Einfluss verschiedener Getreidearten und Herstellungsverfahren auf den Gehalt immunogener Substanzen in Brot sowie *in vivo* auf die Verträglichkeit an der Maus und im Menschen

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- **Zimmermann J**., Hubel P., Pfannstiel J., Afzal M., Longin CFH., Hitzmann B., Götz H., Bischoff SC. Comprehensive proteome analysis of bread deciphering the allergenic potential of bread wheat, spelt and rye. J Proteomics. 2021; 247:104318

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- Kaden-Volynets V., Günther C., **Zimmermann J**., Beisner J., Becker C., Bischoff S.C. Deletion of the Casp8 gene in mice results in ileocolitis, gut barrier dysfunction and malassimilation, which can be partially attenuated by inulin or butyrate. Am J Physiol Gastrointest Liver Physiol. 2019; 317:G493-G507

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- Bischoff S.C., **Zimmermann J**. Weizenunverträglichkeiten – Hype oder Herausforderung? Aktuelle Ernährungsmedizin 2018; 43:459-466
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Zusammenfassung

Inzwischen gibt es drei Krankheitsbilder, die durch Verzehr von Getreide getriggert werden. Die Zöliakie und die Weizenallergie sind eingehend untersucht, während die Nicht-Zöliakie Weizensensitivität (NCWS), deren Inzidenz ansteigt, viele Fragen aufwirft. Die Symptomatik ist unspezifisch, es fehlen diagnostische Marker und die Pathophysiologie ist unklar. Die Diagnostik der NCWS erfolgt lediglich auf Basis des Ausschlusses von Weizenallergie und Zöliakie. Als Auslöser stehen neben bakteriell fermentierbaren Kohlenhydraten (FODMAPs) ausgewählte Getreideproteine wie Gluten oder α-Amylase-Trypsin-Inhibitoren (ATIs) im Fokus der Forschung. Ziel der vorliegenden Arbeit war es zum einen, den Einfluss der Wahl des Getreides (Weichweizen, Dinkel, Roggen) und der Herstellung von Brot (Vermahlungsgrad und Wahl zwischen Hefe- und Sauerteig) auf das Vorliegen potenziell immunogener Proteine auf Basis einer Proteomanalyse zu untersuchen. In einem zweiten Schritt sollte die Verträglichkeit ausgewählter Brote in einem transgenen Mausmodell mit intestinalen Entzündungen und in einer Humanstudie an Patienten mit NCWS und subjektiver Dinkeltoleranz auf ihre Verträglichkeit untersucht werden. Dadurch sollten mögliche Auslöser der NCWS eingegrenzt und zugrundeliegende Mechanismen untersucht werden.

Auf Basis einer quantitativen Proteomics Methode (nano-UHPLC-ESI-MS basiert) konnten mit der Identifizierung von mehr als 4000 verschiedenen Proteinen in Brot- und Mehlproben zum ersten Mal umfassende Proteomdaten für Brot und Mehl generiert werden. Zudem konnten wir zeigen, dass auch Roggen- und Dinkelproteine anhand der Weizendatenbank (*Triticum aestivum*) identifiziert werden, was auf eine hohe Sequenzhomologie zwischen den Getreidearten hindeutet. Zudem wurde auf Basis der Pfam Annotierung eine Liste mit bekannten und potenziell immunogenen Getreideproteinen erstellt, die für weitere Analysen herangezogen wurde. Dabei zeigte sich, dass weder die absolute Anzahl noch die Abundanz dieser Proteine abhängig vom Mahlgrad des Mehls oder dem Fermentationsprozess des Teiges waren, was bedeutet, dass sie bei der Brotherstellung nicht selektiv abgebaut werden. Jedoch konnten in Dinkel- und Weizenproben im Vergleich zu Roggenproben solche Proteine in höherer Anzahl und höherer relativer Menge identifiziert werden.

Der Einfluss ausgewählter Brote von der Proteomanalyse wurde *in vivo* an einem Mausmodell untersucht. Eine Hypothese zur Entstehung von NCWS basiert auf einer latenten oder manifesten Vorerkrankung des Darmes wie z.B. eine Störung der Darmbarriere als Ursache für die Symptome nach Getreideverzehr. Aus diesem Grund wurde für die vorliegende Arbeit ein

Mausmodell (Casp8$^{\Delta IEC}$) gewählt, das durch den Knockout des Caspase-8 Gens charakterisiert ist, was zu einem Verlust von Panethzellen und somit einer Schädigung der Darmbarriere führt, woraus intestinale Entzündungen resultieren. Als Kontrolltiere dienten Wurfgeschwister ohne Caspase-8 Gen-Knockout (Casp8^{fl}). Untersucht wurden 6 verschiedene Brote, darunter jeweils ein Hefeteigbrot aus Weizen, Roggen und Dinkel (I, II, III), jeweils ein Sauerteigbrot aus Weizen mit- und ohne Backmittel (IV und VI) sowie ein Hefeteigbrot aus Weizenvollkornmehl (VI). Als Kontrolle dienten eine reisbasierte glutenfreie Diät und eine Diät mit 5% zugesetztem Gluten. Insbesondere Hefeteigbrot, nicht aber Gluten, führte bei Casp8$^{\Delta IEC}$ Mäusen zu einer Verschlimmerung der Entzündungen im Kolon und einer erhöhten Sterblichkeit, während die Wurfgeschwister ohne intestinale Entzündungen (Casp8^{fl}) alle Diäten bzw. Brote gleich gut tolerierten. Die Ergebnisse lassen vermuten, dass andere Inhaltsstoffe als Gluten Entzündungen verstärken und diese insbesondere in Hefeteigbrot aus Weizen- und Roggenbrot zu finden sind.

In einer Subgruppe von NCWS Patienten wird Dinkelbrot subjektiv besser vertragen als Weizenbrot, was sowohl auf die Genetik als auch auf die unterschiedliche Herstellung von Weizen- und Dinkelbrot zurückzuführen sein könnte. Um das Phänomen zu verifizieren und ggf. zugrundeliegende Mechanismen zu identifizieren, wurde eine klinische Studie an Patienten dieser Subgruppe durchgehführt. Ziel der verblindeten Studie war es zu untersuchen, ob Dinkelbrot tatsächlich besser vertragen wird als Weizenbrot und ob die Herstellung (16h Teigführung oder 1h Teigführung +Backmittel) einen Einfluss hat. Zusätzlich wurden den Teilnehmern zwei weitere Brote verabreicht, die mit 1,5 % Oligofruktose (+FODMAP) oder 5 % Weizengluten angereichert waren. Nach jedem Brot (jeweils 4 Tage nach 3 Tagen Washout) wurden die gastrointestinalen Symptome mit dem Irritable Bowel Syndrome Severity Scoring System (IBS-SSS) Fragebogen bewertet. Außerdem wurden extraintestinale Symptome und verschiedene Blut- und Stuhlparameter analysiert. Es zeigte sich, dass Dinkelbrot im Vergleich zu Weizenbrot nach verblindetem Verzehr nicht besser vertagen wurde und dass FODMAP-reiches Brot im Vergleich zu FODMAP-armem Brot nicht schlechter vertragen wurde.

Im Rahmen des Promotionsprojekts wurden somit erstmals Daten einer umfassenden Proteomanalyse von Mehl und korrespondierenden Broten publiziert und eine Liste mit Allergenen erstellt, die auch für zukünftige Allergenanalysen herangezogen werden kann. Durch Untersuchungen am Mausmodell und am Menschen wurden neuen Erkenntnissen zum Einfluss der Getreideart und der Art der Brotherstellung auf die Proteinzusammensetzung und die Verträglichkeit generiert.

Abstract

There are now three disease entities that are triggered by the consumption of grains. Coeliac disease and wheat allergy have been studied in detail, while non-celiac wheat sensitivity (NCWS), whose incidence is increasing, raises many questions. The symptoms are non-specific, diagnostic markers are lacking and the pathophysiology remains unclear. The diagnosis of NCWS is only based on the exclusion of wheat allergy and coeliac disease. Besides bacterially fermentable carbohydrates (FODMAPs), selected cereal proteins such as gluten or α-amylase trypsin inhibitors (ATIs) are possbile triggers and thus in the focus of research. The aim of the present work was firstly to investigate the influence of the choice of grain (soft wheat, hereafter referred to as „wheat“, spelt, rye) and the production of bread (degree of milling and choice between yeast and sourdough) on the presence of potentially immunogenic proteins on the basis of a proteome analysis. In a second step, the tolerability of selected breads was to be investigated in a transgenic mouse model with intestinal inflammation and in a human study of patients with NCWS and subjective spelt tolerance. This should narrow down possible triggers of NCWS and investigate underlying mechanisms.

Based on a quantitative proteomics method (nano-UHPLC-ESI-MS based), comprehensive proteome data for bread and flour could be generated for the first time with the identification of more than 4000 different proteins in bread and flour samples. In addition, we were able to show that rye and spelt proteins are also identified using the soft wheat database (*Triticum aestivum*), which indicates a high degree of sequence homology between the different species. In addition, a list of known and potentially immunogenic cereal proteins was compiled on the basis of the Pfam annotation, which was used for further analyses. This showed that neither the absolute number nor the abundance of these proteins were dependent on the degree of milling of the flour or the fermentation process of the dough, which means that they are not selectively degraded during bread production. However, such proteins could be identified in higher numbers and higher relative amounts in spelt and wheat samples compared to rye samples.

The influence of selected breads from the proteome analysis was investigated *in vivo* in a mouse model. One hypothesis for the development of NCWS is based on a latent or manifest previous disease of the intestine such as a disturbance of the intestinal barrier as the cause of the symptoms after consumption of cereal products. For this reason, in the present work a mouse model ($Casp8^{\Delta IEC}$) was chosen that is characterized by the knockout of the caspase-8 gene, which

leads to a loss of Paneth cells and thus damage to the intestinal barrier, resulting in intestinal inflammation.

Littermates without caspase-8 gene knockout served as controls ($Casp8^{fl}$). Six different breads were investigated, including one yeast fermented bread each from wheat, rye and spelt (I, II, III), one wheat sourdough bread with and without bread improver (IV and VI) and one yeast fermented bread from wholegrain wheat flour (VI). A rice-based gluten-free diet and a diet with 5% added gluten served as controls. Specifically, yeast fermented bread, but not gluten, exacerbated colonic inflammation and increased mortality in $Casp8^{\Delta IEC}$ mice, whereas littermates without intestinal inflammation ($Casp8^{fl}$) tolerated all diets or breads equally well. The results suggest that ingredients other than gluten increase inflammation and that these are particularly found in yeast fermented bread made from wheat and rye bread.

In a subgroup of NCWS patients, spelt bread is subjectively better tolerated than wheat bread, which could be due to genetics as well as the different production of wheat and spelt bread. In order to verify the phenomenon and possibly identify underlying mechanisms, a clinical study was conducted on patients in this subgroup. The aim of the blinded study was to investigate whether spelt bread is actually better tolerated than wheat bread and whether the production (16h fermentation or 1h fermentation + bread improver) has an influence. In addition, the participants were given two further breads enriched with 1.5 % oligofructose (+FODMAP) or 5 % wheat gluten. After each bread (4 days each + 3 days washout), gastrointestinal symptoms were assessed using the Irritable Bowel Syndrome Severity Scoring System (IBS-SSS) questionnaire. Extraintestinal symptoms and various blood and stool parameters were also analysed. It was found that spelt bread was not better tolerated than wheat bread after blinded consumption and that FODMAP-rich bread was not worse tolerated compared to FODMAP-poor bread.

Within the framework of the PhD project, data from a comprehensive proteome analysis of flour and corresponding breads were published for the first time and a list of allergens was compiled, which can also be used for future allergen analyses. New findings on the influence of the cereal and the type of bread production on the protein composition and tolerance were generated through studies on in mice and on humans.

1. Einleitung

Getreide stellt seit etwa 10 000 Jahren als Grundnahrungsmittel für uns Menschen eine wichtige Quelle für Energie, Ballaststoffe und Mikronährstoffe dar [1]. Gleichzeitig gibt es Getreideunverträglichkeiten, die dazu führen, dass bestimmte Bestandteile von Weizen und anderen glutenhaltigen Getreidegattungen von Betroffenen nicht vertragen werden.

Während Weizenallergie und Zöliakie eingehend untersuchte, getreideabhängige Erkrankungen sind, deren Prävalenz und Pathomechanismen bekannt sind, bestehen bezüglich der Nicht-Zöliakie-Weizensensitivität (NCWS) viele Unklarheiten [2]. Bei dieser Krankheitsform treten unspezifische gastrointestinale und extraintestinale Symptome im Zusammenhang mit dem Verzehr glutenhaltiger bzw. weizenhaltiger Lebensmittel auf, obwohl eine Weizenallergie und eine Zöliakie ausgeschlossen wurden [3–5]. Die Inzidenz dieser Unverträglichkeit steigt in der westlichen Welt an [6] und liegt wahrscheinlich zwischen 1-15% [7–11]. Die Beschwerden ähneln denen der Zöliakie und Weizenallergie und umfassen intestinale Symptome wie Durchfall, Bauchschmerzen, Blähungen und Verstopfung [12] oder extraintestinale Symptome wie Kopfschmerzen, Müdigkeit, Ekzeme, Gelenk-/Muskelschmerzen oder Angstzustände [5]. Die „Salerno Experts' Criteria", die 2015 von einem internationalen Konsortium erarbeitet wurden, stellen nach wie vor die einzigen offiziellen Diagnosekriterien der NCWS dar und empfehlen, die NCWS durch eine verblindete, placebokontrollierte, orale Provokation mit Weizenbrot oder mit Weizen angereicherten Nahrungsmitteln nach vorheriger Glutenkarenz zu verifizieren [12]. Die Überlappung der Symptome mit denen anderer Erkrankungen und das Fehlen valider Marker, macht den Diagnoseprozess langwierig und komplex. Auch die Pathomechanismen der NCWS sind noch unbekannt. Es wird jedoch angenommen, dass eine Aktivierung des Immunsystems und intestinale Entzündungen beteiligt sind [13–17]. Zudem gibt es Hinweise auf eine beeinträchtigte Darmbarriere [18] und Veränderungen im intestinalen Mikrobiom der Betroffenen [19]. Die Bezeichnung Nicht-Zöliakie-Glutensensitivität („non-celiac gluten sensitivity", NCGS) wurde in Nicht-Zöliakie-Weizensensitivität geändert („non-celiac wheat sensitivity", NCWS), da in doppelverblindeten Untersuchungen das Klebereiweiß nur bei einem Drittel der Patienten als Auslöser für die klinische Symptomatik identifiziert werden konnte [20]. Welche Getreidekomponenten die Beschwerden der Betroffenen auslösen, bleibt bislang unklar. Als Auslöser von Unverträglichkeitsreaktionen stehen insbesondere Getreideproteine im Fokus, da von Proteinen wie Gluten, α-Amylase Trypsininhibitoren (ATIs) und Lektinen bekannt ist, dass sie das Immunsystem aktivieren können. Auch fermentierbare Oligo-, Di- und Monosaccharide sowie

Polyole (FODMAPs) könnten die Beschwerden der NCWS Patienten triggern [21]. Diese Kohlenhydrate, die neben anderen Lebensmitteln auch in Getreide enthalten sind, sind nur zu einem geringen Teil (5 – 15 %) im Dünndarm resorbierbar [22], wodurch große Mengen bis ins Kolon gelangen. Dort werden sie von der Mikrobiota verstoffwechselt, was zur Entstehung von Gasen wie Wasserstoff und Kohlenstoffdioxid führt [23]. Zudem sind manche Fruktane osmotisch aktiv, woraus ein Wassereinstrom ins Darmlumen resultiert [24]. Die Gase und das Wasser führen zu einer Volumenzunahme im Darm, die in manchen Individuen zu reizdarmähnlichen Beschwerden wie abdominalen Schmerzen und Blähungen führen kann**.** FODMAPs aus Getreide sind jedoch zumindest als alleinige Auslöser der NCWS sehr unwahrscheinlich. Sie sind nicht weizenspezifisch und werden von den Patienten auch unter glutenfreier Diät weiterhin aufgenommen. Auch zeigten Longin et al., dass die Menge in Getreideprodukten wie Brot für das Auslösen von Symptomen eher vernachlässigbar zu sein scheint [25]. Zudem haben FODMPAs z. B. als Ballaststoffe einen positiven Einfluss auf die intestinale Mikrobiota [26]. Aus diesem Grund fokussierten wir uns im vorliegenden Projekt insbesondere auf die Analyse der Getreideproteine als mögliche Auslöser der NCWS.

Brotweizen (*Triticum aestivum* ssp. *aestivum*), im Folgenden als "Weizen" bezeichnet, und Dinkelweizen (*Triticum aestivum* ssp. *spelta*), im Folgenden als "Dinkel" bezeichnet, sind hexaploide Unterarten der Gattung Weizen (*T. aestivum*) und damit biologisch eng miteinander verwandt, während Roggen, der neben Weizen und Dinkel für die Brotherstellung verwendet wird, einer anderen Gattung angehört (*Secale cereale*). Trotz der großen genetischen Ähnlichkeit hat Dinkel einen höheren durchschnittlichen Rohproteingehalt und ein höheres Verhältnis von Gliadin zu Glutenin als Weizen [27,28]. In eigenen Proteomanalysen konnten wir zeigen, dass sich ein Drittel der Proteine zwischen Weizen- und Dinkelmehl unterscheidet [29]. Auch die Herstellungsprozesse von Brot wie Mahlen, Kneten, Fermentieren, Erhitzen und die Zugabe von Backmittel könnte die Zusammensetzung von Brot und das Vorhandensein möglicher Auslöser von NCWS im Brot beeinflussen [30–32]. Da die äußere Schicht des Korns andere Proteine enthält als das Endosperm, weist Vollkornmehl eine andere Proteinzusammensetzung auf als Auszugsmehl [33]. Darüber hinaus kommt es während der Teigruhe zu biochemischen Veränderungen in der Proteinfraktion des Teigs aufgrund der Wirkung mikrobieller und endogener Getreideenzyme. Mittels SDS-PAGE wurde gezeigt, dass Sauerteigbrot im Vergleich zu Hefeteigbrot und Weizenmehl weniger proinflammatorische ATIs enthält, was vermutlich auf die Reduktion von Disulfidbindungen zurückzuführen ist [34,34]. Eine klinische Studie konnte jedoch nicht zeigen, dass Reizdarmpatienten Sauerteigbrot besser vertragen als Hefeteigbrot [34].

Backmittel enthalten oft zusätzliche Proteasen, die Proteine wie Gluten abbauen. In Kombination mit der thermischen Verarbeitung des Teigs können solche Herstellungsverfahren das Proteom von Brot verändern und somit das Vorhandensein potenzieller Auslöser der NCWS beeinflussen [31,32].

Folgende Fragestellungen sollten in der vorliegenden Arbeit geklärt werden:

- Wie beeinflussen die Herstellungsweise von Brot (Vermahlungsgrad, Teigführung, Verwendung von Backmittel) und die Wahl des Getreides (Weizen, Dinkel, Roggen) die Proteinzusammensetzung?
- Welchen Einfluss haben die genannten Faktoren auf potentielle Allergene?
- Wie wirken sich verschiedene Brotsorten in einem Mausmodell mit vulnerablem Darm aus?
- Gibt es beim Menschen einen objektiven Unterschied in Bezug auf die Verträglichkeit von Dinkel- und Weizenbrot?

2. Manuskripte

2.1 Proteomanalyse von Brot

Getreideproteine sind Auslöser von Zöliakie und Weizenallergie [11,35]. Zudem stehen sie im Verdacht, weitere Unverträglichkeiten wie NCWS auszulösen [13,36]. Eine Analyse des Proteoms von Brot bildet deshalb die Basis für die Suche nach den genauen Triggern dieser noch unverstandenen Krankheit. In früheren Studien wurde bereits die Proteinzusammensetzung von Mehl und Brot mittels verschiedener Proteomik-Ansätze (2-D-Gelelektrophorese, gerichtete Proteinquantifizierung oder Shotgun-Proteomik) durchgeführt [29,37–41]. Diese Studien konzentrierten sich jedoch entweder auf die Proteinzusammensetzung von Mehl [37,38,41] oder fokussierten sich auf spezifische Proteingruppen wie ATIs, Gluten oder Lupine in Brot [39–41].

In der vorliegenden Arbeit dagegen erfolgte eine systematische Untersuchung des kompletten Proteoms von Brot und Mehl mit besonderem Augenmerk auf immunogene Proteine. Dafür wurde mittels Nano-UHPLC-ESI-MS und labelfreier Quantifizierung (LFQ) das Proteom von sechs verschiedenen Mehlen (jeweils Vollkorn- und Auszugsmehl) aus Weizen, Roggen und Dinkel untersucht sowie von 14 Brotsorten, die daraus hergestellt wurden (Tabelle 1).

Tabelle 1. Übersicht über die 14 Brotsorten für die Analyse

Getreide	Vermahlung	Herstellung
Weizen	Auszugsmehl oder Vollkornmehl	Sauerteig oder Hefeteig
Dinkel		
Roggen		
Weizen	Auszugsmehl oder Vollkornmehl	Sauerteig + Backmittel

Auf Basis der Ergebnisse dieses Projektes sollte geklärt werden, ob die Wahl des Getreides (Weizen, Dinkel, Roggen) oder bestimmte Herstellungsmechanismen von Brot dessen Proteinzusammensetzung verändern und ob dadurch bekannte oder potentielle immunogene Proteine abgebaut oder angereichert werden. Der Artikel wurde 2021 in der Fachzeitschrift Journal of Proteomics veröffentlicht [30].

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Comprehensive proteome analysis of bread deciphering the allergenic potential of bread wheat, spelt and rye

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ABSTRACT

Background/Objectives: Cereal products like flour and bread are known to trigger diseases such as wheat allergy, celiac disease and non-celiac wheat sensitivity (NCWS). Some of these diseases are caused by allergenic proteins, the expression of which might vary depending on the grain type and manufacturing processes. Therefore, we examined the protein composition and abundance of potentially allergenic proteins in flours from bread wheat, spelt and rye, and corresponding breads.

Materials and methods: Using Nano-LC-ESI-MS/MS and label free quantification (LFQ) we analyzed the proteome of six different bread flours (wholegrain and superfine flours from rye, spelt and bread wheat) and 14 bread types (yeast and sourdough fermented breads from all flours and wheat breads plus/minus bread improver). Potentially allergenic proteins in flours and breads were functionally categorized using the Pfam database and relatively quantified by LFQ.

Results: We could show that almost equal numbers of proteins can be identified in rye- and spelt samples compared to wheat samples using the Uniprot bread wheat protein database, indicating high sequence conservation between cereals. In total, 4424 proteins were identified in the 20 flour and bread samples. The average number of identified proteins in flour (2719 ± 243) was slightly higher than in bread (2283 ± 232; $P < 0.001$). In wheat- and spelt wholegrain flour higher protein numbers (wheat: 2891 ± 90; spelt: 2743 ± 140) were identified on average than in superfine flour (wheat: 2562 ± 79; $P = 0.009$; spelt: 2431 ± 140; $P = 0.004$). Neither the absolute number nor the abundance distribution of potentially allergenic proteins were dependent on the flour type or the fermentation process, but known allergenic proteins like gliadins showed higher relative abundance in spelt- and wheat samples, compared to rye samples.

Conclusion: We provide comprehensive proteome data for six flour types and related breads showing that the grain species have greater influence on proteome composition than milling and fermentation processes. Our data indicate that allergenic proteins are not selectively degraded during bread production and are more abundant in bread wheat and spelt compared to rye.

Significance: Our proteomics study revealed that bread contains a number of potentially and proven allergenic proteins. Most likely allergenicity is not dependent on milling or conventional fermentation processes, but on the grain type. Relative abundance of allergenic proteins was higher in spelt- and wheat samples than in rye samples. Considering rye bread as better suited to atopic individuals predisposed to react to cereal allergens, clinical trials are warranted to verify this assumption.

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1. Introduction

Besides rice and maize, bread wheat is the most widely consumed food grain in the world. Wheat flour is the most important ingredient in home baking and is the framework for almost every commercially baked product. At the same time, wheat proteins are known to be capable of triggering wheat allergy and celiac disease in certain individuals [1,2]. In addition, it is suspected that wheat proteins may trigger the symptoms of NCWS (non-celiac wheat sensitivity), another grain-associated clinical entity [3,4].

