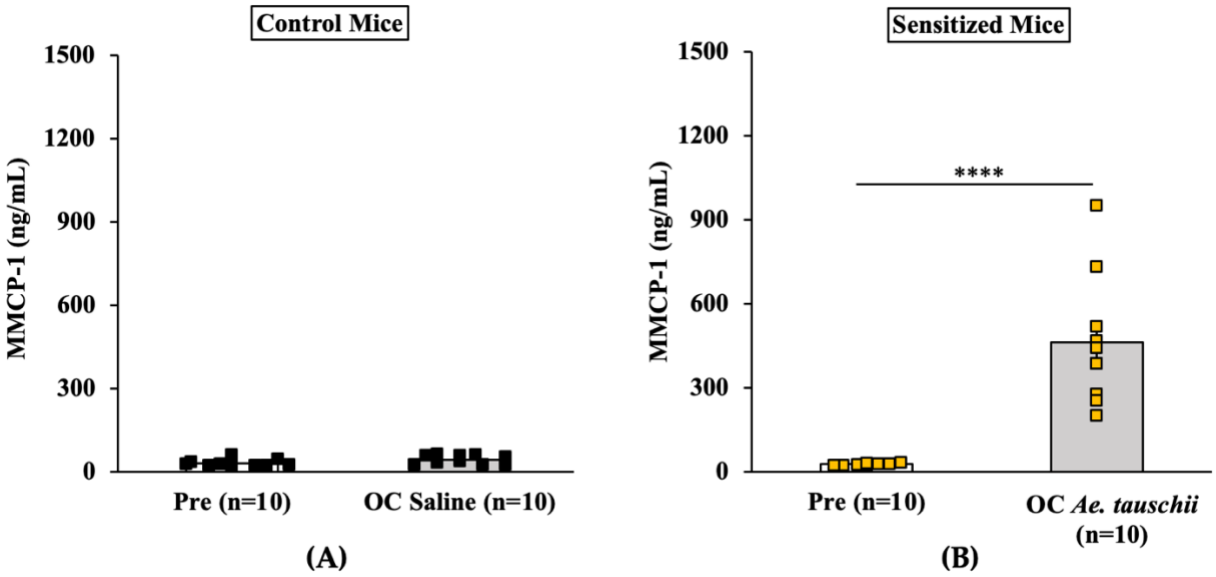

Figure 6.9. Oral challenge with SSPE from *Ae. tauschii* elicits robust mucosal mast cell response (MMCR) in Balb/c mice. Mice were sensitized and orally challenged with *Ae. tauschii* SSPE, or saline as described in Materials and Methods. Plasma levels of mucosal mast cell protease (MMCP)-1 levels (ng/mL) in pre and at 1-hour post challenge were measured by ELISA. (A) MMCP-1 levels in control mice challenged with saline. (B) MMCP-1 levels in allergic mice challenged with *Ae. tauschii* SSPE. ***$p < 0.005$, student's t-test.

6.4.4. Comparative map of the intrinsic allergenicity sensitization potential of diploid, tetraploid, and hexaploid wheat

We used the sIgE data from the above validation studies and our previously reported durum wheat study (Gao et al 2022) for preparing a comparative sensitization map. The sIgE

antibody levels elicited by respective wheats were determined by subtracting the baseline (pre) sIgE levels from the 8th response (8R) sIgE levels. The resulting comparative map of the intrinsic allergenicity sensitization potential of the four genetically distinct wheats is shown in **Figure 6.10**. *T. durum* and *T. aestivum* SSPEs elicited almost identical sIgE levels and *Ae. tauschii* elicited slightly lower sIgE levels. *T. monococcum* (genome AA) SSPE elicited significantly lower sIgE levels than the other three wheats.

