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Recent breakthrough in gluten contamination

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Recent breakthrough in gluten contamination

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EDITORIAL
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Editorial: Recent Breakthrough in Gluten Contamination

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Keywords: celiac disease, gluten contamination, gluten-free diet, gliadin, ELISA

Editorial on the Research Topic

Recent Breakthrough in Gluten Contamination

INTRODUCTION

Gluten contamination is a serious health issue for celiac disease (CD) patients. Exposure to a small amount of gluten (>10 mg/daily) can trigger an intense immunological reaction sufficient to restore clinical symptoms. Less than 20 mg/kg gluten in food is considered a safe amount of gluten as established by the regulatory authorities. However, substantial gluten contamination in commercial gluten-free products has been reported in recent years. It is extremely important to quantify accurately the amount of gluten in food products. Immunological techniques (i.e., antibody-based ELISA) are generally considered reliable methods to quantify gluten in food products. However, due to certain limitations, they do not often achieve the necessary accuracy, especially in the case of hydrolyzed and high heat-processed food samples. In the last decades, several non-immunological methods, such as DNA- and proteomics-based methods have been evaluated for gluten quantification in food products. Although these methods showed their efficiencies, due to some drawbacks, they are not regularly in use. Hence, gluten contamination, despite these efficient tools, remains a significant issue. There is certainly an unmet need to develop a reliable gluten quantification method with high accuracy and precision, especially in challenging food matrices. This Research Topic was aimed to provide comprehensive information about current approaches to accurately quantify gluten in food products and their biological proxy (i.e., urine from CD patients).

In this special issue a total of eight articles have been published (five pieces of original research, two brief research reports, and one opinion paper). The opinion paper by Scherf et al. was submitted on behalf of the Prolamin Working Group (PWG) as a statement on the final rule by the U.S. Food and Drug Administration (FDA) regarding gluten-free labeling for foods containing fermented or hydrolyzed ingredients. The rule acknowledged the absence of a scientifically valid analytical method to accurately quantify gluten in such food products, and thus the compliance with requirements for the use of gluten-free claims for these foods will be evaluated based on evidence that the food or ingredient used is gluten-free before fermentation or hydrolysis.

Gluten-free labeling is used on food packages to communicate the absence of gluten ingredients and show that any unintentional gluten in the food is below the threshold value. A survey of gluten content in foods labeled as gluten-free can be a helpful tool for risk assessment and improving the quality of life for CD and gluten-sensitive patients. In a brief research report, Calderón de la Barca et al. analyzed the cost comparison of gluten-free labeled foods from north-western Mexico with their equivalent counterpart, which may contain gluten. Further, the authors reported the findings from select gluten-free labeled foods analyzed for gluten content by ELISA, and immunoreactivity with CD patient IgA. Good manufacturing practices, including the use of dedicated or

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clean equipment, can significantly help in reducing the gluten cross-contact in foods. In the second brief report, Thompson et al. reported the gluten content in fries from different restaurants that did not contain gluten in their ingredients but were fried in shared fryers used to prepare other products containing wheat as a gluten source. With recent advancements in gluten detection methods, testing is no longer restricted to the laboratories but has reached the hands of consumers. This makes gluten detection convenient at the place of food consumption, such as at home and restaurants. However, the proficiency of the method and user may differ in laboratory *vs.* food consumption sites. In an original research, Marić and Scherf studied one such portable gluten sensor using food samples containing varying gluten content. The authors reported the performance of the sensor and discuss the variability in results from select samples when analyzed by different users.

In recent years, proteomics has been increasingly used for gluten detection as well as characterization. Three other original articles of this special issue used such analytical tools to assess gluten. The use of oats in the diet of celiac patients has been a continued topic of interest, partly due to the debated safe level of consumption. Gell et al. studied the variability among oat proteins from different varieties and various countries and developed an estimation method for ranking the avenin-epitope content, which may have an application in the selection of oat variety. Nye-Wood et al. used LC-MS to compare the protein profile of wheat flour containing markedly reduced allergenic gluten with traditional wheat flour. The authors report findings on changes in the amount of gliadin and glutenin specific proteins, and allergenic epitopes proportion in the novel wheat flour. Escobar-Correas et al. studied the proteome of various ryegrass cultivars using LC-MS to identify gluten-like peptides and a possible approach to distinguish ryegrass and wheat gluten.

Finally, in an original article, Costantino et al. encompass the role of telemedicine and urinary gluten peptides detection in assessing dietary compliance for CD patients during the COVID-19 pandemic. Sars-Cov2 pandemic has negatively affected national health systems worldwide and telemedicine has proven to be a reliable tool to deliver health care in certain situations, e.g., CD follow-up.

In summary, the articles in this special issue provide insights on gluten assessment to ensure safe food choices are available for gluten-intolerant consumers. The topics covered range from gluten measurement to its complex proteomic analysis using various analytical tools.

AUTHOR CONTRIBUTIONS

AV prepared a draft concept of the special issue. GS and FV supplemented and corrected the concept. AV, GS, and FV prepared the list of authors for the special issue manuscripts and were the main corresponding editors. All authors contributed to the article and approved the submitted version.

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OPINION
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Statement of the Prolamin Working Group on the Determination of Gluten in Fermented Foods Containing Partially Hydrolyzed Gluten

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On August 12, 2020, the U.S. Food and Drug Administration (FDA) has finalized a rule related to gluten-free labeling for foods containing fermented, hydrolyzed ingredients. The FDA believes that there is no scientifically valid analytical method effective for determining gluten in fermented or hydrolyzed foods. In the absence of an analytical method, the FDA has decided to evaluate gluten-free claims on these foods based only on evidence that the food or ingredient used is gluten-free before fermentation or hydrolysis. For example, barley-based beers from which gluten is removed during brewing using special filtration, adsorption and/or enzymatic treatment are therefore excluded from bearing a gluten-free label.

The Prolamin Working Group (PWG) acknowledges that the FDA rule is a regulatory act and might have to take into consideration several aspects other than scientific evidence, including risk assessment. Nevertheless, the PWG thinks that science has to be the most important driver for regulatory acts in risk management.

In contrast, in the EU such beers are currently allowed to bear a gluten-free label. As required by Regulation (EU) No 1169/2011 of 25 October 2011 on the provision of food information to consumers, the ingredients list must include "barley malt" in highlighted lettering, because gluten-containing cereals are listed in Annex II of the Regulation as substances or products causing allergies or intolerances. The maximum gluten level to bear a gluten-free claim is set in Regulation (EU) No 828/2014 of 30 July 2014 on the requirements for the provision of information to consumers on the absence or reduced presence of gluten in food. On this legal basis, non-governmental Organizations such as the Association of European Coeliac Societies (AOECS) have developed the European Licensing System with guidelines that have to be met to allow using the crossed grain symbol for gluten-free food on the label.

This difference of regulation is a topic of much debate at the moment, because of the divergent opinions between the FDA and the EU. Today, enzyme-linked immunosorbent assays (ELISAs) are the methods of choice for gluten quantitation and are widely used in routine analysis of gluten in food. The R5 antibody, the most prevalent monoclonal antibody used in gluten analysis, was developed by Enrique Mendez, a member of the PWG and is available as a Sandwich ELISA for intact gluten and a competitive ELISA for partially hydrolyzed gluten. The R5 Sandwich ELISA has been endorsed by Codex Alimentarius as a Type 1 method to determine gluten in food. However, the Sandwich format is not suitable to determine gluten in products containing partially hydrolyzed gluten, and competitive ELISAs are required for this type of analysis. Competitive ELISAs based on either R5, G12 or DQ2.5-glia-α3 antibodies are currently available, even if their use is not yet fully approved by official food control authorities. The following statements on the R5 ELISA might be also valid for other competitive ELISAs. Based on the available scientific studies, the PWG thinks that the competitive R5 ELISA is suitable to determine the gluten content of fermented foods containing partially hydrolyzed gluten from wheat, rye or barley, such as beer. The PWG acknowledges that this method has limitations because it might miss smaller peptide fragments and might not have the perfect standard to account for the vast variety of different fermentation procedures common in food processing. However, this method has been validated by an international collaborative study under the guidance of the PWG in 2013. Beer spiked with partially hydrolyzed gluten, naturally gluten-contaminated starch syrup and dried sourdough were used as matrices. The collaborative study was successful and the R5 competitive ELISA was subsequently approved by the respective expert panels as AACCI Method 38-55.01 and AOAC Official Method of Analysis (Final Action OMA 2015.05) because the method was shown to be accurate, precise and specific for its intended purpose. An additional study was initiated with internationally known experts to show the reliability of the R5 competitive ELISA method for the investigation of beer samples. A subcommittee of the American Society of Brewing Chemists recommended that the method for gluten determination by R5 competitive ELISA be included in their Methods of Analysis. The PWG therefore considers that this method is currently state-of-the-art to quantitate gluten in fermented foods.

The FDA thinks that the R5 competitive ELISA method is not suitable for the detection and quantitation of gluten in any fermented or hydrolyzed food because of different hydrolytic conditions in the food to be analyzed and in the material used for calibration. This opinion is scientifically correct but following this reasoning would imply that a different calibrator has to be prepared for each sample matrix, i.e., for each type of beer, even from the same producer. Thus, standardization of the method would be impossible and results would not be comparable between different laboratories or manufacturers. This would be a step back in gluten analysis. Furthermore, the same issue also applies to other methods used for gluten analysis, e.g., sandwich ELISA or liquid chromatography tandem mass spectrometry (LC-MS/MS). In this field of research, calibrators are always a compromise between different possibilities.

The most important alternative method to detect gluten in fermented foods is LC-MS/MS. This technique is currently able to detect and quantify gluten fragments (peptides) in beers but so far there is no validated routine method to give absolute values for the gluten content based on these fragments. The PWG has not seen any values in mg/kg of a CD-active peptide that would really allow to make a good statement if these traces would be relevant to CD patients or if the contents are so low that they fall below the 20 mg/kg threshold for gluten-free foods. Even if modern MS equipment is able to detect femtomolar amounts of peptides, a proper risk assessment of the detected contents is not available.

It is often said that the R5 competitive ELISA does not provide information on the immunogenicity of the detected fragments. However, this is also true for alternative analytical methods for gluten quantitation, and, in addition, this is not the intended use of the method. Food analytical methods can only determine the content of an analyte and are relevant for the decision if this analyte is below or above a threshold set by legislation. Immunogenicity can only be evaluated in clinical studies.

The PWG thinks that more scientific studies are needed to (i) better understand MS-detection of residual gluten in beer and other fermented foods and also (ii) to ensure that the R5 competitive ELISA picks up each peptide fragment it should. Also, MS detection and quantitation of gluten fragments is not feasible for food manufacturers, in particular small companies, because it is too expensive and too demanding in terms of time and skill of the operators. Thus, it is difficult to recommend it as a routine testing method also having in mind that a proper method for absolute gluten quantitation by MS is not available so far.

AUTHOR CONTRIBUTIONS

KS and PK wrote the first draft of the manuscript. All authors contributed to revising and editing the manuscript and approved the final manuscript.

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BRIEF RESEARCH REPORT
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Gluten-Free Foods Cooked in Shared Fryers With Wheat: A Pilot Study Assessing Gluten Cross Contact

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Introduction: Consumers with celiac disease are discouraged from eating fried foods cooked in shared fryers with wheat-containing foods at restaurants based on presumed gluten exposure. The purpose of the present study is to assess gluten levels of fries free of gluten-containing ingredients cooked in shared fryers with wheat.

Methods: 20 orders of fries were purchased from 10 different restaurants. Restaurants confirmed that fries and oil were free of gluten-containing ingredients. All restaurants confirmed that their fryers were used to cook wheat-containing foods. Fries were sent to Bia Diagnostics and tested in 1-gram duplicates using the R7001 sandwich R5 ELISA and the R7021 competitive R5 ELISA. A microwave control also was run.

Results: The sandwich ELISA found gluten in 9/20 fry orders (7 to > 80 ppm). The competitive ELISA found gluten in 3/20 fry orders (14 to > 270 ppm). In the microwave control (60-ppm gluten mixture of wheat flour and canola oil), the unheated mixture tested at a mean level of 64 ppm gluten using the sandwich ELISA and 137 ppm gluten using the competitive ELISA. The mixture heated to 190°C tested at a mean level of 55 ppm gluten using the sandwich ELISA and < 10 ppm and 16 ppm gluten using the competitive ELISA.

Discussion: Based on test results, 25% of fry orders would not be considered gluten-free.

Summary: Gluten cross contact may occur when gluten-free foods are cooked in shared fryers with wheat. ELISAs may underperform when analyzing for gluten that has been heated.

Keywords: gluten, wheat, cross contact, shared fryers, competitive R5 ELISA

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INTRODUCTION

Dietitians have long been discouraging consumers with celiac disease (CD) from ordering gluten-free foods cooked in the same deep fryer as gluten-containing foods at restaurants. This recommendation is based on presumed gluten exposure vs. evidence-based research that gluten cross contact occurs. To the best of the authors' knowledge there is no published

data on gluten levels of gluten-free foods after cooking in shared fryers. The lack of evidence of cross contact contributes to confusion among consumers, especially when gluten-free foods cooked in shared fryers (e.g., fries) are marked as gluten-free on some restaurant menus. The purpose of the present study is to help inform consumer recommendations by assessing gluten levels of fries free of gluten-containing ingredients cooked in shared fryers with wheat.

METHODS

A convenience sample of 20 orders of fries was purchased from 10 different restaurants in California and Ohio. Prior to purchase, restaurants confirmed that fries and oil were free of gluten-containing ingredients. Restaurants also were asked specifically if their fries or oil contained any wheat, malt or gluten ingredients. Fries were ordered plain with salt only. All restaurants confirmed that their fryers were used to cook wheat-containing products (e.g., fried chicken/fish, onion rings, fried sandwiches). Because the gluten level in a shared fryer may vary, two separate orders of fries were purchased from each restaurant on consecutive Saturday afternoons.

Each order of fries was placed unopened into a coded bag. Fries were mailed to Bia Diagnostics, LLC, Colchester, VT, USA (ISO Accredited Lab). Each individual order of fries was homogenized using a blender and tested in 1-gram duplicates using the Ridascreen Gliadin R7001 sandwich R5 enzyme-linked immunosorbent assay (ELISA) and extracted with the cocktail solution (Art. No. R7006) following the kit manufacturer's directions (R-biopharm, Darmstadt, Germany) (1). Samples were also tested in 1-gram duplicates using the Ridascreen Gliadin R7021 competitive R5 ELISA and extracted with ethanol following the kit manufacturer's directions (2). A total of 80 extractions were tested (4 extractions from each sample).

To assess whether the sandwich and competitive ELISAs are fit for purpose to test for the presence of gluten in products heated in oil, a microwave control was run. A 60 mg/kg (ppm) gluten mixture of wheat flour and canola oil was prepared by Bia Diagnostics and tested for gluten before and after heating in a microwave to 190°C/374°F (within temperature range recommended by the U.S. Department of Agriculture for deep frying chicken) (3). Samples were tested in duplicate using the sandwich and competitive R5 ELISAs.

RESULTS

Fries

The sandwich R5 ELISA found quantifiable levels of gluten in 9 of 20 (45%) orders of fries ranging from 7 to > 80 mg/kg (ppm) (above the highest standard) (**Table 1**). Five orders (25%) of fries tested above 20 mg/kg (ppm) of gluten. Fries from 6 of the 10 (60%) restaurants were found to contain quantifiable levels of gluten in at least 1 of the 2 orders, with fries from 4 of these 6 restaurants found to contain levels above 20 mg/kg (ppm) of gluten in at least 1 of the 2 orders. The competitive R5 ELISA found gluten in 3 of the 20 (15%) orders of fries ranging from 14 to > 270 mg/kg (ppm) gluten (above the highest standard).

Microwave Control

The unheated oil and wheat flour mixture tested at a mean level of 64 mg/kg (ppm) of gluten using the sandwich R5 ELISA and 137 mg/kg (ppm) of gluten using the competitive R5 ELISA (**Table 1**). The oil and wheat flour mixture heated to 190°C/374°F tested at a mean level of 55 mg/kg (ppm) of gluten using the sandwich R5 ELISA and < 10 mg/kg (ppm) and 16 mg/kg (ppm) of gluten using the competitive R5 ELISA.

DISCUSSION

Testing found varying levels of gluten in the fry samples, including samples tested from the same restaurant. The gluten level in a shared fryer at any given time likely varies depending upon several factors, including previously cooked foods, oil change frequency, and filtration system. The impact of these factors on the gluten level in fryer oil is worthy of further research.

While orders were placed only with restaurants confirming that fries were free of gluten-containing ingredients, it was not feasible given the real world nature of this study to verify gluten-free status by testing uncooked fries. However, a study on gluten levels of packaged foods not labeled gluten-free but appearing to be free of gluten containing ingredients, found that <5% contained levels of gluten at or above 20 mg/kg (ppm) (5). While it is possible that some gluten present in the tested fries could have been from the uncooked fries themselves vs. cross contact due to the presence of wheat in the shared oil, this seems relatively unlikely. In future studies, it would be useful to partner with restaurants to test raw ingredients in addition to testing finished food products.

A microwave vs. a deep fryer was used for the control. Using a fryer in the lab proved challenging due to difficulty in maintaining a homogeneous flour and oil mixture, preventing precipitation of the flour, and preventing burning of the flour on the heating element. This was true even when the flour and oil mixture was placed in a beaker.

Based on test results, 5 of the 20 (25%) orders of fries would not be considered gluten-free (4); 15 (75%) of the fry orders would be considered gluten-free. Gluten cross contact in fries may add substantial amounts of gluten to the diet, depending upon the amount of fries consumed (**Figure 1**).

It may be the case that all ELISAs underperform when analyzing for gluten that has been heated (7). This may be due to a decrease in solubility of the gluten (i.e., ability of gluten to dissolve in solution to be extracted) as a result of exposure to high temperatures (8). Increased temperatures also may result in denaturation (i.e., change in structure) of the gluten present in samples, reducing their affinity to the antibodies used in the ELISA methods (9). The impact of processing, including heating, on gluten has yet to be fully elucidated.

Results using the sandwich R5 ELISA may underestimate gluten levels in the cooked fries (1, 10). According to R-Biopharm, "In processed food (e.g., heat treatment, dehydration,

TABLE 1 | Gluten levels mg/kg (ppm) in samples tested.

Gluten levels of restaurant fries cooked in shared fryers with wheat-containing foods					
Test code	**Sample tested**	**Sandwich* Extraction 1**	**Sandwich Extraction 2**	**Competitive** Extraction 1**	**Competitive Extraction 2**
F1A	Plain fries, salt only	< 5	< 5	< 10	< 10
F1B	Plain fries, salt only	< 5	< 5	< 10	< 10
F2A	Plain fries, salt only	< 5	< 5	< 10	< 10
F2B	Plain fries, salt only	18	19	< 10	< 10
F3A	Plain fries, salt only	45	28	19	14
F3B	Plain fries, salt only	52	62	29	31
F4A	Plain fries, salt only	< 5	< 5	< 10	< 10
F4B	Plain fries, salt only	< 5	< 5	< 10	< 10
F5A	Plain fries, salt only	11	7	< 10	< 10
F5B	Plain fries, salt only	11	9	< 10	< 10
F6A	Plain fries, salt only	< 5	< 5	< 10	< 10
F6B	Plain fries, salt only	< 5	< 5	< 10	< 10
F7A	Plain fries, salt only	19	15	< 10	< 10
F7B	Plain fries, salt only	65	> 80	> 270	> 270
F8A	Plain fries, salt only	28	23	< 10	< 10
F8B	Plain fries, salt only	< 5	< 5	< 10	< 10
F9A	Plain fries, salt only	< 5	< 5	< 10	< 10
F9B	Plain fries, salt only	< 5	< 5	< 10	< 10
F10A	Plain fries, salt only	< 5	< 5	< 10	< 10
F10B	Plain fries, salt only	24	22	< 10	< 10
Gluten levels mg/kg (ppm) of wheat flour and oil mixture in microwave control					
Temp.	**Sample tested**	**Sandwich Extraction 1**	**Sandwich Extraction 2**	**Competitive*** Extraction 1**	**Competitive Extraction 2**
Unheated	Wheat flour & oil mixture (60 mg/kg)	72	55	165	109
Heated to 190°C/374°F	Wheat flour & oil mixture (60 mg/kg)	49	60	16	< 10

*The lower limit of quantification for the sandwich R5 ELISA is 5 mg/kg (ppm) of gluten. The R7001 assay is a Codex Alimentarius Type 1 Method and an AOAC Official Method of Analysis (1). It is also one of two assays that FDA has stated they will use if testing is necessary as part of gluten-free rule enforcement (4).

**The lower limit of quantification for the competitive R5 ELISA is 10 mg/kg (ppm) of gluten. The R7021 is an AOAC Official First Action Method (2). Gluten protein fragments cannot be adequately detected using a sandwich ELISA. When gluten protein fragments are suspected, a competitive ELISA is recommended.