There are several known ways in which grain proteins can trigger discomfort in susceptible persons. On the one hand, specific gluten peptides lead to activation of specific immune cells like CD4 + T-lymphocytes, macrophages or dendritic cells in celiac disease patients, which in turn leads to damage of the small intestinal epithelium [5]. Wheat allergy patients, on the other hand, react to gluten and non-gluten proteins like β-amylase, α-Amylase-Trypsin inhibitors (ATIs) and surface-active proteins such as nonspecific lipid transfer proteins (LTPs) and puroindolines with a release of histamine from basophils and mast cells [6,7]. Based on in vitro studies and animal experiments, ATIs could additionally stimulate intestinal immune cells via the activation of Toll-like receptor-4 and could thus trigger intestinal inflammation or exacerbate pre-existing inflammation in patients without wheat allergy or celiac disease [3,8].

Detailed characterization of the protein composition of bread and flour is essential to evaluate their allergenic potential. Based on early biochemical work the wheat flour proteome can be classified in four protein fractions including albumins, globulins, prolamins and glutelins that can be sequentially extracted from flour using different solvents [9]. Prolamins and glutelins together comprise the storage protein fraction of the grain (gluten) and comprise up to 80% of total protein content [10]. The albumin- and globulin-fraction comprise IgE-binding proteins [11]. Previous grain flour proteome studies revealed grain-specific differences, e.g. between wheat and rye [12,13], but also between wheat and spelt [14]. Prandi et al. [15] showed that spelt contains less immunogenic or toxic peptide epitopes compared to other wheat variants. Even if rye is less closely related to wheat, spelt, einkorn and emmer, rye proteins can also trigger celiac disease and wheat allergy [16].

Milling, kneading, fermentation and heating impact the characteristics of bread and might also affect the bread proteome to different extends. Since the outer layer of the grain contains other proteins than the endosperm, it is likely that different types of flour (f.e. wholegrain- and superfine flour) have different protein compositions [17]. In addition, biochemical changes occur in the protein fraction of the dough during fermentation due to the action of microbial and endogenous wheat enzymes [18]. Sourdough wheat bread contains less pro-inflammatory tetrameric and dimeric ATIs compared to yeast-fermented bread and wheat flour, which might be due to disulphide bond reduction [3,19]. Rizello et al. [20] further showed that specific sourdough microorganisms can also lead to a decrease of other allergenic proteins in wheat- and rye bread. However, a clinical study could not prove that IBS patients tolerate sourdough fermented wheat bread better than yeast fermented wheat bread [19]. Bread improvers used to facilitate the manufacturing process often contain additional proteases that break down proteins like gluten. In combination with thermal processing of the dough, such manufacturing methods can modify the bread proteome [21,22].

Several studies analyzed the composition of grain flour proteomes and bread proteomes using different proteomics approaches like 2-D gel electrophoresis, shotgun proteomics and targeted protein quantification [14,23–27]. These studies focused either on the protein composition of flour [14,23,24] or analyzed specific protein groups like ATIs, gluten or lupines in bread [25–27]. To the best of our knowledge, no study systematically investigated the comprehensive proteome of bread and corresponding flours with particular attention to different allergenic proteins so far. In this study, we examined the influence of different manufacturing processes on the bread proteome of wheat, spelt and rye and characterized the proteome of the corresponding flours in detail using a label free quantification (LFQ) proteomics approach.

2. Material and methods

2.1. Breadmaking

For preparing the different breads analyzed in our study we used grains from bread wheat, spelt and rye (Schapfenmühle, Ulm, Germany), from which superfine flour (SF) (Quadrumat junior mill, Brabender, Duisburg) and wholegrain flour (WG) (Ultra-centrifugal mill ZM 200 sieve insert 0,5 mm; Retsch, Haan) was produced.

Bread from different grains (bread wheat, spelt, rye), different types of flour (SF or WG) and different fermentation (yeast dough or sourdough) were produced (in total 12 bread types). In addition, 100 g bread improver (MeisterMarken Ulmer Spatz, type Weißback Super, CSM Deutschland GmbH, Bremen, Germany) was added to wheat sourdough breads from SF or WG flours, respectively (2 additional bread types). Bread improver was composed of buttermilk powder (24%); wheat flour; soy flour; emulsifier (soya lecithins, mono- and diglycerides of fatty acids); wheat malt flour; dextrose; salt; acidity regulator (calcium acetate); sour whey powder; acidulant (citric acid, lactic acid); wheat swell flour; flour treatment agent: ascorbic acid; enzymes. This approach resulted in a total of 14 bread types (Supplementary Table 1).

For preparing the sourdough a conventional sourdough starter (TK Starter, Ernst Böcker GmbH&Co.KG; Minden) was used. A mother sourdough 30% was prepared composed of sourdough starter 136.5 g, flour 1322 g, water 1542 g, and leavened for 20 h at 26 °C. Next, the mother dough was added to the remaining ingredients of the breads. The composition of the breads from sourdough and yeast dough each is given in Table 1.

For manufacturing the breads, the ingredients were mixed and kneaded in total for six minutes. The bread dough from SF flour was kneaded 3 min with low (25 Hz) and 3 min with high intensity (50 Hz) whereas bread dough from WG flour was kneaded 2 min with low and 4 min with high intensity. Afterwards the doughs were leavened at 30 °C and 80% humidity for 20 min. Subsequently the doughs were formed. The time for bench rest at room temperature was 10 min before the dough experienced a last maturation step in the loaf pan for 80 min (bread wheat and spelt) and 70 min (rye). Baking temperature was 220 °C and baking time was 40 min for breads from SF flour and 50 min for breads from WG flour (bread wheat and spelt). Breads from rye were baked for 60 and 70 min, respectively (Fig. 1). After the baking process breads were cut into slices of 2 cm, freeze-dried (Delta 1–24 LSC, Martin Christ Gefriertrocknungsanlagen GmbH; Osterode am Harz), milled and stored at −20 °C until analysis.

2.2. Protein extraction for proteome analysis

20 mg flour or bread were suspended in lysis buffer containing 2% SDS (sodium dodecyl sulfate), 20 mM DTT (dithiothreitol) and 150 mM Tris–HCl pH 8.5 and incubated for 10 min at 95 °C. After centrifugation at 4 °C for 30 min at 13,700 rpm the supernatant was removed, and proteins were precipitated using chloroform–methanol precipitation [28]. Protein pellets were resuspended in 6 M urea in 50 mM Tris–HCl pH 8.5, and protein concentrations were determined by the Bradford assay [29].

2.3. In-solution digest of proteins and peptide purification

10 μg flour- or bread protein extract in 60 μl 6 M urea, 50 mM Tris HCl (pH 8.5) were used for in solution digests. DTT was added to a final concentration of 10 mM for the reduction of cysteines. Samples were incubated for 30 min at 56 °C under shaking at 1000 rpm in an Eppendorf Thermomixer. Alkylation of cysteines was performed by

Table 1
Protein families containing potentially immunoresponsive and allergenic proteins identified in wheat according to the allergome database and the AllFam database.

No.	Pfam ID	Pfam name	Identified proteins in our dataset	Defined group name
1	PF00079	Serine Proteinase Inhibitors	12	Serpins
2	PF00085	Thioredoxin	18	Thioredoxin
3	PF00112	Papain family cysteine protease	1	PCP
4	PF00121	Triosephosphate isomerase	4	TIM
5	PF00141	Peroxidase	29	Peroxidase
6	PF00182	Chitinase class I; Chitin recognition protein	10	Chitinase
7	PF00190	Cupin	22	Cupin
8	PF00197	Trypsin and protease inhibitor	2	Kunitz
9	PF13016	Cys-rich Gliadin N-terminal	29	Gliadin
10	PF03157	High molecular weight glutenin (HMW) subunit	3	Glutenin
11	PF14368	Probable lipid transfer	15	LTP
12	PF00234	Protease inhibitor/seed storage/LTP family	47	Inhibitor/ Storage
13	PF00235	Profilin	1	Profilin
14	PF00314	Thaumatin family	15	Thaumatin
15	PF00560	Leucine rich repeat N-terminal domain	2	LRR
16	PF03227	Gamma interferon inducible lysosomal thiol reductase	3	GILT
17	PF00450	Serine carboxypeptidase	10	Serine Carbox
18	PF00321	Plant thionin	2	Thionin
19	PF01373	Glycosyl hydrolase family 14	1	GlycoHydro
20	PF00534	Glycosyl transferases group 1	14	GlycoTransfer
21	PF00161	Ribosome inactivating protein	6	RIP

Only protein families with representatives that can elicit immune response by ingestion are shown (for further details see Materials and Methods). Source: Nr. 1–19: [37]; 20–21 [42]. Abbreviations: Triosephosphate isomerase (TIM); Papain-like cysteine protease (PCP); Leucine rich repeat N-terminal domain (LRR); Gamma interferon inducible lysosomal thiol reductase (GILT).

adding 30 mM iodoacetamide and incubation for 45 min at room temperature (RT) in the dark. Alkylation was stopped by add-ing 50 mM DTT and samples were incubated for another 10 min at RT. Five hundred nanogram (500 ng) LysC protease (Roche) in 50 mM Tris HCl pH 8.5 was added and samples were digested overnight at 30 °C. Next, the urea in the reaction mixture was diluted to 2 M by adding the appropriate amount of 50 mM Tris HCl pH 8.5. One microgram (1 μg) trypsin (Roche) in 50 mM Tris HCl pH 8.5 was added and digestion was continued for 4 h at 37 °C. The digest was stopped by adding 3 μl 10% TFA (trifluoroacetic acid). Next, peptide mixtures were concentrated and desalted on C18 stage tips26 and dried vacuum [30]. Dried samples were dissolved in 30 μl 0.1% TFA. Aliquots of 3 μl were subjected to nano-LC-MS/MS analysis.

2.4. Nano-LC-MS/MS

Mass spectrometry analysis was performed as previously described [14]. Briefly the measurements were performed on an UltiMate 3000 RSLCnano system (Thermo Fisher Scientific, Bremen, Germany) coupled to a Q-Exactive HF-X mass spectrometer (Thermo Fisher Scientific) using a Nanospray Flex ion source (Thermo Fisher Scientific). Tryptic digests were concentrated and desalted on a precolumn (300 μm × 5 mm, Acclaim PepMap100 C18, 5 μm particle size, 100 Å pore size) and separated on a nanoEase MZ HSS T3 analytical column (25 cm × 75 μm, 1.8 μm particle size, 100 Å pore size, Waters) operated at constant temperature of 35 °C. Peptides were separated at a flow rate of 300 nL/min using a 90 min gradient with the following profile: 2% - 55% solvent B in 90 min, 55% - 95% solvent B in 5 min and maintained at 90% solvent B for 5 min. 0.5% acetic acid (solvent A) and 0.5% acetic acid in acetonitrile/H2O (80/20, *v*/v, solvent B) were used as solvents.

The Q Exactive HF-X was operated under the control of XCalibur 4.3.73 software (Thermo Fisher Scientific). MS spectra (m/z = 300–1800) were detected in the Orbitrap at a resolution of 60,000 (at m/z = 200) using a maximum injection time (MIT) of 100 ms and an automatic gain control (AGC) value of 1 x 10E6. Internal calibration of the Orbitrap analyzer was performed using lock-mass ions from ambient air as described in [31]. Data dependent MS/MS spectra were generated for the 10 most abundant peptide precursors in the Orbitrap using higher-energy C-trap dissociation (HCD) fragmentation at a resolution of 15,000, a normalized collision energy of 27 and an intensity threshold of 1.6 x 10E6. Only ions with charge states from +2 to +5 were selected for fragmentation using an isolation width of 1.6 Da. For each MS/MS scan, the AGC was set at 2 x 10E5 and the MIT was 50 ms. Fragmented precursor ions were dynamically excluded for 30 s within a 10 ppm mass window to avoid repeated fragmentation.

2.5. MS data analysis and protein quantification

Processing of the raw files and protein identification were performed with MetaMorpheus software suite version 0.0.307 (https://github.com/smith-chem-wisc/MetaMorpheus) [32]. First, mass re-calibration was performed by the calibrate task with default settings. In a second step the re-calibrated spectra were searched against the wheat proteome sequence downloaded from UniProt [33] (*Triticum aestivum*, 2019). Search in MetaMorpheus was performed in the classic search operation mode against the automatically computed reversed sequences as decoy database. Mass tolerance of 5 ppm (parts per million) for MS spectra und 20 ppm for MS/MS spectra were used. Trypsin was specified as enzyme and three missed cleavages were allowed. Minimal peptide length was set to five and maximal peptide length was limited to 30 amino acids. The 'Common Fixed'- (Carbamidomethyl on cysteines) and 'Common Variable' -option (Oxidation on methionine) for modifications were chosen and the 'Match between runs' feature was enabled. The peptide q-value cut of was set to 0.01 and the minimum allowed peptide score was set to five. For protein quantification the FlashLFQ option in the MetaMorpheus software suite was enabled with a 5 ppm peak finding tolerance for MS1 scans [34].

The bread wheat data base (Uniprot *Triticum aestivum*; Taxon identifier 4565) was used not only for the wheat analyses, but also for the spelt and rye analyses of proteins, because specific databases for spelt and rye proteins are not available in public repositories so far.

For the downstream statistical analysis, the MetaMorpheus Protein groups output file was load into the Perseus software frame (Version 1.6.14.0) [34]. Only protein groups with a q-value <0.01 and with at least one valid FlashLFQ (Label free quantification) in the data set were considered for downstream analysis. Additionally, known contaminants and proteins groups that match to the decoy database during search were excluded. In total 4424 protein groups remained in the data set. For further quantitative analysis, LFQ intensities were log2-transformed.

2.6. Downstream analysis of the data

For each sample, the LFQ (label-free quantification) intensities of three replicates were measured. LFQ intensities were log2-transformed for further analysis. The dataset was filtered for Q values <0.01 and for intensities in at least one sample, leading to 4424 identified proteins.

For analyzing the 100 most abundant proteins, the average LFQ intensities were sorted by descending order in Microsoft Excel software and the 100 proteins with the highest LFQ intensities were picked for analysis. Protein abundances were calculated based on the average LFQ

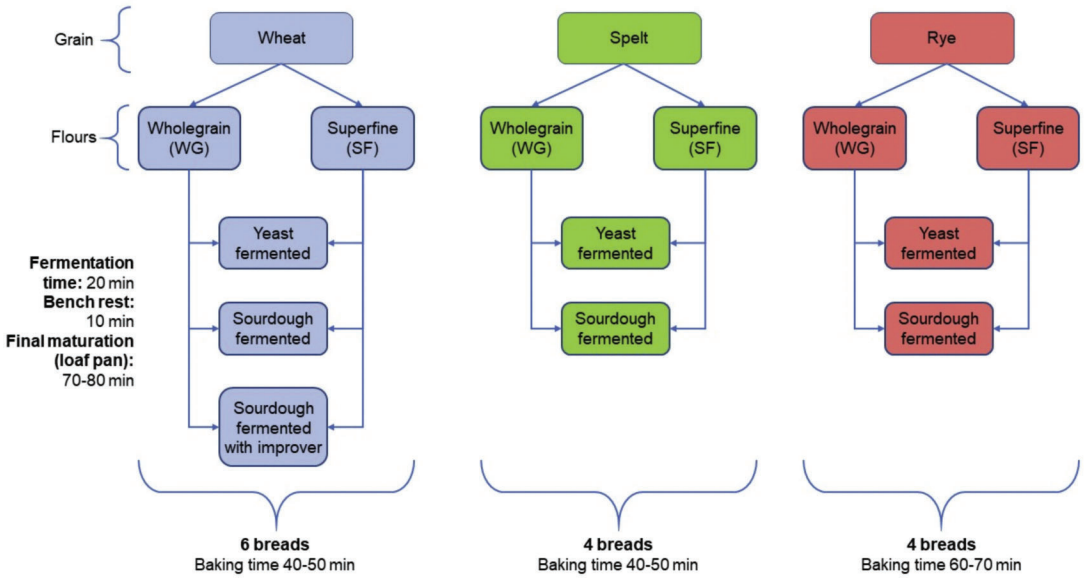


Fig. 1. Flours and corresponding bread types investigated in this study.
In total, 14 breads and 6 corresponding flours derived from 3 grains were examined. Further details see text.

intensity of all three replicates per sample.

Bar charts, Swarm plots and Pie charts were constructed using Graphpad Prism software 9.0 (GraphPad Software, San Diego, California USA, http://www.graphpad.com). Venn diagrams were created using R software (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org) [35] and Lucidchart (www.lucidchart.com).

2.7. Systems analysis and functional annotation

Hierarchical clustering was performed by Perseus software Version 16.14 (Perseus computational platform, Martinsried, Germany, http://www.maxquant.net/perseus/) [36]. For the functional annotation analysis of differentially regulated protein groups, we considered proteins with at least 3 valid LFQ values in one experimental condition ($n = 3$) which additionally show at least 70% valid LFQ values in the whole dataset. The remaining missing values were imputed by Quantile Regression Imputation of Left-Censored data based on the R script "ImputeR" (tune.sigma = 1). Significance of protein abundance changes between the different conditions were computed by a permutation-based Welch's *t*-Test (FDR = 0.05, S0 = 0.5, 250 randomizations) which required at least 3 valid LFQ values (unimputed) in one of the tested conditions (Perseus). Protein groups with significant abundance changes were used for hierarchical cluster analysis in Perseus. Therefore, each row in the data matrix was z-scored separately for wheat, spelt and rey samples on bases of the unimputed LFQ intensities. The normalized LFQ data were used as imput for the row clustering. Euclidean distance was used as measure for the row clustering in Perseus. Row clusters which show distinct behavior in their average protein profile were selected manually and associated annotated terms (from the Interpro and Pfam database) were computed by a Fisher exact test using the initially identified 4424 proteins in this study as reference set. Annotation terms were filtered for an enrichment factor > 5.

The six samples with bread improver were excluded from this analysis, as the bread improver contains proteins from several foreign species which could potentially lead to altered quantification and false identification of a bread wheat proteom subset through peptides with homologues sequences.

2.8. Definition of allergenic wheat proteins

Juhász et al. 2018 [37] identified wheat proteins associated with adverse allergic and immune responses in people with wheat associated disorders. These proteins were identified based on information in a database of allergen families (AllFam; www.meduniwien.ac.at/allfam) [38] supplemented by the data in the AllergenOnline FARRP database (www.allergenonline.org) [39]. The set of proteins defined by Juhász et al. was used for the identification of seed-borne allergens and immunoresponsive proteins in flour and bread samples, but only proteins with "ingestion" as route of exposure were considered. This criterion was fulfilled by 330 proteins, of which 290 could be assigned to 22 Pfam families. 40 remaining proteins that were not belonging to any Pfam family were excluded. We defined all proteins in these 22 Pfam families as "proven or potentially allergenic/immunoresponsive proteins".

Furthermore, we used the allergome database [40–42] to identify additional allergens. The allergome platform is currently the most comprehensive collection of allergen data. It contains identified and characterized allergenic proteins, published in peer-reviewed journals, including those in the official WHO/IUIS Allergen Nomenclature list. We were able to identify additional allergens in the allergome database that could be assigned to 2 additional Pfam families finally resulting in 24 Pfam families in total with potentially and proven allergenic/immunoresponsive proteins. In the following these proteins will be called "allergenic proteins".

2.9. Statistics

Statistical analysis was performed by Graphpad Prism 9 (GraphPad Software, San Diego, California). Data were tested for normal distribution by Shapiro Wilk test. Milling effects (WG or SF flour) were analyzed using an independent-samples students *t*-test. Effects of grain type (rye, spelt, bread wheat) or different manufacturing processes (sourdough fermentation, yeast fermentation, bread improver) were analyzed using one-way analysis of variance (ANOVA). Level of significance (α) was set at 0.05.

3. Results

3.1. Quantitative protein analysis of flour and bread

In total, 4424 unique proteins were identified in all flour and bread samples (6 flours, 14 breads) by our proteomics approach. Numbers of

identified proteins varied between samples, ranging from 2271 to 3143 different proteins per sample (Fig. 2A, B). In average, flour samples (2719 ± 243; mean ± SD of 6 flours) contained higher numbers of different proteins than bread samples (2283 ± 232; mean ± SD of 14 bread types; $P < 0.0001$) (Fig. S1). To consider differences between the different grain types, the number of identified proteins was compared across flours and breads from bread wheat (2407 ± 295 mean ± SD of 2 flours and 3 bread types), spelt (2393 ± 237 mean ± SD of 2 flours and 2 bread types) or rye (2443 ± 402 mean ± SD of 2 flours and 2 bread types) but no statistically significant differences were observed between the different grain types. Spelt and rye proteins were identified in almost equal numbers compared to bread wheat (hereafter referred to as wheat) proteins using the bread wheat protein database (Uniprot *Triticum aestivum*; Taxon identifier 4565).

In whole grain (WG) flour and bread samples from wheat and spelt higher numbers of unique proteins were identified than in corresponding superfine (SF) flour samples. This was expected since in the production of SF flour the aleurone layer of the grain, which is rich in proteins, is removed. However, for rye flour and bread samples the picture was different, as in all SF samples higher numbers of proteins were identified compared to WG samples (Fig. 2 A, B).

Comparing different grain types, approximately two thirds of the identified proteins were common between flour (61%) and half of the identified proteins were common between bread (46%) from wheat, spelt and rye (Fig. 2 C, D). The overlap of identified proteins was always higher between wheat and spelt samples (12% in flour, 20% in bread) than between wheat and rye samples (6% in flour and 3% in bread) or rye and spelt samples (5% in flour and 4% in bread). 12% of the proteins in rye flour and 15% of the proteins in rye bread were only identified in rye, but not in flour and bread samples from wheat and spelt, leading to a different protein composition of flour and bread samples from rye.

3.2. Influence of manufacturing processes on protein composition

To analyze possible effects of milling and the manufacturing processes on protein composition of flour and bread in more detail we compared the distribution of all proteins that had LFQ intensities between SF and WG flours (Fig. 3 A, C, E) and between different bread types (Fig. 3 B, D, F), respectively. Comparing SF and WG flour, we could show that in all three different grain types around 80% of the proteins were common. 15% of the proteins were unique in wheat WG flour and 13% of proteins were unique spelt WG flour. Lower numbers of unique proteins were identified in wheat and spelt SF flour (5% and 6%, respectively). In rye flour differences between WG flour and SF flour were less pronounced, 8% of the proteins could only be found in WG flour and 6% in SF flour.

In addition, we investigated the influence of the fermentation process on the protein composition of breads and compared the numbers of identified proteins in sourdough fermented (S) and yeast fermented (Y) breads. In wheat samples we also analyzed sourdough fermented bread with improver (SB) (Fig. 3B, 2D, F). The majority of proteins (66%–80%) could be identified in all the different bread types within one grain type (wheat, spelt or rye). The number of common proteins for wheat breads was slightly lower because three different bread types were compared, while for spelt- and rye bread only two different bread types were compared. No significant differences in the number of unique proteins were observed comparing WG or SF sourdough fermented bread to yeast fermented bread (Fig. 3 B, D, F and Fig. S2 A-C). Comparing WG bread and WG flour, the number of unique proteins in flour (10–13%) was higher compared to the unique proteins in the different breads (2–5%) (Fig. S2 D—F).