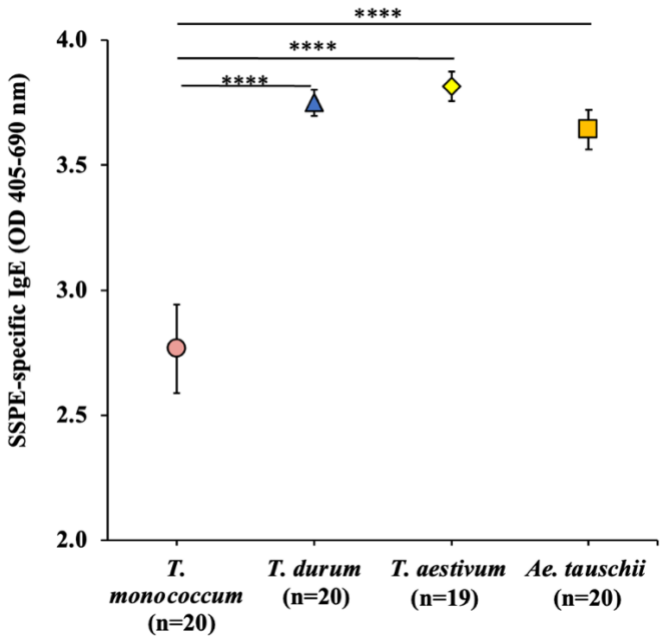

Figure 6.10. Comparative map of the intrinsic allergenicity sensitization potential of diploid, tetraploid, and hexaploid wheat. The changes in SSPE-specific IgE antibody levels after the 8th skin exposure to SSPEs from the respective wheats are shown in the figure. ****$p <$ 0.001, one-way ANOVA and Tukey's post hoc tests.

6.4.5. Comparative map of the intrinsic allergenicity disease elicitation potential of diploid, tetraploid, and hexaploid wheat

We used the absolute changes in the rectal temperature data upon oral allergen challenge from the above validation studies of *T. monococcum*, *T. aestivum* and *Ae. tauschii*, and our previously reported durum wheat (*T. durum*) studies (Gao et al 2022) for preparing a comparative disease elicitation map. **Figures 6.11 A and B** show the disease elicitation potential map at 15 and 20 minutes post oral allergen challenge with a 15 mg SSPE dose. **Figures 6.11 C and D** show the disease elicitation potential map at 15 and 20 minutes post oral allergen challenge with a 20 mg dose. As evident, *Ae. tauschii* elicited the least HSR compared to the other wheat species. *T. monococcum* elicited lower HSR responses compared to *T. aestivum* and *T. durum* at 15 mg dose, but not at 20 mg dose (**Figure 6.11A-D**). Similar disease elicitation potential maps were obtained for 25 and 30 minutes post oral allergen challenge time points (data not shown).