***In the microwave control, the results of the unheated sample are overestimates when assessed using the competitive ELISA. The competitive ELISA is intended to analyze the presence of protein fragments. Generally, results using the competitive will be higher as compared to the sandwich when assessing intact gluten. The competitive ELISA requires only a single epitope to detect gluten while a sandwich ELISA requires two (2).

etc.), proteins may be altered or fragmented, this may have an impact on the recovery/cross reactivity" (1). Results using the competitive R5 ELISA also may underestimate gluten levels in the cooked fries for the same reason (2, 10). In the microwave control, the gluten level appeared to fall to almost unquantifiable levels when the mixture was heated to 190°C/374°F as compared to the unheated sample. According to R-Biopharm, "Heat treated samples that are extracted with ethanol show a reduced recovery" (2). Ethanol is the extraction solution used with the competitive ELISA (2). For this reason, R-Biopharm recommends that heat treated samples be extracted with the cocktail solution and analyzed with the sandwich ELISA (2). However, the sandwich R5 ELISA is not recommended for foods when gluten proteins may have become fragmented due to processing (1). There is a need for improved analytical methods for gluten analysis to address foods that may be both heat treated and contain fragmented gluten.

The impact of heat on the ability of ELISAs to accurately detect and quantify gluten is an area that requires additional research. As pointed out by Panda and Garber, the limitations of ELISAs are further compounded by the lack of clinical information regarding the immunopathogenicity of gluten peptide fragments as compared to intact gluten protein (10). While the solubility, fragmentation, or denaturation of gluten may impact the ability of ELISAs to accurately detect and quantify it, this doesn't

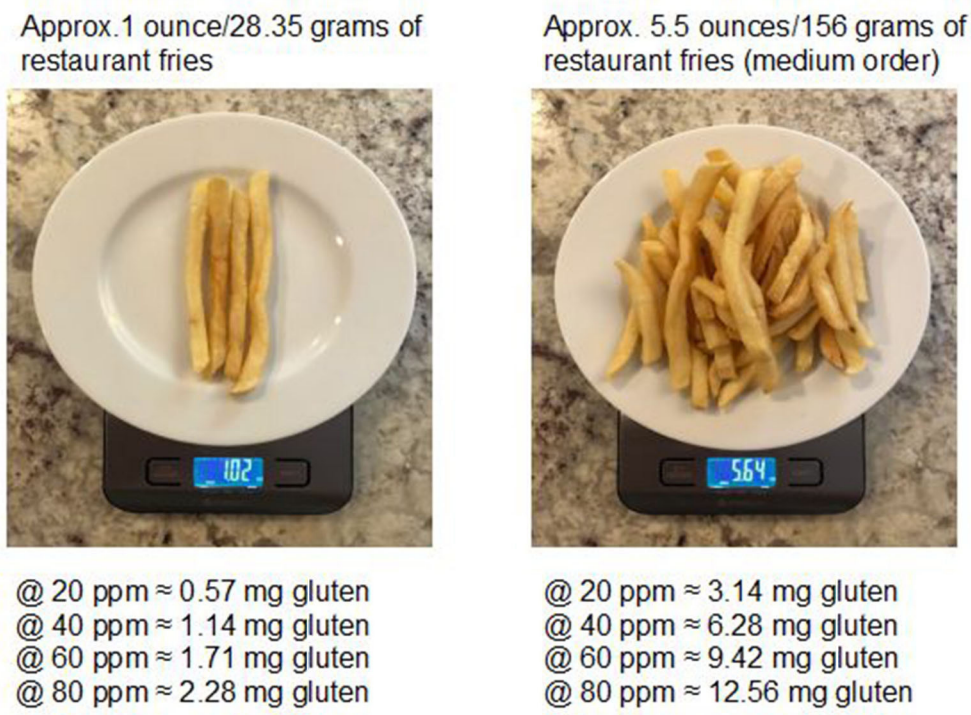

FIGURE 1 | Milligram amount of gluten in restaurant fries at various ppm levels*, **. * Each 1 ounce/28.35 g portion of fries at a gluten level of 20 ppm contains 0.57 mg of gluten.** 10 mg of gluten per day is considered by experts to be a tolerable amount for most individuals with celiac disease (6).

mean that gluten is rendered "safe" for persons with CD. As stated by Sharma et al., while assays may underestimate gluten content in processed foods due to incomplete extraction, this does not mean gluten isn't present in amounts deemed unsafe for consumers (9).

SUMMARY

Results of this assessment suggest that gluten cross contact may occur when gluten-free foods are cooked in shared fryers with wheat. While a much larger study may be warranted, it remains prudent to advise consumers with CD to avoid foods cooked in shared fryers. It is impossible for a consumer to know how much gluten is in fryer oil and how much gluten may end up in an order of fries. Shared holding trays, scoops, and fryer baskets also are sources of potential cross contact. The gluten levels reported in this investigation may be underestimates due to the limitations of the analytical methods available for gluten analysis of foods heated to high temperatures.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

LE-M performed the laboratory analysis. TT wrote the first draft of the manuscript. All authors contributed to the conception, design of the study, analysis of the data, manuscript revision, read, and approved the submitted version.

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Conflict of Interest: TT is the owner and founder of Gluten Free Watchdog, LLC. LE-M is an employee of Bia Diagnostics, LLC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Nutritional and Gastroenterological Monitoring of Patients With Celiac Disease During COVID-19 Pandemic: The Emerging Role of Telemedicine and Point-of-Care Gluten Detection Tests

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Background and Aims: Since the beginning of the coronavirus disease 2019 (COVID-19) pandemic, telemedicine has been supporting many patients with chronic diseases worldwide. However, data on celiac disease (CeD) nutritional and gastroenterological remote monitoring are scanty. The aims of our study were to verify patients' trust in telemedicine and to evaluate the feasibility of telemedicine in nutritional monitoring.

Material and Methods: We used telemedicine in place of the scheduled but not provided follow-up visits during the first lockdown of the COVID-19 pandemic. Patients received a phone call, and televisits were conducted for CeD patients with mild or moderate symptoms and/or with blood alterations. The patient's adherence to the gluten-free diet (GFD) was evaluated according to the Celiac Dietary Adherence Test (CDAT). When gluten contamination was suspected, a point-of-care gluten detection test was prescribed. The patient's trust in telemedicine was assessed, through an adapted version of the Patient Trust Assessment Tool (PATAT) questionnaire, as the percentage of patients giving a score of at least 4 out of 5 on a Likert scale for three selected key statements: "I can trust televisit," "I can trust that possible problems with the telemedicine service will be solved properly," and "I feel at ease when working with this website."

Results: One hundred and twelve CeD patients were phone called; among symptomatic patients, 39 out of the 42 scheduled (92.9%) televisits were performed. Among the 39 visits, 34 (87.2%) questionnaires were compiled. The patients included in the study obtained a CDAT score from 7 to 13 (11 $\pm$ 2). Gluten detection tests were prescribed to 11 patients, resulting positive in 2. Trust in the telemedicine service was achieved in 94.1, 88.2, and 97.1% for the three selected key statements of the PATAT questionnaire.

Conclusion: During the COVID-19 pandemic, telemedicine showed to be feasible and the majority of patients trusted the combined gastroenterological and nutritional televisits. Gluten detection tests demonstrated to be useful tools for the patient and for the caregiver to confirm adherence to the GFD remotely.

Keywords: telemedicine, telehealth, nutrition, celiac disease, televisits, gluten free diet, gluten detection test, COVID-19

INTRODUCTION

Telehealth is defined by the American Telemedicine Association (ATA) as "technology-enabled health and care management and delivery systems that extend capacity and access" (1). It includes not only health care delivery (often identified as telemedicine) in terms of disease diagnosis and treatment but also several other activities and services, such as prevention, education, and public health promotion (2). Telehealth is able to improve and increase access to health care, extending interaction with distantly located patients and enabling both patients and health care providers (HCPs) to have more flexible scheduling and greater efficiency in terms of cost and time (3).

Since the first reported cases of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-related pneumonia in China in December 2019, in just a few weeks, the virus had spread worldwide, leading to unforeseen consequences on every aspect of our daily working and social life as well as to radical changes in health care delivery. Italy was the first European country to experience the outbreak, and in particular, Lombardy and the areas of Bergamo, Lodi, and Milan were the very first "red areas" identified in the Country[1]. On March 9, 2020, the Italian government decided to place the whole country in a strict lockdown for almost 2 months. Moreover, in almost every Italian hospital, scheduled outpatient visits had been canceled, medical services were discontinued, and the medical staff were referred to perform other duties. In this scenario, telemedicine emerged as the ideal (and only) solution to overcome the impossibility of carrying out regular visits, allowing the continuation of patient assistance (4).

In the literature, only a few studies, before and during the coronavirus disease 2019 (COVID-19) pandemic, have evaluated the role and perception of telehealth and, in particular, televisits in gastroenterology and even fewer in celiac disease (CeD) (5, 6) and nutrition (7). These studies generally showed a positive result in favor of telemedicine, as televisits with patients in their homes for nutritional counseling appear to be appropriate since nutritional advice can be easily submitted to patients during such face-to-face consultation (6). Despite these studies and those performed in other fields mostly reporting positive outcomes in terms of satisfaction and cost-effectiveness, none of them investigate a perspective about patients' trust on telemedicine and televisits. Furthermore, the lack of data about the interactions among SARS-CoV-2 infection and CeD underlines the necessity to maintain a constant follow-up of patients (8).

CeD is a chronic autoimmune disease with a prevalence around 1%. The gluten-free diet (GFD) is the only effective treatment. However, a full adherence to the GFD is very difficult to achieve, and thus, patients should be followed up regularly for the assessment of symptoms and dietetic adherence. The newly introduced technologies for the detection of gluten in food and biological samples (urine and stool) can support CeD monitoring. Among them, urinary detection of gluten immunogenic peptides (GIP) is a self-administered point-of-care test with the aim to reveal unconscious gluten ingestions. It has been demonstrated that urinary GIP test is sensitive, specific, and effective to monitor GFD adherence (9, 10). Furthermore, questionnaires could be useful during CeD follow-up; the Celiac Dietary Adherence Test (CDAT) is a clinically relevant easily administered questionnaire which helps in the standardized evaluation of GFD adherence (11).

Aims

The aims of our study were to verify CeD patients' trust in telemedicine and to evaluate the feasibility of telemedicine in CeD gastroenterological and nutritional monitoring.

METHODS

Patients

During the general lockdown in Italy for the COVID-19 pandemic, we embraced telemedicine for our patients with CeD. From March 2020 to May 2020, phone calls were made in place of the previously scheduled but not carried out follow-up visits at our tertiary referral center *"Centre for Prevention and Diagnosis of Celiac Disease"* (Gastroenterology Unit, *Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico*, Milan, Italy).

Televisits were proposed in addition to phone calls for every patient with symptoms (e.g., diarrhea, abdominal pain, weight loss) or in those with altered blood test results. In other cases, visits were postponed. Televisits were also offered to patients for nutritional counseling concerning CeD and GFD.

Video calling solutions from *Google* (*Hangouts* or *Meet*) or *Microsoft Teams* were used according to each patient's preference. Patients who did not have internet connection or were unable to use a smartphone device were necessarily excluded *a priori*.

With the term telemedicine, we intend phone calls, televisits, and remote point-of-care diagnostic test for gluten detection and GFD monitoring.

[1]http://www.salute.gov.it/portale/nuovocoronavirus/dettaglioNotizieNuovoCoronavirus.jsp?lingua=italiano&menu=notizie&p=dalministero&id=4370 (accessed September 14, 2020).

Gluten-Free Diet Adherence

The patient's adherence to the GFD was evaluated according to the CDAT. The CDAT is a clinically relevant, easily administrated seven-item instrument which allows the standardized evaluation of GFD adherence. It is a sensitive tool developed using standard psychometric techniques. CDAT is based on a score ranging from 7 to 35 for seven questions, each on a five-point scale, with higher scores denoting worse GFD adherence (11).

In case of uncertain gluten contamination or presence of intestinal/extraintestinal symptoms, it was suggested to patients to self-verify the dosage of gluten urinary peptides following the manufacturer's instruction (9, 10). Gluten detection tests use G12 monoclonal antibody (MoAb) able to detect GIP in urine by the immunochromatographic technique. The positive result indicates that gluten intake was detected within the last 24–48 h (12).

Trust in Telemedicine

Patients' trust in televisits was assessed through an adapted version of the Patient Trust Assessment Tool (PATAT) questionnaire (**Table 1**). The questionnaire investigated five trust areas: care organization, care professionals, treatment, technology, and telemedicine services (13). It was translated into Italian and formulated online on the *EUSurvey* platform by our center. After the televisit, each patient received an email containing the questionnaire URL and provided their informed consent before compiling the anonymous questionnaire. The questionnaire was formulated through the *EUSurvey* platform, which is widely used for clinical research questionnaires in Europe.

Patients' trust in telemedicine was expressed as a percentage of patients >75% giving a score of at least 4 out of 5 on a Likert scale for three selected key statements: "I can trust televisit" (5.1), "I can trust that possible problems with the telemedicine service will be solved properly" (5.2), and "I feel at ease when working with this website" (5.4).

This study was approved by our local ethics committee (number 550/2020).

TABLE 1 | Clinical and demographic characteristics of celiac patients who accepted the televisit.

	CeD (*n* = 39)
Age, years, median (range)	42.0 (20–73)
Female, *n* (%)	34 (87.2%)
Age at diagnosis (years)	31.0 (2–61)
Disease duration (years)	11.0
Refractory CeD	1 (2.6%)
Familiarity for CeD, *n* (%)	11 (28.2%)
Comorbidities, *n* (%)	25 (64.1%)

CeD, celiac disease.

Statistical Analysis

The demographic data were described as median (range) or unless otherwise indicated. The patients' trust was expressed as total number and percentage. The continuous variables were compared using independent Student's *t*-test. Fisher's exact test was used to evaluate the distribution of categorical variables. The statistical analysis was performed by SPSS software ver. 26.0 (IBM, Armonk, NY, US). Following the previously reported data on patients' trust in telemedicine (14, 15)/*post hoc* power analysis was performed. A power (β – 1) >80% with a two-sided 5% significance level was considered acceptable (16) (G*Power package ver. 3.1.9.4, University of Dusseldorf, http://www.gpower.hhu.de.).

RESULTS

During the March 2020 lockdown, we phone called 112 CeD patients scheduled to undergo routine gastroenterological and nutritional visits.

Thus, we scheduled 42 televisits (37.5%) for every patient with symptoms or altered blood test results; among them, 39 (92.9%) televisits were successfully performed. Three patients refused to perform the televisit and preferred the in-person visit (**Figure 1**).

The baseline characteristics of the patients who accepted televisits are shown in **Table 1**. No demographic differences were observed between the group undergoing televisit and the postponed patients (data not shown).

All included patients reported a very good adherence to the GFD, expressed as CDAT score ranging from 7 to 13 (11 ± 2). Eleven patients reported the presence of symptoms despite referring adherence to GFD but with a suspect of

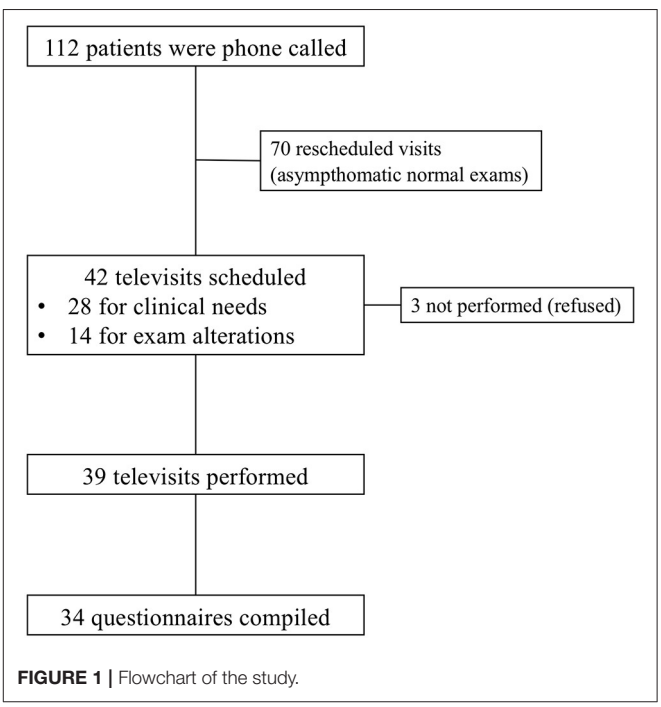

FIGURE 1 | Flowchart of the study.

possible contaminations. To those patients, we prescribed gluten detection tests which resulted positive in two cases (18.2%). Thirty-four (87.2%) questionnaires about trust in telemedicine were compiled after the nutritional and gastroenterological televisits (**Figure 1**, **Table 2**).

Regarding trust in the telemedicine service, items 5.1, 5.2, and 5.4 received a score of least 4 in 94.1, 88.2, and 97.1% of the patients, respectively (**Figure 2**). Findings from the PATAT questionnaire are reported in **Table 2**. According to the previously reported patients' trust in telemedicine, ranging from 50 to 60%, the estimated power was >80% in case of comparison with the trust showed by the analyzed cohort of CeD subjects.

The questionnaire results showed that, during the COVID-19 pandemic, CeD patients who were followed at our center agreed to receive a televisit in spite of the traditional in-person visit and they trusted televisits.

DISCUSSION

Telemedicine has been often recognized as a valuable tool with great potential. Nevertheless, its role is still marginal in daily clinical practice, as communication with patients occurs largely *via* emails and texts, even though patients often find these forms of communication unsatisfactory due to the offline interaction and the delay in response (17). It should also be considered that up until December 2020 (15), there were no laws or regulations by the Italian National Health System officially directing or recognizing telemedicine as a tool to perform and deliver health care.

Recently, a study conducted by the Universities of Padua and Salerno among adult CeD patients aimed to assess their perception of COVID-19 effects. When asked about their opinion on remote telemedicine visits, most of them responded that they

TABLE 2 | The patient trust assessment tool (PATAT) as responded by 34 CeD patients.

		Percentage of patients giving a score ≤3	Percentage of patients giving a score ≥4
1	**Trust in the care organization**		
1.1	The Celiac Centre Polyclinic of Milan has a good reputation.	0%	100%
1.2	At the Celiac Centre Polyclinic of Milan, they handle my personal information carefully.	2.9%	97.1%
1.3	At the Celiac Centre Polyclinic of Milan, they take action when something goes wrong.	2.9%	97.1%
1.4	At the Celiac Centre Polyclinic of Milan, I feel at ease.	0.0%	100%
1.5	At the Celiac Centre Polyclinic of Milan, they take my specific needs into account.	2.9%	97.1%
2	**Trust in care professional**		
2.1	I trust my doctor's judgement about my medical care.	0%	100%
2.2	My doctor provides me with all the information on all potential medical options.	0%	100%
2.3	My doctor keeps all my medical information private.	2.9%	97.1%
2.4	I always follow my doctor's advice.	5.9%	94.1%
2.5	My doctor does not do everything they should about my medical care.	85.3%	14.8%
3	**Trust in treatment**		
3.1	The treatment I receive is effective.	8.8%	91.2%
3.2	It is clear to me what the treatment I receive entails.	2.9%	97.1%
3.3	Together, my doctor and I made the choice for this treatment.	17.4%	73.5%
3.4	The treatment I receive is not helping me enough.	97.1%	2.9%
3.5	It has been explained well to me what my treatment entails.	2.9%	97.1%
4	**Trust in technology**		
4.1	When I use Google/Microsoft video service, I am in control.	5.9%	94.1%
4.2	Everything that I do on Google/Microsoft video service remains private.	8.8%	91.2%
4.3	The personal information that is stored at Google/Microsoft will not get lost.	23.5%	76.5
4.4	Google/Microsoft video service is easy to use.	5.9%	94.1%
4.5	Legal policy and technological safeguards make Google/Microsoft video service a safe environment.	8.8%	91.2%
5	**Trust in telemedicine service**		
5.1	I can trust this telemedicine service.	5.9%	94.1%
5.2	I can trust that possible problems with this telemedicine service will be solved properly.	11.8%	88.2%
5.3	I can trust this service less than other online services.	82.3%	17.7%
5.4	I feel at ease when working with Google/Microsoft video service.	2.9%	97.1%
5.5	I do not like to enter my personal data on Google/Microsoft.	79.4%	21.6%

Trust in telemedicine was expressed as a percentage of patients >75% giving a score of at least 4 out of 5 on a Likert scale for three selected key statements: "I can trust televisit" (5.1), "I can trust that possible problems with the telemedicine service will be solved properly" (5.2), and "I feel at ease when working with this website" (5.4).