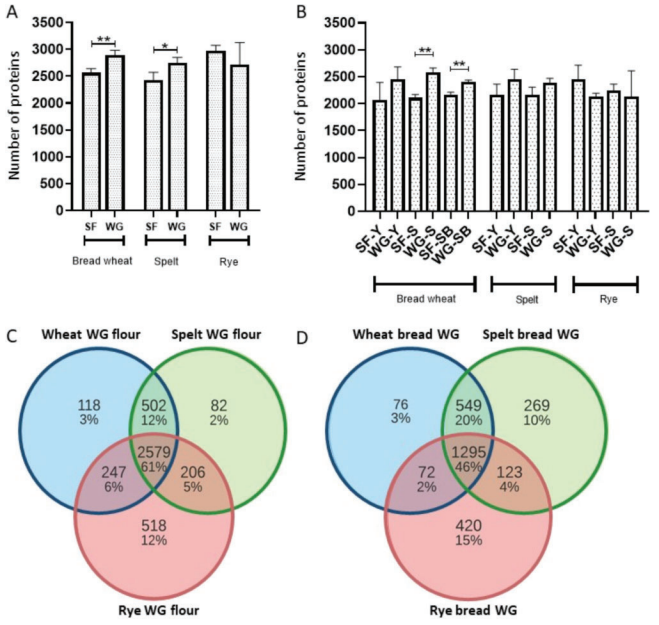


Fig. 2. Total number of proteins detected in flours (A) and breads (B) by nano-LC-ESI-MS/MS as well as overlap of proteins between flours (C) and breads (D) of wheat, spelt and rye. (A): Number of different proteins in wholegrain (WG) and superfine (SF) flour from wheat, spelt and rye. (B): Number of different proteins in yeast fermented bread (Y) and sourdough fermented bread (S) from SF and WG flour. Two sourdough wheat breads additionally contained bread improver (SB). Means of three replicates and the standard deviation (SD) are shown. $*P < 0.05$; $**P < 0.01$, $***P < 0.001$. (C) and (D): Venn Diagrams showing the overlap of common and unique proteins in WG flour and WG bread of wheat, spelt and rye. Percentage values were rounded without decimal places.

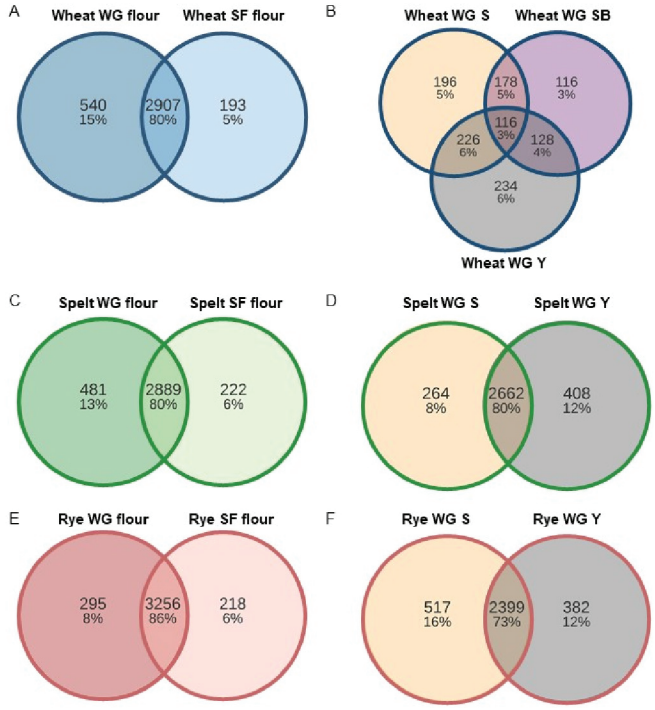


Fig. 3. Overlap of proteins between different flour and bread types, respectively. (A, C, E): Venn diagrams show the overlap between WG and SF flours in wheat, spelt and rye. (B, D, F): Overlap between different manufactured breads (Y = yeast fermented; S = sourdough fermented; SB = sourdough fermented with bread improver) from wheat, spelt and rye (percentage values were rounded to zero decimal places).

3.3. Functional classification and annotation of proteins in flour and bread

We used label-free quantification (LFQ) analysis to gain further insights into the differences in protein composition between different flour types and breads. After filtering the complete dataset for proteins that had at least 70% valid values (LFQ intensities) in all samples, 1737 proteins remained. Welch's *t*-tests were performed for all relevant comparisons of bread and flour types using a cut-off of $P < 0.01$ and an enrichment factor > 5 to identify differentially abundant proteins. We compared WG and SF samples of wheat, spelt and rye using mean values of bread and flour samples. In addition, we compared yeast- to sourdough fermented bread. Proteins that showed significant abundance changes in at least one comparison were ranked using unsupervised hierarchical clustering (Fig. 4). Two dominant clusters emerged from the hierarchical clustering analysis: The first cluster comprised 45 proteins that showed lower abundance in sourdough fermented bread compared to yeast fermented bread and flour (Fig. 4, cluster A, orange). The second cluster comprised 107 proteins that showed higher abundance in WG samples compared to SF samples (Fig. 4, cluster B, blue).

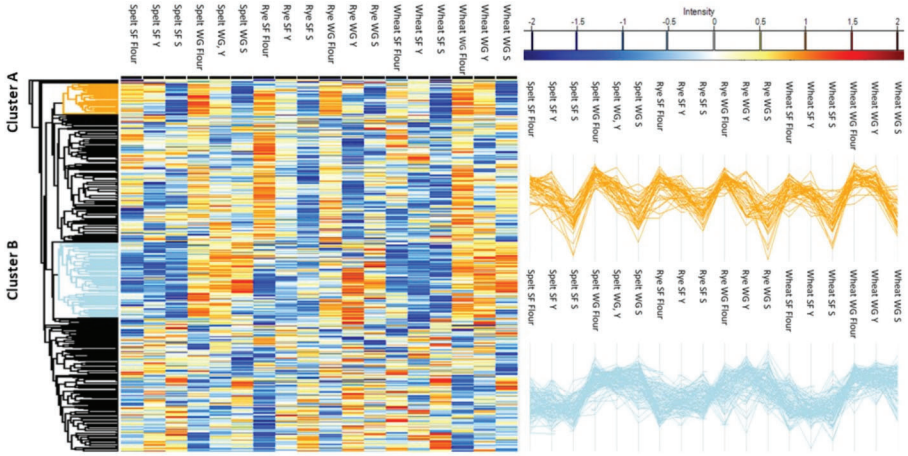
The two dominant protein clusters identified by hierarchical clustering analysis were further analyzed using functional annotation enrichment analysis. 887 of the 1737 proteins with >70% valid values had PfamID annotation. Based on these annotations functional annotation enrichment analysis using Fisher's exact test was performed. Cluster A included proteins belonging to the Linker-histones Pfam family and the Ribosomal proteins S19 and L28e, which were depleted in sourdough breads compared to corresponding WG and SF flours as well as corresponding yeast fermented breads. In cluster B the protein groups Cupin_1, SMP (Seed maturation protein), Barwin and Glyoxalase showed higher abundances in WG samples (bread + flour) compared to SF samples (bread + flour).

3.4. Allergenic proteins in bread and flour

Next, we focused on the presence of potentially and proven allergenic proteins in bread and flour that may cause an immune response by dietary intake. Based on available literature and allergen databases (see material and methods), we defined 24 Pfam domain families that included proven and potentially allergenic proteins. 2105 proteins in our dataset were annotated with a Pfam ID. 246 of these proteins could be assigned to 21 of the above mentioned Pfam domain families (Table 1). The remaining 3 Pfam domain families were not present in the set of proteins identified in our study. The 21 Pfam domain families with 246 representatives in our dataset comprise proven as well as potentially allergenic wheat proteins. 54 of these 246 were proven allergenic proteins according to the allergome database [42] and the database of Juhasz et al. [37] known to be involved in celiac disease or wheat allergy. The allergen list can also be used for the identification of potentially allergenic proteins in wheat-related species like rye or spelt, because they share common ancestry and over 70% sequence similarity [37].

The abundance of potentially allergenic proteins was further analyzed using histograms comparing distributions of LFQ intensities of allergenic proteins (Table 1; 246 proteins including proven and potential allergens) and the remaining proteins that were summarized as "Others" (4177 proteins). In WG flour of wheat, spelt and rye, the distribution of LFQ intensities of allergenic proteins was shifted towards higher LFQ intensities compared to "other" proteins (Fig. 5A), while rye flour showed lower abundances of potentially allergenic proteins compared to wheat and spelt. For deeper understanding of the distribution of allergenic proteins in the different datasets, we calculated the average LFQ intensity of allergenic proteins and others. Allergenic proteins were further divided into potentially allergenic proteins and proven allergenic proteins as described above. To exclude, that differences regarding the LFQ intensities of allergenic proteins in distinct samples are based on a lower number of detected allergenic proteins, for further comparison we exclusively considered the LFQ intensity of proteins detected in all the samples that were compared to each other.

2579 proteins with PfamID were detected in at least one replicate of WG flour of wheat, spelt and rye (Fig. 5B). 168 of these proteins belonged to the potentially allergenic proteins shown in Table 1. Of these 119 proteins were potential allergens according to their Pfam ID, while 49 proteins were proven allergens. The reaming proteins (n = 2411) were summarized as "others". We could observe significantly lower abundances of potential and proven allergens in rye flour compared to wheat and spelt flour, while differences between wheat and spelt were statistically not significant (Fig. 5B). Accordingly, we analyzed different manufactured wheat breads from WG flour (Y, S, SB) and the corresponding flour. 129 potential- and 52 proven allergens were found in all four sample sets, but no significant differences between allergen numbers in the different bread types and the corresponding



Cluster	Proteins/ cluster	Pfam Name	Proteins/ group	Enrichment factor	P-value
A) Depleted in sourdough bread	45	Ribosomal_S19	2	38.6	>0.001
		Ribosomal_L28e	2	38.6	>0.0001
		Linker_histone	4	28.95	>0.0001
B) Enriched in WG	107	Barwin	3	16.234	>0.0001
		SMP	4	12.175	>0.0001
		Glyoxalase	6	8.1168	0.0038
		Cupin_1	15	7.5757	>0.0001

Fig. 4. Protein clusters characteristic for flour or bread types. Heat map (left) and profile plot (right) of z-scored protein abundances (log2 LFQ intensity) include 1737 differentially abundant proteins identified by Welch's *T*-Test. Mean values (three replicates) of normalized LFQ intensities for each sample after hierarchical clustering of different fermentation conditions (yeast and sourdough) and different milling types (WG and SF) in wheat, spelt and rye samples are shown. Enriched proteins are shown in red while depleted proteins are shown in blue. Samples with bread improver were excluded from this analysis, because the commercial bread improver contained unknown proteins from plant sources. Fisher's exact test was performed to identify significantly enriched or depleted Pfam groups in the two most prominent clusters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

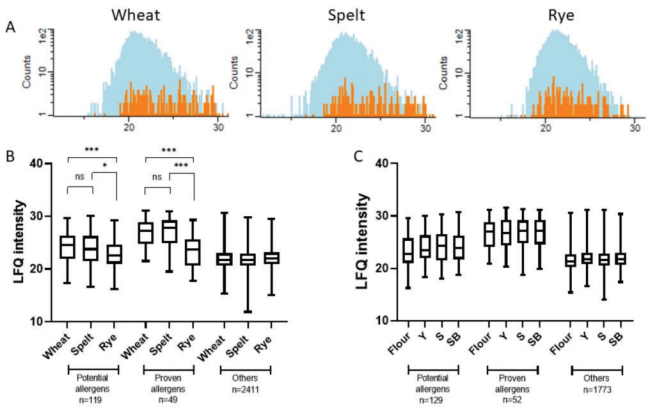


Fig. 5. Relative abundance of allergenic proteins. (A) Histograms show the distribution of proven or potentially allergenic proteins (orange, $n = 246$) compared to other proteins (blue, $n = 4178$) in WG flour of wheat, spelt and rye. (B + C): Allergenic proteins were further divided in "potential allergens" and "proven allergens". Box plots show abundances of potential allergens and proven allergens in different flour or bread types. Only proteins were considered that were identified in at least one replicate of WG flour from wheat, spelt and rye (B) or in each WG wheat bread type and the flour (C). Statistical significance by one-way ANOVA: $*P < 0.05$; $**P < 0.01$, $***P < 0.001$. Abbreviations: Y, yeast fermented; S, sourdough fermented; SB, sourdough fermented with bread improver. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

flour could be observed (Fig. 5C). To get further insight, which allergen groups were responsible for the differences observed between wheat, spelt and rye we analyzed the differences in LFQ intensities within each of the 21 allergen groups. For that purpose, we calculated the mean of the LFQ intensities of the 21 allergen groups in the three replicates of WG flour of wheat, spelt and rye (Fig. S3A). In two of the allergen groups, namely the gliadin group and the inhibitor/storage group, significant differences in the LFQ intensities between wheat and rye as well as between spelt and rye were observed (Fig. S3A, S3B). Considering these two allergen groups in different bread types (Fig. S3C) and in WG and SF flour from wheat (Fig. S3D), no significant differences in allergen abundancies could be observed.

Another group of potential allergens are amylase trypsin inhibitor proteins (ATIs) that may be involved in triggering the symptoms of NCWS. These proteins belong as well as some gluten proteins to the Inhibitor/Storage Pfam family. We identified nine different ATI proteins in our dataset. The relative abundance of all nine ATI proteins was reduced in rye samples compared to wheat and spelt samples (Fig. S4).

Also other potential grain components like fructans have been described as being of relevance in adverse reactions to cereal products like bread [43]. Fructans are produced in the vacuole by the action of specific enzymes (fructosyltransferases) that transfer fructose from sucrose to the growing fructan chain [44]. Rye is known to contain more fructans than wheat and spelt [45]. Searching for fructosyltransferases in rye, wheat and spelt bread we found only one out of 23 fructosyltransferases that are listet in uniprot in our dataset (Sucrose:fructan-6-fructosyltransferase; UniProtKB - A0A3B6I6G4). This protein could be found in all rye samples, but only in few wheat and spelt samples, with a tendency towards higher LFQ intensities in rye than in wheat and spelt (Fig. S5).

4. Discussion

We show for the first time comprehensive proteome data for three different grain flours (wheat, spelt and rye) derived from commercially available sources and for corresponding breads. We detected more than 4000 different proteins with up to 3143 different proteins per sample and we found that flour and bread samples contained around 1300 common proteins that were identified in all samples.

Our results revealed Almost two third that the Uniprot bread wheat protein database that we used as reference allows the identification not only of spelt proteins, as shown earlier by our group [14], but also of rye proteins. This suggests that protein sequences are highly conserved between wheat (both bread wheat and spelt) and rye, which is in line with the well-known fact that wheat and wheat-related species like rye have a common ancestry and share over 70% sequence similarity [37]. Even a single, non-isobaric amino acid substitution within a peptide would lead to a difference to the wheat reference sequence and would result in loss of this peptide in a database search. Although we cannot exclude that some rye- and spelt proteins are not identified by our approach, our results suggest that rye- and spelt proteins can be identified in comparable numbers to wheat proteins using the bread wheat proteome database. Almost two third of the proteins in WG flour and half of the proteins in WG bread could be identified in the samples of all three grain types. Wheat and spelt shared more common proteins than wheat and rye or spelt and rye, indicating that the diploid species rye (*Secale cereale*) is biologically more distant to wheat and spelt that belong to the same hexaploid species (*Triticum aestivum*).

We were able to identify up to 13% more unique proteins in flours compared to the corresponding breads and the average number of identified proteins in flour was slightly higher than in bread. Loss of proteins during processing of the flours to produce bread could be due to partial or complete degradation of proteins by bacterial or fungal proteases during the dough fermentation process [47]. Alternatively, protein extraction from bread might be less efficient than that from flour. Several procedures might account for a reduced number of proteins in breads compared to the corresponding flour. During baking dough is transformed into crumb through gelatinization of the starch granules in the dough and coagulation of the proteins, whereby a stable gluten network is built that encloses the gelatinized starch granules [48] and might hinder protein extraction. The extractability of wheat proteins was shown to decrease during the bread-making due to aggregation and polymerization of proteins based on disulfide bonds that are formed during heat treatment of bread [49]. Also heating during baking process might play a role by degrading particular proteins. Previous studies showed that the solubility of the most abundant gluten proteins and possibly other proteins decreases through heat treatment [50].

Confirming earlier findings [51], we identified more different proteins in WG compared to SF flour from wheat and spelt. Most likely this is due to the fact that the aleurone layer of the grain, which is rich in proteins, is removed with the outer layer of the bran during the milling process [52]. In contrast, in rye samples more proteins could be detected in SF compared to WG flour. An explanation for this contradictory finding might be the higher abundance of pentosans present in rye compared to wheat and spelt [53,54]. Pentosans are polysaccharides present in all plant cells containing cellulose. They are mainly located in the external parts of rye kernel [55] and therefore present in higher amount in rye WG flour. Especially the presence of insoluble pentosans could complicate the extraction of proteins from rye WG flour samples. These observations could likewise be confirmed for the corresponding breads. Hierarchical cluster analysis revealed that particularly proteins of the Cupin and Barwin protein families were significantly reduced in SF samples (flour and bread). The Cupin superfamily includes wheat globulin storage proteins as well as germins located predominantly in the seed bran [50,56,57]. Since also Barwin proteins are known as plant pathogenesis-related proteins and especially expressed during fungal infections [58], it is likely that they are expressed preferentially in the outer layers of the kernel.

Of particular interest in our proteome analysis were potentially allergenic proteins that might cause adverse reactions to bread and other cereals. We defined 24 Pfam domain families that included proven and potentially allergenic proteins. 2105 proteins in our dataset were annotated with a Pfam ID. 246 of these proteins could be assigned to one of the above mentioned Pfam domain families. 54 of these proteins have

been confirmed to be involved in celiac disease or wheat allergy [37,42], whereas 192 have been categorized as potentially allergenic according to their Pfam annotation. Most allergenic proteins in food belong to only a few protein families and superfamilies based on their structural and functional properties [38,59]. Moreover, food allergens must be heat stable and resistant to digestive enzymes [59]. Further, Codex Alimentarius guidelines indicate that proteins with sequence identities to known allergens exceeding 35% in any segment of at least 80 amino acids may be allergenic [60].

The 246 proteins showed higher LFQ intensities on average than the 4178 other proteins in all analyzed samples. Neither the absolute number nor the distribution of these potentially allergenic proteins were dependent on the flour type or the fermentation process, respectively. Remarkably, significantly more potential and proven allergenic proteins could be found in spelt- and wheat flour, compared to rye flour. This observation can be explained by lower amounts of gluten proteins present in rye compared to wheat [12]. Accordingly, when comparing the 21 allergen groups we defined in our study in wheat, spelt and rye wholegrain flour samples, we only observed significant differences between the abundancies of “gliadins” and “Inhibitor/Storage” proteins, two allergen groups to which gluten proteins belong to.

Apart from primary amino acid sequences, immune responses to proteins are also dependent on protein folding and tertiary structure, which explains why not all proteins within one allergen family are allergens. Therefore, the clinical impact of our findings needs to be confirmed in patient studies with challenge tests. Of the 54 proven allergenic proteins found in our dataset, 49 are found in at least one of three replicates of wheat, spelt and rye WG flour; however, with lower LFQ intensities in rye, further explaining why rye might be less relevant for allergy than wheat or spelt. Also well-known allergens like ATIs, from which we could identify 9 in our dataset, were more abundant in wheat and spelt flours and breads, respectively, according to our data. On the other hand, rye is thought to contain more fructans compared to wheat and spelt [45]. Confirming this, we found one fructosyltransferase constantly in rye and only occasionally in wheat and spelt samples. The clinical relevance of this finding needs to be confirmed.

Numerous studies examined the degradation of allergenic proteins during bread-making using 2D-gel electrophoresis. De Angelis et al. [61] observed differences between the composition of allergenic proteins of yeast and sourdough fermented breads. They suggested that traditional protocols of bread-making such as long-time fermentation of sourdough (24 h at 37 °C) with selected starters result in extensive degradation of IgE-binding wheat albumins and globulins. Seven proteins were identified by MALDI-TOF mass spectrometry. Three of these proteins could also be identified in our dataset, namely xylanase inhibitor protein 1, endogenous α -amylase-subtilisin inhibitor (WASI) and ATI 0.19. Comparing the LFQ intensities of these three proteins between wheat flour, yeast fermented bread and sourdough fermented bread, we were not able to detect any differences (data not shown). This might be due to the shorter dough fermentation time (100 min for rye and 110 min for wheat and spelt at 30 °C) used in our study. Using SDS-PAGE and immunoblot analysis, Rizello et al. [20] showed a stronger proteolytic effect of some IgE-binding proteins in sourdough fermented compared to yeast fermented bread from wheat and rye. In contrast to these findings, we did not observe such differences, most likely due to different baking conditions. This assumption is strengthened by results from Loponen et al. [62], who used comparable fermentation times as we did in the present study and showed that short-time fermentation (2 h) of sourdough did not result in degradation of gluten proteins [63].

To a subset of sourdough fermented breads, we added a typical bread improver used in conventional bakeries to analyze its impact on the bread proteome. We could neither detect significant differences regarding the number of identified proteins nor the relative abundance of allergenic proteins related to the presence or absence of the improver (see supplementary table 3). The fact that we did not observe any differences in the relative abundance of allergenic proteins between different bread types, or between bread and flour, indicates that there is no specific degradation of allergenic proteins during the fermentation process of the present study.

Apart from allergenic proteins, other proteins might be modified or even removed by the manufacturing process. Although less relevant for allergies, this might affect the quality of bread. Indeed, we found that up to 16% of these other proteins were found uniquely in one of the bread types suggesting that the manufacturing process affects these proteins. At present, it is unclear how this alters bread quality. Hierarchical cluster analysis showed that the abundances of histone- and ribosomal proteins were reduced in sourdough fermented bread compared to yeast fermented bread and flour. Maybe this is due to histone protein degradation by lactic acid bacteria for obtaining pyrimidines and purines. Many lactobacilli are auxotrophic for both purine- and pyrimidine bases and therefore, they inherit a salvage-pathway system based on trans-N-deoxyribosylases to utilize external DNA bases for growth [64].

In conclusion, our results show that allergenic proteins are not selectively degraded during conventional bread production, neither in yeast nor in sourdough fermented bread nor in sourdough fermented bread with improver.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2021.104318.

References

[1] K.E.C. Grimshaw, T. Bryant, E.M. Oliver, J. Martin, J. Maskell, T. Kemp, E.N. Clare Mills, K.D. Foote, B.M. Margetts, K. Beyer, G. Roberts, Incidence and risk factors for food hypersensitivity in UK infants: results from a birth cohort study, Clin. Transl. Allergy 6 (2015) 1.
[2] A. Fasano, I. Berti, T. Gerarduzzi, T. Not, R.B. Colletti, S. Drago, Y. Elitsur, P.H. R. Green, S. Guandalini, I.D. Hill, M. Pietzak, A. Ventura, M. Thorpe, D. Kryszak, F. Fornaroli, S.S. Wasserman, J.A. Murray, K. Horvath, Prevalence of celiac disease in at-risk and not-at-risk groups in the United States: a large multicenter study, Arch. Intern. Med. 163 (2003) 286–292.
[3] V.F. Zevallos, V. Raker, S. Tenzer, C. Jimenez-Calvente, M. Ashfaq-Khan, N. Rüssel, G. Pickert, H. Schild, K. Steinbrink, D. Schuppan, Nutritional wheat amylase-trypsin inhibitors promote intestinal inflammation via activation of myeloid cells, Gastroenterology 152 (2017), 1100–1113.e12.
[4] A. Fasano, A. Sapone, V. Zevallos, D. Schuppan, Nonceliac gluten sensitivity, Gastroenterology 148 (2015) 1195–1204.
[5] L.M. Sollid, Coeliac disease: dissecting a complex inflammatory disorder, Nat. Rev. Immunol. 2 (2002) 647–655.
[6] G. Ricci, L. Andreozzi, F. Cipriani, A. Giannetti, M. Gallucci, C. Caffarelli, Wheat allergy in children: a comprehensive update, Medicina (Kaunas) 55 (2019) 400.
[7] S. Tanabe, Analysis of food allergen structures and development of foods for allergic patients, Biosci. Biotechnol. Biochem. 72 (2008) 649–659.
[8] Y. Junker, S. Zeissig, S.-J. Kim, D. Barisani, H. Wieser, D.A. Leffler, V. Zevallos, T. A. Libermann, S. Dillon, T.L. Freitag, C.P. Kelly, D. Schuppan, Wheat amylase trypsin inhibitors drive intestinal inflammation via activation of toll-like receptor 4, J. Exp. Med. 209 (2012) 2395–2408.
[9] T. Osborne, The Vegetable Proteins, 2nd ed., Longmans, Green and Co, London, 1924.
[10] P.R. Shewry, N.G. Halford, D. Lafiandra, Genetics of wheat gluten proteins, Adv. Genet. 49 (2003) 111–184.
[11] T. Takizawa, H. Arakawa, K. Tokuyama, A. Morikawa, Identification of allergen fractions of wheat flour responsible for anaphylactic reactions to wheat products in infants and young children, Int. Arch. Allergy Immunol. 125 (2001) 51–56.
[12] B. Lexhaller, M.L. Colgrave, K.A. Scherf, Characterization and relative quantitation of wheat, rye, and barley gluten protein types by liquid chromatography-tandem mass spectrometry, Front. Plant Sci. 10 (2019) 1530.
[13] M.L. Colgrave, H. Goswami, K. Byrne, M. Blundell, C.A. Howitt, G.J. Tanner, Proteomic profiling of 16 cereal grains and the application of targeted proteomics to detect wheat contamination, J. Proteome Res. 14 (2015) 2659–2668.