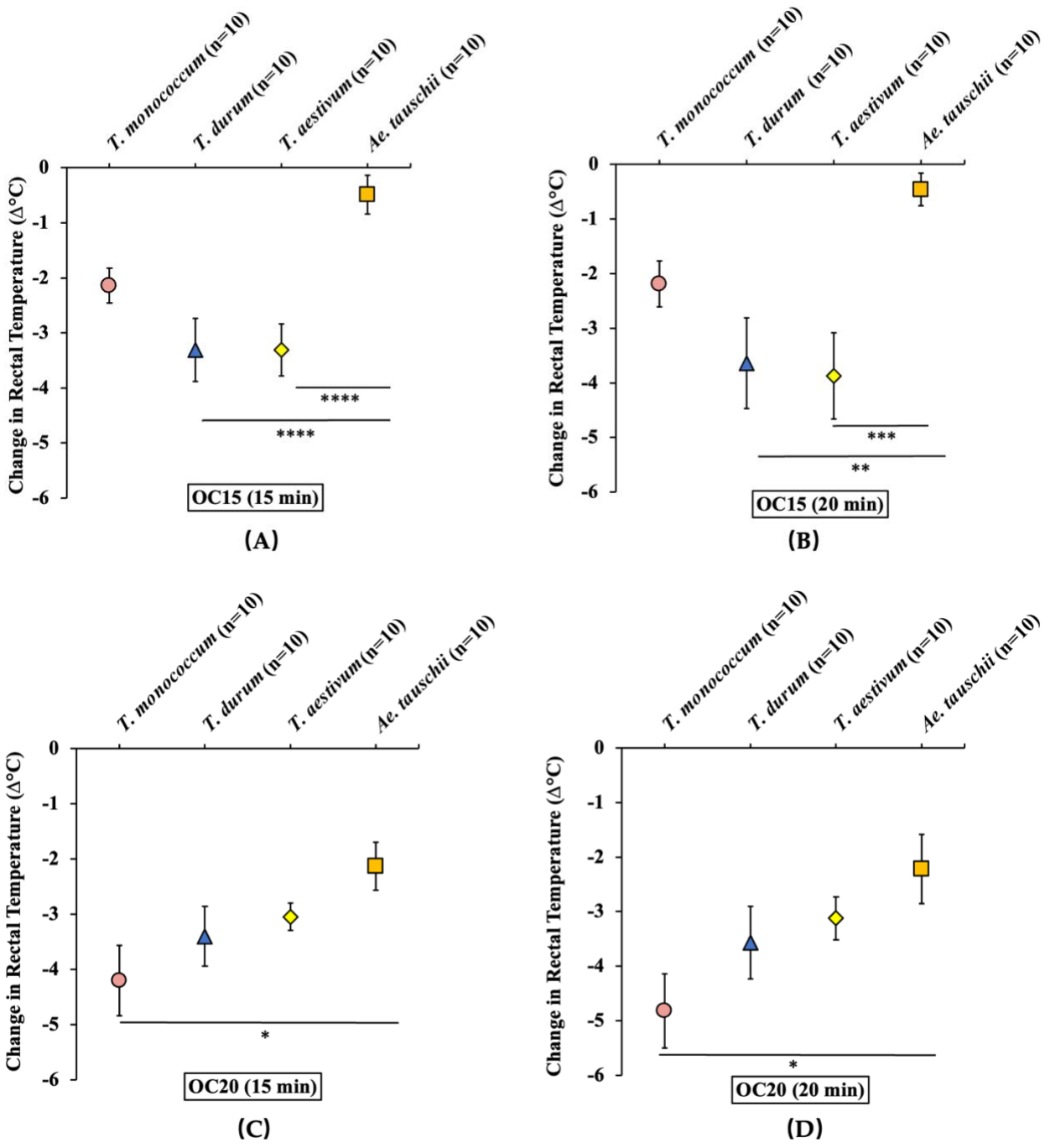

Figure 6.11. Comparative map of the intrinsic allergenicity disease elicitation potential of diploid, tetraploid, and hexaploid wheat. (A-B) HSRs at 15 minutes and 20 minutes after oral challenge doses of 15 mg SSPE of diploid, tetraploid, and hexaploid wheat. (C-D) HSRs at 15 min and 20 minutes after oral challenge doses of 20 mg of diploid, tetraploid, and hexaploid wheat. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$, one-way ANOVA and Tukey's post hoc tests.

6.4.6. Comparative map of the mucosal mast cell response (MMCR) elicitation potential of diploid, tetraploid, and hexaploid wheat

We used the MMCP-1 data upon oral allergen challenge from the above validation studies of *T. monococcum*, *T. aestivum* and *Ae. tauschii*, and our previously reported durum wheat (*T. durum*) studies (Gao et al 2022) for preparing a comparative MMCR elicitation potential map. **Figures 6.12 A and B** show the MMCR elicitation potential maps at 15 and 20 mg oral allergen challenge doses, respectively. As evident, *T. aestivum* elicited the highest MMCR, followed by *T. monococcum* and *T. durum* which were comparable to each other. *Ae. tauschii* elicited the lowest MMCR, which was significantly lower than that of *T. aestivum*, but not of the *T. monococcum* or *T. durum.*