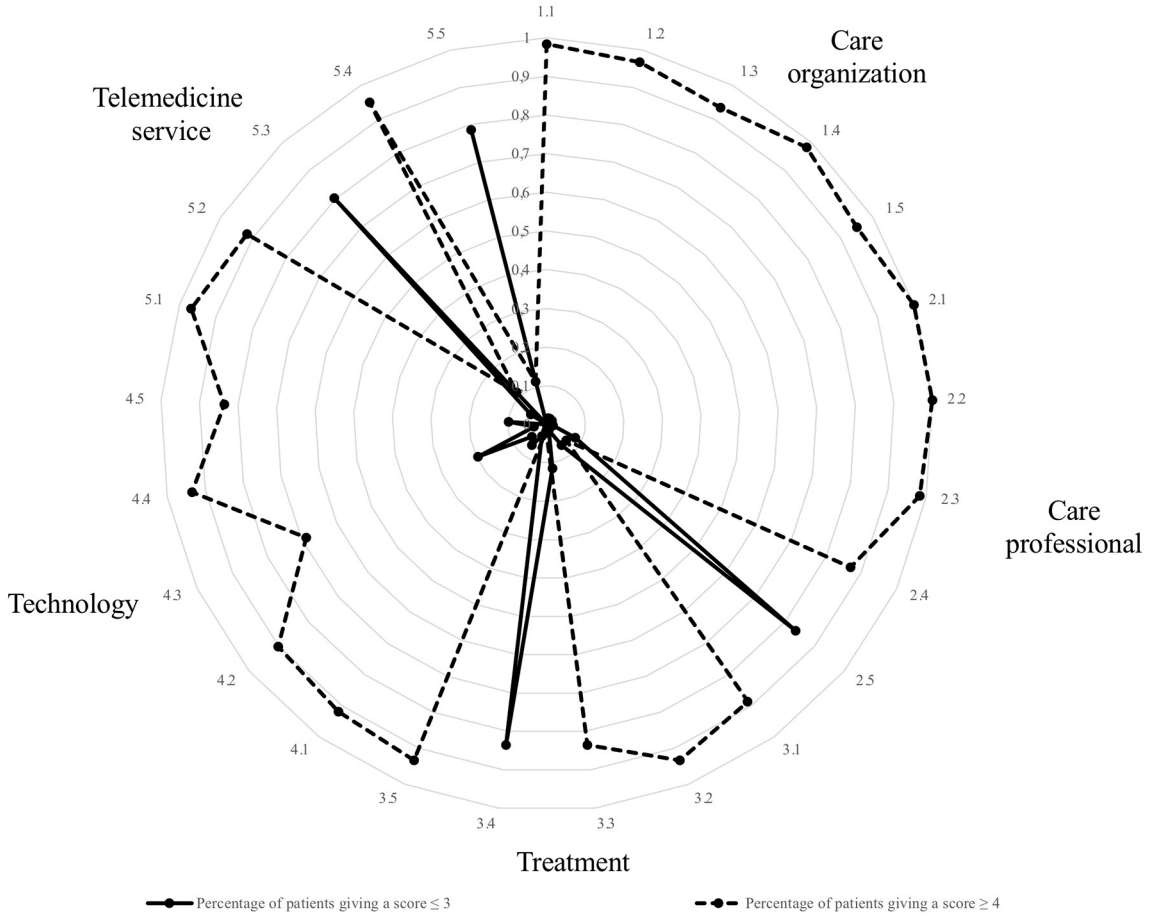

FIGURE 2 | Radar chart of the trust scores from celiac patients assessed through an adapted version of the Patient Trust Assessment Tool (PATAT) questionnaire. Five trust areas were investigated: care organization (1.1–1.5), care professionals (2.1–2.5), treatment (3.1–3.5), technology (4.1–4.5), and telemedicine services (5.1–5.5). The dotted line refers to the patients giving a score ≥4 (out of 5) on a Likert scale. The continuous line refers to the patients giving a score ≤3. Statements 2.5, 3.4, 5.3, and 5.5 were negative. Regarding trust in telemedicine services, items 5.1, 5.2, and 5.4 received a score of least 4 in 95, 90, and 84% of the cases, respectively.

were "happy with it" (86%) and part of them explicitly requested it (∼17%) (6).

The experience gathered during the COVID-19 pandemic will probably further reinforce pre-COVID data on the effectiveness and good performance of telemedicine and push for a more widespread adoption in regular clinical practice even beyond the emergency pandemic. Our study demonstrated the good feasibility and the positive attitude of CeD patients toward telemedicine. This positive trust rate in televisits among CeD patients is a fundamental prerequisite for having confidence in proposing this approach and achieving a successful implementation. The high percentage of patients observed in the study is certainly due to the high number of patients who trust the *Center for Prevention and Diagnosis of Celiac Disease* and its doctors. It is likely that such trust can instill confidence in the proposed telemedicine service even if trust in the technology itself does not have such high values and that the pandemic has helped increase the usual trust in the performance performed away from a doctor's office. A possible limitation of our study is that the vast majority of the patients who had the televisits due to CeD were women, and males are less inclined to follow-up and carry out with medical appointments. However, similar results were observed in a previous study on patients with inflammatory bowel diseases and there was a male sex predominance (18).

To date, thanks to advanced communication technologies, most commonly computers and mobile phones, HCPs can interact long distance with patients *via* synchronous modalities (mainly live videoconferencing allowing for interactive consultation and immediate interventions) and asynchronous modalities (2). However, the spread of telemedicine may be limited by patients who are not familiar with digital technologies. In our experience, in-person visits were maintained for those who did not have internet access and/or technological devices or were not capable of using them. Alternatively, a patient's caregiver could help for this portion of patients eventually excluded from remote assistance.

Nevertheless, our study may change our propensity leading HCPs to more consistently adopt telemedicine and expand its use

beyond the traditional ideal setting (e.g., young patient, digital workers) and beyond the emergency pandemic context.

Considering that strict adherence to a GFD is the only therapy in patients with CeD (excluding patients with refractory celiac disease), remote monitoring could play an important role. Gastroenterological and nutritionist televisits or phone triage could be performed using validated easy-to-administer questionnaires, which also seem to be an optimal tool even in an emergency setting such as the COVID-19 pandemic. Furthermore, in selected cases, the use of point-of-care and self-administered tests to monitor GFD (such as urinary GIP) could give an important support. A proportional fraction of GIP absorbed reaches the circulation and is excreted in the urine, allowing for a better evaluation of patients' adherence to GFD compared to serology (which has low specificity and sensitivity in determining both adherence to the gluten-free diet and healing of the intestinal mucosa) and to frequent repetition of biopsies (19). It has been demonstrated that they are a valuable aid for celiac patients to monitor their GFD, improving adherence and checking for accidental gluten ingestion (9, 12). Moreover, these point-of-care tests are easy-to-use, low-cost, reliable, and accurate tools to verify possible gluten contamination even during remote televisits.

Considering telemedicine innovation and its potential risks (privacy issues or possible medical errors), only few televisits have been performed in our study. Therefore, the expression of patients' trust in televisit was evaluated in a small group, and it is a good representative of those who had the televisit. Nevertheless, since the aim of our study was not to analyze what influenced the level of trust among different groups, it may represent well the general trust in telemedicine in a larger CeD population.

In the near future, it is likely that telemedicine is going to be used for visiting patients who are asymptomatic and with a mild disease as well as for maintaining normal visits for patients with mild or severe disease or with symptoms. In addition, telemedicine could be used as a triage visit in order to select patients who request further exams or in-person visits as well as those who do not.

Whether this trust in telemedicine will last when the COVID-19 pandemic will eventually be over and how telemedicine should be better deployed will surely be further analyzed.

CONCLUSION

During the COVID-19 pandemic, telemedicine has been a powerful and convenient tool for patients with CeD to gain access to remote assistance; at the same time, it has potentially contained SARS-CoV-2 spreading among patients and HCPs. We had the possibility to perform a televisit in more than 90% of symptomatic patients, and the majority of these patients trusted the combined gastroenterological and nutritional televisits. Gluten detection tests demonstrated to be useful tools for the patient and for the caregiver to confirm adherence to the GFD or accidental gluten contamination remotely.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, on reasonable request to the corresponding author.

ETHICS STATEMENT

This study, involving human participants, was reviewed and approved by Ethics Committee Milano Area 2, n. 550/2020. The patients/participants provided their informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AC and DN designed the study, carried out the acquisition, analysis and interpretation of data. AC, NN, LR, and DN wrote the manuscript. VL, LS, and AS gave material support for video-consultations. LE and MV supervised the study and critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Gluten-Free Labeling Is Misused Frequently in Foods Marketed in Northwestern Mexico

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Background: Patients with celiac disease (CD) require a gluten-free (GF) diet, including industrialized products containing ≤20 mg gluten/kg. The market status of GF food products is almost unknown in Mexico. Therefore, we studied the GF-labeled products on the northwestern Mexican market and analyzed their gluten content.

Methods: We searched for GF type of foods in three different supermarkets of each chain in Mexicali Baja California and Hermosillo Sonora and corroborated the price, origin, and GF certification of each item using internet sites. We quantified the gluten in the foods using the sandwich R5-enzyme-linked immunosorbent assay (ELISA) and detected their immune-reactivity for IgA from patients with CD.

Results: The study included >263 different GF-labeled foodstuffs, and 55% of them were made in Mexico. The Mexican items were principally flours, sausages, bread and bakery, milk-type products, and tortillas, while pasta, snacks, and breakfast cereals were mainly imported. The cost ratio of GF products to the conventional mean was 3.5, ranging principally from 1 to 13. The most common GF-labeled foods were flours and pasta (34), cookies and snacks (32), breakfast cereals, sausages, and milk-type products (18–20). Although 36% of the products were certified, 17.4% of the analyzed samples contained >20 mg gluten/kg, mainly the non-certified ones and those made in Mexico. IgA from patients with CD reacted *in vitro* against gluten proteins from the contaminated GF-labeled products.

Conclusion: The accessibility of GF products in the northwestern Mexican market is wide; however, such products are expensive, and some could be risky for patients with CD because they contain gluten, which is recognized by the immune systems of these patients.

Keywords: gluten-free labeling, foods, celiac disease, analysis, mexican market

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INTRODUCTION

Wheat gluten proteins contribute viscoelasticity and extensibility to the dough used in the preparation of bread and other widely consumed foodstuffs all over the world. Although wheat-containing foods are innocuous for the majority of people, gluten exacerbates the signs and symptoms in those with celiac disease (CD), an autoimmune enteropathy that develops in ∼1%

of any population. Additionally, other wheat-related disorders (1), such as wheat allergies and non-celiac wheat sensitivity, affect 5–6% of the population; some of these disorders could be related to wheat proteins other than gluten. The food industry has developed dietary gluten-free (GF) foods for people suffering from CD and other intolerances. According to the *Codex Alimentarius*, GF foods consist of ingredients that do not contain wheat, rye, or barley, and the gluten level they contain must not exceed 20 mg/kg, in total, or <100 mg/kg in processed form (2).

In spite of the Code*x Alimentarius* and guidelines given by different governments regarding GF foodstuffs for their marketing, some of the products are not GF, as demonstrated for products in Spain (3), Italy (4), Turkey (5), India (6), and others. However, there is scarce information about the Mexican market for GF-labeled foods and their safety, and their regulation is deficient. The northwestern Mexican states of Baja California and Sonora both share borders with California and Arizona in the USA, and the residents used to cross the border to shop. Due to COVID-19, the border had been closed last year, and the northwestern markets were similar and could be representative of the markets throughout Mexico.

Therefore, the study aimed to describe and classify GF-labeled foodstuffs available in the northwestern Mexican market, compare their costs with those of their conventional counterparts, quantify the gluten in food products using the *Codex*-recommended enzyme-linked immunosorbent assay (ELISA) kit, and detect the immune-reactivity of IgA from patients with CD.

METHODS

Collection of GF-Labeled Foods

First, all the GF-labeled food products found in the markets of the two capital cities of northwest Mexico, Hermosillo Sonora, and Mexicali Baja California, were registered from November 2020 to February 2021. Screening was carried out in three different supermarkets of each chain and specialized food shops in the two cities. Pictures were taken to verify the data for cost, quantity, origin, GF certification, and composition, from internet sites of the corresponding brands. Additionally, the costs of GF foods were compared with those of their conventional counterparts on a per weight basis. The total cost in terms of Mexican pesos and the grams of the presentation of each foodstuff were considered. With these data, the cost per 100 g product was calculated to make comparisons between GF and conventional products. Subsequently, it was calculated as a ratio of the relative cost of GF products with respect to their equivalent conventional ones, expressing the result as a GF/CONV ratio. We obtained the means and ranges of the cost ratio for different types of products.

The products were randomly selected and purchased for analysis of about 33% of each product type in order to reach a confidence level of 95%. The product types selected for this study include the following: breakfast cereals; oats and granola; pasta; cookies; flours; bread, bakery, and breading; sweet and salty snacks; and fresh and dried tortillas. The rest of the product types, such as sausages, dressings, or milk-type products, were not analyzed because they were not transported and produced in the same facilities as gluten-containing cereals or their derivatives.

After identification, a representative sample of each product was taken by quartering for analysis. Samples were finely ground in a kitchen blender, avoiding cross-contamination by careful washing and drying of the blender cup and accessories that come in contact with the sample after processing each sample. All samples were stored at −20°C until further analysis.

Gluten Analysis

The GF foods were assayed by the Ridascreen Gliadin R7001 sandwich R5 ELISA, as proposed by the *Codex Alimentarius*. Each finely ground sample (250 mg) was extracted with 2.5 mL of a solution (cocktail) containing 250 mM 2-mercaptoethanol and 2 M guanidine hydrochloride in phosphate-buffered saline (7), following the recommendations of the manufacturer (R-Biopharm AG, Darmstadt, Germany). In products with chocolate or cocoa, 0.25 g of skimmed milk powder was added to the cocktail solution during the extraction procedure.

SDS-Gel Electrophoresis, Electro-Blotting, and Immuno-Detection

Sample extraction was followed as described for ELISA analysis by Ridascreen Gliadin R7001. Electrophoresis and immuno-detection were carried out as previously described in the laboratory (8). Briefly, GF extracts were mixed (1:1, v/v) with × 5 extraction buffer (0.3 M Tris-HCl [pH 6.8], 0.35 M SDS, 50% [v/v] glycerol, 0.05% [w/v] bromophenol blue, 0.05% [w/v] β-mercaptoethanol, and 1,200 μL of water), vortexed for 20 min, heated at 95°C for 10 min, and centrifuged at 12,000 g for 10 min. The prepared samples were loaded onto 12% (w/v) polyacrylamide gels, and electrophoresis under denaturing and reducing conditions was performed at 200 V for 45 min. The gel was stained with Coomassie blue and silver stain, and the mirror gel was electro-transferred to membranes by semi-dry blotting. The membrane was incubated overnight with a 50x-diluted sera pool, from three patients with CD (two adults and one child), in TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, and 5 mM NaN_3). After washing, incubation was conducted with HRP-conjugated rabbit anti-human IgA (DAKO, Glostrup, DK), 1:2,000 (v/v) in TBST, and then, the membrane was washed, and the HRP activity was developed with DAB (3,3′-diaminobenzidine tetrahydrochloride; Sigma, St Louis, MO) and the reaction was stopped by washing with water.

Statistical Analysis

Descriptive statistics of the data were performed. Differences in cost between the GF-labeled products and their conventional counterparts were evaluated with paired *t*-tests. $P < 0.05$ were considered significant.

RESULTS

Variety, Certification, and Costs

There were at least 263 different GF-labeled products in the northwestern Mexican market. The majority of them were the same in both studied locations (Sonora and Baja California) due

TABLE 1 | Gluten-Free (GF)-labeled product types, certification, and comparative costs with homologous conventional products ($n = 263$).

Product type	Quantity	Certified *n* (%)	Cost ratio GF/CONV Mean (range)	*p*-value
Breakfast cereals	18	10 (55.5)	2.2 (1.0–5.0)	< 0.0001*
Oats and granola	14	8 (57.1)	3.0 (1.3 – 8.8)	0.0012*
Pasta products	34	23 (67.6)	7.5 (4.1–20)	< 0.0001*
Cookies	32	15 (46.8)	3.0 (1.0–9.3)	< 0.0001*
Grains	9	0	1.9 (1.0–2.8)	0.0005*
Flours	34	6 (17.6)	5.4 (1.0–2.8)	< 0.0001*
Bread, bakery and breading	18	0	4.3 (1.7–9.1)	0.0001*
Sweet and salty snacks	32	14 (43.7)	1.7 (1.1–3.4)	< 0.0001*
Fresh and dried tortilla	15	6 (40)	3.8 (1.0–13)	0.0263*
Sausages	20	2 (10)	1.4 (1.0–2.0)	0.0002*
Milk-type and milk foods	18	5 (27.7)	3.0 (1.0–6.1)	0.0014*
Sauces and dressings	12	3 (25)	2.7 (1.0–6.4)	0.0030*
Others	7	2 (28.5)	3.5 (1.2–9.0)	0.0357*

*GF, Gluten free; CONV, Conventional; n, sample size. *Paired t-test (costs), $p < 0.05$ were considered significant.*

to the common supermarket chains. As shown in **Table 1**, the most common GF products were flours and pasta, each one with 34 different products, followed by cookies and snacks, with 32 different products, and breakfast cereals, bread and bakery, sausages, and milk-type products, with around 18–20 different products each. Local or foreign institutions certified 36% of the total marketed GF-labeled foods. Two of the Mexican CD associations have certification lists accessible only for associates, with the following labels: ACELMEX and SSG (*Seguro Sin Gluten*); the rest are registered international certifications such as *Federación de Asociaciones de Celiacos de España* (FACE), European ELS, and the Gluten-Free Certification Organization (GFCO). The cost ratio of GF to conventional product mean was 3.5, ranging from 1 to 9, except for some flours and pasta products, which cost up to 20 times more than the corresponding conventional products.

Places of Origin and Brands

Figure 1 shows the places of origin of the most common GF-labeled products and their types in the studied markets, as well as the variety of brands per type and origin. While breakfast cereals, pasta, and snacks were principally imported products, cookies and flours were both Mexican and imported, and sausages, bread and bakery, and milk-type products were mostly Mexican. The imported products were mainly from the USA, followed by European countries, such as Spain, Italy, and Romania, and even some Latin-American countries, such as Ecuador and Costa Rica. Additionally, **Figure 1** shows that most of the GF products are sold under a wide variety of brands, some of which only produce one or two different GF foodstuffs.

Gluten Contamination

In respect to gluten contamination, **Table 2** presents the nine analyzed product types, accounting for 206 different foodstuffs, from which a subsample of 86 products was analyzed for gluten content. Gluten was undetectable in 77% of the analyzed products but 5–16 mg/kg of gluten was detected in 6% of the products. Of the 86 food products analyzed, 15 (17.4%) had gluten contents >20 mg/kg, the majority of them originated in Mexico, one was from Ecuador, and another was from the USA (**Table 2**). Two of the contaminated samples presented with a GF certification by a Mexican CD association. Nine of the gluten-contaminated products contained between 20 and 100 mg/kg of gluten: one breakfast cereal product, two oat- and granola-type products, one pasta product, one presentation of cookies, three flour products, and one bread product. Three products (one cookie and two different brands of biscuits) contained 100–150 mg/kg gluten, while two bread and bakery products and one tortilla brand contained between 948 and 12,279 mg/kg of gluten. These last three products, as well as two of them with around 100 mg/kg gluten, were from a non-certified brand, which declared that potato and rice or corn flours with flaxseed ferment as their main ingredients. Another contaminated cake contained, according to its label, oat flour, banana, carrot, and several other minor grains. The gluten-contaminated breakfast cereal declared on its label quinoa and chia, with apple and cinnamon. The two oat products contaminated with gluten declared that they contained only oat flakes. The label of the gluten-containing pasta declared that it contained chickpea and chia. One of the gluten-contaminated cookies contained coconut, almond, cinnamon, and vanilla, and the other cookie product declared only rice, sugar, and additives. One of the gluten-contaminated flour products consisted of quinoa flour; there were two products with almond, oat, and rice flours, chocolate, and additives; and three products with oat, tapioca, and chickpea.

Reactivity of Human IgA Against Gluten in GF-Labeled Foods

Figure 2A shows the electrophoretic patterns of the proteins from seven GF-labeled foods with the highest gluten content stained with silver stain and Coomassie blue. All of them were extracted from the same quantity of product, and the sample in lane 2 appears to contain more protein with more defined subunits, given the intensity of the stain. Bands, although

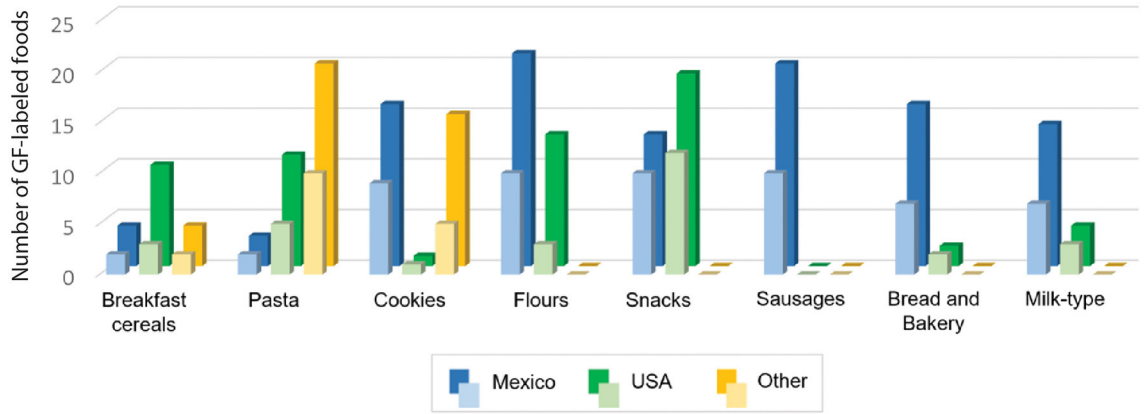

FIGURE 1 | The total number of products (bold colors) and the number of different brands of each product type (light colors), according to their country of origin.