[14] M. Afzal, J. Pfannstiel, J. Zimmermann, S.C. Bischoff, T. Würschum, C.F.H. Longin, High-resolution proteomics reveals differences in the proteome of spelt and bread wheat flour representing targets for research on wheat sensitivities, Sci. Rep. 10 (2020) 14677.
[15] B. Prandi, T. Tedeschi, S. Folloni, G. Galaverna, S. Sforza, Peptides from gluten digestion: a comparison between old and modern wheat varieties, Food Res. Int. 91 (2017) 92–102.
[16] S. Bischoff, S.E. Crowe, Gastrointestinal food allergy: new insights into pathophysiology and clinical perspectives, Gastroenterology 128 (2005) 1089–1113.
[17] F.M. Dupont, W.H. Vensel, C.K. Tanaka, W.J. Hurkman, S.B. Altenbach, Deciphering the complexities of the wheat flour proteome using quantitative two-dimensional electrophoresis, three proteases and tandem mass spectrometry, Proteome Sci. 9 (2011) 10.
[18] B. Simonato, G. Pasini, M. Giannattasio, A.D. Peruffo, F. de Lazzari, A. Curioni, Food allergy to wheat products: the effect of bread baking and in vitro digestion on wheat allergenic proteins. A study with bread dough, crumb, and crust, J. Agric. Food Chem. 49 (2001) 5668–5673.
[19] R. Laatikainen, J. Koskenpato, S.-M. Hongisto, J. Loponen, T. Poussa, X. Huang, T. Sontag-Strohm, H. Salmenkari, R. Korpela, Pilot study: comparison of sourdough wheat bread and yeast-fermented wheat bread in individuals with wheat sensitivity and irritable bowel syndrome, Nutrients 9 (2017) 1215.
[20] C.G. Rizzello, M. de Angelis, R. Coda, M. Gobbetti, Use of selected sourdough lactic acid bacteria to hydrolyze wheat and rye proteins responsible for cereal allergy, Eur. Food Res. Technol. 223 (2006) 405–411.
[21] P.J. Davis, C.M. Smales, D.C. James, How can thermal processing modify the antigenicity of proteins? Allergy 56 (Suppl. 67) (2001) 56–60.
[22] K.-E. El Mecherfi, S.D. Todorov, M.A. Cavalcanti de Albuquerque, S. Denery-Papini, R. Lupi, T. Haertlé, B. Dora Gombossy de Melo Franco, C. Larré, Allergenicity of fermented foods: emphasis on seeds protein-based products, Foods 9 (2020) 792.
[23] M. Afzal, M. Sielaff, V. Curella, M. Neerukonda, K. El Hassouni, D. Schuppan, S. Tenzer, C.F.H. Longin, Characterization of 150 wheat cultivars by LC-MS-based label-free quantitative proteomics unravels possibilities to design wheat better for baking quality and human health, Plants (Basel) 10 (2021) 424.
[24] M. Sielaff, V. Curella, M. Neerukonda, M. Afzal, K. El Hassouni, U. Distler, D. Schuppan, Longin CFH, S. Tenzer, Hybrid QconCAT-Based targeted absolute and data-independent acquisition-based label-free quantification enables in-depth proteomic characterization of wheat amylase/trypsin inhibitor extracts, J. Proteome Res. 20 (2021) 1544–1557.
[25] R. Pilolli, M. de Angelis, A. Lamonaca, E. de Angelis, C.G. Rizzello, S. Siragusa, A. Gadaleta, G. Mamone, L. Monaci, Prototype gluten-free breads from processed durum wheat: use of monovarietal flours and implications for gluten detoxification strategies, Nutrients 12 (2020) 3824.
[26] S. Islam, W. Ma, G. Yan, L. Gao, R. Appels, Differential recovery of lupin proteins from the gluten matrix in lupin-wheat bread as revealed by mass spectrometry and two-dimensional electrophoresis, J. Agric. Food Chem. 59 (2011) 6696–6704.
[27] U. Bose, A. Juhász, J.A. Broadbent, K. Byrne, C.A. Howitt, M.L. Colgrave, Identification and quantitation of amylase trypsin inhibitors across cultivars representing the diversity of bread wheat, J. Proteome Res. 19 (2020) 2136–2148.
[28] D. Wessel, U.I. Flügge, A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids, Anal. Biochem. 138 (1984) 141–143.
[29] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[30] J. Rappsilber, M. Mann, Y. Ishihama, Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips, Nat. Protoc. 2 (2007) 1896–1906.
[31] J.V. Olsen, L.M.F. de Godoy, G. Li, B. Macek, P. Mortensen, R. Pesch, A. Makarov, O. Lange, S. Horning, M. Mann, Parts per million mass accuracy on an orbitrap mass spectrometer via lock mass injection into a C-trap, Mol. Cell. Proteomics 4 (2005) 2010–2021.
[32] R.M. Miller, R.J. Millikin, C.V. Hoffmann, S.K. Solntsev, G.M. Sheynkman, M. R. Shortreed, L.M. Smith, Improved protein inference from multiple protease bottom-up mass spectrometry data, J. Proteome Res. 18 (2019) 3429–3438.
[33] The UniProt Consortium, UniProt: the universal protein knowledgebase in 2021, Nucleic Acids Res. 49 (2021) D480–D489.
[34] R.J. Millikin, S.K. Solntsev, M.R. Shortreed, L.M. Smith, Ultrafast peptide label-free quantification with FlashLFQ, J. Proteome Res. 17 (2018) 386–391.
[35] R Core Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2018.
[36] S. Tyanova, T. Temu, P. Sinitcyn, A. Carlson, M.Y. Hein, T. Geiger, M. Mann, J. Cox, The Perseus computational platform for comprehensive analysis of (prote) omics data, Nat. Methods 13 (2016) 731–740.
[37] A. Juhász, T. Belova, C.G. Florides, C. Maulis, I. Fischer, G. Gell, Z. Birinyi, J. Ong, G. Keeble-Gagnère, A. Maharajan, W. Ma, P. Gibson, J. Jia, D. Lang, K.F.X. Mayer, M. Spannagl, J.A. Tye-Din, R. Appels, O.-A. Olsen, Genome mapping of seed-borne allergens and immunoresponsive proteins in wheat, Sci. Adv. 4 (2018) eaar8602.
[38] C. Radauer, M. Bublin, S. Wagner, A. Mari, H. Breiteneder, Allergens are distributed into few protein families and possess a restricted number of biochemical functions, J. Allergy Clin. Immunol. 121 (2008), 847–52.e7.
[39] Food Allergy Research and Resource Program, Available from, http://www.allergenonline.org/.
[40] A. Mari, E. Scala, Allergome: a unifying platform, Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M (2006) 29–39 (discussion 39-40).
[41] A. Mari, C. Rasi, P. Palazzo, E. Scala, Allergen databases: current status and perspectives, Curr Allergy Asthma Rep 9 (2009) 376–383.
[42] Allergome. http://www.allergome.org, July 09, 2020. Available from, http://www.allergome.org/.
[43] G.I. Skodje, V.K. Sarna, I.H. Minelle, K.L. Rolfsen, J.G. Muir, P.R. Gibson, M. B. Veierød, C. Henriksen, K.E.A. Lundin, Fructan, rather than gluten, induces symptoms in patients with self-reported non-celiac gluten sensitivity, Gastroenterology 154 (2018), 529–539.e2.
[44] I. Vijn, S. Smeekens, Fructan: more than a reserve carbohydrate? Plant Physiol. 120 (1999) 351–360.
[45] K. Whelan, O. Abrahmsohn, G.J.P. David, H. Staudacher, P. Irving, M.C.E. Lomer, P.R. Ellis, Fructan content of commonly consumed wheat, rye and gluten-free breads, Int. J. Food Sci. Nutr. 62 (2011) 498–503.
[47] C.G. Rizzello, M. de Angelis, R. Di Cagno, A. Camarca, M. Silano, I. Losito, M. de Vincenzi, M.D. de Bari, F. Palmisano, F. Maurano, C. Gianfrani, M. Gobbetti, Highly efficient gluten degradation by lactobacilli and fungal proteases during food processing: new perspectives for celiac disease, Appl. Environ. Microbiol. 73 (2007) 4499–4507.
[48] N.G. Heredia-Sandoval, M.Y. Valencia-Tapia, A.M. La Calderón de Barca, A. R. Islas-Rubio, Microbial proteases in baked goods: modification of gluten and effects on immunogenicity and product quality, Foods 5 (2016).
[49] H. Singh, A study of changes in wheat protein during bread baking using SE-HPLC, Food Chem. 90 (2005) 247–250.
[50] P.R. Shewry, N.G. Halford, Cereal seed storage proteins: structures, properties and role in grain utilization, J. Exp. Bot. 53 (2002) 947–958.
[51] G.G. Heshe, G.D. Haki, A.Z. Woldegiorgis, H.F. Gemede, Effect of conventional milling on the nutritional value and antioxidant capacity of wheat types common in Ethiopia and a recovery attempt with bran supplementation in bread, Food Sci. Nutr. 4 (2015) 534–543.
[52] B. Pedersen, K.E. Knudsen, B.O. Eggum, Nutritive value of cereal products with emphasis on the effect of milling, World Rev. Nutr. Diet. 60 (1989) 1–91.
[53] C.G. Biliaderis, M.S. Izydorczyk, O. Rattan, Effect of arabinoxylans on bread-making quality of wheat flours, Food Chem. 53 (1995) 165–171.
[54] Michniewicz J. Biliaderis, C.G, Bushuk W., Water-insoluble pentosans of wheat: composition and some physical properties, Cereal Chem. 67 (1990) 434–439.
[55] K. Buksa, R. Ziobro, A. Nowotna, H. Gambuś, The influence of native and modified arabinoxylan preparations on baking properties of rye flour, J. Cereal Sci. 58 (2013) 23–30.
[56] A.L. Mitchell, T.K. Attwood, P.C. Babbitt, M. Blum, P. Bork, A. Bridge, S.D. Brown, H.-Y. Chang, S. El-Gebali, M.I. Fraser, J. Gough, D.R. Haft, H. Huang, I. Letunic, R. Lopez, A. Luciani, F. Madeira, A. Marchler-Bauer, H. Mi, D.A. Natale, M. Necci, G. Nuka, C. Orengo, A.P. Pandurangan, T. Paysan-Lafosse, S. Pesseat, S.C. Potter, M.A. Qureshi, N.D. Rawlings, N. Redaschi, L.J. Richardson, C. Rivoire, G. A. Salazar, A. Sangrador-Vegas, Sigrist CJA, I. Sillitoe, G.G. Sutton, N. Thanki, P. D. Thomas, Tosatto SCE, S.-Y. Yong, R.D. Finn, InterPro in 2019: improving coverage, classification and access to protein sequence annotations, Nucleic Acids Res. 47 (2019) D351–D360.
[57] S.B. Altenbach, C.K. Tanaka, W.J. Hurkman, W.H. Vensel, Expression of globulin-2, a member of the cupin superfamily of proteins with similarity to known food allergens, is increased under high temperature regimens during wheat grain development, J. Cereal Sci. 49 (2009) 47–54.
[58] C. Caruso, M. Nobile, L. Leonardi, L. Bertini, V. Buonocore, C. Caporale, Isolation and amino acid sequence of two new PR-4 proteins from wheat, J. Protein Chem. 20 (2001) 327–335.
[59] E.N.C. Mills, J.A. Jenkins, M.J.C. Alcocer, P.R. Shewry, Structural, biological, and evolutionary relationships of plant food allergens sensitizing via the gastrointestinal tract, Crit. Rev. Food Sci. Nutr. 44 (2004) 379–407.
[60] R.E. Goodman, Practical and predictive bioinformatics methods for the identification of potentially cross-reactive protein matches, Mol. Nutr. Food Res. 50 (2006) 655–660.
[61] M. de Angelis, C.G. Rizzello, E. Scala, C. de Simone, G.A. Farris, F. Turrini, M. Gobbetti, Probiotic preparation has the capacity to hydrolyze proteins responsible for wheat allergy, J. Food Prot. 70 (2007) 135–144.
[62] R. Di Cagno, M. de Angelis, S. Auricchio, L. Greco, C. Clarke, M. de Vincenzi, C. Giovannini, M. D'Archivio, F. Landolfo, G. Parrilli, F. Minervini, E. Arendt, M. Gobbetti, Sourdough bread made from wheat and nontoxic flours and started with selected lactobacilli is tolerated in celiac Sprue patients, Appl. Environ. Microbiol. 70 (2004) 1088–1096.
[63] J. Loponen, M. Mikola, K. Katina, T. Sontag-Strohm, H. Salovaara, Degradation of HMW glutenins during wheat sourdough fermentations, Cereal Chem. J. 81 (2004) 87–93.
[64] M. Kilstrup, K. Hammer, P. Ruhdal Jensen, J. Martinussen, Nucleotide metabolism and its control in lactic acid bacteria, FEMS Microbiol. Rev. 29 (2005) 555–590.

2.2 Studie an einem Mausmodell für entzündliche Darmerkrankungen

Die Hintergründe und Mechanismen der NCWS sind bislang unbekannt. Eine Hypothese zur Entstehung basiert auf einer latenten oder manifesten Vorerkrankung des Darmes wie z.B. eine Störung der Darmbarriere oder eine intestinale Dysbiose als Ursache für die Symptome nach Weizenverzehr [42,43]. Aus diesem Grund wurde in der vorliegenden Arbeit ein Mausmodell gewählt, das durch den Knockout des Caspase-8 Gens charakterisiert ist, was zu einem Verlust von Panethzellen und somit einer Schädigung der Darmbarriere führt, woraus spontan intestinale Entzündungen resultieren [44]. Die Wahl des Getreides (Weizen, Dinkel, Roggen), der Vermahlungsgrad des Mehls (Vollkorn, Auszugsmehl), die Art der Teigführung (Hefeteig, Sauerteig) sowie der Zusatz von Backmitteln können die Zusammensetzung von Brot beeinflussen [30,31] und somit auch den Gehalt an Inhaltsstoffen, die sich positiv oder negativ auf bereits bestehende intestinale Pathologien auswirken [45]. In der vorliegenden Arbeit wurden sechs Brotsorten aus der Proteomanalyse am Mausmodell untersucht (Tabelle 2).

Tabelle 2. Übersicht über die 6 verschiedenen Brotsorten

Getreide	**Vermahlung**	**Herstellung**
Weizen	Auszugsmehl	Hefeteig
	Auszugsmehl	Sauerteig
	Auszugsmehl	Sauerteig + Backmittel
	Vollkornmehl	Hefeteig
Dinkel	Auszugsmehl	Sauerteig
Roggen	Auszugsmehl	Sauerteig

Ziel dieser Studie war es, die Auswirkungen verschiedener Brotsorten und einer Diät mit 5% Gluten an einem Mausmodell mit bestehender Darmbarrierestörung und -entzündung zu untersuchen und mit der Wirkung in gesunden Mäusen zu vergleichen, um daraus Rückschlüsse über den Einfluss der Wahl des Getreides und der Brotherstellung auf dessen Verträglichkeit zu ziehen. Auch die Ergebnisse der Proteomanalyse sollte auf ihre klinische Relevanz in diesem Mausmodell untersucht werden. Der Artikel wurde im Januar 2022 von der Fachzeitschrift Journal of Digestive Diseases an Science akzeptiert.

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ORIGINAL ARTICLE

Consumption of Yeast-Fermented Wheat and Rye Breads Increases Colitis and Mortality in a Mouse Model of Colitis

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Abstract

Background Cereals are known to trigger for wheat allergy, celiac disease and non-celiac wheat sensitivity (NCWS). Inflammatory processes and intestinal barrier impairment are suspected to be involved in NCWS, although the molecular triggers are unclear.

Aims We were interested if different bread types influence inflammatory processes and intestinal barrier function in a mouse model of inflammatory bowel disease.

Methods Epithelial caspase-8 gene knockout ($Casp8^{\Delta IEC}$) and control ($Casp8^{fl}$) mice were randomized to eight groups, respectively. The groups received different diets for 28 days (gluten-free diet, gluten-rich diet 5 g%, or different types of bread at 50 g%). Breads varied regarding grain, milling and fermentation. All diets were isocaloric.

Results Regardless of the diet, $Casp8^{\Delta IEC}$ mice showed pronounced inflammation in colon compared to ileum, whereas $Casp8^{fl}$ mice were hardly inflamed. $Casp8^{fl}$ mice could tolerate all bread types. Especially yeast fermented rye and wheat bread from superfine flour but not pure gluten challenge increased colitis and mortality in $Casp8^{\Delta IEC}$ mice. Hepatic expression of lipopolysaccharide-binding protein and colonic expression of tumor necrosis factor-α genes were inversely related to survival. The bread diets, but not the gluten-rich diet, also decreased colonic tight junction expression to variable degrees, without clear association to survival and inflammation.

Conclusions Bread components, especially those from yeast-fermented breads from wheat and rye, increase colitis and mortality in $Casp8^{\Delta IEC}$ mice highly susceptible to intestinal inflammation, whereas control mice can tolerate all types of bread without inflammation. Yet unidentified bread components other than gluten seem to play the major role.

Keywords Inflammatory bowel disease · Colitis · Wheat hypersensitivity · Bread · Baker's yeast

Introduction

Wheat sensitivities increased extensively in the last years in industrialized western countries [1, 2]. Three types of wheat sensitivities can be distinguished, celiac disease (CD), wheat allergy (WA) and non-celiac wheat sensitivity

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(NCWS), with a prevalence of approximately 1% of adults each [3]. CD and WA are immunological diseases [4] while the origin of NCWS is unclear at present, but an involvement of the immune system and an impaired intestinal barrier are assumed to play a role [5, 6]. NCWS symptoms may also be secondary to low-grade type of chronic inflammation triggered by particular bread consumption [1, 7].

The prevalence of NCWS among patients with inflammatory bowel disease (IBD) seems to be much higher than in the normal population with a prevalence of up to 27.6% [8]. Individuals who report an IBD flare within the past 60 days were significantly more likely to report NCWS suggesting that this "wheat intolerance" or "bread intolerance" could be a transient phenomenon associated with an impaired intestinal barrier function that improves in IBD remission phases [8]. It has been reported that also CD might be related to IBD, since CD is a kind of risk factor for IBD [9] and microscopic colitis [10].

It has been shown that a proportion of patients with IBD benefit from a gluten-free diet [11], and IBD patients with NCWS reported higher disease activity as compared to IBD patients without NCWS [12]. The underlying triggering agents are not necessarily gluten-related, but could be also fermentable carbohydrates (FODMAPs) causing increased gas production [8]. Apart from triggers, manufacturing processes with a lower fermentation time and the use of highly efficient yeast species have been discussed as potentially relevant factors in the pathophysiology of NCWS, the first case of which was published in 1978 [13].

Milling, kneading, fermentation and heating impact the characteristics of bread and might also affect the composition of breads, especially the protein composition [14, 15].

Rizzello et al. [16] showed that specific sourdough microorganisms can lead to a decrease of allergenic proteins in wheat- and rye bread. Bread improvers in combination with thermal processing of the dough could lead to the formation of new allergens [15, 17].

This study aimed to evaluate the effects of different bread types and a gluten enriched diet in control and Casp8$^{\Delta IEC}$ mice, a mouse model with intestinal inflammation. Loss of the caspase-8 gene has been shown to result in a loss of Paneth cells and a reduced expression of antimicrobial peptides, resulting in epithelial damage, an impaired gut barrier, and finally in a higher susceptibility to intestinal inflammation [18].

Methods

Bread Making

For preparing the different breads analyzed in our study we used grains from bread wheat (hereafter referred to as wheat), spelt and rye (Schapfenmühle, Ulm, Germany), from which superfine flour (SF) (Quadrumat junior mill, Brabender, Duisburg) and wholegrain flour (WG) (Ultracentrifugal mill ZM 200 sieve insert 0.5 mm; Retsch, Haan) were produced. Details of bread manufacturing and results of flour analysis are described elsewhere [14]. For this study six different bread types were produced (Table 1). Bread improver (MeisterMarken Ulmer Spatz, type Weißback Super, CSM Deutschland GmbH, Bremen, Germany) was used for one bread type (W SF SB) and was composed of buttermilk powder (24%); wheat flour; soy flour; emulsifier (soya lecithins, mono- and diglycerides of fatty acids); wheat malt flour; dextrose; salt; acidity regulator (calcium acetate); sour whey powder; acidulant (citric acid, lactic acid); wheat swell flour; ascorbic acid; enzymes. Study breads differed regarding oligosaccharide content (Table S1).

Animals

In a preliminary test, we tested if healthy mice tolerate a diet with a high proportion of bread. Eight female Balb/c mice of an age of 11 weeks were fed for 28 days with a diet that consisted of 50 g% (50% of total food weight expressed in gram) of yeast-fermented wheat bread from superfine flower (W SF Y, see also Table 1). Food intake and weight gain were measured every day.

In the main part of our studies, mice with a specific deletion of caspase-8 in the intestinal epithelium (Casp8$^{\Delta IEC}$) bred on a C57BL/6 J genetic background and Cre-negative control littermates with floxed caspase-8 alleles (Casp8^{fl}) were used. They were originally obtained from the laboratory of Christoph Becker (Medical Clinic, Erlangen, Germany) [18] and bred in our laboratory under specific pathogen-free (SPF) conditions. To avoid too serious spontaneous inflammation, 6-week-old female Casp8$^{\Delta IEC}$ and Casp8^{fl} mice as well as Cre-negative littermates were used. Mice genotypes were confirmed by DNA analysis from ear biopsies using polymerase chain reaction (PCR), specific for the respective allele. Before start of our experiments, all mice received ad libitum a gluten free breeding diet (S0514-E750, Ssniff Spezialdiäten GmbH, Soest, Germany). Both Casp8$^{\Delta IEC}$ and Casp8^{fl} were divided into eight groups with eight mice each. Each group was fed with a different diet offered in pellet form ad libitum for 28 days. The eight diets are listed in Table 2.

During the experiments mice were housed in a SPF barrier facility with a fully controlled environment at 22 ± 2 °C and 50% humidity, under a 12-h light/dark cycle accredited by the Association for Assessment and Accreditation for Laboratory Animal Care International. They were kept in collective cages containing two or three mice each. Our study was approved by the local Animal Care and Use Committee (Regional Council Stuttgart, V343/18 EM). All

Table 1 Composition of the six different breads used in the study

Bread type[1]	Grain type[1]	Flour[1]	Dough[1]	Improver [g]	Flour [g]	Yeast [g]	Salt [g]	Fat [g]	Sugar [g]	Water [g]	Ascorbic acid [g]	Mother dough [g]	Total [g]
W SF Y	W	SF	Y	–	5000	50	100	50	50	2770	0.2	–	8020
W SF S	W	SF	S	–	3500	50	100	50	50	1270	0.2	3000	8020
W SF SB	W	SF	S	100	3500	50	100	50	50	1270	0.2	3000	8120
W WG Y	W	WG	Y	–	5000	50	100	50	50	3140	0.2	–	8391
Sp SF Y	Sp	SF	Y	–	5000	50	100	50	50	2705	0.2	–	7955
R SF Y	R	SF	Y	–	5000	50	100	–	–	3005	0.2	–	8155

Abbreviations: W, Wheat; Sp, Spelt; R, Rye; SF, Superfine flour; WG, Wholegrain flour; Y, Yeast fermented; S, Sourdough fermented

experiments were conducted according to the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA).

Dietary Treatment

Eight different custom diets (Ssniff Spezialdiäten GmbH) were produced for use in the experiments; (i) a gluten-free control diet based on rice (GF) and (ii) a gluten-free diet based on rice supplemented with 5 g% wheat gluten (Sigma-Aldrich, St. Louis, USA) (5G), which served as controls, and (iii–xiii) six different bread-diets, which consisted half/half of the corresponding bread (50 g%) and other nutrients except cereals (50 g%) to reach the nutritional requirements for mice. All experimental diets had the same total energy content of 14.6 MJ/kg (Table 2).