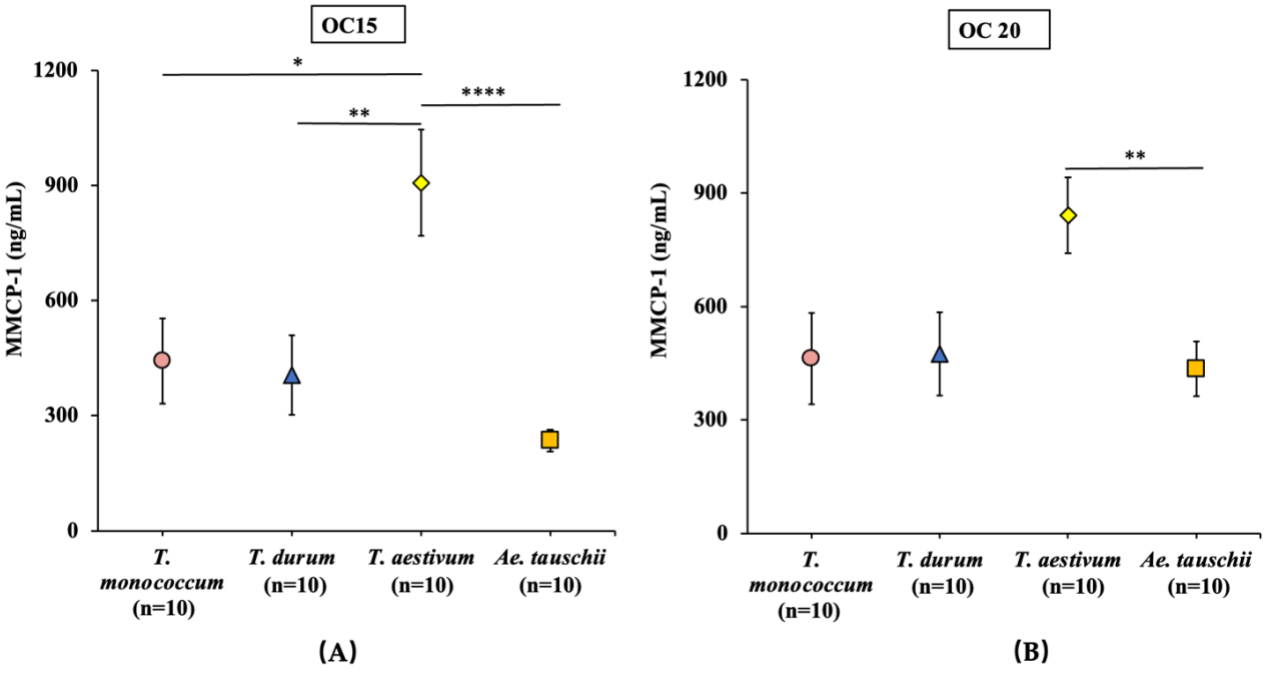

Figure 6.12. Comparative map of the mucosal mast cell response (MMCR) elicitation potential of diploid, tetraploid, and hexaploid wheat. (A) Average MMCR blood level after 15 mg oral SSPE allergen challenge dose. (B) Average MMCR blood level after 20 mg oral allergen challenge dose. *$p < 0.05$, **$p < 0.01$, ****$p < 0.001$, one-way ANOVA and Tukey's post hoc tests. OC: oral challenge. MMCP-1: mucosal mast cell protease-1.

6.5. Discussion

Here, we tested the hypothesis that the salt-soluble protein extracts (SSPEs) from hexaploid *T. aestivum*, tetraploid *T. durum*, and diploid *T. monococcum*, and *Ae. tauschii* wheat exhibit differences in intrinsic allergenicity potential. We were uniquely positioned to test this hypothesis because we recently published a novel adjuvant-free transdermal sensitization/oral elicitation (TS/OE) mouse model using durum wheat that could be used as a pre-clinical tool to address this hypothesis (Gao et al., 2022). We first validated the TS/OE mouse model for the hexaploid wheat *T. aestivum* (common wheat, cv. Ambassador), and the two diploid wheat relatives, *T. monococcum* and *Ae. tauschii*. Then, we developed a comparative map illustrating the intrinsic allergenicity potential of these wheat species of differing ploidy levels. Overall, our data support the hypothesis tested.

This research reports six novel findings: i) validation of the TS/OE mouse model for allergenic sensitization and oral disease elicitation using SSPE from diploid *T. monococcum*; ii) validation of the TS/OE mouse model for allergenic sensitization and oral disease elicitation using SSPE from *T. aestivum*; iii) validation of the TS/OE mouse model for allergenic sensitization and oral disease elicitation using SSPE from *Ae. tauschii*; iv) development of a comparative map of intrinsic allergenicity sensitization potential of *T. aestivum*, *T. durum*, *T. monococcum*, and *Ae. tauschii*; v) development of a comparative map of intrinsic disease elicitation potential of these four wheats; and vi) development of a comparative map of mucosal mast cell response elicitation potential of these four wheats. Thus, this work has not only validated the TS/OE mouse model for genetically distinct hexaploid and diploid wheats, but also provides pre-clinical intrinsic allergenicity potential maps of diploid, tetraploid, and hexaploid wheat.