TABLE 2 | Gluten contamination of GF-labeled products ($n = 86$).

Product type	Analyzed products	Contaminated samples	Origin of contaminated samples
Breakfast cereals	7	1	Ecuador
Oats and granola	6	2	Mexico
Pasta products	14	1	Mexico
Cookies	13	2	Mexico/USA
Grains	4	0	–
Flours	13	3	Mexico
Bread, bakery, and breading	9	5	Mexico
Sweet and salty snacks	13	0	-
Fresh and dried tortilla	7	1	Mexico

n, sample size; USA, United States of America.

diffused (due to degradation by the food treatments) in lanes 3–8, could correspond to subunits of gliadins (35–50 kDa and 66 kDa). IgA in the sera from patients with CD clearly and differentially reacted to the gluten proteins from GF-labeled foods electro-transferred to the membrane and present in lanes 2–8 (**Figure 2B**), in agreement with the ELISA-based gluten assay. Although all the samples in part A show mainly protein subunits between 35 and 50 kDa, the blot in part B shows a differential pattern. While IgA from patients with CD recognized the same 35–50 kDa subunits of prolamins in samples from lanes 2 and 5, in samples 3, 4, 6, 7 and 8, they recognized higher molecular weight protein subunits. Samples from lanes 2 and 5 contained oats, while the rest of the samples were from the same brand and it did not declare any more that potato and rice or corn flours for all the its products.

DISCUSSION

The total number of GF products in the Spanish market exceeds that included in this research by a factor of 8.5 (2,247 vs. 263) (3). A possible reason is that CD emerged in Mexico a short time ago; only 8 years ago, it was still considered as rare (9). Although some GF-labeled breakfast cereals, oats, grains, and cookies were comparable in cost with their conventional counterparts, in general, GF foods were more expensive. Bread and bakery foodstuffs, pasta products, and flours were on average 4.3–7.5 times more expensive than the equivalent conventional products, although some products were up to 20 times more expensive. In general, imported GF foods were the most expensive, but some Mexican products, such as pasta, were also expensive. The mean cost ratios of GF to conventional foods in this study (1.4–7.5) were considerably higher than those reported in Greece (1.2–3.4) (10) and similar to those reported in Spain (1.3–6.9) (3).

Certified and imported GF products contained < 20 mg/kg of gluten except two foreign products and two certified Mexican foodstuffs (**Table 2**). According to the published list of ACELMEX-certified products, some of the brands were formerly certified for GF-labeling, but, currently, they have not renewed their certification. There were common ingredients, such as oats, rice, and quinoa, in some of the gluten-contaminated products. Perhaps the foodstuffs were prepared with some cross-contaminated cereal flours of 20–100 mg/kg, such as those containing oats and rice. The quantity of 948–12,279 mg/kg of gluten corresponds to 1–10% of wheat flour in the total mix, and it is not cross-contamination. Electrophoretic patterns and the recognition of gluten proteins by IgA from patients with CD, as shown in **Figure 2A**, demonstrated that there were gluten

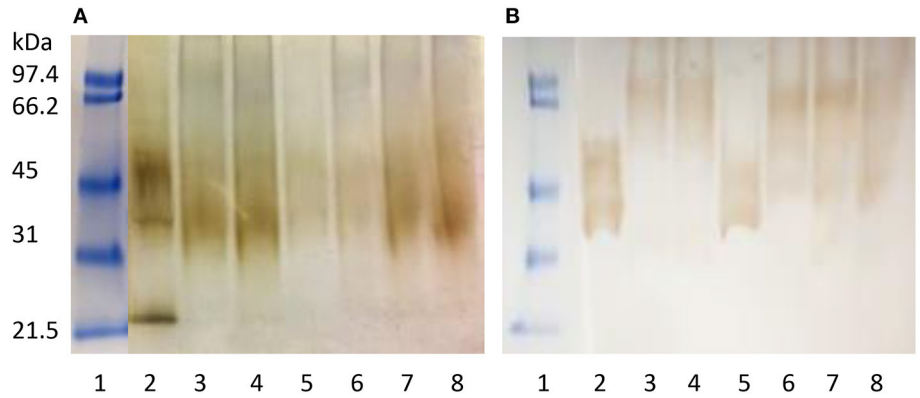

FIGURE 2 | Immuno-reactive proteins in gluten-free (GF)-labeled foods detected with IgA of a pool of sera from three patients with celiac disease (CD). **(A)**: Coomassie blue and silver stained electrophoresis gel and **(B)**: blots after incubation with the sera pool. Lane 1: m.w.std., lanes 2–8: extracts of gluten contaminated foods with 40–12,279 mg/kg gluten.

proteins in some of the analyzed GF-labeled foods. Although the subunits of higher m.w. (around 66 kDa) were represented by faded spots in Part A, the IgA from patients with CD clearly recognized them in Part B because they are the most immunogenic wheat proteins (8). The bread, cake, and tortilla with the highest gluten content and immune reactivity of IgA in patients with CD were from a popular brand with several bakeries and cafeterias in different Mexican cities, in addition to not having any GF certification.

Interestingly, none of the snacks were contaminated with gluten, and the salty ones were mainly fried products. According to Thompson et al. (11), ELISA may underperform when used on heat-treated samples; however, the sandwich-type ELISA combined with extraction using the cocktail solution employed in this study performed well enough to detect gluten contamination in fries analyzed by this method. Additionally, the cocktail solution developed by García et al. (7) has demonstrated to be an excellent extraction procedure.

The percentage of GF-labeled foods in northwest Mexico that were gluten contaminated amounted to 17.4%, with a mean of 1,580 (range: 30–12,279 mg/kg) mg/kg, considerably higher than percentages and means obtained for GF-labeled foods in other locations. In Italy, GF-labeled products with >20 mg/kg of gluten were uncommon (9%) and quantitatively low with a mean of 59 mg/kg (4). In southern India, 9.8% of the products contained > 20 mg/kg of gluten with a mean of 32.5 mg/kg (6). In Turkey, 17.5% of the analyzed GF-labeled samples contained > 20 mg/kg of gluten, although it was principally due to the use of buckwheat flour (5). It is clear from this study that, if we discard the brand with the most contaminated products, the results are comparable with those previously discussed. We hope that GF-labeled foods will improve after strict Mexican regulations are put in place.

As in other countries and given these results, the main concern is the marketing of food products that are GF-labeled, without the necessary tests and certification. This is especially important when the products have natural GF grains as ingredients and are assumed to be safe (12). The only commercially available test approved by the *Codex Alimentarius* is the ELISA R5 test, which was used in this study. The nutrition label information is the only guide that people with CD and other wheat-related diseases have when choosing different foods. The labeling should serve for consumer protection and not constitute a risk, as happened with some of the products evaluated in this study, when the labeling contained unverified information and did not have an official certification that supports the contents containing below the 20 mg/kg of gluten. As a recommendation, it is considered essential that the health professional contributes to the education of patients to learn how to identify, verify, and choose only those GF products that have an official certification, and not based on the possibly misleading labeling information.

The Mexican Health Secretariat is currently updating the official standard NOM-086 on food labeling in compliance with the *Codex Alimentarius*, in order to protect the general population and patients with CD and other patients with wheat-related diseases and to provide safe GF-labeled manufactured food products. This standard underscores the role of the government in the enforcement of the GF certification of these products. The implementation of the regulation and its effective application would allow better control of patients with CD, as well as diversify the options of products supported by certification. This could help reduce costs in the medium and long term, which is another drawback of these types of products (13). In addition to Mexico, other Latin American countries, such as Argentina, Chile, and Brazil, have begun to modify the laws to regulate the labeling of GF products in the last decade (13). However, some of these regulations do not define tolerance limits for gluten content or mention control measures. In Mexico, it is necessary to verify the effective adherence of the food product manufacturing industry to guarantee safety.

In conclusion, the accessibility of GF-labeled industrialized products in the northwest Mexican market is sufficient, although the majority of such products are expensive, with 45% of them being imported from several countries, mainly the USA. However, some of them could be risky for patients with CD due to their gluten content. Brand regulation over the use of GF-labeling is urgently required.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AMC and MM-L designed and conducted the research. AMC wrote the first manuscript version. VL-A made the market study and selected the analyzed sample. VL-A and JV-M carried out all the analyses. All authors read the manuscript and agreed to be accountable for the content of the work.

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A Portable Gluten Sensor for Celiac Disease Patients May Not Always Be Reliable Depending on the Food and the User

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A strict lifelong gluten-free (GF) diet is currently the only known effective treatment for celiac disease (CD), an inflammatory disorder of the small intestine with a worldwide prevalence of about 1%. CD patients need to avoid wheat, rye, and barley and consume GF foods containing <20 mg/kg of gluten. However, strict adherence to a GF diet tends to reduce the quality of life of CD patients compared to the general population and may lead to fear of inadvertent gluten consumption, especially when eating out. To help alleviate risk of gluten exposure, a portable gluten sensor was developed by Nima Labs that allows CD patients to test foods on site prior to consumption. With very limited independent information on the analytical performance of the Nima sensor available so far, our aim was to evaluate the reliability of the sensor using a variety of different foods with defined gluten content. All samples were tested with the sensor and analyzed by enzyme-linked immunosorbent assay as reference method. Of the 119 samples with gluten content ranging from 2 to 101,888 mg/kg tested in total, the sensor showed 80 positive (67.2%), 37 negative (31.1%) and 2 invalid results at the first of three consecutive measurements. The detection rate for samples containing ≥20 mg/kg of gluten was 90%. Samples containing 2 mg/kg of gluten or below consistently tested negative, but samples with a gluten content between 2 to 20 mg/kg of gluten may either test positive or negative. Overall, the performance of the sensor was acceptable in our study, but we observed systematic variation between different users that also appeared to depend on the sample being tested. This highlights the need to improve user education especially regarding the effect of sampling, testing limitations in case of partially hydrolyzed, fractionated or fermented gluten and training users on how to perform the test in a way that gluten will be reliably detected.

Keywords: barley, celiac disease, enzyme-linked immunosorbent assay, gluten-free, rye, sensor, wheat

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INTRODUCTION

Celiac disease (CD) is one of the most common food-induced inflammatory diseases affecting about 1% of the population worldwide (1). It is triggered in genetically susceptible individuals by the storage proteins of wheat, rye, and barley, which are referred to as gluten. The ingestion of gluten-containing cereals leads to small intestinal inflammation with villous atrophy, infiltration

of intraepithelial lymphocytes and subsequently a variety of different intra- and extraintestinal symptoms (2). With a strict lifelong gluten-free (GF) diet as the only effective treatment available so far, CD patients need to avoid products made of wheat, rye, barley and closely related crosses or varieties. Next to naturally GF foods, CD patients may consume specific products bearing a GF label according to national legislation. As laid down in Codex Alimentarius Standard 118-1979, foods labeled "gluten-free" may contain no more than 20 mg of gluten per kg of the product (3). To ensure compliance with the limit, it is essential that GF cereals and pseudo cereals are not mixed with gluten-containing cereals from cultivation to processing into the final product, that GF dishes are prepared separately from gluten-containing dishes in large kitchens and restaurants, and that the methods for gluten analysis are reliably applied by manufacturers of GF products and food control authorities (4).

However, strict adherence to a GF diet is associated with significant restrictions for those affected, which lead to a reduced quality of life compared to the general population (5) and can even provoke anxiety and depression (6). Social activities, eating out and traveling are perceived as particularly problematic, especially in the first years after diagnosis. On these occasions, up to 88% of respondents deliberately accepted dietary transgressions because the GF diet is perceived as too strict, difficult and uncomfortable during social activities (7). Thus, CD patients risk a recurrence of symptoms and consequently an increased risk of long-term complications (8).

So far, methods for analyzing gluten traces in food are designed exclusively for use in specialized laboratories. Because CD patients cannot always be sure that GF foods are really GF, especially when eating out, there is a need for point-of-care (POC) tests. Ideally, small and portable POC tests should provide low-cost, fast, and accurate results with small sample volumes and be easy to perform so that consumers can use them without problems, e.g., in a restaurant. Often, such POC tests are connected to a smartphone app and a social media presence (9).

The POC test for gluten detection used in this study has recently been developed for CD patients by Nima Labs (10). The sensor performs sample preparation, gluten analysis, result interpretation and data transmission within 2–4 min and displays a wheat ear (positive, gluten detected) or smiley (negative, no gluten detected) symbol. Positive was defined by the manufacturer as the sensor detecting 20 mg/kg of gluten or more in sample amounts of 0.1–2 g with a 99.0% probability as true positive. In contrast, negative was defined as <2 mg/kg gluten. Thus, there is a measurement uncertainty in the range of 2–20 mg/kg gluten. The sensor is based on two monoclonal antibodies 13F6 and 14G11 directed against the 33-mer peptide from α-gliadin immobilized on the test line of a lateral flow immunoassay (LFIA). Users are instructed to place a pea-sized portion of the food into a disposable capsule and activate the grinding mechanism when screwing the top of the capsule shut to homogenize the test portion. The extraction solution is added with the last turn. After inserting the capsule into the instrument and pressing the start button, the test portion is mixed with the extraction solution for 30 s and a valve finally allows the extract to flow onto the LFIA. A peak identification algorithm compares the differences in light intensities of the negative LFIA with those of the test line of a positive LFIA, taking into account a control line and a hook line (at very high gluten concentrations).

Various factors such as extraction time, sensitivity and specificity of the two antibodies, cross-reactivities, reproducibility, food matrix, sample weight and sample inhomogeneity were taken into account by the manufacturer. The analysis of 447 food samples gave three false negative results, ten false positive results and 31 invalid results, which occur when a test is not completed correctly, e.g., because the food to be tested absorbs the entire volume of extraction solution, the solution becomes too viscous or the pores of the LFIA become blocked (10). Independent tests with the sensor on 13 different products showed that in 96.5% of the tests the samples with 20 mg/kg of gluten or more were identified as true positives. In some samples, such as bread, pasta and puffed maize, only 47% of the samples with 20 mg/kg of gluten were identified as true positive and the detection rate increased to 88% at 30 mg/kg of gluten and to 97.5% at 40 mg/kg of gluten (11).

Further studies to assess the reliability of the sensor are not yet available, but are essential as false positives restrict CD patients' options to compose their meal and have a negative impact on the GF food industry, while false negatives pose a significant risk to CD patients (12). Points that have not been studied so far include the possibility of a hook effect occurring at high gluten concentrations and the problem of sampling in the case of inhomogeneous distribution of gluten in food and dishes. The sensor was designed to detect intact gluten proteins and there have been no studies to date on whether it also detects fermented or partially hydrolyzed gluten. Since the study by Taylor et al. (11) used wheat flour only to produce defined food samples, there is also a lack of knowledge about the sensitivity and specificity of the sensor to rye and barley.

The main aim of our study was therefore to test the reliability of the portable gluten sensor using homogeneous and inhomogeneous samples with defined gluten content. We used naturally GF raw materials and prepared foods from different categories with defined gluten content by blending in different gluten sources (wheat, rye, and barley flours). Commercially available foods ($n = 21$, nine of them bearing a GF label) containing fermented or partially hydrolyzed gluten were also analyzed. A second aim was to study the influence of sample weight, high gluten content and different users on the results of the sensor.

METHODS

Material

All chemicals, reagents and solvents such as acetonitrile, disodium hydrogen phosphate, dithiothreitol, ethanol, potassium dihydrogen phosphate, 1-propanol, sodium chloride, trifluoroacetic acid (TFA) and urea were at least *pro analysi* or HPLC grade. Cocktail (patented) was from R-Biopharm (Darmstadt, Germany). The Prolamin Working Group (PWG)-gliadin reference material (13) was obtained from the Arbeitsgemeinschaft Getreideforschung e.V. (Association of Cereal Research, Detmold, Germany). Organic grains of wheat

and rye were from denree (Töpen, Germany) and those of barley from Davert (Aschberg, Germany). GF rice flour was from Müller's Mühle (Gengenbach, Germany). All other foods and ingredients used to prepare food samples with defined gluten content were purchased in a local supermarket (Karlsruhe, Germany). Commercially available products with unknown gluten concentrations (nine beers B1-B9, four sauces S1-S4, three potato products P1-P3, two tofu T1-T2, and three sourdough samples D1-D3) from different manufacturers were also bought in a local supermarket (Karlsruhe, Germany). Some of these products had a GF label according to European Commission Implementing Regulation (EU) No. 828/2014.

Determination of Gluten Content in Wheat, Rye, and Barley Flours

Wheat, rye, and barley grains were milled into wholemeal flours using a variable speed rotor mill (Pulverisette 14, Fritsch, Idar-Oberstein, Germany) and a 500 μm sieve. Wheat flour was used without additional sieving. Rye and barley flours were used both without additional sieving and with additional sieving (500 μm) to improve homogeneity of the food samples (designated as rye II and barley II).

After a 2-week rest, the gluten content was determined according to modified Osborne fractionation combined with reversed-phase high-performance liquid chromatography (RP-HPLC) as described by Lexhaller et al. (14). In brief, the flours (100 mg) were extracted sequentially by vortex mixing for 2 min at 22°C and magnetic stirring with salt solution (2 × 1 mL; 0.4 mol/l NaCl with 0.067 mol/L Na_2HPO_4/KH_2PO_4, pH 7.6) for 10 min at 22°C (albumins/globulins), followed by 60% (v/v) ethanol (3 × 0.5 mL) for 10 min at 22°C (prolamins), and glutelin extraction solution [2 × 1 mL; 50% (v/v) 1-propanol/0.1 mol/L Tris-HCl, pH 7.5 containing 2 mol/L urea and 0.06 mol/L (w/v) dithiothreitol] for 30 min at 60°C under argon (glutelins). The suspensions were centrifuged (3,550 × *g*, 25 min, 22°C), the supernatants combined and made up to 2 mL with the extraction solvent, respectively.

The extracts were filtered (Whatman Spartan 13/0.45 RC, GE Healthcare, Freiburg, Germany) and analyzed by RP-HPLC: instrument, UFPLC Prominence with LabSolutions software (Shimadzu, Duisburg, Germany); column, Acclaim 300 C_{18} (particle size 3 μm, pore size 30 nm, 2.1 × 150 mm, Thermo Fisher Scientific, Braunschweig, Germany); temperature, 60°C; injection volume, 20 μL for albumins/globulins, 10 μL for wheat, 20 μL for rye and 40 μL for barley prolamins; 20 μL for wheat and barley and 40 μL for rye glutelins; elution solvents, TFA (0.1%, v/v) in water (A) and TFA (0.1%, v/v) in acetonitrile (B); linear gradient, 0–0.4 min 0% B, 0.5 min 20% B, 7 min 60% B, 7.1–9.0 min 90% B, 9.1–27 min 0% B for albumins/globulins; 0–0.4 min 5% B, 0.5 min 30% B, 18 min 80% B, 18.1–20.1 min 90% B, 20.2–36 min 5% B for prolamins and glutelins; flow rate, 0.4 mL/min; detection, UV absorbance at 210 nm. PWG-gliadin was used for external calibration and the absorbance areas were used to calculate the protein content of the extracts. Gluten content was the sum of prolamin and glutelin content, respectively. Three independent biological replicates were performed for each flour.

Preparation of Foods With Defined Gluten Content

Typical recipes and kitchen utensils were used to ensure practical relevance of our study. All naturally GF raw materials were confirmed to be GF by R5 sandwich ELISA prior to use (prolamin content below the limit of quantitation at 2.5 mg/kg). A GF control was prepared for each food using only GF ingredients. Then, a gluten-containing mixture was made by adding a defined amount of wheat, rye, and barley flour, respectively, to the GF control to reach a target gluten content of 1,000 mg/kg ($mix_{1,000}$). The $mix_{1,000}$ was further blended with the GF control to a target gluten content of 100 mg/kg (mix_{100}). This mix_{100} was subsequently used to adjust the target gluten content to 4 or 5 mg/kg, 10, 20, and 30 mg/kg for high-protein, high-fat and unheated high-starch foods and to 3, 6, 12, 18, and 30 mg/kg for heated high-starch foods (**Table 1**).