Human Equivalence Dose for Gluten

To assess the human equivalence dose (HED) for gluten consumption in our mouse experiments we used the formula HED = mouse dose (mg/kg) × (mouse Km/human Km), whereby Km is the ratio of body weight to body surface area for a given species (37 for humans, 3 for mice) [19]. In our experiments, mice received 125 mg/d of gluten in the 5G diet, or 20–70 mg/d of gluten in the bread diets. Considering an average mouse body weight of 20 g, this corresponds to 35.4 g/d of gluten (5G diet), or 5.6–19.8 g/d of gluten (bread diets) in a 70 kg man, respectively, which is in line with the average daily gluten ingestion in humans is estimated to be between 13 and 30 g/day [7, 20].

Feeding Period, End of the Experiment and Dropouts

Food intake and body weight of the mice were assessed three times a week during the feeding period of 4 weeks. The health status was checked daily by using internally established health scores to evaluate the health status. If mice met the criteria for premature study end like diarrhea or bloody feces, loss of body weight > 15%, or deteriorated general well-being, they were killed before the planned study end and defined as dropouts. Otherwise, the study terminated after 28 days, and mice were killed after blood had been collected from the portal vein. Mice were fasted for 4–6 h and then anesthetized with ketamine-xylazine (100:16 mg/kg body weight) by intraperitoneal injection 1 h after gavage with fluorescein isothiocyanate-dextran 4000 (FITC-D4000) (600 mg/kg body weight, using a stock volume of 50 mg/mL). Liver and gut tissue was collected and immediately frozen in dry ice or stored in neutral-buffered formalin.

Table 2 Proximate nutrient content in the eight experimental diets

Content	Rice-based gluten free (GF)	GF/5G	W SF Y	W SF S	W SF SB	W WG Y	Sp SF Y	R SF Y
Crude protein (%)	22.1	22.1	22.1	22.1	22.1	22.1	22.1	22.1
Crude fat (%)	6.0	6.0	5.2	4.8	4.5	3.8	5.7	3.2
Crude fiber (%)	5.9	5.9	8.0	6.9	6.2	4.2	8.0	3.0
Crude ash (%)	6.0	6.0	6.7	6.8	6.9	7.3	6.9	6.8
Starch (%)	45	45	45	45	45	45	45	45
Sugar (%)	5.4	5.4	5.4	5.4	5.4	5.4	5.4	5.4
Energy (MJ/kg)	14.6	14.6	14.6	14.6	14.6	14.6	14.6	14.6

Data are presented as percent of weight (g%) for all components except energy content, which is expressed in megajoule per kg. Abbreviations see Table 1

Colonoscopy

To detect colitis and tissue necrosis in the course of the study, colonoscopy using a mouse video endoscopic system (Karl Storz Endoscope, Coloview system, Mainz, Germany) was performed as described [21].

Histological Analysis

Colon and ileum samples were rinsed, collected and fixed in 10% neutral-buffered formalin (Sigma-Aldrich, St Louis, USA) for minimum 24 h and subsequently embedded in paraffin. Paraffin sections of 5 µm were cut and de-waxed prior to staining with hematoxylin/eosin (H&E; Merck, Darmstadt, Germany). Tissue sections were evaluated, and images taken by standard light microscopy using an AxioImager Z1 microscope (Carl Zeiss MicroImaging, Jena, Germany). Histological scoring occurred as already described [22]. Total histological score (sum of both the tissue damage score and the infiltration score of inflammatory cells) in ileum and colon tissue resulted in 0–6 points, which were interpreted as a low-grade histological score (LHSc, 0–2 points), a middle grade histological score (MHSc, > 2–4 points), and a high grade score (HHSc, > 4–6 points).

Small Intestinal Permeability

Blood from the portal vein was collected in heparinized 1.5 ml tubes for FITC-D4000 analyses as described elsewhere [23]. In short, fluorescence was measured in 96-well plates (Infinite M200 PRO, Tecan, Crailsheim, Germany) using a fluorimeter (Multi-Detection Microplate Reader, Synergy TM HT, Bio-Tek®, Vermont, USA) with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. FITC-D4000 concentrations were calculated with the help of a standard curve of FITC-D4000 with concentrations ranging from 0 to 250 µg/ml.

Real-Time Quantitative Reverse Transcription-PCR

Total RNA was isolated from liver- and colon tissue using Trizol® reagent (Invitrogen Life Technologies, Carlsbad, USA) following the manufacturer's instructions. Yield and purity of RNA was determined by NanoDrop ND-1000 spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific, Darmstadt, Germany). In addition, RNA integrity was checked using agarose gel electrophoresis. Intact RNA samples with an absorbance ratio OD 260/280 between 1.8 and 2.1 and OD 260/230 greater than 2.0 were used for further analysis. cDNA was synthesized from 1 µg of total RNA using the SuperScript® IV Reverse-Transcriptase (Thermo FisherScientific, Darmstadt, Germany) after DNAse treatment (Promega, Madison, USA). cDNA was stored at − 20 °C until use. Real-time PCR was conducted in Bio-Rad iQ5 Real- Time System. Eva Green Universal PCR Master Mix (Bio-Rad Laboratories, Munich, Germany) was used to prepare the PCR mix. The amplification program for primers: tumor necrosis factor-α (TNFα), mucin-2 (Muc2), zonula occludens-1 (ZO1), Occludin and Claudin-2 (CLDN2) was: 95 °C for 30 s, 40 cycles at 95 °C for 5 s and 60 °C for 30 s. For the primers of lipopolysaccharide-binding protein (LBP), toll-like receptor-4 (TLR4) and myeloid differentiation primary response-88 (MyD88) the program was: 95 °C for 30 s, 40 cycles at 95 °C for 5 s and 62 °C for 30 s. After amplification, a thermal denaturing cycle was added to derive the dissociation curve of the PCR product to verify amplification specificity. The comparative C_T method was used to determine the amount of the target genes, normalized to an endogenous reference (β-actin mRNA expression) and relative to a calibrator ($2^{-\Delta\Delta C}{}_T$). Primer sequences are given in Table 3.

Statistical Analysis

The results are shown as the means ± SEM if not indicated otherwise. Normal distribution was tested using Shapiro–Wilk test. To test the potential effects of the factors of

Table 3 Primer sequences used for RT-PCR

Gene	Forward (5´-3´)	Reverse (5´-3´)
β-Actin	GCT GAG AGG GAA ATC GTG CGT G	CCA GGG AGG AAG AGG ATG CGG
CLDN2	TTC TCT ACA ACA ACT CCA TCC TC	GCA GC CATT TCC TTC TCT CC
LBP	GGT GGC GTG GTC ACT AAT GT	CTC ACT TGT GCC TTG TCT GG
Muc2	GAT GGC ACC TAC CTC GTT GT	GTC CTG GCA CTT GTT GGA AT
MyD88	CAA AAG TGG GGT GCC TTT GC	AAA TCC ACA GTG CCC CCA GA
TNFα	ACC ACC ATC AAG GAC TCA	AGG TCT GAA GGT AGG AAG
TLR4	GAT CTG AGC TTC AAC CCC TTG	TGC CAT GCC TTG TCT TCA AT
Occludin	ATG TCC GGC CGA TGC TCT C	CTT TGG CTG CTG TTG GGT CTG
ZO1	GAA TGT GAG GCA GAT GAC AG	AGG TCT TTG CGG ATG TCC ACG T

Abbreviations: CLDN2, claudin-2; LBP, LPS-binding-protein; Muc2, mucin-2; MyD88, myeloid differentiation primary factor-88; TNFα, tumor necrosis factor-α; TLR4, toll-like receptor-4; ZO1, zonula occludens-1

diet (six bread diets, GF and 5G) and genotype ($Casp8^{\Delta IEC}$ vs. $Casp8^{fl}$) and their interaction, we performed two-way ANOVA with a Tukey's multiple comparison test and inverse transformation of raw data in cases of unequal variance. Survival data were analyzed using the Kaplan–Meier method. Means and percentages of normally distributed variables were reported with their respective 95% confidence intervals (95% CI). A P-value < 0.05 was considered as statistically significant. For statistical analysis and figure presentations we used GraphPad Prism, version 9.2 (Graph Pad, La Jolla, CA) and SPSS, version 25 (IBM, Armonk, NY). For correlation analyses, we calculated Spearman's correlation coefficients with SPSS, version 25 (IBM).

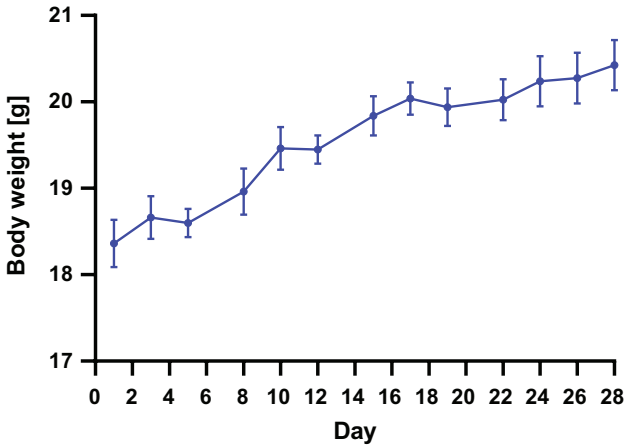

Fig. 1 Growth curves in normal mice. Body weight changes in healthy adolescent mice fed a diet containing 50 g% of yeast-fermented wheat bread from superfine flower defined in Table 1 over 4 weeks. Data are shown as means ± SEM ($n = 8$)

Results

Body Weight Change in Response to a Bread-Rich Diet in Control Mice

In a first set of experiments, we tested tolerance to a diet with a high bread portion (50 g% bread) in healthy young adult mice. The growth curves of all mice examined showed a constant weight gain during the study period of 28 days (Fig. 1). The start weight of the mice was between 17 and 19 g, and after 28 days they weighed between 19 and 22 g. Daily food consumption per mouse was relatively constant between 2.6 and 3.1 g during the 28 days of the experiment. Daily food consumption and weight gain was similar to that in mice fed a standard chow (data not shown).

Survival and Body Weight Change in Response to Different Breads in Caspase-8 Mice

In the next set of experiments, $Casp8^{\Delta IEC}$ mice and $Casp8^{fl}$ control mice of the same genetic background (C57BL/6) were fed six study diets described in Table 1 and 2. Depending on the diet the mice received, a variable portion of $Casp8^{\Delta IEC}$ mice, but not a single $Casp8^{fl}$ control mouse, died during the study. Survival was 100% in $Casp8^{\Delta IEC}$ mice fed with a gluten- and bread-free control diet, and 88% in mice fed with a control diet supplemented with gluten. Survival further decreased to 38–75% depending on the type of bread ingested during the study (Fig. 2A). In total, 20 out of 64 $Casp8^{\Delta IEC}$ mice did not reach the regular end of the study, either because they died or because they must be killed prematurely since they developed major symptoms such as severe weight loss, rectal bleeding and/or diarrhea. The worst outcome was observed in mice fed yeast-fermented superfine flour rye bread (38% survival, $P = 0.049$ compared to GF), but also yeast-fermented spelt and wheat bread were harmful (43% survival for spelt, $P = 0.150$; 63% survival for wheat SF and WG, $P = 0.376$ compared to GF). Neither the genotype ($Casp8^{fl}$ and $Casp8^{\Delta IEC}$) nor the

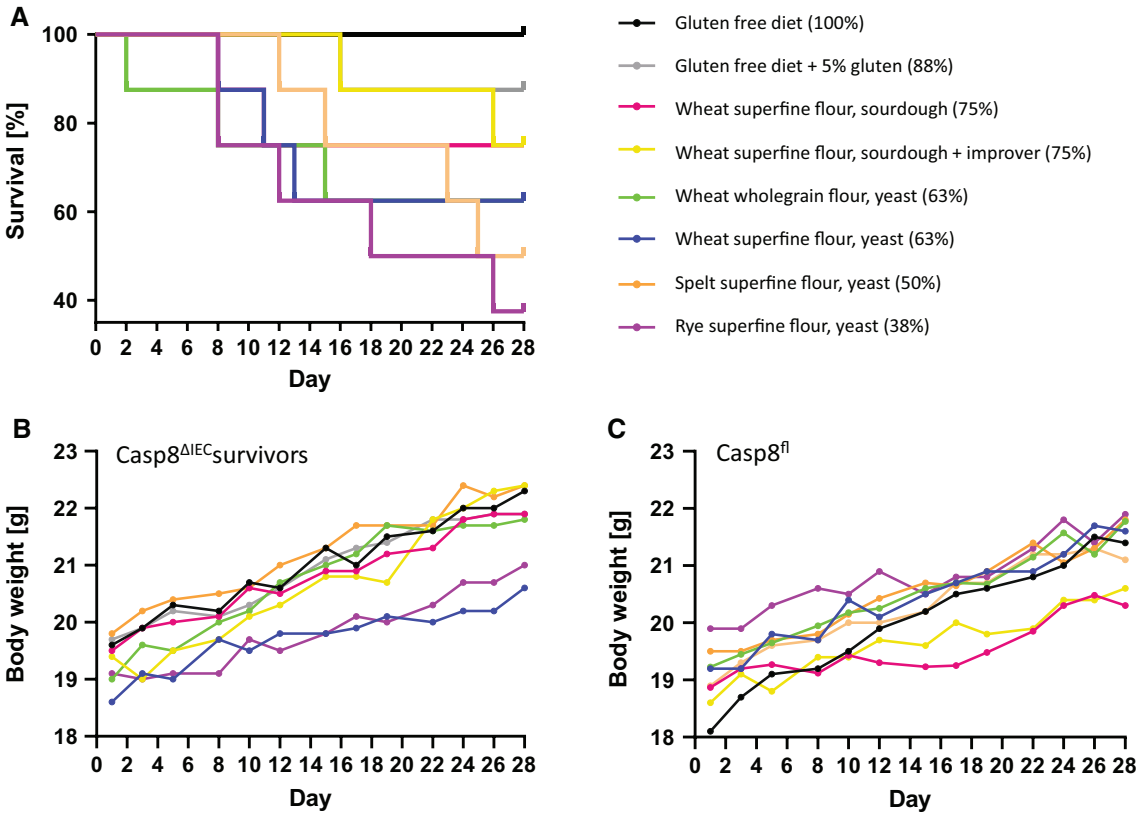

Fig. 2 Survival and growth curves in ileocolitis and control mice. **A** Kaplan–Meier survival curves and survival rates of Casp8$^{\Delta IEC}$ mice that were fed either a rice-based gluten free diet (GF), rice-based diet with 5% gluten (5G) or a diet containing 50 g% of six different breads listed in the figure legends. Survival of Casp8^{fl} control mice was always 100% and not affected by the different diets (data not shown). Survival of Casp8$^{\Delta IEC}$ mice was reduced to 38–88% within 4 weeks depending on the diets offered, but remained 100% in mice fed the GF control diet. There was a significant difference between GF and the rye bread diet ($P=0.049$). **B + C** Growth curves of Casp8$^{\Delta IEC}$ survivors ($n=3$–7 per group) and Casp8^{fl} mice ($n=8$ per group) fed with different diets listed in the figure legend. Each curve shows the mean body weights at given time points for mice of different diet groups. Only data of mice that survived until day 28 are shown

type of diet influenced the daily food intake, which varied between 2.4 and 2.9 g/day, or the liver/body weight ratio, which varied slightly between 3 and 5% (Table S2). Growth curves were similar in Casp8$^{\Delta IEC}$ survivors and Casp8^{fl} mice (Fig. 2B + C) but became flatter when including all Casp8$^{\Delta IEC}$ mice (Table S2). Only the group that received GF had an almost normal weight gain. Body weight changes were variable at the individual mouse level strongly correlated with the genotype ($P<0.001$). Also, the diet influenced body weight gain ($P=0.02$). Post hoc analyses revealed differences in weight changes between GF compared to all bread-fed mouse groups except W SF SB (Table S2).

Intestinal Inflammation

Selective endoscopic imaging revealed severe inflammatory lesions throughout the mucosa in some Casp8$^{\Delta IEC}$ mice (Fig. 3A). Colonic tissue sections showed variable mucosal damage and neutrophil infiltration into the mucosa (Fig. 3B). Scoring the histological changes revealed that Casp8$^{\Delta IEC}$ mice generally had higher inflammation scores than Casp8^{fl} mice, the differences being more pronounced in the colon than in the small intestine (Figs. 3C + D). In general, the scores for inflammation in the ileum were quite low for both Casp8$^{\Delta IEC}$ and Casp8^{fl} mice with no diet-related differences (Fig. 3C). In Casp8$^{\Delta IEC}$ mice, the extent of colitis was much higher compared to control mice, and different between the six diet groups (Fig. 3D). Two-way-ANOVA revealed genotype-related differences regarding the extent of colitis and identified particular diets aggravating the colitis such as yeast-fermented rye and wheat bread as well as sourdough fermented wheat bread as indicated by hashes (Fig. 3D). Also, ileitis was affected by the genotype ($P=0.03$), albeit weakly, without clear discrimination between the diet groups.

Markers of Intestinal Permeability

The expression of tight junction (TJ) proteins, well-recognized as markers of gut barrier function, was measured in

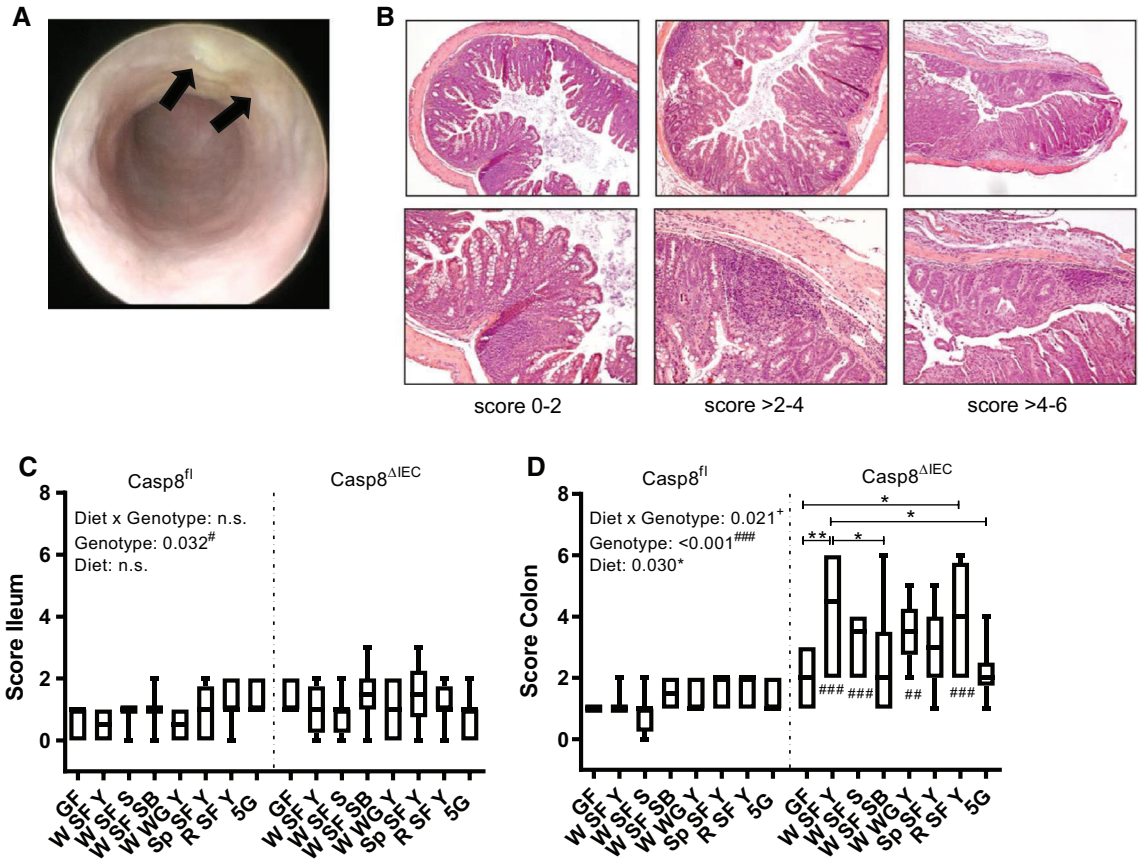

Fig. 3 Inflammation and tissue damage in Casp8$^{\Delta IEC}$ and Casp8^{fl} mice after feeding different bread-rich diets. **A** Mucosal image derived from colonoscopy showing a severely inflamed colon specimen (high grade, score 6, see methods), the black arrows indicate mucosal areas with ulcerations. **B** Representative photomicrographs of colonic tissues stained with hematoxylin and eosin; examples for low (score 0–2), middle (score > 2–4), and high (score > 4–6) grade histological scores are shown. **C**, **D** Results from quantitative scoring of inflammation and tissue damage in the ileum (**C**) and the colon (**D**). Tissue specimen derived from Casp8$^{\Delta IEC}$ and Casp8^{fl} mice fed 8 diets each, as described in Table 1. Abbreviations: GF, Gluten free diet; 5G, diet supplemented with 5 g% gluten; W, wheat; SF, superfine flour; Y, yeast fermented; S, sourdough fermented; SB, sourdough fermented with bread improver; Sp, spelt; R, rye). Statistics: Means ± SEM are shown. Two-way ANOVA was conducted to separate genotype effects, diet effects, and possible interaction between genotype and diet. The results are indicated by text in the upper left corner of panels C and D. If e.g. "Genotype" or "Diet" had a significant effect, we performed the Tukey's multiple comparison test and indicated the results by asterisk on top of the bars for diet effects, or by hash's on bottom of the bars for genotype effects. $^{*/\#}P < 0.05$; $^{**/\#\#}P < 0.01$, $^{***/\#\#\#}P < 0.001$

the colonic mucosa to further study the role of caspase-8 knockout and diet on gut barrier function. No genotype-specific differences could be seen regarding ZO1, CLDN2 and occludin mRNA expression, while the diet had a great influence (two-way-ANOVA, $P < 0.001$). No differences could be observed between 5G and GF diet, while TJ protein expression could be shown to be reduced in all mice fed with sourdough wheat bread (W SF S) compared to control diet (in selected cases) and 5G diet (in all mice). TJ protein expression were also reduced in mice fed with sourdough wheat bread compared to mice fed with yeast wheat bread (W SF Y) suggesting that not only wheat but also the type of formation plays a role. When comparing wholegrain with superfine yeast-fermented wheat bread, wholegrain bread resulted in a lower expression of ZO1 and CLDN2 mRNA. No differences could be found when yeast fermented breads from superfine flour of wheat (W), spelt (Sp) and rye (R) were compared to each other (Fig. 4). However, the expression of selected TJs was reduced in mice fed rye bread compared to the 5G diet.

Measuring FITC-D-4000 concentration in the plasma as marker for the small intestine barrier function, no differences could be found, neither between the different diet groups nor between Casp8^{fl} and Casp8$^{\Delta IEC}$ mice (Figure S1).