We chose wheat species representing the wheat diploid AA, DD, tetraploid AABB and hexaploid AABBDD genomes. We previously developed and characterized the TS/OE mouse model using SSPE from durum wheat variety, Carpio (Sissons, 2008). The *T. aestivum* variety used, Ambassador, is a hexaploid common wheat (genomes AABBDD) that is commonly used for cracker and cookie making (Nagelkirk, 2012). *T. monococcum* is a cultivated form of the A genome species, einkorn that is commercially available and there is significant research interest in characterizing its health promoting properties (Hidalgo & Brandolini, 2014). *Ae. tauschii* is the D genome donor to modern common wheat and no cultivated forms have been developed. It is noteworthy that although we chose one representative variety from each genotype in this study, future research is needed to verify whether or not the chosen variety might represent most other varieties within each wheat species for intrinsic allergenicity potential (Shewry, 2009).

Compared to other animal models (i.e., dog, rat, and swine), mouse models have several advantages including relatively low cost of purchase and management, and ready availability of reagents (Jin et al., 2019). Most previously published mouse models of wheat allergy are adjuvant-based, which reflect a situation of co-exposure to both allergen and adjuvants, and they are very useful to study mechanisms of disease. However, they are not considered suitable to evaluate intrinsic allergenicity of wheat proteins because the adjuvants are thought to enhance sensitivity and reduce specificity (Gao et al., 2019; Gonipeta et al., 2015). For example, the intrinsic allergenicity property of wheat proteins independent of the effect of adjuvant is difficult to decipher from such models. Therefore, they do not reveal the intrinsic allergenicity of wheat proteins. On the contrary, an adjuvant-free mouse model is preferred to address this issue as it will make the data interpretation much easier without the need of differentiating the effect of adjuvant from that of allergens. Gangur and coworkers have developed a novel adjuvant-free

transdermal sensitization and oral elicitation (TS/OE) mouse model of food allergy that is capable of simulating many aspects of human food allergies (Gonipeta et al., 2015). This model has been utilized in assessing the intrinsic allergenicity of multiple food allergens (e.g., shellfish, tree nuts, milk, and sesame) including wheat (Birmingham et al., 2007; Gao et al., 2022; Gonipeta et al., 2010; Navuluri et al., 2006; Parvataneni et al., 2009; Parvataneni et al., 2016). Therefore, we employed the TS/OE mouse model in this study.

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There are two previous studies that demonstrated the potential variations in *in vitro* IgE binding allergenicity among different wheat lines/varieties. Nakamura et al (2005) tested the IgE binding capacity of several wheat varieties that were diploid (*T. monococum*), tetraploid (*T. durum, T. dicoccum, T. polonicum, T. turgidum*), and hexaploid (*T. aestivum, T. compactum, T. spelta*) in nature (Nakamura et al., 2005). Then they used direct IgE ELISA to characterize the allergenicity of 324 wheats, among which several candidates, including Einkorn (*T. monococum*), were identified as less allergenic based on binding to IgE antibodies obtained from wheat-allergic patients. They found that IgE reactivities of tetraploid and hexaploid wheats were higher than that of the diploid Einkorn wheat. These data concur in principle with our findings that *T. monococum* (Einkorn) elicited lower IgE production responses compared to the tetraploid durum wheat in our TS/OE mouse model. Larré et al (2011) compared the IgE-binding capacity of salt-soluble protein extracts (SSPE) from the hexaploid wheat (*T. aestivum*, cultivar Récital) with that of the diploid wheat Engrain (*T. monococcum*, genome AA) (Larré et al., 2011). Although they used different varieties of hexaploid and diploid wheats than that we have used in this mouse model study, results are consistent that diploid A genome wheat species demonstrate lower IgE binding capacity than the hexaploid wheat.

When fractionated, wheat proteins are composed of salt-insoluble glutens and salt-soluble non-gluten proteins. Both protein fractions act as allergens and trigger wheat allergy symptoms in humans (Cianferoni, 2016). In this study, we focused on investigating the allergenicity of non-gluten wheat proteins (i.e., SSPE) as it is under-studied compared to its gluten counterpart. In addition, none of the studies in the past have compared the allergenicity of SSPE or glutens from four genetically different wheats. This gap in knowledge makes it important to examine the intrinsic allergenicity of both SSPEs (non-glutens) as well as glutens from genetically distinct

wheat protein fractions. Here we have validated the TS/OE model and developed intrinsic allergenicity potential maps using SSPEs from four distinct wheats. A similar approach could be used to validate the TS/OE model and develop intrinsic allergenicity potential maps for glutens from diploid, tetraploid, and hexaploid wheat.