High-Protein and High-Fat Foods

Commercially available GF sausage meat (100 g portions) without or with addition of wheat flour was heated in aluminum foil in water at 100°C for 30 min. After cooling to room temperature, the sausage meat was cut and homogenized in an HR 3655/00 blender (Philips, Hamburg, Germany). The final samples made of GF sausage meat and mix_{100} were only blended by hand using mortar and pestle for 30 s with the intent to achieve an inhomogeneous gluten distribution (sample A) (**Figure 1**).

Meat balls (150 g portions) were prepared from minced meat (50% pork, 50% beef), eggs, chopped onions, salt and without or with addition of wheat, rye or barley flour, respectively, as well as a mix of wheat, rye, and barley flour (1 + 1 + 1, w/w/w). The portions were fried for 7 min on each side in sunflower oil. After cooling, the meat balls were homogenized as described above. Homogeneous samples were blended using mortar and pestle for 3 min (samples B–F).

The vegetarian patty contained soy granules soaked in water, GF rice flour, eggs, chopped onions and salt without or with addition of wheat flour. The mass was divided into 150 g portions and further processed as described for the meat balls, with the exception that final blending only lasted for 30 s (sample G).

The salad dressing contained sunflower oil, vinegar, herbs, salt and sugar without or with addition of wheat flour mixed in the blender. Guar gum was slowly added to achieve high viscosity and the salad dressing was further mixed for 30 s with a spatula (sample H).

Unheated High-Starch Foods

GF rice flour was mixed by shaking upside down for 12 h with the appropriate amount of wheat, rye, barley, spelt, durum wheat, emmer and einkorn flours as described in Schopf and Scherf (15) (samples I–Q, **Figure 2**).

Heated High-Starch Foods

Breads were made from a GF flour mix (Dr. Schär, Burgstall/Postal, Italy), water, dry yeast (Frießinger Mühle, Bad Wimpfen, Germany), sunflower oil and salt. All ingredients were kneaded to a homogeneous dough for 5 min at medium speed using a kitchen machine (MUM4405, Bosch, Munich,

TABLE 1 | Overview of high-protein, high-fat and high-starch foods with defined gluten content.

Code	Sample matrix	Gluten source (flour)	Intended gluten distribution	Target gluten content (mg/kg)				
High-protein and high-fat foods								
A	Sausage meat	Wheat	Inhomogeneous		4	10	20	30
B	Meatball	Wheat	Homogeneous		4	10	20	30
C	Meatball	Wheat	Inhomogeneous		4	10	20	30
D	Meatball	Barley	Homogeneous		4	10	20	30
E	Meatball	Rye	Homogeneous		4	10	20	30
F	Meatball	Wheat/rye/barley	Homogeneous		4	10	20	30
G	Vegetarian patty	Wheat	Inhomogeneous		4	10	20	30
H	Salad dressing	Wheat	Inhomogeneous		5	10	20	30
Unheated high-starch foods								
I	Rice flour	Wheat	Homogeneous		5	10	20	30
J	Rice flour	Barley	Homogeneous		5	10	20	30
K	Rice flour	Barley, sieved	Homogeneous		5	10	20	30
L	Rice flour	Rye	Homogeneous		5	10	20	30
M	Rice flour	Rye, sieved	Homogeneous		5	10	20	30
N	Rice flour	Durum wheat	Homogeneous		5	10	20	30
O	Rice flour	Spelt	Homogeneous		5	10	20	30
P	Rice flour	Einkorn	Homogeneous		5	10	20	30
Q	Rice flour	Emmer	Homogeneous		5	10	20	30
Heated high-starch foods								
R	Rice bread, crumb	Wheat	Homogeneous	3	6	12	18	30
S	Rice bread, crust	Wheat	Homogeneous	3	6	12	18	30
T	Rice bread, crumb	Barley	Homogeneous	3	6	12	18	30
U	Rice bread, crust	Barley	Homogeneous	3	6	12	18	30
V	Rice bread, crumb	Rye	Homogeneous	3	6	12	18	30
W	Rice bread, crust	Rye	Homogeneous	3	6	12	18	30

Germany). The dough was divided into 150 g portions and either no flour or wheat, rye or barley flour was added followed by further mixing. Then, breads were baked for 35 min at 180°C, removed from the oven, cooled, separated into crumb and crust and cut into small pieces. The pieces were freeze-dried and subsequently homogenized to a fine powder using the blender (crumb samples R, T and V; crust samples S, U and W, **Figure 3**).

Gluten Analysis Using ELISA

For comparison, all samples were also analyzed by enzyme-linked immunosorbent assay (ELISA) as reference method. All ELISA measurements were performed in a separate fume hood to avoid gluten contamination and surfaces, vials and equipment had been cleaned with 60% ethanol. The gluten content was determined with three replicates by R5 sandwich ELISA (RIDASCREEN Gliadin; R-Biopharm) for samples A-W (**Figures 1**–**3**) or R5 competitive ELISA (RIDASCREEN Gliadin competitive; R-Biopharm) for commercially available products with unknown gluten concentrations (**Figure 4**). The ELISA was performed strictly according to the manufacturer's instructions, respectively. The absorbances were read at 450 nm with a Tecan Infinite 200 PRO microplate reader (Crailsheim, Germany). The cubic spline function implemented in the software RIDASOFT Win.NET (R-Biopharm) was used to calculate the prolamin content in the samples. Values below the limit of quantitation (2.5 mg/kg for prolamin content) were extrapolated using a second order polynomial function. Gluten content was obtained by multiplying the prolamin content by a factor of 2, as stated in the Codex (3). Homogeneity of selected samples (meatball, wheat, at 20 mg/kg of gluten) prepared to be homogeneous and inhomogeneous, respectively, was tested using ten replicates from different parts of the sample container according to standard procedures (16). Mean values, absolute standard deviations and relative standard deviations (RSD) were calculated for all quantitative results.

Gluten Analysis Using the Sensor

All food samples were measured in three replicates using the sensor (Nima Labs Inc., San Francisco, CA, USA) strictly according to the manufacturer's instructions. In case of ambiguous results (one replicate not in agreement) three more replicates were analyzed. Sample quantity (0.1–2.0 g, in 0.2 g steps) was varied using four exemplary samples C, H, T, and U (21.3, 15.9, 8.6, and 18.6 mg/kg of gluten, respectively, according to ELISA). To assess whether a high-dose hook effect might occur, wheat, rye, and barley flours were tested directly, as well as the $mix_{1,000}$ of samples B, H, T, T prior to freeze-drying, U, and U prior to freeze-drying. Four different users tested four more exemplary samples F, R, U, and W (5.6, 14.8, 22.8, and 9.0 mg/kg of gluten, respectively, according to ELISA).

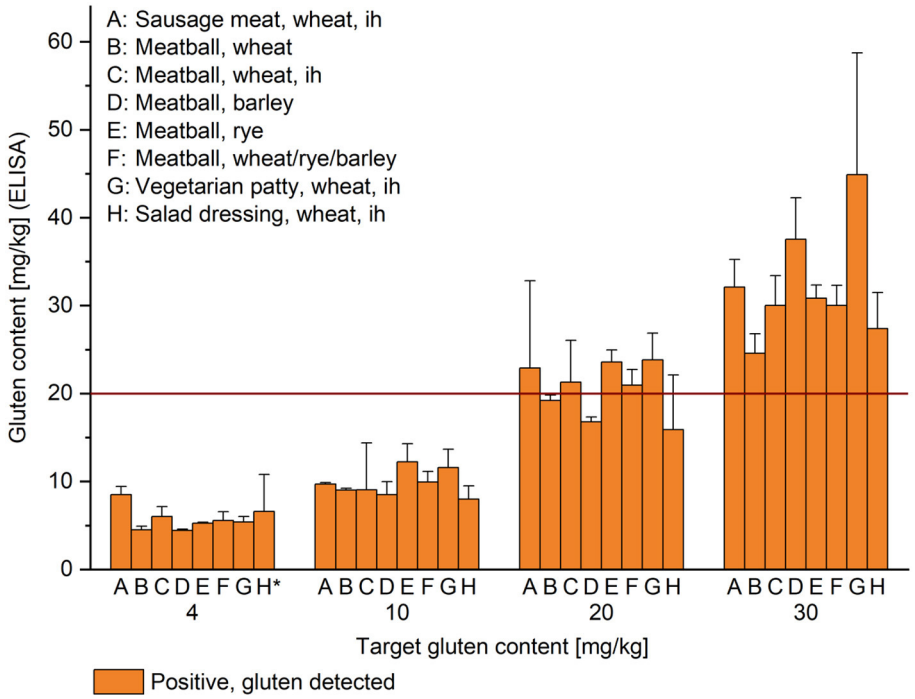

FIGURE 1 | Gluten content of high-protein and high-starch foods. Target gluten content is indicated below the x-axis and gluten content analyzed by R5 sandwich ELISA on the y-axis; given as mean ($n = 3$) + standard deviation. The result of the sensor at the first of three consecutive measurements was gluten detected (orange) in all cases. ih, inhomogeneous, *, target gluten content was 5 mg/kg for sample H. All other samples not designated as inhomogeneous were prepared to be homogeneous. The red horizontal line indicates the threshold for gluten-free products at 20 mg/kg.

RESULTS

Gluten Content in Wheat, Rye, and Barley Flours

The wheat flour contained 3.5% gliadins and 3.3% glutenins, amounting to 6.8% gluten (all values based on flour weight). The gluten content of the rye flour was 2.8%, consisting of 1.9% prolamins and 0.9% glutelins, whereas barley had 3.3% of gluten, composed of 0.8% prolamins and 2.5% glutelins. The second sieving step for rye and barley flours (II) resulted in slight changes of total gluten content, so that the rye flour (II) contained 2.5% of gluten and the barley flour (II) 3.9% of gluten. The gluten content of the flours was also analyzed by R5 sandwich ELISA and the results were 7.6% for wheat, 12.0% for rye and 5.6% for barley. This corresponds to recoveries of 112% for wheat, 429% for rye and 169% for barley. The ELISA results of the $mix_{1,000}$ samples were used for further calculations of the final target gluten concentrations.

Analysis of High-Protein and High-Fat Foods With Defined Gluten Content

Compared to the target gluten content of 4 or 5 mg/kg, 10, 20, and 30 mg/kg for high-protein and high-fat foods, the ELISA results yielded recoveries from 82% (B, meatball, wheat, at 30 mg/kg) to 139% (F, meatball, wheat/rye/barley, at 4 mg/kg) for homogeneous foods. Considering the foods that were intentionally mixed for shorter times to achieve an inhomogeneous gluten distribution, the recoveries were between 91% (C, meatball, wheat, ih, at 10 mg/kg) and 212% (A, sausage meat, wheat, ih, at 5 mg/kg). According to expectations, high RSD of up to 64% in sample H (salad dressing, wheat, ih, at 5 mg/kg) of triplicate determinations were observed for inhomogeneous samples A, C, G, and H (**Figure 1**). In contrast, the RSD were between 2 and 18% over all homogeneous samples. The sensor returned a result of gluten detected for all samples irrespective of the gluten content at the first of three consecutive measurements. Considering the triplicate measurements with the sensor, there were only 3 negative results out of 96 tests in total (3%). These occurred in samples A at 10 mg/kg, D at 10 mg/kg and F at 5 mg/kg and thus were within the range of measurement uncertainty of the sensor.

Analysis of Unheated High-Starch Foods With Defined Gluten Content

The comparison of target gluten content and that measured by ELISA resulted in recoveries from 39% (L, rice/rye, at 5 mg/kg) to 204% (Q, rice/emmer, at 5 mg/kg). Additional sieving helped increase recovery to 72% in the rice/rye mix (M, rice/rye II, at 5 mg/kg). The ELISA gave consistently lower recoveries for rice/durum wheat (N, 58–82%) and rice/einkorn (P, 42–86%) mixtures compared to rice/spelt (O, 122–168%) and rice/emmer (Q, 114–204%). All high-starch foods were prepared with the intention to achieve homogeneity, but most RSD lay between 10

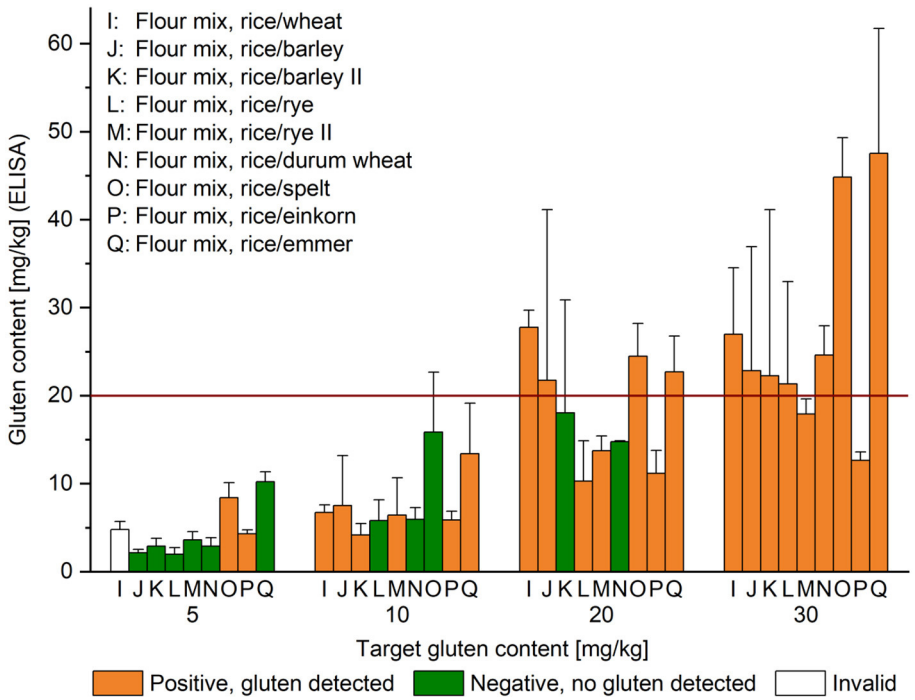

FIGURE 2 | Gluten content of unheated high-starch foods. Target gluten content is indicated below the x-axis and gluten content analyzed by R5 sandwich ELISA on the y-axis; given as mean ($n = 3$) + standard deviation. The result of the sensor at the first of three consecutive measurements was either gluten detected (orange), no gluten detected (green) or invalid (white). Flour mixes designated with (II) were additionally homogenized. The red horizontal line indicates the threshold for gluten-free products at 20 mg/kg.

and 33%. However, RSD up to 89% (J, rice/barley, at 20 mg/kg) were observed, most likely due to different mixing behavior of the dry powders (**Figure 2**). The sensor detected gluten in all samples with a target gluten content of 30 mg/kg and in 7 out of 9 samples with 20 mg/kg. One reason may have been inhomogeneity of sample K (rice/barley II), but this explanation does not apply to sample N (rice/durum wheat), because sample N had an exceptionally low RSD (0.5%) at 20 mg/kg. No gluten was detected in 3 out of 9 samples at 10 mg/kg in the samples containing rye (L), durum wheat (N) and spelt (O). At the 5 mg/kg level, the sensor returned the following results: 1 invalid, 2 gluten detected and 6 no gluten found. Out of the 108 triplicate tests with the sensor, there were 74 positive (68.5%) and 33 negative (30.5%) results, as well as 1 invalid result. Only 3 samples (J, L, and N, at 5 mg/kg) always showed a negative result, whereas either 1 or 2 out of 3 tests came back negative for the other samples with a gluten content from 5 to 20 mg/kg. At the threshold of 20 mg/kg, 2 out of 3 tests were negative for sample K and 1 out of 3 for sample N.

Analysis of Heated High-Starch Foods With Defined Gluten Content

Gluten recoveries assessed by ELISA lay between 28% (T, rice/barley, crumb, at 12 mg/kg) and 185% (S, rice/wheat, crust, at 3 mg/kg). As already reported for the unheated high-starch foods, some heated samples also had high RSD with up to 81% (V, rice/rye, crumb, at 18 mg/kg), but others as low as 4%, with most between 10 and 38%. The gluten sensor found gluten in almost all samples with a target gluten content of 12 mg/kg or higher, except for sample T at 12 and at 18 mg/kg (**Figure 3**). No gluten was detected in any of the samples at the 3 mg/kg level. This was according to expectations for samples T and V that also tested below 2 mg/kg by ELISA. Gluten detection might have been possible for the other samples at this level, because the gluten content analyzed by ELISA was 3.1 mg/kg or higher, but the sensor returned only negative results also after triplicate analysis. Two out of 6 samples (V and W) tested positive at the 6 mg/kg level at the first of three measurements and in 5 out of 6 tests in total. Of the samples that tested negative, the sensor found no gluten in 3 out of 3 replicates in samples T and U, whereas it found no gluten in 2 out of 3 replicates in sample R and in 1 out of 3 replicates in sample S. Overall, of the 90 tests performed, 55 (61%) came back as gluten found, 34 (38%) as no gluten detected and 1 as invalid. As observed before, the sensor detected gluten also well below 20 mg/kg.

Analysis of Foods With Unknown Gluten Content

A selection of commercially available foods with unknown gluten content was also tested with the sensor to study whether it could also detect fermented and partially hydrolyzed gluten. The gluten content was analyzed by competitive ELISA for

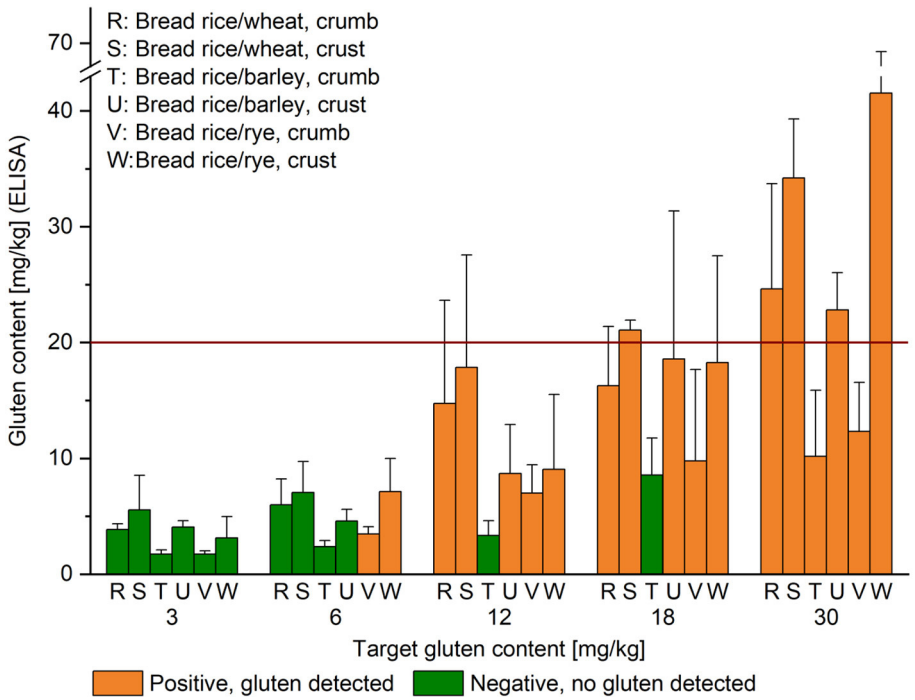

FIGURE 3 | Gluten content of heated high-starch foods. Target gluten content is indicated below the x-axis and gluten content analyzed by R5 sandwich ELISA on the y-axis; given as mean ($n = 3$) + standard deviation. The result of the sensor at the first of three consecutive measurements was either gluten detected (orange) or no gluten detected (green). The red horizontal line indicates the threshold for gluten-free products at 20 mg/kg.