Markers of Intestinal Integrity and Bacterial Translocation

The expression of the mucus gene Muc2 and the proinflammatory TNFα gene were measured in the colon, while the

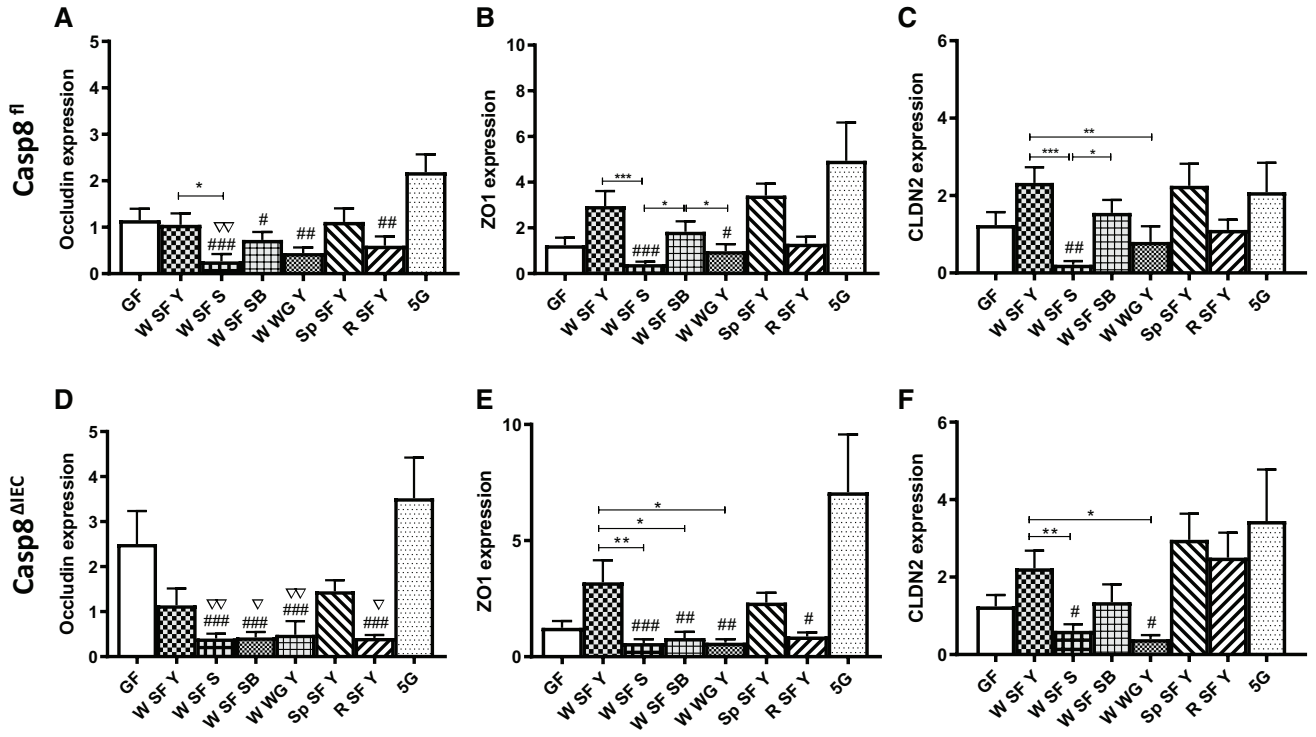

Fig. 4 Relative mRNA expression of tight junction proteins in the colon. Data from Casp8^{fl} (panels **A**–**C**) and Casp8$^{\Delta IEC}$ (panels **D**–**F**) mice fed 8 diets each, as described in Table 1 are shown as means ± SEM. Abbreviations see Fig. 3. Statistics by two-way ANOVA as described for Fig. 3. Genotypic differences between Casp8^{fl} and Casp8$^{\Delta IEC}$ fed the same diet were not found. Dietary differences within the same genotype are indicated by asterisks. Differences between one of the breads and the control diets are indicated by triangles for GF, and by hashes for 5G * $^{\nabla\,\#}P<0.05$; ** $^{\nabla\nabla\,\#\#}P<0.01$, *** $^{\#\#\#}P<0.001$. Abbreviations: ZO1, zonula occludens-1; CLDN2, claudin-2. Other abbreviations see Fig. 3.

expression of LPS associated proteins LBP [24], TLR4 [25] and MYD88 [26] gene expression were analyzed in the liver of the mice. LBP was additionally found to be elevated in the serum of NCWS patients [5]. Gene expression of Muc2 and TNFα in the colon as well as LBP in the liver were strongly related to the genotype ($P<0.001$). Only for Muc2 also an influence of the diet could be found after comparison for multiple testing ($P=0.002$). The dietetic effect was most pronounced, when comparing sourdough bread with GF ($P=0.008$), or when comparing sourdough bread with sourdough bread supplemented with bread improver ($P=0.018$) in control mice (Fig. 5).

Correlations

Our data revealed a positive correlation between survival of the mice and both Muc2 mRNA expression ($r_s=0.332$, $P=0.003$) in the colon and body weight gain ($r_s=0.624$, $P<0.001$). Moreover, an inverse association between survival of the mice and both TNFα mRNA in the colon ($r_s=-0.366$, $P=0.001$) and LBP mRNA in the liver ($r_s=-0.415$, $P=0.001$) was found (Fig. 6).

Discussion

Our mouse studies show that healthy mice not only accept a diet with a high bread portion, containing 50 g% bread but also digest it and develop a constant weight gain. These data confirm previous studies on feeding bread to mice [27–29]. The situation changes dramatically, when bread is offered to unhealthy mice suffering from genetically induced colitis. Loss of the caspase-8 gene in mice has been shown to result in a phenotype characterized by epithelial damage and an impaired gut barrier resulting in ileitis and colitis [18, 22]. When administering diets containing 50 g% of bread, we observed pronounced harmful effects varying depending on the bread type. Bread intake was associated with body weight loss and increased mortality in Casp8$^{\Delta IEC}$ mice but not in control mice. The mechanisms were not related to the amount of food intake, but to the type of food intake, since harmful events were only seen in mice fed bread, neither in mice fed bread free, nor in mice fed bread free but supplemented with gluten. The harmful effects were also related to the genotype of mice since only Casp8$^{\Delta IEC}$ mice died following bread ingestion.

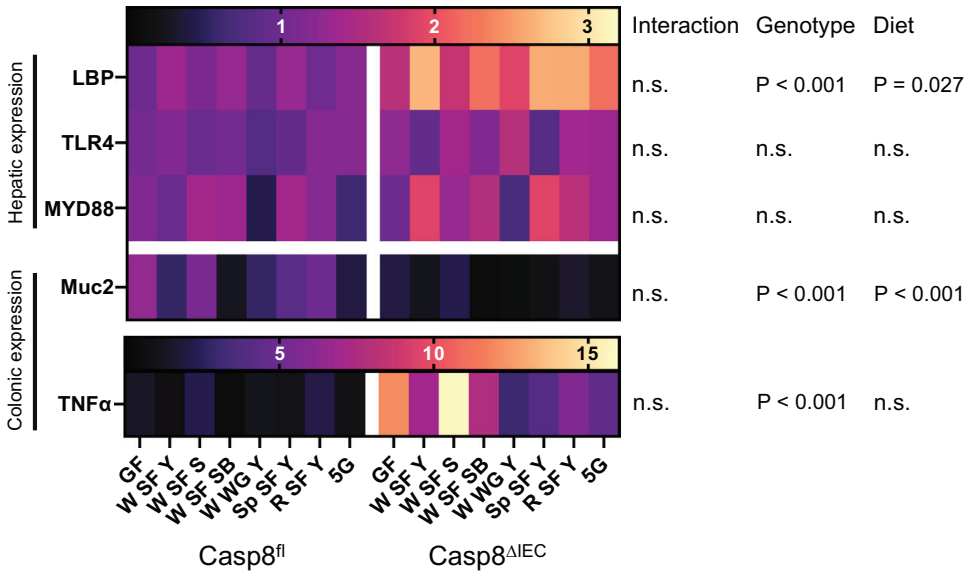

Fig. 5 Heatmap for the expression of gut barrier-related genes. Relative mRNA expression of MYD88, TLR4 and LBP in the liver, and of Muc2 and TNFα in the colon of Casp8^{fl} and Casp8$^{\Delta IEC}$ mice is indicted by two color scales, one for TNFα, one for all other genes. Statistics by two-way ANOVA as described for Fig. 3. Abbreviations: MYD88, myeloid differentiation primary response; TLR4, Toll-like receptor 4; LBP, lipopolysaccharide-binding protein; Muc2, mucin-2; TNFα, tumor necrosis factor-α; n.s., not significant. Other abbreviations see Fig. 3. *Posthoc analysis revealed significant differences between GF and W SF SB ($P=0.008$) and between WSF S and W SF SB ($P=0.018$)

The Casp8$^{\Delta IEC}$ genotype causes gut barrier dysfunction and malabsorption resulting from colitis as shown earlier [18, 22]. The central role of gut barrier dysfunction is underlined by our finding of an increased hepatic expression of LBP in Casp8$^{\Delta IEC}$ mice compared to Casp8^{fl} mice and a negative correlation between hepatic LBP and survival strongly suggesting that systemic LPS translocation from the intestine to the liver contributed to the enhanced mortality of Casp8$^{\Delta IEC}$ mice fed bread. The negative correlation between survival and colonic inflammation scores ($r_s=-0.529$; $P<0.001$, data not shown) further supports our hypothesis that gut barrier dysfunction contributes to the deleterious effects of bread in Casp8$^{\Delta IEC}$ mice, since colonic inflammation is associated with bacterial translocation resulting from intestinal epithelium damage [22, 30]. Our observation that mRNA expression of TJ molecules did not differ between Casp8$^{\Delta IEC}$ and Casp8^{fl} mice does not necessarily argue against our hypothesis, because epithelial damage can occur also independently from changes in TJ protein expression, if inflammation directly induces necroptosis of enterocytes [18, 31]

Previous studies of our group have shown that Casp8$^{\Delta IEC}$ mice spontaneously develop colitis triggered by luminal signals, e.g., bacterial signals derived from the commensal gut microbiome [32, 33]. The present study indicates that not only bacterial components but also components from bread can trigger colitis and possibly ileitis, the latter being minor in the present experiments compared to previous results from our group [22]. The less pronounced ileitis could be due to the lower age of the mice in the present experiments or other experimental conditions.

Which bread components enhance mucosal inflammation in Casp8$^{\Delta IEC}$ mice cannot be fully answered, but our data indicate that gluten does not act as a trigger here. Casp8$^{\Delta IEC}$ mice fed a bread-free diet (GF), or a bread-free diet supplemented with gluten at an even higher amount than that in the breads, did not show such deleterious effects, even though gluten worsens intestinal barrier function [34–36] and induces inflammation in celiac disease and possibly other gastrointestinal diseases [37].

Regarding the different bread types in more detail, rye bread was unexpectedly harmful in Casp8$^{\Delta IEC}$ mice regarding weight gain, survival and colitis. A particular factor that could cause the pronounced impairment of body weight gain and the reduced survival in mice fed rye bread could be small molecules such as phytic acid which is found in higher amounts in rye compared to wheat and spelt [38] and is able to reduce the absorption of minerals such as iron, zinc and calcium, and thereby promotes nutritional deficiencies [39–41].

Another obviously relevant factor for the harmful effects of breads in in Casp8$^{\Delta IEC}$ mice is yeast, since yeast-fermented breads (rye > spelt > wheat) caused less survival and less body weight gain compared to sourdough breads. It has been suggested that in Casp8$^{\Delta IEC}$ mice a distinct local microbiota might drive regional inflammation via activation

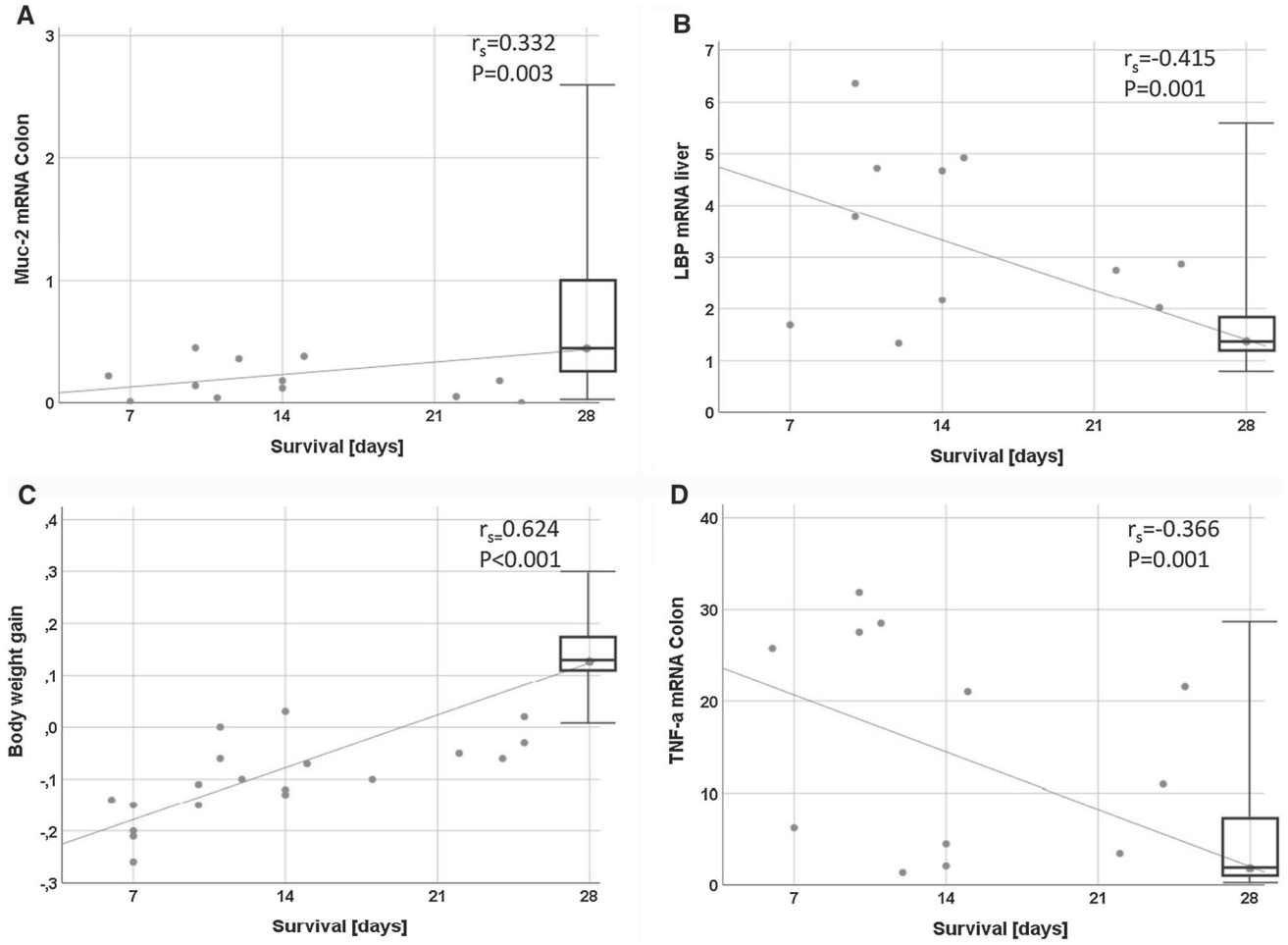

Fig. 6 Correlations between survival and biomarkers or body weight, respectively. Survival time is indicated on the *x*-axis, LBP, Muc2 and TNFα gene expression (panels **A**–**C**) and body weight (panel **D**) in the *y*-axis. Regression curves, the Spearman's rank correlation coefficients (r_s) and the *P* values are indicated. Abbreviations: see Fig. 5

of TLRs or indirectly through the release of cytokines [18, 31]. Possibly, yeast fermented breads modulate the intestinal microbiota in Casp8$^{\Delta IEC}$ mice in a way leading to a more pronounced colonic inflammation. Indeed, grain components of potential relevance for microbiota modulation such as FODMAPs [42] or cereal proteins like ATIs [7, 43], lectins [44, 45], and gliadins [46, 47], which have been shown to worsen intestinal gut inflammation, can be degraded to a higher degree by sourdough fermentation compared to yeast fermentation [16, 48–50].

However, when analyzing the breads regarding these potential triggers we could show that the amount of mono- and disaccharides was the same, and that the amount of oligosaccharides was only slightly different between the breads without association to inflammation or survival. In addition, in a previous proteome analysis we could show no clear difference regarding the amount of the above-mentioned proteins or other potential inflammatory proteins between yeast and sourdough fermented breads, or between wheat and spelt breads, while rye bread had the lowest overall content of such components compared to wheat and spelt breads [14]. Thus, it is unlikely that these compounds were major contributors to inflammation or mortality in Casp8$^{\Delta IEC}$ mice.

Our data suggest yet unknown inflammatory molecules in yeast-fermented bread not detected via proteome or FODMAP analysis that mediate the negative effects of yeast-fermented breads. Alternatively, yeast-fermented bread might lack some protective factors such as inactivated bacterial cells or bacterial metabolites that are generated during bacterial fermentation and have health-promoting effects via mucosal healing and immunomodulatory, anti-inflammatory or antibacterial properties [51, 52].

The difference between sourdough and yeast fermentation could be even more pronounced if fermentation time would be prolonged from about 2 h in our study to about 16 h. Overall, the rather small difference in harmful effects of different breads seen in our study, could be also due to the fact that the strong genotype-related pathologies that

occurred in Casp8$^{\Delta IEC}$ may have masked some subtle differences between the diets.

In conclusion, our study shows that healthy mice tolerated bread well, while bread-rich diets resulted in serious harmful effects in Casp8$^{\Delta IEC}$ mice. In such mice characterized by intestinal inflammation and loss of barrier function, bread-rich diets caused an aggravation of inflammation and loss of barrier function, reduced weight gain and increased mortality. The harmful effects of the breads occurred to a variable degree depending on the bread type, while gluten supplementation without bread was generally well tolerated. Candidates other than gluten like proinflammatory peptides and small bioactive molecules in bread must be considered to influence intestinal barrier impairment and inflammation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10620-022-07462-3.

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Author's contribution All authors contributed to the study conception and design. SCB conceived and designed research; JZ, LDF and VK-V performed experiments; BH produced breads; JZ analyzed data and prepared figures; JZ and SCB interpreted results of experiments and drafted manuscript; all authors approved final version of manuscript.

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Declarations

Conflict of interest The authors declare no conflict of interest with regard to the present paper.

References

1. Fasano A, Sapone A, Zevallos V, Schuppan D. Nonceliac gluten sensitivity. *Gastroenterology.* 2015;148:1195–1204.
2. Catassi C, Gatti S, Lionetti E. World perspective and celiac disease epidemiology. *Dig Dis.* 2015;33:141–146.
3. Fasano A, Berti I, Gerarduzzi T et al. Prevalence of celiac disease in at-risk and not-at-risk groups in the United States: a large multicenter study. *Arch Intern Med.* 2003;163:286–292.
4. Battais F, Richard C, Jacquenet S et al. Wheat grain allergies: an update on wheat allergens. *Eur Ann Allergy Clin Immunol.* 2008;40:67–76.
5. Uhde M, Ajamian M, Caio G et al. Intestinal cell damage and systemic immune activation in individuals reporting sensitivity to wheat in the absence of coeliac disease. *Gut.* 2016;65:1930–1937.
6. Sapone A, Lammers KM, Casolaro V et al. Divergence of gut permeability and mucosal immune gene expression in two gluten-associated conditions: celiac disease and gluten sensitivity. *BMC Med.* 2011;9:23.
7. Zevallos VF, Raker V, Tenzer S et al. Nutritional Wheat Amylase-Trypsin Inhibitors promote intestinal inflammation via activation of myeloid cells. *Gastroenterology.* 2017;152:1100–1113.e12.
8. Limketkai BN, Sepulveda R, Hing T et al. Prevalence and factors associated with gluten sensitivity in inflammatory bowel disease. *Scand J Gastroenterol.* 2018;53:147–151.
9. Shah A, Walker M, Burger D et al. Link between Celiac Disease and Inflammatory Bowel Disease. *J Clin Gastroenterol.* 2019;53:514–522.
10. Williams JJ, Kaplan GG, Makhija S et al. Microscopic colitis-defining incidence rates and risk factors: a population-based study. *Clin Gastroenterol Hepatol.* 2008;6:35–40.
11. Herfarth HH, Martin CF, Sandler RS, Kappelman MD, Long MD. Prevalence of a gluten-free diet and improvement of clinical symptoms in patients with inflammatory bowel diseases. *Inflamm Bowel Dis.* 2014;20:1194–1197.
12. Aziz I, Branchi F, Pearson K, Priest J, Sanders DS. A study evaluating the bidirectional relationship between inflammatory bowel disease and self-reported non-celiac gluten sensitivity. *Inflamm Bowel Dis.* 2015;21:847–853.
13. Ellis A, Linaker BD. Non-coeliac gluten sensitivity? *The Lancet.* 1978;311:1358–1359.
14. Zimmermann J, Hubel P, Pfannstiel J et al. Comprehensive proteome analysis of bread deciphering the allergenic potential of bread wheat, spelt and rye. *J Proteomics.* 2021;247:104318.
15. El Mecherfi KE, Todorov SD, Cavalcanti de Albuquerque MA et al. Allergenicity of fermented foods: Emphasis on seeds protein-based products. *Foods.* 2020;9:792.
16. Rizzello CG, de Angelis M, Coda R, Gobbetti M. Use of selected sourdough lactic acid bacteria to hydrolyze wheat and rye proteins responsible for cereal allergy. *Eur Food Res Technol.* 2006;223:405–411.
17. Davis PJ, Smales CM, James DC. How can thermal processing modify the antigenicity of proteins? *Allergy.* 2001;56:56–60.
18. Günther C, Martini E, Wittkopf N et al. Caspase-8 regulates TNF-α-induced epithelial necroptosis and terminal ileitis. *Nature.* 2011;477:335–339.
19. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. *J Basic Clin Pharm.* 2016;7:27–31.
20. van Overbeek FM, Uil-Dieterman IG, Mol IW et al. The daily gluten intake in relatives of patients with coeliac disease compared with that of the general Dutch population. *Eur J Gastroenterol Hepatol.* 1997;9:1097–1099.
21. Becker C, Fantini MC, Neurath MF. High resolution colonoscopy in live mice. *Nat Protoc.* 2006;1:2900–2904.

22. Kaden-Volynets V, Günther C et al. Deletion of the Casp8 gene in mice results in ileocolitis, gut barrier dysfunction, and malassimilation, which can be partially attenuated by inulin or sodium butyrate. *Am J Physiol Gastrointest Liver Physiol.* 2019;317:G493–G507.
23. Volynets V, Reichold A, Bárdos G et al. Assessment of the intestinal barrier with five different permeability tests in healthy C57BL/6J and BALB/cJ mice. *Dig Dis Sci.* 2016;61:737–746.
24. Zweigner J, Schumann RR, Weber JR. The role of lipopolysaccharide-binding protein in modulating the innate immune response. *Microbes Infect.* 2006;8:946–952.
25. Poltorak A, He X, Smirnova I et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science.* 1998;282:2085–2088.
26. Liu S, Gallo DJ, Green AM et al. Role of toll-like receptors in changes in gene expression and NF-kappa B activation in mouse hepatocytes stimulated with lipopolysaccharide. *Infect Immun.* 2002;70:3433–3442.
27. Carcea M, Turfani V, Narducci V et al. Bread for the aging population: The effect of a functional wheat-lentil bread on the immune function of aged mice. *Foods.* 2019;8:510.
28. Derbise A, Echenique-Rivera H, Garcia-Lopez M et al. Bread feeding is a robust and more physiological enteropathogen administration method compared to oral gavage. *Infect Immun.* 2020;88:e00810–e819.
29. Arias M, Cobo M, Jaime-Sánchez P et al. Gut microbiota and systemic inflammation changes after bread consumption: The ingredients and the processing influence. *J Funct Foods.* 2017;32:98–105.
30. Guo Y, Zhou G, He C, Yang W, He Z, Liu Z. Serum Levels of Lipopolysaccharide and 1,3-β-D-Glucan Refer to the Severity in Patients with Crohn's Disease. *Mediators Inflamm.* 2015;2015:843089.
31. Günther C, Buchen B, He G-W et al. Caspase-8 controls the gut response to microbial challenges by Tnf-α-dependent and independent pathways. *Gut.* 2015;64:601–610.
32. Stolzer I, Kaden-Volynets V, Ruder B et al. Environmental microbial factors determine the pattern of inflammatory lesions in a murine model of Crohn's Disease-like inflammation. *Inflamm Bowel Dis.* 2020;26:66–79.
33. Volynets V, Louis S, Pretz D et al. Intestinal barrier function and the gut microbiome are differentially affected in mice fed a western-style diet or drinking water supplemented with fructose. *J Nutr.* 2017;147:770–780.
34. Wagner SJ, Schmidt A, Effenberger MJP et al. Semisynthetic diet ameliorates Crohn's disease-like ileitis in TNFΔARE/WT mice through antigen-independent mechanisms of gluten. *Inflamm Bowel Dis.* 2013;19:1285–1294.
35. Drago S, El Asmar R, Di Pierro M et al. Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. *Scand J Gastroenterol.* 2006;41:408–419.
36. Sander GR, Cummins AG, Henshall T, Powell BC. Rapid disruption of intestinal barrier function by gliadin involves altered expression of apical junctional proteins. *FEBS Lett.* 2005;579:4851–4855.
37. Sapone A, Bai JC, Ciacci C et al. Spectrum of gluten-related disorders: consensus on new nomenclature and classification. *BMC Med.* 2012;10:13.
38. Michniewicz J. Water-Insoluble Pentosans of Wheat: Composition and Some Physical Properties. *Cereal Chem.* 1990;5:434–439.
39. Oberleas D, Muhrer ME, O'Dell BL. Effects of Phytic Acid on Zinc Availability and Parakeratosis in Swine. *J. Anim. Sci.* 1962;21:57–61.
40. Torre M, Rodriguez AR, Saura-Calixto F. Effects of dietary fiber and phytic acid on mineral availability. *Crit Rev Food Sci Nutr.* 1991;30:1–22.
41. Lopez HW, Duclos V, Coudray C et al. Making bread with sourdough improves mineral bioavailability from reconstituted whole wheat flour in rats. *Nutrition.* 2003;19:524–530.
42. Zhou S-Y, Gillilland M, Wu X et al. FODMAP diet modulates visceral nociception by lipopolysaccharide-mediated intestinal inflammation and barrier dysfunction. *J Clin Invest.* 2018;128:267–280.
43. Pickert G, Wirtz S, Matzner J et al. Wheat Consumption Aggravates Colitis in Mice via Amylase Trypsin Inhibitor-mediated Dysbiosis. *Gastroenterology.* 2020;159:257–272.e17.
44. Gong T, Wang X, Yang Y et al. Plant Lectins Activate the NLRP3 Inflammasome To Promote Inflammatory Disorders. *J Immunol.* 2017;198:2082–2092.
45. Pusztai A. Characteristics and consequences of interactions of lectins with the intestinal mucosa. *Arch Latinoam Nutr.* 1996;44:10S–15S.
46. Harris KM, Fasano A, Mann DL. Cutting edge: IL-1 controls the IL-23 response induced by gliadin, the etiologic agent in celiac disease. *J Immunol.* 2008;181:4457–4460.
47. Zhang L, Andersen D, Roager HM et al. Effects of Gliadin consumption on the Intestinal Microbiota and Metabolic Homeostasis in Mice Fed a High-fat Diet. *Sci Rep.* 2017;7:44613.
48. de Angelis M, Rizzello CG, Scala E et al. Probiotic preparation has the capacity to hydrolyze proteins responsible for wheat allergy. *J Food Prot.* 2007;70:135–144.
49. Huang X, Schuppan D, Rojas Tovar LE et al. Sourdough Fermentation Degrades Wheat Alpha-Amylase/Trypsin Inhibitor (ATI) and Reduces Pro-Inflammatory Activity. *Foods.* 2020;9:943.
50. Loponen J, Gänzle MG. Use of Sourdough in Low FODMAP Baking. *Foods.* 2018;7:96.
51. Thakur BK, Saha P, Banik G et al. Live and heat-killed probiotic Lactobacillus casei Lbs2 protects from experimental colitis through Toll-like receptor 2-dependent induction of T-regulatory response. *Int Immunopharmacol.* 2016;36:39–50.
52. Aguilar-Toalá JE, Garcia-Varela R, Garcia HS et al. Postbiotics: An evolving term within the functional foods field. *Trends in Food Science & Technology.* 2018;75:105–114.