Many studies have shown that food processing can alter the allergenicity of food proteins including those of wheat (Gao et al., 2021; Masthoff et al., 2013; Ortiz et al., 2016; Vanga et al., 2017). For instance, wheat allergens under novel processing methods may have new epitopes generated or hidden epitopes revealed, either of which may increase their allergenicity. The intrinsic allergenicity potential maps could be developed using processed wheats applying the approach presented here. By comparing the allergenicity potential maps of native vs. processed wheat proteins, it is possible to determine the quantitative effects of processing on intrinsic allergenicity within each genotype of wheat. Such work has the potential to identify and tailor specific processing conditions to produce hypo/non-allergenic wheat products within a particular genetic background. At the same time, using comparative potential maps of allergenicity, it is also possible to identify potentially hyper-allergenic wheats.

In summary, we report the first utilization of an adjuvant-free mouse model as a pre-clinical testing tool in assessing the natural variation in the intrinsic allergenic potential among diploid, tetraploid and hexaploid wheat. We demonstrate for the first-time differences in the intrinsic allergenicity potential among wheat species of different ploidy levels. This pre-clinical comparative mapping strategy may be used to identify potentially hyper/hypo/non-allergenic wheat varieties and in assessing the changes to intrinsic wheat allergenicity due to processing from respective wheat genotypes.

6.6. Conclusions

We demonstrate for the first-time similarities and differences in the intrinsic allergenicity potential among the four selected genetically distinct wheats. This pre-clinical comparative mapping strategy may be used to identify potentially hyper/hypo/non-allergenic wheat varieties created by genetic modification and assess changes in allergenicity of processed wheats from respective wheat genetic backgrounds.

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CHAPTER 7 FUTURE DIRECTIONS

7.1. Test various wheat varieties/lines/cultivars and GM wheats for intrinsic allergenicity using the validated TS/OE mouse model

There are thousands of genetically distinct wheat varieties/cultivars/lines are currently consumed worldwide (Shewry, 2009). The validated TS/OE mouse model described in this dissertation can be used as a pre-clinical testing tool to evaluate the differences in their allergenicity so that hypo/hyper/non-allergenic wheats can be identified. Furthermore, the model can also be used for safety assessment of GM wheats when they are developed.

7.2. Test the effect of food processing and industrial processing on wheat allergenicity

As discussed in Chapter 3, food and industrial processing have significant impact on wheat allergenicity. The validated TS/OE mouse model can be used to test their effects on intrinsic wheat allergenicity *in vivo*. This model will be useful to develop novel processing methods to reduce wheat allergenicity.

7.3. Developing improved immunotherapies to prevent and treat wheat allergy

There is no vaccine for wheat allergy at present. There is no cure for wheat allergies also. The TS/OE mouse model described in this dissertation can be used to develop novel and improved methods to prevent and treat life-threatening wheat food allergy.

7.4. Testing the role of environmental factors in the development of wheat allergy

Numerous studies have shown that the use of antacids/antiulcer agents, endocrine-disrupting compounds can facilitate the development of food allergies (Mitre et al., 2018; Savage et al., 2012; Tobar et al., 2016; Untersmayr & Jensen-Jarolim, 2008). However, the specific roles that these agents and compounds played in wheat food allergy remains unknown. Therefore, future efforts could investigate the effect of environmental factors on wheat food allergy using this mouse model.

7.5. Testing the role of genetic factors in wheat allergy

Previous studies show that several genetic factors may play in the development of wheat allergy in humans (Hur et al., 2013; Iga et al., 2013; Mizuno et al., 2015; Noguchi et al., 2019). The TS/OE mouse model can be used to elucidate the role of various genetic factors (e.g., using gene knockout, targeted cell gene knockouts, and transgenic approaches for cytokines, cytokine receptors, signaling molecules etc.) in wheat food allergy associated systemic anaphylaxis.

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