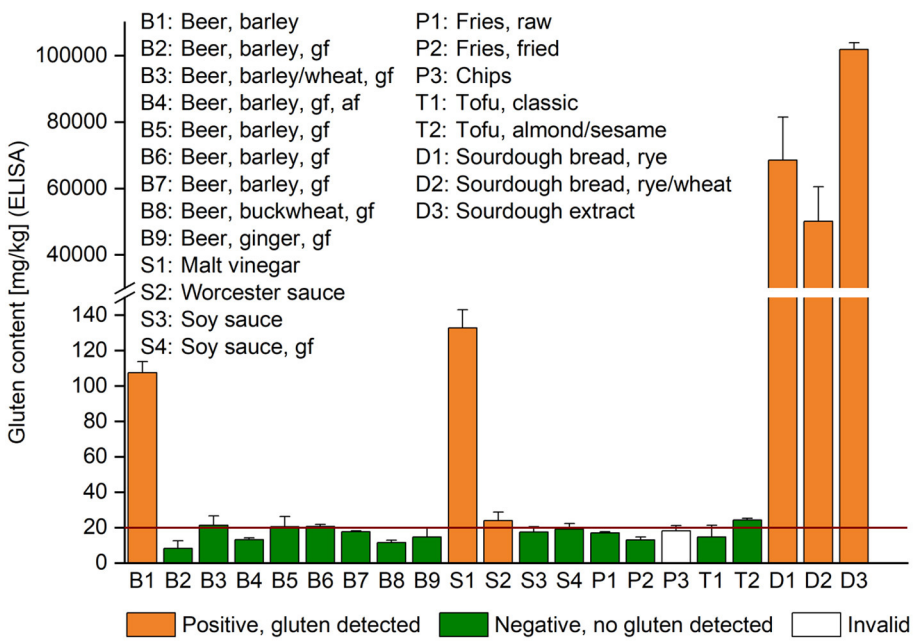

FIGURE 4 | Gluten content of commercially available products with unknown gluten concentrations. The content analyzed by R5 competitive ELISA is given as mean ($n = 3$) + standard deviation. The result of the sensor at the first of three consecutive measurements was either gluten detected (orange), no gluten detected (green) or invalid (white). af, alcohol-free, gf, product with a GF label. The red horizontal line indicates the threshold for gluten-free products at 20 mg/kg.

comparison (**Figure 4**). One regular barley-based beer (B1) had a gluten content of 107 mg/kg and it tested positive using the sensor. All other barley-based beers (B2-B7) had a GF label according to European legislation and they tested negative using the sensor. The ELISA values were between 8.2 mg/kg (B2) and 21.3 mg/kg (B3). Two beers from naturally GF raw materials were also included (B8, B9), but to our surprise, the ELISA still detected 11.6 and 14.7 mg/kg of gluten, whereas the sensor did not. Among the sauces, both sauces with gluten concentrations above 20 mg/kg (S1, S2) tested positive using the sensor, whereas the other two with 17.5 mg/kg (S3) and 19.1 mg/kg of gluten (S4) did not. All potato and tofu samples returned a negative or invalid result using the sensor, while the ELISA detected between 13.1 mg/kg (P2) and 24.2 mg/kg (T2) of gluten. None of these samples had a GF label. All three sourdough samples had extremely high gluten concentrations of 50,097 mg/kg or higher and they were clearly identified as gluten-containing samples using the sensor.

Influence of Sample Weight and High Gluten Content on the Results of the Sensor

There was no evidence for a dependence of the results on the sample weight from 0.1 to 1.5 g for samples C and H, because the sensor detected gluten in all cases. For sample C, even higher sample weights of up to 2.0 g were possible and the intensity of the test line relative to that of the control line increased with increasing sample weight. In case of sample H, 1.5 g was the maximum, because otherwise the viscosity became too high. Starch-rich foods T and U had a smaller working range from 0.3 to 0.9 g, because the capsules could not be closed anymore with higher amounts and the result using only 0.1 g came back negative.

The sensor detected gluten in all analyses of samples containing 1,000 mg/kg or even higher, as in the wheat, rye, and barley flours (**Figure 5**). However, while the intensity of the control line was mostly comparable, the intensity of the hook line was comparatively weak. The test line appeared intensely in all samples containing 1,000 mg/kg and it did not appear to make a difference whether the sample had been freeze-dried or not. When wheat or rye flours were tested directly, the test line was barely discernible, whereas barley flour seemed to be detected more clearly compared to wheat and rye.

Influence of Different Users on the Results of the Sensor

The results of the sensor showed systematic variability between different users that also appeared to depend on the sample (**Figure 6**). While all four users detected gluten in sample F in 11 out of 12 measurements, only one user consistently detected gluten in sample W, whereas all others did not. The results were even less reliable for samples R and U, because two users detected gluten in sample R, whereas two did not. For sample U, the results indicated that three out of four users detected gluten using the sensor in <33% of cases.

DISCUSSION

Of the 119 samples with gluten content ranging from 2 to 101,888 mg/kg tested in total, the sensor showed 80 positive (67.2%), 37 negative (31.1%) and 2 invalid results at the first of three consecutive measurements. When considering all three replicates amounting to 357 tests in total, the percentages remained similar, because there were 241 positive (67.5%), 113 negative (31.7%) and 3 invalid results. Therefore, we decided to focus on the first measurement, because users are unlikely to analyze the same food more than once due to time and cost limitations. About 50% of adults and 86% of teenagers agreed that the test was time-consuming (17) and some commented that the price per capsule was too high (18).

Our detection rate of 90% for samples containing ≥20 mg/kg was comparable to the 87.5% reported by Taylor et al. (11) but somewhat lower than the 99% (confidence interval 97.8–100%) claimed by the manufacturer (10). The sensor should report a GF result for samples containing <2 mg/kg and this was also the case in our study for samples R and T and for the GF raw materials (results not shown). However, samples with up to 18.0 mg/kg (K, intact gluten in the rice/barley flour mix) and 24.2 mg/kg (T2, most likely with partially hydrolyzed gluten) also returned a GF result. In case of sample K, this is deemed acceptable, because the gluten content was still below the regulatory threshold of 20 mg/kg, but not for sample T2. Regarding different sources of gluten, the sensor detected gluten from all species tested, but it appeared to be less sensitive to durum wheat (sample N).

Due to its sandwich design using two antibodies, the manufacturer acknowledges that the sensor may incorrectly show a negative result when fermented foods such as beer, soy sauce and malt extracts/flavorings are tested. Despite this, the test reported gluten in barley-based beer, malt vinegar, Worcester sauce and sourdough extracts. From the samples tested, it appeared that the sensor did detect partially hydrolyzed gluten in foods, but with lower sensitivity compared to intact gluten. This issue needs to be communicated very clearly to the users, because it is not always easy for them to determine whether a composite food containing gluten of unknown origin may contain partially hydrolyzed, fermented or fractionated gluten and may thus cause false-negative results. However, when users where asked to recall the device's testing limitations, nearly half of those asked could not correctly identify these limitations (17). This deficit in user knowledge and education needs to be addressed adequately to help prevent giving a false sense of security. A recent systematic review identified increased patient education/physician-patient communication and increased knowledge of a GF diet as the two most significant facilitators contributing to improved adherence to a GF diet, while lower knowledge of CD and restaurant dining/supermarket shopping were the two most significant barriers (19).

The result of "gluten found" in 56% of samples with <20 mg/kg was according to expectations (10, 11), but it is still likely to cause confusion and also unnecessary anxiety among users, because even samples with a GF label may test positive using the sensor. As trust in the results of the sensor was generally high, ranging from 77 to 100% in adults and

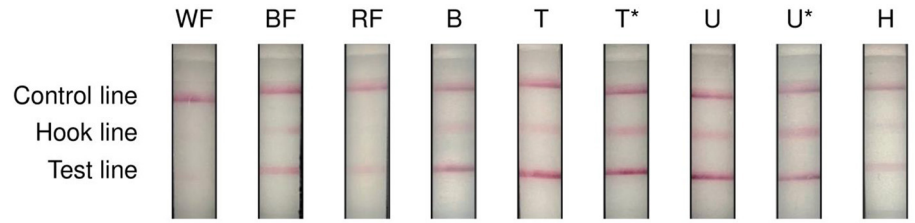

FIGURE 5 | Results of the sensor when testing high gluten concentrations. WF, wheat flour with 6.8% of gluten, BF, barley flour with 3.3% of gluten, RF, rye flour with 2.8% of gluten, B, meatball wheat, T, bread rice/barley, crumb, T*, bread rice/barley, crumb prior to freeze-drying, U, bread rice/barley, crust, U*, bread rice/barley, crust prior to freeze-drying, H, salad dressing, wheat, inhomogeneous. Samples B, T, T*, U, U* and H contained 1,000 mg/kg of gluten.

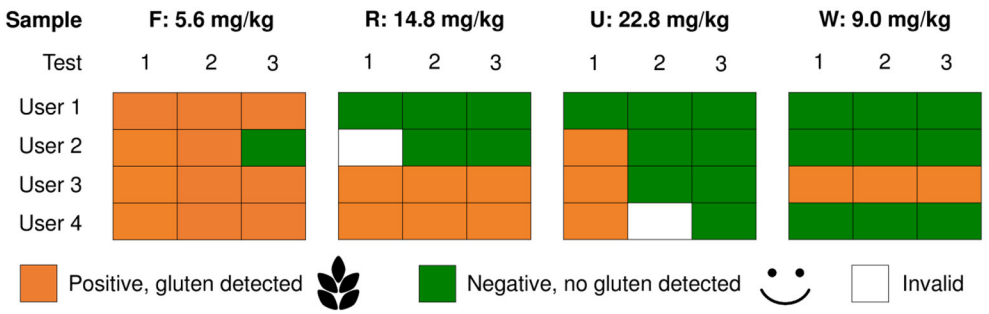

FIGURE 6 | Results of the sensor depending on the user. Samples F (meatball, wheat/rye/barley), R (bread rice/wheat, crumb), U (bread rice/barley, crust) and W (bread rice/rye, crust) were tested three times (test 1, 2 and 3) each by four different users.

teenagers, respectively, over 65% of users reported that the sensor indicated "gluten found" for foods that they had thought to be GF (17). Consequently, they did not eat these foods and might therefore limit an already restrictive diet even more. Developing a qualitative test with high diagnostic accuracy that classifies samples with a gluten content below 20 mg/kg as GF and those above as gluten-containing is certainly demanding. However, this should be encouraged for further improvements, because the result finally leads CD patients in their decision making whether to consume the food or not.

Using a gluten sensor may affect individual CD patients in different ways. More than 90% of both adults and teenagers agreed that it helped them follow a GF diet and gave peace of mind. CD quality of life (QOL) scores improved for adults, but remained unchanged for teenagers. In contrast, 43% of teenagers reported that using the sensor made them anxious (17). Future studies could be designed in a way to evaluate if using a gluten sensor contributes to more accurate gluten avoidance by CD patients compared to those that do not have access to any portable device. The connection between user experience with the sensor, CD QOL and long-term mucosal healing needs to be investigated further, as also suggested by Wolf et al. (18), especially in light of the ongoing debate of how strict a GF diet needs to be.

On the one hand, recent findings indicate that occasional and voluntary low level gluten consumption was not associated with the onset of CD symptoms, serology or histology in a group of asymptomatic adult CD patients (20). Moreover, strict compliance to a GF diet has been reported to decrease QOL compared to the general population and may be low in patients, especially during social events (21). On the other hand, the CD QOL score tended to be higher in patients adhering to the GF diet compared to non-compliant subjects (22). Therefore, the benefits and potential risks of using a portable gluten sensor need to be carefully evaluated and weighed to provide tailored individual recommendations to help CD patients manage their GF diet in the least restrictive way possible.

Acknowledged limitations of our study include a focus on protein- and starch-rich foods, small sample size and subsampling of foods, some of which had inhomogeneous gluten distribution. Further, all users were non-CD patients and they knew of their study participation. This introduces a bias toward very careful use of the sensor in an analytical laboratory setting as opposed to a real life setting. Therefore, the performance of the sensor is likely to be more reliable in our well-controlled study conditions compared to daily routine use, e.g., in a restaurant or a canteen.

Overall, the performance of the sensor was acceptable in our study, but the systematic variation observed between different users was concerning. This could be related to difficulties with inserting a food sample of appropriate size or difficulty in closing the capsules without using the wrench, as has been reported by users (18). Further testing of the same samples with a higher number of different users would also be helpful to identify systematic factors affecting the

results obtained with the sensor and improve instructions for use.

For some samples, repetitive testing gave inconsistent results, most likely due to inhomogeneous distribution of gluten in the sample. Correct sampling directly affects testing reliability, but it is difficult to issue clear guidance for composite foods, such as those present in a real life restaurant setting. This is a general point that limits the applicability of handheld or smartphone-based devices in the hands of CD or food allergy patients.

In conclusion, the gluten sensor may be useful for CD patients to test foods for peace of mind, especially when eating out. The handheld device comes with a charging cable and is easy to carry during travel. However, user education is of critical importance and has to be improved, because users need to be aware of testing limitations, such as the effect of sampling and the potential occurrence of partially hydrolyzed, fractionated or fermented gluten.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AM: formal analysis, investigation, data curation, and writing—review and editing. KS: conceptualization, funding acquisition, resources, supervision, and writing—original draft. All authors reviewed and approved the final manuscript.

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ORIGINAL RESEARCH
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Perennial Ryegrass Contains Gluten-Like Proteins That Could Contaminate Cereal Crops

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Background: To ensure safe consumption of gluten-free products, there is a need to understand all sources of unintentional contamination with gluten in the food chain. In this study, ryegrass (*Lolium perenne*), a common weed infesting cereal crop, is analysed as a potential source of gluten-like peptide contamination.

Materials and Methods: Ten ryegrass cultivars were analysed using shotgun proteomics for the presence of proteins from the prolamin superfamily. A relative quantitative assay was developed to detect ryegrass gluten-like peptides in comparison with those found in 10 common wheat cultivars.

Results: A total of 19 protein accessions were found across 10 cultivars of ryegrass for the protein families of PF00234-Tryp_alpha_amyl, PF13016-Gliadin, and PF03157-Glutenin_HMW. Protein and peptide homology searches revealed that gliadin-like peptides were similar to avenin and gamma-gliadin peptides. A total of 20 peptides, characteristic of prolamin superfamily proteins, were selected for liquid chromatography mass spectrometry (LC-MS) with multiple reaction monitoring (MRM). Only two of the monitored peptides were detected with high abundance in wheat, and all others were detected in ryegrass. Glutenin and alpha-amylase/trypsin inhibitor peptides were reported for the first time in ryegrass and were noted to be conserved across the Poaceae family.

Conclusion: A suite of gluten-like peptides were identified using proteomics that showed consistent abundance across ryegrass cultivars but were not detected in wheat cultivars. These peptides will be useful for differentiating wheat gluten contamination from ryegrass gluten contamination.

Keywords: wild grass, cereal, ryegrass, gluten, wheat, proteomics, LC-MS/MS

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INTRODUCTION

Gluten proteins are the most abundant proteins found in commercial cereal grains, including wheat, barley, rye and oats (1). Consumption of these proteins will trigger gluten-related disorders (GRD) in ∼100 million people globally (2, 3). At least six GRDs (4) have been described; these may be autoimmune, allergic, or neither and are caused by a mix of environmental and genetic factors

(4, 5). Coeliac disease (CD) is the most recognised GRD, which is currently diagnosed based on serology and small intestinal biopsies and is estimated in 0.7–1.4% of the global population (2). Affected individuals are genetically susceptible to generate an autoimmune inflammatory response in the small intestine when exposed to gluten proteins. Long-term exposure results in chronic intestinal inflammation and villi degradation for these individuals (6).

The only treatment for GRDs is to avoid the intake of trigger proteins, i.e., commit to a life-long gluten-free diet (GF-diet), although this is far from simple or easy. Complications arise through the difficulty in avoiding gluten-containing additives, inadvertent gluten intake, and food contamination. Gluten is frequently included in nongrain-based items such as sausages, salad dressing, imitation meats, and even some medications (7, 8). Therefore, the GF-diet mainly consists of fruits, vegetables, legumes, meat, seafood, nuts, dairy and bakery products, which include GF cereals or pseudocereals, such as rice, corn, quinoa and millet (9, 10). Nevertheless, these products may contain hidden gluten due to unintentional contamination through the food supply chain.

Cross-contamination within the food supply chain can happen at different stages, such as production, milling, export and retail. There are multiple possibilities from the moment the grain is grown until the GF-flour is packed, including agricultural co-mingling through crop rotation, storage and transport. Some studies provide examples in the contamination of GF-oats with gluten-containing cereals during harvest (11–13). One further aspect in this regard concerns the contamination of GF products due to weeds growing in the field, a topic that is under-researched. Weed management in crops is a challenging task. Farmers spend thousands of dollars each year in an effort to control weed invasion; however, this outcome is not always completely accomplished, and farmers must deal with weed seed contamination, which can become a serious problem for GF-cereals and other crops that are supposed to be free from gluten, such as pulses (14).

One of the most common weed seeds found in cereal samples is ryegrass (genus *Lolium*) (14). This grass is the most common weed infesting cereal grain fields in Australia and has small dense seeds that are difficult to eliminate during automated grain cleaning (14, 15). Ryegrass belongs to the same Poaceae grass family as wheat and other gluten-containing crops, wherein the storage proteins primarily comprise gluten-like proteins. Ryegrass has been subjected to Western blotting followed by liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) analysis, demonstrating that it contains proteins with structural similarity with gluten proteins (15).

Herein, a combination of discovery and targeted proteomic analyses were undertaken to confirm the presence of gluten-like proteins in ryegrass cultivars. A targeted LC-MS/MS assay was developed and used to assess relative levels of target peptides across 10 ryegrass cultivars and 10 wheat cultivars to identify the differences in peptide abundance patterns and to identify possible ryegrass-specific peptide markers.

This study investigates whether gluten contamination can potentially originate from sources other than traditional cereal grains, such as field contaminants. Understanding the possible origins of gluten contamination and establishing identification and quantification methods for these new protein species could help to provide a more accurate characterisation of food and assure food safety for the population affected by GRDs.

MATERIALS AND METHODS

Sample Material

Grain samples from 10 ryegrass and 10 wheat cultivars were obtained (**Supplementary Table 1**) from the Australian pasture collection and Australian Winter Cereals Collection (Tamworth, Australia). All samples were manually inspected to exclude foreign seed contamination. Flour samples were obtained by milling with a Metefem Hungarian Mill (model FQD2000, Hungary).

Protein Extraction and Digestion

Methods were performed according to the study of Bose et al., with minor changes (16). Flour (20 mg) was weighed (four replicates of wheat and three replicates of ryegrass) and mixed with 200 μL of 55% isopropanol and 2% dithiothreitol (IPA/DTT). Samples were vortexed and sonicated for 5 min and then incubated on a thermo heating mixer block (Thermo Scientific) at 300 rpm at 65°C for 45 min. The mixtures were centrifuged for 15 min at 20,800 ×g, and the supernatants were transferred to Protein LoBind Tubes (Eppendorf). The protein content was quantified *via* Bradford assay, and 200 μg of protein was loaded onto 10-kDa molecular weight cut-off (MWCO) filters (Millipore, Sydney, Australia). Aliquots were adjusted to 200 μL with UA buffer (8 M urea in 0.1 M Tris-HCl, pH 8). The filter content was washed two times with 200 μL UA buffer followed by centrifugation at 20,800 × g for 15 min at room temperature (RT).

Alkylation of cysteines was performed by the addition of 100 μL of 50 mM iodoacetamide (in UA buffer) and incubation in the dark at RT, with 300 rpm shaking for 20 min. Samples were centrifuged for 15 min at 20,800 × g and then washed with 200 μL UA buffer and centrifuged again, as previously described. Flowthrough was discarded and the buffer was changed to 50 mM AmBic (ammonium bicarbonate, pH 8.0) by washing the filters two times with 200 μL of this digestion buffer followed by centrifugation. Filters were transferred to fresh collection tubes. Protein digestion was achieved by adding 200 μL of trypsin (Promega, Wisconsin, USA) prepared as 200 μg/mL in 50 mM AmBic (pH 8.0) containing 1 mM $CaCl_2$ and incubated overnight in the dark at 37°C with 300 rpm shaking. Sample filters were centrifuged at 20,800 × g for 15 min, followed by two washes with 200 μL of AmBic (pH 8.0) and centrifugation. Filtrates were evaporated to dryness in a Savant SpeedVac concentrator (Thermo Scientific, USA). Peptides were reconstituted in 0.1% formic acid to a protein concentration of 1 μg/μL for LC-MS/MS analysis.

Mass Spectrometry and Protein Identification

Liquid chromatography mass spectrometry was performed using an Ekspert nanoLC415 (Eksigent, Dublin, CA) coupled to a TripleTOF® 6600 mass spectrometer (SCIEX, Redwood City,

CA, USA). The specifications of the acquisition parameters have been previously described (15, 16).

Discovery data was generated for 10 ryegrass cultivars, and the spectra were searched against the Poaceae grass family subset of the UniProt database (version 2021/01) appended with the common repository of adventitious proteins. The database from a higher taxonomic group was used due to poor representation of the *Lolium perenne* proteome (748 nonredundant protein sequences). The UniProt UniRef 100 redundancy reduction was applied to remove Poaceae proteins with 100% sequence identity, leading to a total of 1,953,474 protein sequences.

ProteinPilot v5.0.3 software (SCIEX) encompassing the Paragon and ProGroup algorithms (17) was used to identify peptides, infer proteins, and generate false discovery rate (FDR) reports. Results from discovery analysis were curated using an in-house script (git-hub/Sophia-006[1]) (18). To ensure quality in the identification of gluten proteins and peptides, the following curation parameters were applied: 1% FDR or 99% peptide confidence, requisite tryptic and semi-tryptic peptides, up to two missed cleavages, and variable modifications of glutamine to pyro-glutamic acid, carbamidomethyl cysteine, and oxidation of methionine.

Protein summaries (**Supplementary Data 1**) were analysed to identify proteins of interest, i.e., gluten proteins. In this regard, a protein family (Pfam) search was performed that encompassed searching for three specific domains: Gliadin (PF13016), Glutenin_HMW (PF03157), and Tryp_alpha_amyl (PF00234). The Pfam search was performed using profile hidden Markov models 3 (HMMER3) (19). Protein and peptide homology searches were performed using the BLAST algorithm and Peptide Search tool available at UniProt[2], respectively.