2.3 Klinische Studie zur Verträglichkeit von Weizen- und Dinkelbrot

Dinkel (*Triticum aestivum* ssp. *spelta*) wurde zu Beginn des 20. Jahrhunderts durch den eng verwandten, aber ertragreicheren Brotweizen/Weichweizen/Weizen (*Triticum aestivum* ssp. *aestivum*) ersetzt [46], was dazu führte, dass Dinkel heute deutlich hinter Weizen zurückbleibt, was den Verzehr z.B. in Form von Backwaren betrifft [47].

Trotz der engen Verwandschaft der beiden Weizenunterarten wird in einer Subgruppe von NCWS Patienten Dinkelbrot subjektiv besser vertragen als Weizenbrot [48]. Diese Unterschiede wurden jedoch kaum wissenschaftlich untersucht. Auch bleibt unklar auf was die Unterschiede zurückzuführen sind. Dinkel hat z.B. einen höheren durchschnittlichen Rohproteingehalt und eine andere Glutenzusammensetzung als Weizen [27,28]. Zudem werden Weizenteige in der Regel anders verarbeitet als Dinkelteige. Für Dinkelbrot wird der Auszugsmehl vom Typ 630 anstelle von 550 verwendet und die Teigführung von Dinkelteigen ist aufgrund der traditionellen Herstellung oder zur Verbesserung der eher schlechteren technologischen Eigenschaften von Dinkel länger, als die von Weizenteigen [27].

Ziel der klinischen Studie war es, den Unterschied zwischen Weizen- und Dinkelbroten hinsichtlich ihrer Verträglichkeit in der beschriebenen Subgruppe zu objektivieren und herauszufinden, ob der mögliche Unterschied auf die Wahl zwischen Weizen- und Dinkel oder auf die Brotherstellung zurückzuführen ist. Dafür wurden Weizen- und Dinkelbrote jeweils nach einem "traditionellen" Rezept mit langer Teigführung (16h) ohne Backmittel (T) und einem "konventionellen" Rezept mit kurzer Teigführung (1h) und dem Zusatz von Backmittel (C) hergestellt und Personen verabreicht, die laut eigener Aussage Dinkel-, aber kein Weizenbrot vertragen. Der Hauptzielparameter war der Gesamtscore des Irritable Bowel Syndrome Severity Scoring System (IBS-SSS), anhand dessen gastrointestinale Symptome erfasst werden. Weiter wurden extraintestinale Symptome und verschiedene Blut- und Stuhlparameter analysiert. Um herauszufinden, ob FODMAPs oder Gluten potenzielle Auslöser für die Symptome in dieser Patientengruppe sind, wurden den Teilnehmern verblindet zudem zwei weitere Brote verabreicht, die mit 1,5 % Oligofruktose (+FODMAP) oder 5 % Weizengluten (+G) angereichert waren.

Wheat bread does not induce more severe symptoms than spelt bread in suspected non-celiac wheat sensitivity patients

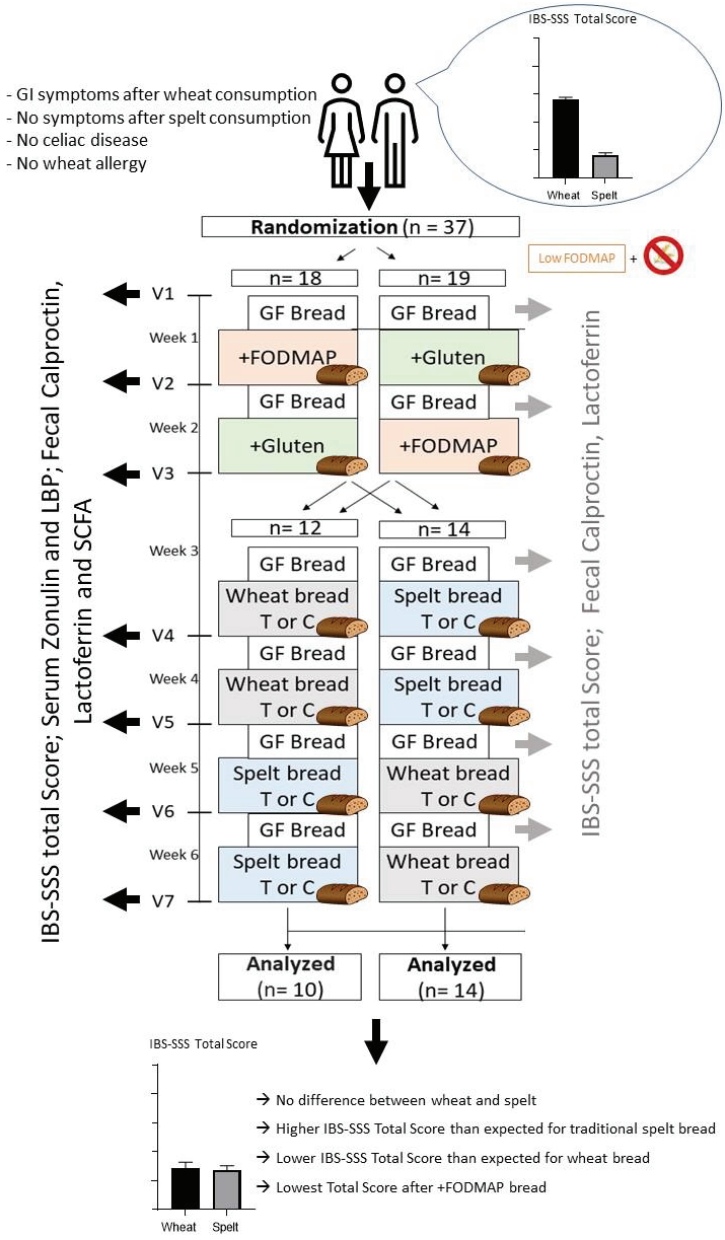
Julia Zimmermann, Anna Schweinlin, Maryam Basrai, Friedrich Longin, Stephan C. Bischoff

Background/Aims: Individuals with non-celiac wheat sensitivity (NCWS) often report better tolerance of spelt (*Triticum aestivum* ssp. spelta) compared to wheat (*Triticum aestivum* ssp. aestivum). This experience has neither been validated nor explained on a molecular level. Therefore, we performed a blinded wheat and spelt bread challenge in individuals with self-reported wheat sensitivity without spelt sensitivity.

Methods: Twenty-four adults with a history of NCWS but spelt tolerance were challenged in a crossover-design over six weeks with six different study breads at 300 g per day for 4 days followed by a washout phase of 3 days with gluten-free bread. Study breads comprised spelt and wheat breads, each made traditionally (T) or conventionally (C), a gluten-free bread with 1.5% added oligosaccharides (+FODMAP), and a gluten-free bread with 5% added wheat gluten (+G). Main outcome parameter was the total score of the Irritable Bowel Syndrome - Severity Scoring System (IBS-SSS).

Results: According to the IBS-SSS total score, discomfort after blinded consumption of wheat bread was lower (P = 0.027 for T and P = 0.073 for C) and after consumption of spelt bread higher than expected by participants (P =0 .002 for T and P = 0.015 for C). There were no differences between wheat and spelt, but the +FODMAP bread was better tolerated than traditional spelt bread after normalization to the IBS-SSS total score of the previous wash-out phase (P = 0.003)

Conclusion: There were no differences in tolerability between wheat and spelt breads but in terms of expected symptoms, indicating a strong nocebo effect for wheat and a placebo effect for spelt in case of blinded consumption. ClinicalTrials.gov (NCT04401956).



Zusammenfassungs-Grafik. 24 Probanden mit Weizenintoleranz ohne Zöliakie oder Weizenallergie aber subjektiver Dinkeltoleranz verzehrten verblindet sechs verschiedene Brote. Ein glutenfreies Brot, dem FODMAPs (+FODMAP) zugefügt wurden, ein glutenfreies Brot mit zugesetztem Gluten (+G) und jeweils ein traditionelles (T) und ein konventionelles (C) Dinkel- und Weizenbrot. In der dreitägigen Auswaschphase erhielten sie glutenfreies Brot. Der Hauptzielparameter war der Gesamtscore des Irritable Bowel Syndrome - Severity Scoring System (IBS-SSS) Fragebogens, Nebenzielparameter waren extraintestinale Symptome sowie Entzündungs- und Darmbarrieremarker in Blut- und Stuhlproben. Es zeigte sich, dass Weizenbrot besser und Dinkelbrot schlechter vertragen wurde, als von den Probanden vor der Studie erwartet. Es gab keinen signifikanten Unterschied hinsichtlich der Verträglichkeit zwischen Weizen und Dinkelbrot, traditionelles Brot wurde nicht besser vertragen als konventionelles Brot. Nach Normalisierung der Scores auf die Werte der vorangegangenen

Auswaschphase wurde das Brot mit zugesetzten FODMAPs sogar besser vertragen als das traditionelle Dinkelbrot.

3. Diskussion

Die Proteomanalyse ausgewählter Brote und Mehle mittels Massenspektrometrie zeigte, dass nicht nur Weizenproteine, sondern auch Dinkelproteine und Roggenproteine anhand der Weizendatenbank (*Triticum aestivum* aestivum) identifiziert werden können, was auf eine hohe Sequenzhomologie zwischen den Getreidearten hinweist und der Beobachtung entspricht, dass Weizen und weizenverwandte Gattungen wie Roggen, eine gemeinsame Abstammung und über 70 % Sequenzähnlichkeit aufweisen [49]. Somit konnten im Zuge dieser Arbeit mit der Identifizierung von mehr als 4000 verschiedenen Proteinen zum ersten Mal umfassende Proteomdaten für Brot und Mehl generiert und publiziert werden. Fast zwei Drittel der Proteine in Vollkornmehl und die Hälfte der Proteine in Vollkornbrot konnten in den Proben aller drei Getreidearten identifiziert werden. Weizen und Dinkel wiesen dabei mehr gemeinsame Proteine auf als Weizen und Roggen oder Dinkel und Roggen, was darauf zurückzuführen ist, dass die diploide Art Roggen (*Secale cereale*) biologisch weiter von Weizen und Dinkel entfernt ist, die der derselben hexaploiden Art (*Triticum aestivum*) angehören.

Bis zu 13 % der Proteine konnten ausschließlich in den Mehlen und nicht in den entsprechenden Broten identifiziert werden und auch die durchschnittliche Anzahl der identifizierten Proteine in Mehl war höher als in Brot. Dies könnte dadurch erklärt werden, dass das Backen zu einer Verkleisterung der Stärkekörner und Denaturierung der Proteine führt, wodurch ein stabiles Proteinnetzwerk gebildet wird, das die Stärkekörner umschließt und die Extrahierbarkeit der Weizenproteine verringert [50]. Der Verlust von Proteinen während der Brotherstellung könnte zudem auf den teilweisen oder vollständigen Abbau der Proteine durch mehleigene oder mikrobielle Proteasen während der Teigruhe zurückzuführen sein [51,52].

In Übereinstimmung mit früheren Untersuchungen konnten wir mehr verschiedene Protein in Vollkorn- im Vergleich zu Auszugsmehl aus Weizen und Dinkel identifizieren, was wahrscheinlich auf das Fehlen der proteinreichen Aleuronschicht in Auszugsmehl zurückzuführen ist [53,54]. Im Gegensatz dazu konnten in Roggenproben mehr Proteine in Auszugsmehl als in Vollkornmehl nachgewiesen werden. Eine Erklärung für diese widersprüchlichen Befunde könnte die höhere Menge an Pentosanen in Roggen im Vergleich zu Weizen und Dinkel sein [55]. Diese Polysaccharide befinden sich hauptsächlich in den äußeren Teilen des Roggenkorns [56] und

könnten somit die Extraktion von Proteinen aus Roggenvollkornmehl erschweren. Die oben genannten Beobachtungen konnten auch für die entsprechenden Brote bestätigt werden.

Unsere auf nano-UHPLC-ESI-MS basierten Proteomdaten haben den Vorteil, dass Analysen im Proteommaßstab durchgeführt werden konnten, während bei Methoden, die auf Gelelektrophorese beruhen, alle Proteine einzeln z.B. mittels Matrix-assisted laser desorption time-of-flight-Massenspektrometrie (MALDI-TOF) identifiziert werden müssen. Von besonderem Interesse war bei unserer Analyse die Identifizierung und relative Quantifizierung immunogener Proteine, die zu unerwünschten Reaktionen auf Brot z.B. bei NCWS Patienten führen können. Hierfür wurde im Zuge dieser Arbeit eine Allergenliste definiert, bei der auf Basis der Pfam Annotierung Getreidellaergene aus drei Datenbanken in funktionelle Gruppen eingeteilt wurden. Aus 21 dieser Gruppen konnten Proteine in unserem Datenset identifiziert werden, was insgesamt 246 potenziell immunogene Getreideproteinen entsprach. Davon war bei 54 eine allergene/immunogene Wirkung bereits nachgewiesen [49,57], während die anderen aufgrund struktureller/funktioneller Ähnlichkeiten mitaufgenommen wurden. Die 246 Proteine zeigten im Durchschnitt höhere LFQ-Intensitäten als die 4178 anderen Proteine in den analysierten Proben. Es konnte zudem gezeigt werden, dass diese Proteine nicht selektiv während der Teigruhe abgebaut werden, weshalb weder die absolute Anzahl noch die Verteilung vom Mahlgrad bzw. vom Fermentationsprozess (Hefeteig/Sauerteig) abhingen. Auch bei den Broten mit Backmittel konnten weder signifikante Unterschiede hinsichtlich der Anzahl der identifizierten Proteine noch in Bezug auf die relative Häufigkeit der immunogenen Proteine nachgewiesen werden. Dies widerspricht einigen vorangegangenen Untersuchungen, in denen gezeigt wurde, dass allergene Proteine insbesondere während der Sauerteigfermentation abgebaut werden [45,58]. Jedoch unterschieden sich die Herstellungsprozesse der Brote in der Literatur von den Broten in dieser Arbeit in der Hinsicht, als deutlich längere Teigruhezeiten bei erhöhter Temperatur (24 Stunden bei 37°C) verwendet wurden, während die Teigruhe der Brote der vorliegenden Arbeit nur ca. 1,5 h bei 30°C betrug. Die geringere Temperatur und die deutlich kürzere Teigruhe verringerten wahrscheinlich den Proteinabbau bei der Brotherstellung. Diese Vermutung wird durch die Ergebnisse von Loponen et al. [59] bestätigt, die zeigten, dass eine kurze Sauerteigfermentation (2 h) nicht zu einem Abbau von Glutenproteine führte.

Bezüglich der LFQ-Intensität und somit der relativen Häufigkeit der 246 Proteine unterschieden sich Weizen und Dinkel kaum, während in Roggen deutlich geringere Mengen dieser Proteine identifiziert werden konnten. Auf Basis dieser Ergebnisse könnte geschlossen werden, dass

Roggen für Unverträglichkeiten weniger relevant sein könnte, als Weizen und Dinkel. Auch bekannte Allergene wie ATIs, von denen 9 im Datensatz der vorliegenden Arbeit identifiziert werden konnten, waren abundanter in Weizen- und Dinkelmehl bzw. –brot als in Roggen. Andererseits wird angenommen, dass Roggen im Vergleich zu Weizen und Dinkel mehr Fruktane enthält, die zu den FODMAPs zählen [60]. Dies wurde dadurch bestätigt, dass Fruktosyltransferasen, wichtige Enzyme für die Fruktansynthese [61], deutlich abundanter in Roggen im Vergleich zu Weizen- und Dinkelproben waren.

Insgesamt zeigten die Ergebnisse der Proteomanalyse, dass allergene Proteine während der Brotherstellung nicht selektiv abgebaut werden, weder in hefe-, noch in sauerteigfermentiertem Brot oder durch den Zusatz von Backmitteln. Während es nahezu keinen Unterschied zwischen Weizen und Dinkel gab, waren allergene/immunogene Proteine in Roggenproben weniger abundant als in Weizen und Dinkel.

Die klinische Relevanz dieser Ergebnisse wurde anschließend in einer tierexperimentellen Studie untersucht, in der jeweils ein hefeteigfermentiertes Brot aus Weizen, Dinkel und Roggen, ein sauerteigfermentiertes Brot aus Weizen, ein sauerteigfermentiertes Brot aus Weizen mit Backmittel und ein hefeteigfermentiertes Brot aus Weizenvollkornmehl untersucht wurden. Eine Hypothese zur Entstehung von NCWS basiert auf einer latenten oder manifesten Vorerkrankung des Darmes wie z.B. eine Störung der Darmbarriere als Ursache für die Symptome nach Weizenverzehr [42]. Aus diesem Grund wurde für diese Arbeit die $Casp8^{\Delta IEC}$ Mauslinie als etabliertes Mausmodell für einen vorgeschädigten Darm gewählt. Diese Mauslinie ist durch den Knockout des Caspase-8 Gens charakterisiert, der zu einem Verlust von Panethzellen und einer Schädigung der Darmbarriere führt, woraus eine hohe Anfälligkeit für intestinale Entzündungen resultiert [44,62]. Als Kontrolltiere dienten Wurfgeschwister ohne Caspase-8 Gen-Knockout ($Casp8^{fl}$). Die Tiere nahmen in der Studie eine tägliche humane Äquivalenzdosis von ca. 25 g Gluten/Tag mit der 5G Diät und 6-20 g/Tag mit den Brotdiäten auf, was in etwa der durchschnittlichen Glutenaufnahme beim Menschen von 13-30 g/Tag entspricht [13,63]. Insbesondere Roggen- und Weizenbrot (Auszugsmehl, Hefeteig), nicht aber die 5G Diät führten bei $Casp8^{\Delta IEC}$ Mäusen zu einer Verschlimmerung der Entzündungen im Colon und einer erhöhten Gewichtsabnahme und Sterblichkeit, während die Wurfgeschwister ohne intestinale Entzündungen ($Casp8^{fl}$) alle Diäten gleich gut tolerierten. Dies bedeutet, dass die Hypothese aus der Proteomanalyse, nach der Roggenbrot möglicherweise verträglicher ist als Weizen- und Dinkelbrot, nicht verifiziert werden konnte. Aufgrund der drastischen Gewichtsabnahme unter der

Roggenbrotdiät trotz gleichem Kaloriengehalt wurde vermutet, dass der erhöhte Gehalt an Phytinsäuren in Roggenbrot die Aufnahme von Nährstoffen wie Eisen und Zink verringern und dadurch zu einer Mangelernährung der Tiere beigetragen haben könnte [64–66].

Es konnte zudem ein leicht positiver Effekt der Sauerteigführung gezeigt werden, da die Sterblichkeit und Gewichtsabnahme der Tiere in den beiden Gruppen mit Sauerteigbrot (+/- Backmittel) geringer waren als in der Gruppe mit den Hefeteigbroten. In vorangegangenen Untersuchungen wurde gezeigt, dass bei Casp8$^{\Delta IEC}$ Mäusen eine bestimmte lokale Mikrobiota die Entzündung über die Aktivierung von Toll-like Rezeptoren oder indirekt durch die Freisetzung von Zytokinen fördern könnte [44,62]. Möglicherweise modulieren Hefeteigbrote die Darmmikrobiota in Casp8$^{\Delta IEC}$ Mäusen in einer Weise, die zu einer ausgeprägteren Dickdarmentzündung führt, denn Getreidebestandteile, wie z.B. FODMAPs [67] oder auch Getreideproteine wie ATIs [13,68], Lektine [69,70] und Gliadine [71,72] können von potenzieller Bedeutung für die Modulation der Mikrobiota sein und nachweislich Darmentzündungen verschlimmern.

Welche Brotbestandteile genau die Entzündung bei Casp8$^{\Delta IEC}$ Mäusen verstärken, konnte in der Studie nicht vollständig geklärt werden. Die Daten der vorliegenden Arbeit deuten jedoch darauf hin, dass Gluten nicht als Auslöser wirkt, da Casp8$^{\Delta IEC}$ Mäuse, die mit höheren Menge Gluten als in den Broten (5G), gefüttert wurden, weniger krank wurden, als unter den Brotdiäten. Auch bezüglich der FODMAP Gehalte konnte gezeigt werden, dass sich die Menge an Oligosacchariden nur geringfügig zwischen den Broten unterschied und ohne, dass ein Zusammenhang mit den beobachteten Entzündungen oder dem Überleben der Tiere bestand. Aufgrund der Tatsache, dass im verwendeten Roggenbrot in der vorangegangenen Proteomanalyse geringere Mengen an ATIs, Gliadine und Lektine nachgewiesen werden konnten als in den Weizen- und Dinklebroten, deutet darauf hin, dass andere, noch unbekannte Trigger, die insbesondere in hefeteigfermentierten Broten aus Roggen, Weizen und Dinkel vorkommen, die Entzündung der Casp8$^{\Delta IEC}$ Mäuse verschlimmerten.

Auch denkbar wäre der Einfluss sogenannter Postbiotics, die in unserer Proteomanalyse nicht detektiert werden können, da es sich hierbei um mikrobielle Proteine handelt, die anhand der Weizendatenbank nicht erfasst werden. Postbiotics müssen abgesehen davon nicht notwendigerweise zu den Proteinen gehören. Zu ihnen gehören inaktivierte Bakterienzellen oder bakterielle Stoffwechselprodukte, die z.B. während der bakteriellen Fermentation bei der Sauerteigführung entstehen. Ihnen werden immunmodulatorische, entzündungshemmende oder

antibakterielle Eigenschaften zugeschrieben [73,74], was auf eine schützende Funktion von Sauerteigbrot hindeuten könnte.