Proteins that contained gliadin, glutenin_HMW, and Tryp_alpha_amyl domains were selected for targeted assay development. These protein sequences were gathered into a FASTA file that was used to construct a table of peptide multiple reaction monitoring (MRM) transitions using Skyline Software (MacCoss Lab Software, Washington, USA) (20). MRM transitions were determined for each peptide. Peptides were selected for MRM based on the following criteria: (1) tryptic or semi-tryptic; (2) identified with 95% confidence; (3) unmodified or common modifications only; and (4) high peak signal intensity. Transitions for each selected peptide were prioritised from the acquired discovery data, including precursor ion (Q1) and fragment ion (Q3) m/z, and rolling collision energy (**Supplementary Data 2**). In total, 20 peptides were selected for MRM experiments. Four semi-tryptic peptides were included; their fully tryptic versions were not included in the final MRM due to insufficient evidence in the discovery data. Three MRM transitions were monitored per peptide based on the intensity and lack of interferences.

Digested peptides were separated with a Shimadzu Nexera UHPLC (Rydalmere, Australia) and analysed with a 6500 QTRAP mass spectrometer (SCIEX), as described previously (15, 16). Relative quantitation was achieved using scheduled MRM scanning experiments with a 60-s detection window for each MRM transition with retention time as determined in preliminary MRM experiments and a 0.6-s cycle time. Peaks were integrated using Skyline, wherein all three transitions were required to coelute at the same retention time (min) with a signal-to-noise ratio (S/N) > 3. The peak areas for the three monitored MRM transitions were summed. The mean, SD, and co-efficient of variation (CV) were calculated for technical replicates for each peptide (**Supplementary Data 3**). Batch and injection order effects were removed from the data by monitoring external standards interspersed with the unknown samples while retaining the differences between ryegrass and wheat samples.

Graphs were generated in the R statistical computing environment using the ggplot package (21) and Morpheus[3] (Broad Institute, Cambridge MA, USA).

RESULTS

Gluten Protein Identification Yield From Shotgun Proteomics

Ten varieties of *L. perenne* were studied (**Supplementary Table 1**) to confirm the suspected presence of gluten in this species. For the identification of gluten proteins, high-resolution data acquisition and database searching was performed using a UniProt database from a higher taxonomic group belonging to the family Poaceae. The total number of proteins identified with 99% confidence between the 10 varieties of ryegrass varied between 160 and 316 (**Figure 1A**). Additionally, peptides discovered with tryptic digestion varied between 205 to 503 (**Figure 1A**).

Next, a protein family domain search was performed for each of the cultivars with the aim to find proteins containing domains characteristic of gliadin, glutenin_HMW, and tryp_alpha_amyl domain families. The results revealed that between three to eight candidate proteins were found for each of the cultivars (**Supplementary Data 4**). A total of 19 protein accessions were found among the 10 varieties of ryegrass. A table showing positive identification of proteins representing each domain family across the varieties is provided in **Supplementary Data 4**. For the tryp_alpha_amyl-domain containing protein family (**Figure 1B**), the candidate protein with the highest number of identification occurrences was UniProt accession C3TX90 (present in nine varieties), followed by proteins T1WIP3 (present in six varieties) and A8VZG4 (present in three varieties). The lowest number of identification occurrences were for A0A0P0Y6A6 (present in two varieties) and for proteins D7F5I5, Q84VT9, and T1WI30 (present in one variety). The gliadin-domain containing protein family (**Figure 1B**) was represented by the proteins G8ZCU8 (present in all the varieties) and I4EP61 (present in six varieties). Further gliadin-domain containing members were detected between one and three varieties (I4EP57, F2X0K8, I4EP62, J9QGY5, B6DQD5, Q84U17, F2X322, and B6UKP4). The glutenin_HMW-domain containing protein family (**Figure 1B**) was the family with the lowest representation

[1]https://github.com/Sophia-006/FDR_proteins_peptides.git
[2]Uniprot.org
[3]https://software.broadinstitute.org/morpheus/

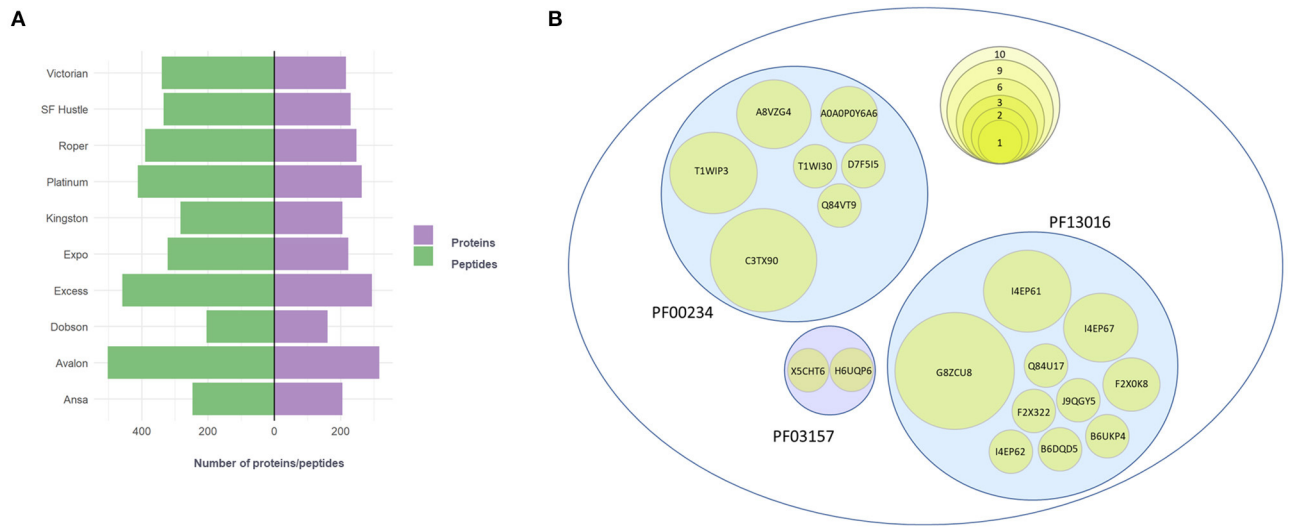

FIGURE 1 | (A) Number of proteins (purple) and peptides (green) detected at 1% FDR (99% confidence) for 10 ryegrass cultivars. **(B)** Proteins identified with the protein family search (Pfam) that belong to three specific families: PF00234-Tryp_alpha_amyl, PF13016-Gliadin, and PF03157-Glutenin_HMW. The size of the circle represents the number of identification occurrences among 10 cultivars of ryegrass.

with two candidate proteins, H6UQP6 and X5CHT6, detected in one ryegrass variety.

Of note, these domains could represent multiple protein types due to the mixed nature of gluten-type proteins, i.e., families which comprise a range of proteins that have the Tryp_alpha_amyl domain or the gliadin-domain are not necessary α-amylase/trypsin inhibitor proteins or gliadins. As such, additional protein and peptide homology searches were performed to determine the full-length homology of these proteins.

Gluten proteins tend to have conserved regions, and further bioinformatic analysis is necessary to determine which peptides could function as peptide markers to differentiate between grass species. As a result, candidate proteins and peptides were subject to searches to identify non-ryegrass orthologues, i.e., to determine the potential for these peptide sequences to be observed in additional species. In consideration of the limitations of mass spectrometric detection, search settings included leucine and isoleucine equivalence (isobaric amino acids) and a requisite C-terminal arginine or lysine (trypsin cleavage site).

Results of the searches are shown in **Table 1**, and a detailed table specifying the species within the tribe identity match can be found in **Supplementary Table 2**. Peptides SQILQQSSCQVMR (G8ZCU8), CPAIHSVVQAIILQK (I4EP61), QFLVQQCSPVAEVPFLR (I4EP61), and QQAQFEGMR (I4EP57) were found to belong exclusively to avenins present in oats, while peptides QQCCQQLAQIPQQLR (F2X0K8) and APFASIVASIGGQE (F2X322) belong to gamma-gliadins that are present in wheat. An additional gamma-gliadin peptide, APFASIVAGIGGQYR (B6DQD5), was found in species of the Triticeae tribe.

Two peptides, QQCCQQLAQIPEQSR and SQMLQQSSCHVIR, from the protein J9QGY5, which is a low molecular weight glutenin (LMW-GS), belong exclusively to the wild grass species *Dasypyrum villosum* of the Triticeae tribe. HMW glutenin (HMW-GS) peptides DVSAKCRPVAVSQVAR, ELQESSLEACRQVVDQQLAGR, and QLQCERELQESSLEACR (X5CHT6) were moderately conserved and found across species of the Triticeae tribe. The peptide DGSFYPGEATPPQQLQQR (H6UQP6) was exclusively found in *Elymus libanoticus*, a species that belongs to the Triticeae tribe.

Peptide EGMEVFPGCR (T1WIP3) was found exclusively in the species *Elymus grandis* (Triticeae); peptide LTAASVPAVCK (T1WI30) was found in several species of the Triticeae tribe. The peptide LLQQQLNPCR (A8VZG4) was found in an α-gliadin sequence of species *Dasypyrum hordeaceum* (Triticeae). For A0A0P0Y6A6, which presented protein homology to a lipid-transfer protein, the conserved peptide TACNCLK was found in species of families Poaceae, Fabaceae, Asteraceae, among others; however, the peptide CGVSIPYTISPSIDCSR was exclusively found in species of rice of tribe Oryzeae. The two peptides DPYYEQCPMRK and SDLYGPNLQGEVTMLMER (C3TX90), from a puroindoline-like protein were found exclusively in *Brachypodium sylvaticum* of tribe *Brachypodieae*. Peptide QLSQIAPQCR (D7FSI5), characteristic of puroindolines and hordoindolines, was present in several species of the Triticeae tribe.

Gluten-Like Peptide Quantification in Wheat and Ryegrass

Peptides characteristic of gluten-like proteins were selected for LC-MRM-MS analysis. Based on the results obtained from homology searches, 20 peptides from 13 proteins were measured with a relative quantitative analysis (**Supplementary Data 5**). The selected peptides were measured in 10 cultivars of perennial ryegrass (*L. perenne*) to determine the abundance of gluten

TABLE 1 | Protein and peptide homology search results.

Peptide sequence	Protein accession	Protein family domain	Protein homology (from BLAST)	Peptide search 100% identity match (Tribe)
SQILQQSSCQVMR	G8ZCU8	PF13016	Avenin	Aveneae
CPAIHSVVQAIILQK	I4EP61	PF13016	Avenin	Aveneae
QFLVQQCSPVAEVPFLR	I4EP61	PF13016	Avenin	Aveneae
AFALQALPAMCDVYVPPHCSVA	I4EP61	PF13016	Avenin	Aveneae
QQAQFEGMR	I4EP57	PF13016	Avenin	Aveneae
QQCCQQLAQIPQQLR	F2X0K8	PF13016	Gamma-gliadin	Triticeae
APFASIVASIGGQE	F2X322	PF13016	Gamma-gliadin	Triticeae
APFASIVAGIGGQYR	B6DQD5	PF13016	Gamma-gliadin	Triticeae
QQCCQQLAQIPEQSR	J9QGY5	PF13016	LMW-glutenin	Triticeae
SQMLQQSSCHVIR	J9QGY5	PF13016	LMW-glutenin	Triticeae
DVSAKCRPVAVSQVAR	X5CHT6	PF03157	HMW-glutenin	Triticeae
ELQESSLEACRQVVDQQLAGR	X5CHT6	PF03157	HMW-glutenin	Triticeae
ELQESSLEACR	X5CHT6	PF03157	HMW-glutenin	Triticeae
QLQCERELQESSLEACR	X5CHT6	PF03157	HMW-glutenin	Triticeae
DGSFYPGEATPPQQLQQR	H6UQP6	PF03157	HMW-glutenin	Triticeae
RCCDELSAIPAYCR	Q84VT9	PF00234	Trypsin inhibitor	Triticeae
LQCVGSQVPEAVLR	T1WIP3	PF00234	Dimeric alpha-amylase inhibitor	Triticeae
EGMEVFPGCR	T1WIP3	PF00234	Dimeric alpha-amylase inhibitor	Triticeae
LLQQQLNPCR	A8VZG4	PF00234	Alpha-gliadin	Triticeae
LTAASVPAVCK	T1WI30	PF00234	Dimeric alpha-amylase inhibitor	Triticeae
TACNCLK	A0A0P0Y6A6	PF00234	Nonspecific lipid-transfer protein	Highly conserved. Families: Poaceae, Fabaceae, Asteraceae
CGVSIPYTISPSIDCSR	A0A0P0Y6A6	PF00234	Nonspecific lipid-transfer protein	Oryzeae
DPYYEQCPMRK	C3TX90	PF00234	Puroindoline-like protein	Brachypodieae
SDLYGPNLQGEVTMLMER	C3TX90	PF00234	Puroindoline-like protein	Brachypodieae
QLSQIAPQCR	D7FSI5	PF00234	Puroindoline, Hordoindoline	Triticeae

Peptide sequence, protein accession and protein family are specified. Protein homology refers to all protein types where the peptide was found. Peptide search results refer to all tribes where the peptide was found with a 100% identity match.

candidates. At the same time, these same peptides were measured in 10 wheat varieties to compare the abundance between these two species, aiming to discover peptides that can differentiate ryegrass from wheat.

The heatmap (**Figure 2**) shows the logarithmic relative abundance of the measured peptides across 10 ryegrass cultivars ($n = 3$) and 10 wheat cultivars ($n = 4$). The complete Euclidean linkage method was used for hierarchical clustering, which shows clear stratification of ryegrass and wheat. Protein family domain membership for each protein is also specified within the graph. The analysis revealed the peptides measured by LC-MRM-MS in ryegrass cultivars had low abundance or were not detected in wheat.

Relative Quantitation of Gluten Proteins Across Ten Cultivars of Ryegrass

Except for the peptides mentioned in the section Gluten-like peptide quantification in wheat and ryegrass above, the remaining peptides were only quantified in ryegrass. Their summary MRM peak area results are shown in **Figures 3**–**5** according to their protein family domain membership. The biological variation between cultivars is presented in **Supplementary Data 6**.

Results for the gluten-like peptides of family PF13016 Gliadin domain-containing proteins (akin to avenin-like proteins and gamma-gliadins) are shown in **Figure 3**. Peptide SQILQQSSCQVMR (**Figure 3A**) from protein G8ZCU8 showed high variability across ryegrass cultivars with the highest abundance in the cultivar Victorian. Peptide QQAQFEGMR (**Figure 3B**) from protein I4EP57 showed variability among all cultivars of ryegrass with a biological co-efficient of variation (CV) of 32%. Peptide AFALQALPAMCDVYVPPHCSVA (I4EP61) varied across the ryegrass cultivars and showed high values; however, CPAIHSVVQAIILQK and QFLVQQCSPVAEVPFLR (I4EP61) (**Figure 3C**) showed lower values but were consistently found among all ryegrass cultivars, with a biological CV of <20%. The peptide QQCCQQLAQIPQQLR (**Figure 3D**) from protein F2X0K8 shows moderate variance across the cultivars, showing higher values for Dobson and Victorian. Peptide APFASIVASIGGQE from F2X322 (**Figure 3E**) was consistent across all ryegrass cultivars showing a CV of 20%. Peptide APFASIVAGIGGQYR from protein B6DQD5 was variable among ryegrass cultivars and was higher in cultivars Expo

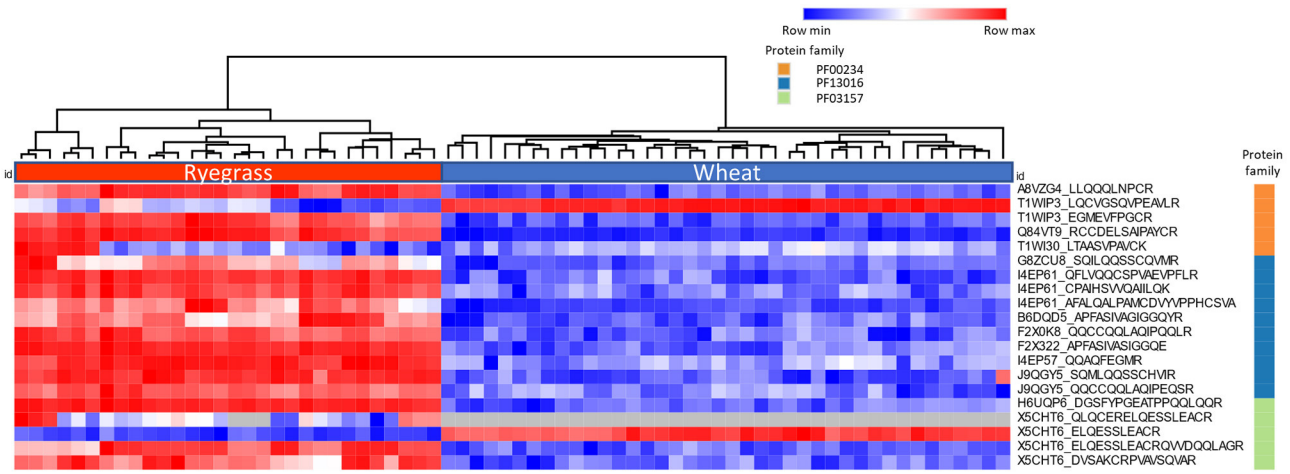

FIGURE 2 | The Heatmap and hierarchical clustering show the relative abundance of the peptides representing two major groups detected across 10 ryegrass cultivars ($n = 3$) and 10 wheat cultivars ($n = 4$). The colour in each cell represents the log (peak area) of each peptide monitored (red = max value, blue = min value, grey = NA). The column to the right indicates the protein family membership for the peptides (orange = PF00234-Tryp_alpha_amyl family, blue = PF13016-Gliadin, green = PF03157-Glutenin_HMW).

and SF Hustle. From protein J9QGY5, two peptides were measured (**Figure 3G**): QQCCQQLAQIPEQSR was not only more abundant but also more variable across cultivars and was higher in the Dobson cultivar and SQMLQQSSCHVIR was in lower abundance but was consistent (CV <25%) across ryegrass cultivars.

Figure 4 shows the quantitation of peptides from proteins belonging to the family PF03157 HMW-glutenin. Peptides belonging to two proteins, namely X5CHT6 and H6UQP6, were measured. Three peptides were quantified from protein X5CHT6, each showing high variability across the cultivars. Peptide DVSAKCRPVAVSQVAR showed higher abundance in Kingston, Platinum, Roper and SF Hustle; peptide ELQESSLEACRQVVDQQLAGR was highest in abundance in Dobson, Excess and SF Hustle, while peptide QLQCERELQESSLEACR was the highest in Victorian (**Figure 4A**). The second HMW glutenin, protein H6UQP6, was quantified using peptide DGSFYPGEATPPQQLQQR with low biological variance across cultivars with a CV < 15% (**Figure 4B**).

The detection of peptides from PF00234 Tryp_alpha_amyl domain-containing proteins are shown in **Figure 5**; these proteins were also shown to have homology to gluten-like proteins (**Table 1**). One peptide EGMEVFPGCR was observed to be variable across ryegrass cultivars. This peptide is found in protein T1WIP3, a dimeric alpha-amylase inhibitor (**Figure 5A**). Peptide LLQQQLNPCR was variable across ryegrass cultivars and is present in protein A8VZG4, which is an α-gliadin (**Figure 5B**). Another peptide LTAASVPAVCK showed variable abundance with high levels in cultivars Roper and Victorian. This peptide is found in protein T1WI30, a dimeric alpha-amylase inhibitor (**Figure 5C**). Peptide RCCDELSAIPAYCR (detected as protein Q84VT9, a trypsin inhibitor) showed good signal intensity but varied across the ryegrass cultivars (**Figure 5D**).

DISCUSSION

Ryegrass (genus *Lolium*) has been identified as one of the most challenging weeds for cropping systems due to its ability to resist herbicides, consequently affecting different farming practises (22). Furthermore, preliminary investigation of ryegrass proteins has revealed its potential as a source of gluten contamination (15, 23, 24). In this regard, an isolation and purification method for the prolamin fraction of ryegrass grains, which was named loliin, was established as early as the 1930s (23). This study preceded the study of Shewry in 1986, who characterised prolamins from different grasses, including *L. perenne*, and established homology of ryegrass γ-prolamins to those from wheat, barley and rye. More recently, Colgrave et al. revealed the reactivity of ryegrass prolamins to the anti-gliadin antibody and identified possible antigenic proteins through LC-MS analysis of gel-separated proteins (15).

Herein, we report the identification of prolamin super-family peptides in ryegrass cultivars and their quantitation when compared with wheat, with the aim to measure differences in peptide abundance and identify potential peptide markers of ryegrass contamination. To this end, grain proteins from 10 cultivars of diploid perennial ryegrass (*L. perenne*) were processed to enrich for seed storage proteins and measured by LC-MS/MS in the search for gluten-like sequences.