Der Unterschied zwischen Sauerteig- und Hefeteig könnte möglicherweise noch deutlicher ausfallen, wenn die Fermentationszeit von etwa 1,5 Stunden in unserer Studie, auf 16 Stunden verlängert würde. Zudem könnten subtilere Unterschiede zwischen den einzelnen Brotdiäten durch die starken genotypbedingte Pathologie, der Casp8$^{\Delta IEC}$ Mäuse überlagert worden sein, weshalb sich für zukünftige Untersuchungen Mauslinien mit weniger stark ausgeprägter Vorerkrankung noch besser eignen.

Zusammenfassend zeigte die tierexperimentelle Studie, dass gesunde Mäuse Brot gut vertragen, während eine brotreiche Ernährung bei Casp8$^{\Delta IEC}$ Mäusen bereits bestehende pathologische Veränderungen wie eine Störung der Darmbarriere, Entzündungen im Colon verschlimmerte und die Gewichtszunahme reduzierte, woraus eine erhöhte Sterblichkeit der Tiere resultierte. Die schädliche Wirkung der Brote trat je nach Brotsorte in unterschiedlichem Maße auf, während eine glutenreiche Diät ohne Brot deutlich besser vertragen wurde. Andere Faktoren als Gluten, wie noch unbekannte proinflammatorische Peptide und mikrobielle Moleküle in Brot, könnten für diese Beobachtungen relevant sein.

Weder in der Proteomanalyse noch in der tierexpetimentellen Untersuchung konnten Unterschiede zwischen Weizen und Dinkel nachgewiesen werden. Bäcker und Müller berichten jedoch von Konsumenten, die angeben, Dinkelbrot besser zu vertragen als Weizenbrot [48]. Dies wurde wissenschaftlich bislang kaum untersuch. Ziel der klinischen Studie im Rahmen dieser Arbeit war es, die genannte Beobachtung zu objektivieren und zu klären, ob mögliche Unterschiede auf die Genetik oder auf die Brotherstellung zurückzuführen sind. Da auch die Sorte und die Anbaubedingungen die Zusammensetzung der Mehle beeinflussen, wurden für die vorliegende Studie Sorten- und Anbaumischungen verwendet.

Aufgrund der vorwiegend gastrointestinalen Beschwerden der Betroffenen wurde als Hauptzielparameter der Gesamtscore des IBS-SSS gewählt, ein Fragebogen anhand dessen reizdarmähnliche Beschwerden erfasst und quantifiziert werden können [75]. Die positive Erwartungshaltung der Probanden gegenüber Dinkel und die negative Erwartungshaltung gegenüber Weizen, die vor der Studie evaluiert wurde, erforderte die Verblindung zwischen Weizen- und Dinkelbrot. Auch auf die Herstellungsweise der Brote konnten die Probanden keine Rückschlüsse ziehen. Der Verzehr erfolgte wie in vorangegangenen Untersuchungen [76] an vier aufeinanderfolgenden Tagen, was als ausreichend für eine Symptomprovokation in der

vorliegenden Studie angesehen wurde, da die Teilnehmer ein Einsetzen der Symptome innerhalb von 0,5-72 Stunden angaben. Eine dreitägige Auswaschphase mit glutenfreiem Brot verhinderte die Überlappung der Symptome zwischen den Studienbroten. Auch hielten die Teilnehmer eine ansonsten glutenfreie und FODMAP-arme Ernährung ein, um den Einfluss anderer Getreideprodukte oder FODMAPs auf die durch die Brote hervorgerufenen Symptome zu verhindern. Die tägliche Menge an Brot umfasste insgesamt 300 g, wodurch eine übliche Glutenprovokation von 15 g Gluten pro Tag gewährleistet wurde [13,63]. Um herauszufinden, ob FODMAPs oder Gluten potenzielle Auslöser für die Symptome in dieser Patientengruppe sind, wurden den Teilnehmern verblindet zudem zwei weitere Brote verabreicht, die mit 1,5 % Oligofruktose (+FODMAP) oder 5 % Weizengluten angereichert waren.

Es zeigte sich, dass nach verblindetem Verzehr Weizenbrot besser und Dinkelbrot schlechter vertragen wurde, als von den Probanden vor der Studie erwartet. Es gab keinen signifikanten Unterschied hinsichtlich der Verträglichkeit zwischen Weizen und Dinkelbrot, traditionell hergestelltes Brot wurde nicht besser vertragen als konventionell hergestelltes Brot und nach Normalisierung der Scores auf die Werte der vorangegangenen Auswaschphase, wurde das Brot mit zugesetzten FODMAPs sogar besser vertragen als das traditionelle Dinkelbrot. Die Ergebnisse der klinischen Studie an einer Subgruppe von NCWS Patienten zeigen bei verblindetem Verzehr einen Noceboeffekt im Hinblick auf Weizen und einen Placeboeffekt in Hinblick auf Dinkel. Die gute Verträglichkeit des +FODMAP Brotes könnte auf einen positiven Effekt von Oligofruktose hindeuten, der möglicherweise auf einen durch die Darmmikrobiota vermittelten Effekt zurückzuführen ist [26]. Die Ergebnisse der klinischen Studie entsprechen somit den Ergebnissen aus der Proteomanalyse der vorliegenden Arbeit, die auf eine sehr ähnliche Proteinzusammensetzung von Weizen- und Dinkelbrot hindeuten [30].

Literaturverzeichnis

[1] Sousa T de, Ribeiro M, Sabença C, Igrejas G. The 10,000-Year Success Story of Wheat! Foods. 2021;10.

[2] Brouns F, van Rooy G, Shewry P, Rustgi S, Jonkers D. Adverse Reactions to Wheat or Wheat Components. Compr Rev Food Sci Food Saf. 2019;18:1437–52.

[3] Barbaro MR, Cremon C, Stanghellini V, Barbara G. Recent advances in understanding non-celiac gluten sensitivity. F1000Res. 2018;7.

[4] Ierardi E, Losurdo G, Piscitelli D, Giorgio F, Amoruso A, Iannone A, et al. Biological markers for non-celiac gluten sensitivity: a question awaiting for a convincing answer. Gastroenterol Hepatol Bed Bench. 2018;11:203–8.

[5] Carroccio A, Mansueto P, Iacono G, Soresi M, D'Alcamo A, Cavataio F, et al. Non-celiac wheat sensitivity diagnosed by double-blind placebo-controlled challenge: exploring a new clinical entity. Am J Gastroenterol. 2012;107:1898-906; quiz 1907.

[6] Sapone A, Bai JC, Ciacci C, Dolinsek J, Green PHR, Hadjivassiliou M, et al. Spectrum of gluten-related disorders: consensus on new nomenclature and classification. BMC Med. 2012;10:13.

[7] van Gils T, Nijeboer P, IJssennagger CE, Sanders DS, Mulder CJJ, Bouma G. Prevalence and Characterization of Self-Reported Gluten Sensitivity in The Netherlands. Nutrients. 2016;8.

[8] Potter MDE, Walker MM, Jones MP, Koloski NA, Keely S, Talley NJ. Wheat Intolerance and Chronic Gastrointestinal Symptoms in an Australian Population-based Study: Association Between Wheat Sensitivity, Celiac Disease and Functional Gastrointestinal Disorders. Am J Gastroenterol. 2018;113:1036–44.

[9] Aziz I, Lewis NR, Hadjivassiliou M, Winfield SN, Rugg N, Kelsall A, et al. A UK study assessing the population prevalence of self-reported gluten sensitivity and referral characteristics to secondary care. Eur J Gastroenterol Hepatol. 2014;26:33–9.

[10] Barbaro MR, Cremon C, Wrona D, Fuschi D, Marasco G, Stanghellini V, et al. Non-Celiac Gluten Sensitivity in the Context of Functional Gastrointestinal Disorders. Nutrients. 2020;12.

[11] Lebwohl B, Ludvigsson JF, Green PHR. Celiac disease and non-celiac gluten sensitivity. BMJ. 2015;351.

[12] Catassi C, Elli L, Bonaz B, Bouma G, Carroccio A, Castillejo G, et al. Diagnosis of Non-Celiac Gluten Sensitivity (NCGS): The Salerno Experts' Criteria. Nutrients. 2015;7:4966–77.

[13] Zevallos VF, Raker V, Tenzer S, Jimenez-Calvente C, Ashfaq-Khan M, Rüssel N, et al. Nutritional Wheat Amylase-Trypsin Inhibitors Promote Intestinal Inflammation via Activation of Myeloid Cells. Gastroenterology. 2017;152:1100-1113.e12.

[14] Volta U, Bardella MT, Calabrò A, Troncone R, Corazza GR. An Italian prospective multicenter survey on patients suspected of having non-celiac gluten sensitivity. BMC Med. 2014;12:85.

[15] Barbaro MR, Di Sabatino A, Cremon C, Giuffrida P, Fiorentino M, Altimari A, et al. Interferon-γ is increased in the gut of patients with irritable bowel syndrome and modulates serotonin metabolism. Am J Physiol Gastrointest Liver Physiol. 2016;310:G439-47.

[16] Picarelli A, Borghini R, Di Tola M, Marino M, Urciuoli C, Isonne C, et al. Intestinal, Systemic, and Oral Gluten-related Alterations in Patients With Nonceliac Gluten Sensitivity. J Clin Gastroenterol. 2016;50:849–58.

[17] Sapone A, Lammers KM, Casolaro V, Cammarota M, Giuliano MT, Rosa M de, et al. Divergence of gut permeability and mucosal immune gene expression in two gluten-associated conditions: celiac disease and gluten sensitivity. BMC Med. 2011;9:23.

[18] Uhde M, Indart AC, Yu XB, Jang SS, Giorgio R de, Green PHR, et al. Markers of non-coeliac wheat sensitivity in patients with myalgic encephalomyelitis/chronic fatigue syndrome. Gut. 2019;68:377–8.

[19] Garcia-Mazcorro JF, Rivera-Gutierrez X, Cobos-Quevedo ODJ, Grube-Pagola P, Meixueiro-Daza A, Hernandez-Flores K, et al. First Insights into the Gut Microbiota of Mexican Patients with Celiac Disease and Non-Celiac Gluten Sensitivity. Nutrients. 2018;10.

[20] Zanini B, Baschè R, Ferraresi A, Ricci C, Lanzarotto F, Marullo M, et al. Randomised clinical study: gluten challenge induces symptom recurrence in only a minority of patients who meet clinical criteria for non-coeliac gluten sensitivity. Aliment Pharmacol Ther. 2015;42:968–76.

[21] Skodje GI, Sarna VK, Minelle IH, Rolfsen KL, Muir JG, Gibson PR, et al. Fructan, Rather Than Gluten, Induces Symptoms in Patients With Self-Reported Non-Celiac Gluten Sensitivity. Gastroenterology. 2018;154:529-539.e2.

[22] Fedewa A, Rao SSC. Dietary fructose intolerance, fructan intolerance and FODMAPs. Curr Gastroenterol Rep. 2014;16:370.

[23] Cummings JH, Englyst HN. Fermentation in the human large intestine and the available substrates. Am J Clin Nutr. 1987;45:1243–55.

[24] Barrett JS, Gearry RB, Muir JG, Irving PM, Rose R, Rosella O, et al. Dietary poorly absorbed, short-chain carbohydrates increase delivery of water and fermentable substrates to the proximal colon. Aliment Pharmacol Ther. 2010;31:874–82.

[25] Longin CFH, Beck H, Gütler A, Gütler H, Heilig W, Zimmermann J, et al. Influence of wheat variety and dough preparation on FODMAP content in yeast-leavened wheat breads. Journal of Cereal Science. 2020;95:103021.

[26] Halmos EP, Christophersen CT, Bird AR, Shepherd SJ, Gibson PR, Muir JG. Diets that differ in their FODMAP content alter the colonic luminal microenvironment. Gut. 2015;64:93–100.

[27] Schober TJ, Clarke CI, Kuhn M. Characterization of Functional Properties of Gluten Proteins in Spelt Cultivars Using Rheological and Quality Factor Measurements. Cereal Chemistry Journal. 2002;79:408–17.

[28] Pruska-Kedzior A, Kedzior Z, Klockiewicz-Kaminska E. Comparison of viscoelastic properties of gluten from spelt and common wheat. Eur Food Res Technol. 2008;227:199–207.

[29] Afzal M, Pfannstiel J, Zimmermann J, Bischoff SC, Würschum T, Longin CFH. High-resolution proteomics reveals differences in the proteome of spelt and bread wheat flour representing targets for research on wheat sensitivities. Sci Rep. 2020;10:14677.

[30] Zimmermann J, Hubel P, Pfannstiel J, Afzal M, Longin CFH, Hitzmann B, et al. Comprehensive proteome analysis of bread deciphering the allergenic potential of bread wheat, spelt and rye. J Proteomics. 2021;247:104318.

[31] El Mecherfi K-E, Todorov SD, Cavalcanti de Albuquerque MA, Denery-Papini S, Lupi R, Haertlé T, et al. Allergenicity of Fermented Foods: Emphasis on Seeds Protein-Based Products. Foods. 2020;9.

[32] Davis PJ, Smales CM, James DC. How can thermal processing modify the antigenicity of proteins? Allergy. 2001;56 Suppl 67:56–60.

[33] Dupont FM, Vensel WH, Tanaka CK, Hurkman WJ, Altenbach SB. Deciphering the complexities of the wheat flour proteome using quantitative two-dimensional electrophoresis, three proteases and tandem mass spectrometry. Proteome Sci. 2011;9:10.

[34] Laatikainen R, Koskenpato J, Hongisto S-M, Loponen J, Poussa T, Huang X, et al. Pilot Study: Comparison of Sourdough Wheat Bread and Yeast-Fermented Wheat Bread in Individuals with Wheat Sensitivity and Irritable Bowel Syndrome. Nutrients. 2017;9.

[35] Grimshaw KEC, Bryant T, Oliver EM, Martin J, Maskell J, Kemp T, et al. Incidence and risk factors for food hypersensitivity in UK infants: results from a birth cohort study. Clin Transl Allergy. 2015;6:1.

[36] Fasano A, Sapone A, Zevallos V, Schuppan D. Nonceliac gluten sensitivity. Gastroenterology. 2015;148:1195–204.

[37] Afzal M, Sielaff M, Curella V, Neerukonda M, El Hassouni K, Schuppan D, et al. Characterization of 150 Wheat Cultivars by LC-MS-Based Label-Free Quantitative Proteomics Unravels Possibilities to Design Wheat Better for Baking Quality and Human Health. Plants (Basel). 2021;10.

[38] Sielaff M, Curella V, Neerukonda M, Afzal M, El Hassouni K, Distler U, et al. Hybrid QconCAT-Based Targeted Absolute and Data-Independent Acquisition-Based Label-Free Quantification Enables In-Depth Proteomic Characterization of Wheat Amylase/Trypsin Inhibitor Extracts. J Proteome Res. 2021;20:1544–57.

[39] Pilolli R, Angelis M de, Lamonaca A, Angelis E de, Rizzello CG, Siragusa S, et al. Prototype Gluten-Free Breads from Processed Durum Wheat: Use of Monovarietal Flours and Implications for Gluten Detoxification Strategies. Nutrients. 2020;12.

[40] Islam S, Ma W, Yan G, Gao L, Appels R. Differential recovery of lupin proteins from the gluten matrix in lupin-wheat bread as revealed by mass spectrometry and two-dimensional electrophoresis. J Agric Food Chem. 2011;59:6696–704.

[41] Bose U, Juhász A, Broadbent JA, Byrne K, Howitt CA, Colgrave ML. Identification and Quantitation of Amylase Trypsin Inhibitors Across Cultivars Representing the Diversity of Bread Wheat. J Proteome Res. 2020;19:2136–48.

[42] Uhde M, Ajamian M, Caio G, Giorgio R de, Indart A, Green PH, et al. Intestinal cell damage and systemic immune activation in individuals reporting sensitivity to wheat in the absence of coeliac disease. Gut. 2016;65:1930–7.

[43] Leccioli V, Oliveri M, Romeo M, Berretta M, Rossi P. A New Proposal for the Pathogenic Mechanism of Non-Coeliac/Non-Allergic Gluten/Wheat Sensitivity: Piecing Together the Puzzle of Recent Scientific Evidence. Nutrients. 2017;9.

[44] Günther C, Martini E, Wittkopf N, Amann K, Weigmann B, Neumann H, et al. Caspase-8 regulates TNF-α-induced epithelial necroptosis and terminal ileitis. Nature. 2011;477:335–9.

[45] Rizzello CG, Angelis M de, Coda R, Gobbetti M. Use of selected sourdough lactic acid bacteria to hydrolyze wheat and rye proteins responsible for cereal allergy. Eur Food Res Technol. 2006;223:405–11.

[46] Longin CFH, Würschum T. Genetic variability, heritability and correlation among agronomic and disease resistance traits in a diversity panel and elite breeding material of spelt wheat. Plant Breed. 2014;133:459–64.

[47] Zentralverband des Deutschen Bäckerhandwerk. Der Deutsche Brotkorb; 2017. [29.01.20222]; Available from: https://www.baeckerhandwerk.de/baeckerhandwerk/zahlen-fakten/brotverbrauch-und-brotkorb-der-deutschen/.

[48] Vu NT, Chin J, Pasco JA, Kovács A, Wing LW, Békés F, et al. The prevalence of wheat and spelt sensitivity in a randomly selected Australian population. Cereal Research Communications. 2015;43:97–107.

[49] Juhász A, Belova T, Florides CG, Maulis C, Fischer I, Gell G, et al. Genome mapping of seed-borne allergens and immunoresponsive proteins in wheat. Sci Adv. 2018;4:eaar8602.

[50] Singh H. A study of changes in wheat protein during bread baking using SE-HPLC. Food Chemistry. 2005;90:247–50.

[51] Heredia-Sandoval NG, Valencia-Tapia MY, La Calderón de Barca AM, Islas-Rubio AR. Microbial Proteases in Baked Goods: Modification of Gluten and Effects on Immunogenicity and Product Quality. Foods. 2016;5.

[52] Rizzello CG, Angelis M de, Di Cagno R, Camarca A, Silano M, Losito I, et al. Highly efficient gluten degradation by lactobacilli and fungal proteases during food processing: new perspectives for celiac disease. Appl Environ Microbiol. 2007;73:4499–507.

[53] Heshe GG, Haki GD, Woldegiorgis AZ, Gemede HF. Effect of conventional milling on the nutritional value and antioxidant capacity of wheat types common in Ethiopia and

a recovery attempt with bran supplementation in bread. Food Sci Nutr. 2016;4:534–43.

[54] Pedersen B, Knudsen KE, Eggum BO. Nutritive value of cereal products with emphasis on the effect of milling. World Rev Nutr Diet. 1989;60:1–91.

[55] Biliaderis CG, Izydorczyk MS, Rattan O. Effect of arabinoxylans on bread-making quality of wheat flours. Food Chemistry. 1995;53:165–71.

[56] Buksa K, Ziobro R, Nowotna A, Gambuś H. The influence of native and modified arabinoxylan preparations on baking properties of rye flour. Journal of Cereal Science. 2013;58:23–30.

[57] http://www.allergome.org. Allergome. [July 09, 2020]; Available from: http://www.allergome.org/.

[58] Angelis M de, Rizzello CG, Scala E, Simone C de, Farris GA, Turrini F, et al. Probiotic preparation has the capacity to hydrolyze proteins responsible for wheat allergy. J Food Prot. 2007;70:135–44.

[59] Loponen J, Mikola M, Katina K, Sontag-Strohm T, Salovaara H. Degradation of HMW Glutenins During Wheat Sourdough Fermentations. Cereal Chemistry Journal. 2004;81:87–93.

[60] Whelan K, Abrahmsohn O, David GJP, Staudacher H, Irving P, Lomer MCE, et al. Fructan content of commonly consumed wheat, rye and gluten-free breads. Int J Food Sci Nutr. 2011;62:498–503.

[61] Vijn I, Smeekens S. Fructan: more than a reserve carbohydrate? Plant Physiol. 1999;120:351–60.

[62] Günther C, Buchen B, He G-W, Hornef M, Torow N, Neumann H, et al. Caspase-8 controls the gut response to microbial challenges by Tnf-α-dependent and independent pathways. Gut. 2015;64:601–10.

[63] van Overbeek FM, Uil-Dieterman IG, Mol IW, Köhler-Brands L, Heymans HS, Mulder CJ. The daily gluten intake in relatives of patients with coeliac disease compared with that of the general Dutch population. Eur J Gastroenterol Hepatol. 1997;9:1097–9.

[64] Lopez HW, Duclos V, Coudray C, Krespine V, Feillet-Coudray C, Messager A, et al. Making bread with sourdough improves mineral bioavailability from reconstituted whole wheat flour in rats. Nutrition. 2003;19:524–30.

[65] Oberleas D, Muhrer ME, O'Dell BL. Effects of Phytic Acid on Zinc Availability and Parakeratosis in Swine. J. Anim. Sci. 1962;21:57–61.

[66] Torre M, Rodriguez AR, Saura-Calixto F. Effects of dietary fiber and phytic acid on mineral availability. Crit Rev Food Sci Nutr. 1991;30:1–22.

[67] Zhou S-Y, Gillilland M, Wu X, Leelasinjaroen P, Zhang G, Zhou H, et al. FODMAP diet modulates visceral nociception by lipopolysaccharide-mediated intestinal inflammation and barrier dysfunction. J Clin Invest. 2018;128:267–80.

[68] Pickert G, Wirtz S, Matzner J, Ashfaq-Khan M, Heck R, Rosigkeit S, et al. Wheat Consumption Aggravates Colitis in Mice via Amylase Trypsin Inhibitor-mediated Dysbiosis. Gastroenterology. 2020;159:257-272.e17.

[69] Gong T, Wang X, Yang Y, Yan Y, Yu C, Zhou R, et al. Plant Lectins Activate the NLRP3 Inflammasome To Promote Inflammatory Disorders. J Immunol. 2017;198:2082–92.

[70] Pusztai A. Characteristics and consequences of interactions of lectins with the intestinal mucosa. Arch Latinoam Nutr. 1996;44:10S-15S.

[71] Harris KM, Fasano A, Mann DL. Cutting edge: IL-1 controls the IL-23 response induced by gliadin, the etiologic agent in celiac disease. J Immunol. 2008;181:4457–60.

[72] Zhang L, Andersen D, Roager HM, Bahl MI, Hansen CHF, Danneskiold-Samsøe NB, et al. Effects of Gliadin consumption on the Intestinal Microbiota and Metabolic Homeostasis in Mice Fed a High-fat Diet. Sci Rep. 2017;7:44613.

[73] Thakur BK, Saha P, Banik G, Saha DR, Grover S, Batish VK, et al. Live and heat-killed probiotic Lactobacillus casei Lbs2 protects from experimental colitis through Toll-like receptor 2-dependent induction of T-regulatory response. Int Immunopharmacol. 2016;36:39–50.

[74] Aguilar-Toalá JE, Garcia-Varela R, Garcia HS, Mata-Haro V, González-Córdova AF, Vallejo-Cordoba B, et al. Postbiotics: An evolving term within the functional foods field. Trends in Food Science & Technology. 2018;75:105–14.

[75] Betz C, Mannsdörfer K, Bischoff SC. Validierung des IBS-SSS. Z Gastroenterol. 2013;51:1171–6.

[76] Dale HF, Hatlebakk JG, Hovdenak N, Ystad SO, Lied GA. The effect of a controlled gluten challenge in a group of patients with suspected non-coeliac gluten sensitivity: A randomized, double-blind placebo-controlled challenge. Neurogastroenterol Motil. 2018.

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Eidesstattliche Versicherung über die eigenständig erbrachte Leistung gemäß § 18 Absatz 3 Satz 5 der Promotionsordnung der Universität Hohenheim für die Fakultäten Agrar-, Natur- sowie Wirtschafts- und Sozialwissenschaften

1. Bei der eingereichten Dissertation zum Thema *„Einfluss verschiedener Getreidearten und Herstellungsverfahren auf den Gehalt immunogener Substanzen in Brot sowie in vivo auf die Verträglichkeit an der Maus und im Menschen“* handelt es sich um meine eigenständig erbrachte Leistung.

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