Shotgun proteome measurement and database searching were used for the initial discovery of gluten-like protein sequences. The protein search database comprised the public sequences of the Poaceae family proteins from UniProt, a higher taxonomic group for wheat and ryegrass representing diverse genetic

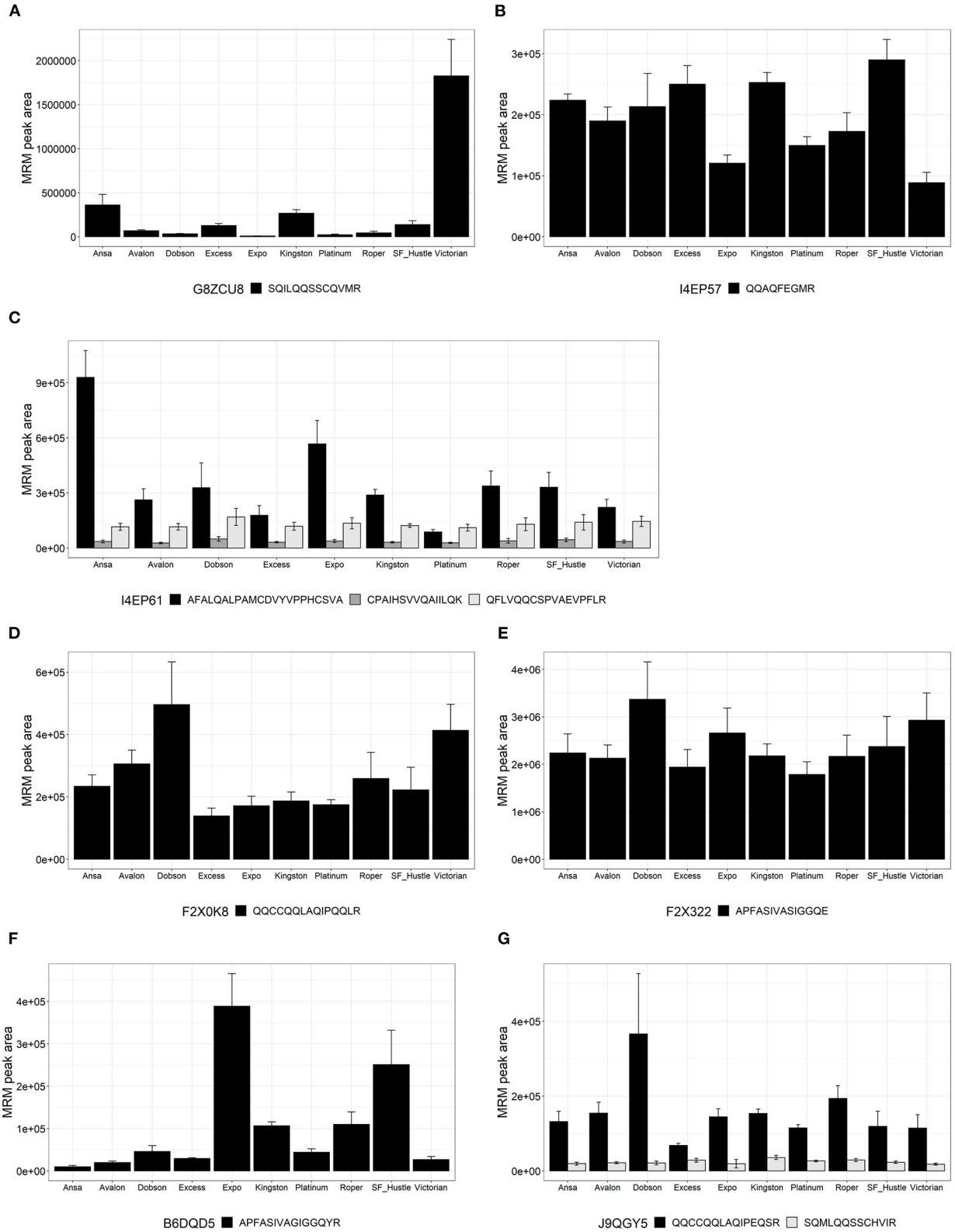

FIGURE 3 | Relative quantitation expressed as multiple reaction monitoring (MRM) peak area for peptides from proteins of the family PF13016 across 10 cultivars of *Lolium perenne*. Data are presented as the mean ± SD ($n = 3$) with one to three peptides from each protein. **(A)** Avenin protein (G8ZCU8); **(B)** Avenin (I4EP57); **(C)** Avenin (I4EP61); **(D)** Gamma-gliadin (F2X0K8); **(E)** Gamma-gliadin (F2X322); **(F)** Gamma-gliadin (B6DQD5); and **(G)** LMW-glutenin (J9QGY5).

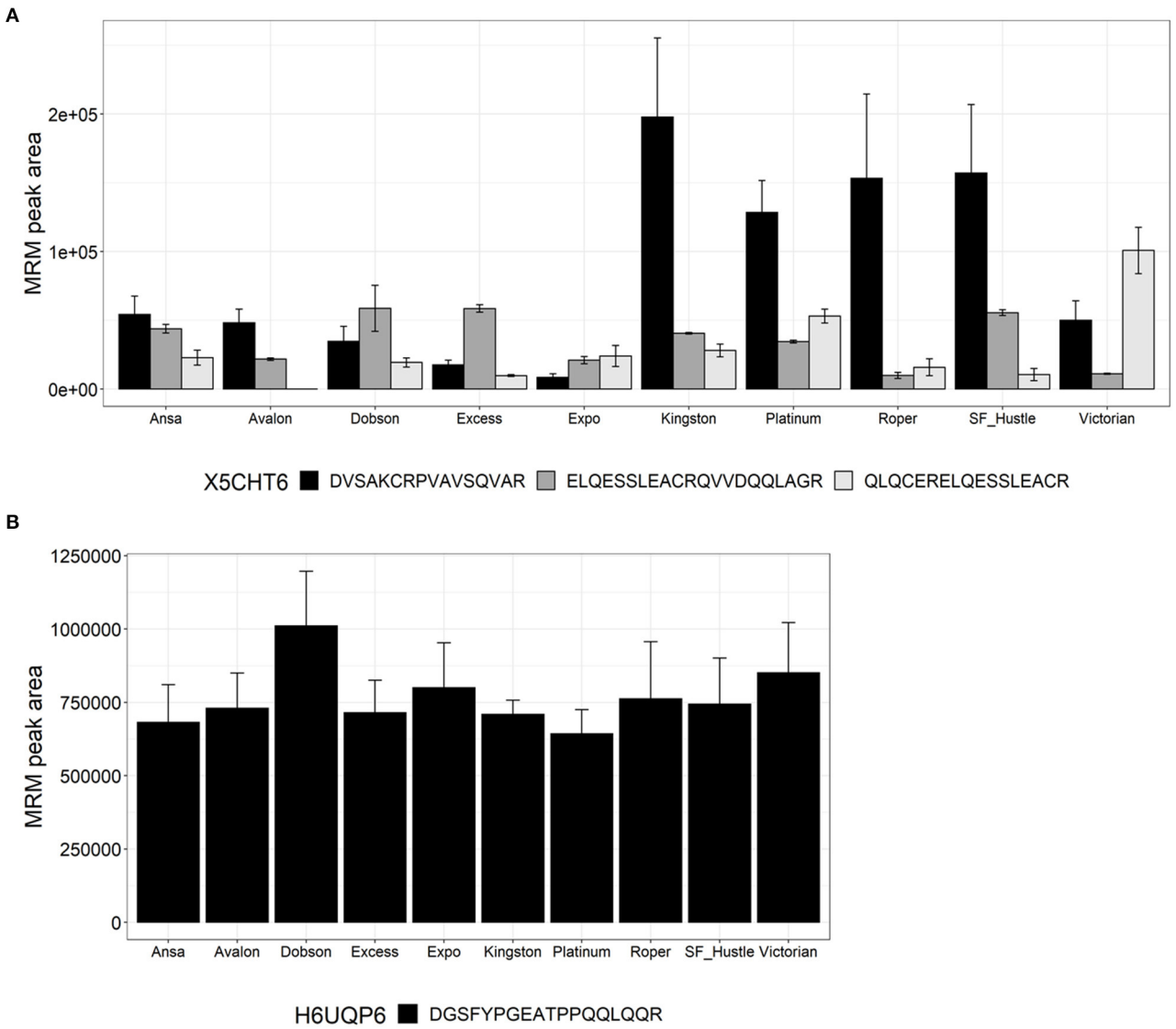

FIGURE 4 | Relative quantitation of glutenin peptides across the 10 varieties of *L. perenne* from proteins of the family PF03157. Data are presented as the mean ± SD ($n = 3$) with one to three peptides from each protein. **(A)** HMW-glutenin (X5CHT6); and **(B)** HMW-glutenin (H6UQP6).

variations. Previous proteomics studies in *L. perenne* have used a different database approach where *Brachypodium distachyon* sequences were searched to successfully characterise drought response in the leaves from this grass (25); because of the focus on this compartment, no gluten-like proteins were identified in this study. No other studies besides Colgrave et.al. reported proteome measurement in ryegrass seeds (15).

The discovery strategy herein was complemented by a protein family domain search identifying three to eight gluten-like proteins in each ryegrass cultivar (**Supplementary Data 4**). This number is lower when compared with the frequency of gluten proteins identified when searching traditional cereal grain data (e.g., wheat, rye, barley) against the same Poaceae subset of the UniProt-KB database, which typically varies between 5 and 47 gluten proteins (26, 27). However, the gluten-like protein sequences in ryegrass may differ from those in wheat and other Poaceae members. Therefore, further investigation is needed to precisely characterise the prolamins in ryegrass. Nevertheless, the strategy to determine gluten-like proteins through detecting the protein family domains for Gliadin, Glutenin_HMW, and Tryp_alpha_amyl revealed 19 protein accessions representing these families, including: 10 gliadin-domain containing proteins, 2 glutenin_HMW domain containing proteins, and 7 Tryp_alpha_amyl domain-containing proteins (**Figure 1**). Due to a paucity of data (protein sequences) in the *Lolium* subset of the UniProt database (748 nonredundant protein sequences), the identification of gluten-like peptides is likely not exhaustive and has led to the detection of proteins from

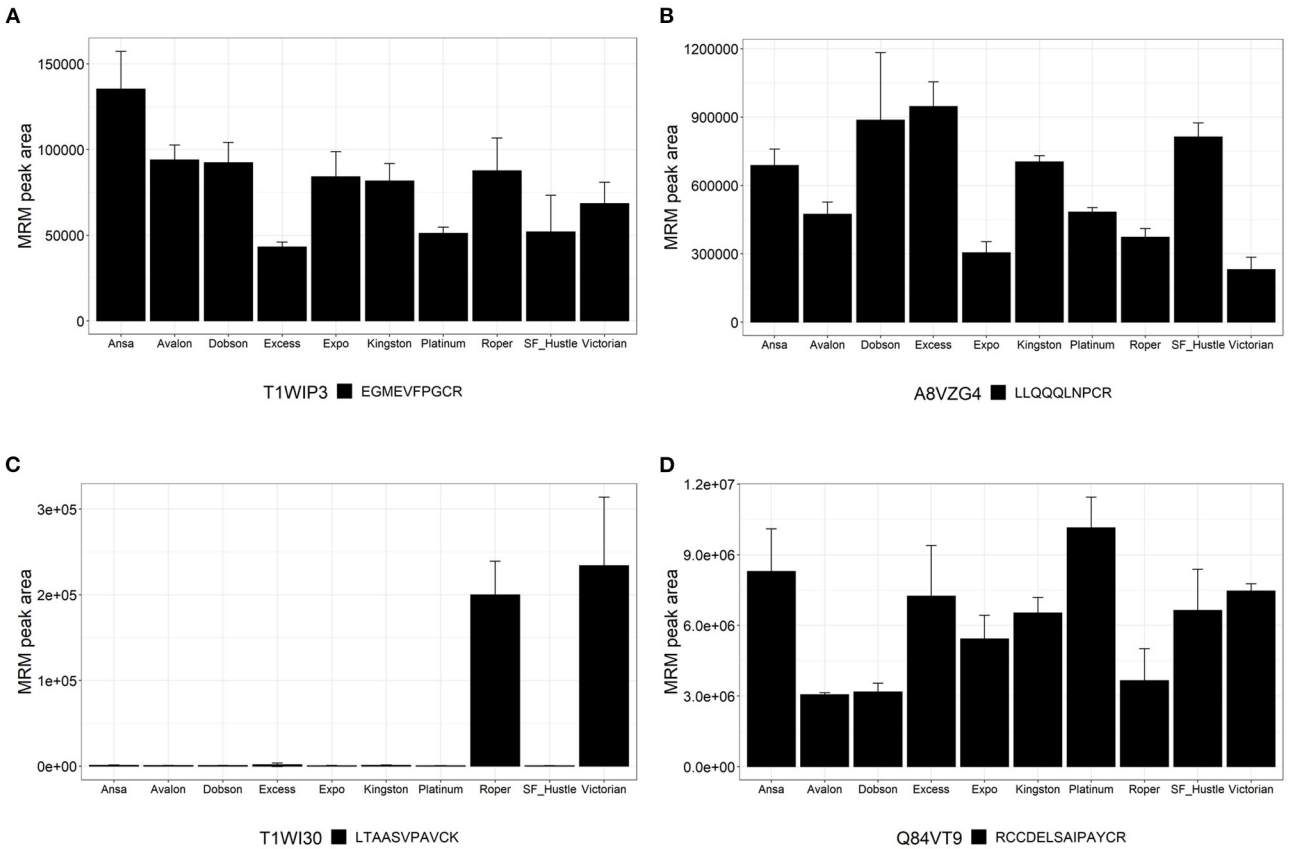

FIGURE 5 | Relative quantitation of peptides across the 10 varieties of *L. perenne* from proteins of the Pfam family PF00234. Graphs show the MRM peak area for each cultivar. Data are presented as the mean ± SD ($n = 3$) with one peptide from each protein. **(A)** Dimeric alpha-amylase inhibitor (T1WIP3); **(B)** Alpha-gliadin (A8VZG4); **(C)** Dimeric alpha-amylase inhibitor (T1WI30); and **(D)** Trypsin inhibitor (Q84VT9).

other species, i.e., orthologous proteins. The implementation of genomic and/or transcriptomic sequencing efforts would allow more ryegrass gluten-like proteins to be discovered.

A targeted MRM assay was developed for specifically measuring peptides found in proteins with homology to gliadins, glutenins, and ATIs. Prior to measuring these peptides, we expected that some targets would be detected at high levels in ryegrass and not in wheat, since target peptides were identified from ryegrass discovery proteomics. The results of this study revealed clear differences between the peptide content in ryegrass and wheat, with peptides predominant in ryegrass, regardless of belonging to the same family of Poaceae.

Gluten Relative Quantitation and Potential Markers

Gliadin and HMW Glutenin Family

In this study, nine peptides were identified from seven protein sequences, characteristic of prolamin proteins, and were measured by LC-MRM-MS. Six of these proteins belong to the Gliadin domain containing protein family and one to the Tryp_alpha_amyl family. Peptide search analysis revealed that five peptides (SQILQQSSCQVMR, CPAIHSVVQAIILQK, QFLVQQCSPVAEVPFLR, AFALQALPAMCDVYVPPHCSVA, and QQAQFEGMR) were primarily detected in avenin proteins (**Table 1**, **Supplementary Table 2**). In ryegrass, the peptides detected in all cultivars with consistent levels were QFLVQQCSPVAEVPFLR and CPAIHSVVQAIILQK (**Figure 3C**). These peptides are not found in wheat, barley, or rye; therefore, these peptides are possible markers for differentiating ryegrass contamination from traditional gluten-containing grains. However, they are also common to *Avena sp*. and thus will not discriminate ryegrass from oats (**Supplementary Table 2**). Another peptide, LLQQQLNPCR (**Figure 5B**), from protein A8VZG4, a Tryp_alpha_amyl domain containing protein family member, was present in an α-gliadin of the species *D. hordeaceum* of the Triticeae tribe. However, this peptide may not be an ideal peptide marker due to its lack of uniform signal. Three peptides (QQCCQQLAQIPQQLR, APFASIVASIGGQE, and APFASIVAGIGGQYR, **Figures 3D–F**) were matched back to γ-gliadin sequences from species of the Triticeae tribe; interestingly, though these specific peptides were not detected in the wheat cultivars tested. These peptides showed variable abundance across the ryegrass cultivars, and although not detected in the wheat cultivars examined herein, these peptides will not make ideal markers for ryegrass contamination due to their presence in known wheat γ-gliadin sequences.

The nomenclature of prolamin super-family proteins is diverse depending on the cereal of origin. In wheat, these proteins are gliadins; in rye, they are secalins; in barley, hordeins, and oats, they are avenins. The phylogenetic relationship between prolamin proteins from different species has been demonstrated with homology comparisons between avenin sequences and α- and γ-gliadins from wheat, B-hordeins from barley, and γ-secalins from rye (28–30). Herein, we showed that ryegrass has gliadin-like peptides that share a certain level of similarity to avenins and γ-gliadins. Moreover, this agrees with a comparative genomic study that revealed conserved genetic maps in terms of orthology and collinearity in the lineage of the Triticeae (wheat), Aveneae (oat), and Poeae tribes (ryegrass) (31–33), although phylogeny studies place *Lolium* closer to *Avena* than to *Triticum* and *Hordeum* (34–37).

The other fraction of prolamins is constituted by glutenins, which are present as high molecular weight (HMW) and low molecular weight (LMW) subunits that join to make multimeric proteins held by disulphide bonds (1). Immune reactive epitopes have also been reported for HMW and LMW glutenins (38–43).

In this study, seven peptides were measured from three glutenin-like protein sequences. None of these peptides have been reported previously in ryegrass (15). Two peptides QQCCQQLAQIPEQSR and SQMLQQSSCHVIR (**Figure 3G**) from protein J9QGY5 (Gliadin-domain containing protein family) were found exclusively in LMW-GS of the species *D. villosum* of the Triticeae tribe. Both peptides were experimentally detected in ryegrass but not in wheat; the peptide SQMLQQSSCHVIR showed consistent levels across the ryegrass cultivars. Five peptides were characteristic of HMW-GS. These included four peptides (ELQESSLEACR, DVSAKCRPVAVSQVAR, ELQESSLEACRQVVDQQLAGR, and QLQCERELQESSLEACR, **Supplementary Figures 1C,D**, **Figure 4**) matching to protein X5CHT6. These were determined to be conserved within the Triticeae tribe and had variable abundance within the analysed ryegrass cultivars. The peptide ELQESSLEACR (**Supplementary Figures 1C,D**) was highly abundant in the wheat cultivars. Peptide DGSFYPGEATPPQQLQQR was found consistently in ryegrass samples and was not detected in the wheat extracts; it was exclusively found in a protein sequence from *E. libanoticus*, which is a wild species of the Triticeae tribe.

Two peptides characteristic of LMW glutenin (QQCCQQLAQIPEQSR and SQMLQQSSCHVIR) and one peptide characteristic of HMW glutenin (DGSFYPGEATPPQQLQQR) are found within Triticeae tribe; however, they were not present in wheat protein sequences. As such, these sequences may be candidate peptides to differentiate wheat and ryegrass. Nevertheless, this study reports candidate glutenin peptides for the first time in ryegrass cultivars.

Tryp_Alpha_Amyl Family

Alpha-amylase/trypsin inhibitor proteins are not considered as gluten proteins; however, recent research suggests that some GRDs are not triggered by gluten proteins but by other types of proteins with similar structures, including ATI proteins (44, 45). These ATI proteins are involved in plant defence; however, they also activate the innate immune system and trigger pro-inflammatory cytokines (46–49). It is believed that ATIs can affect individuals with a sensitive type of GRD (46, 47).

ATI proteins were identified in this study, and diagnostic peptides were measured by LC-MS. The strategic approach consisted of searching peptides characteristic of proteins with the protein family domain PF00234, which lead to the initial recognition of several proteins and peptides; however, a deeper analysis using protein and peptide homology searches revealed that some of these peptides were found in other members of the PF00234-domain containing proteins, namely nsLTPs and puroindolines. Ultimately, four peptides characteristic of ATI proteins were measured from three target proteins. Two peptides (LQCVGSQVPEAVLR and EGMEVFPGCR, **Supplementary Figures 1A,B**, **Figure 5A**) from protein T1WIP3 were conserved for species of the Triticeae tribe, which coincides with the quantitative measurements showing a higher abundance in the wheat cultivars. Peptide LTAASVPAVCK (**Figure 5C**) from the protein T1WI30 had variable abundance across the ryegrass cultivars and was found in sequences from several species of the Triticeae tribe. Consequently, these peptides are unlikely candidates for markers of ryegrass presence. The last peptide, RCCDELSAIPAYCR (**Figure 5D**), from protein Q84VT9 showed high abundance in ryegrass and was not detected in wheat. This peptide was found exclusively in alpha-amylase/trypsin inhibitor proteins of species *Hordeum vulgare*; therefore, it is not present in wheat databases and is a candidate peptide to differentiate ryegrass from wheat but not barley.

The results described herein for the identification of ATI proteins provide evidence for peptide sequence similarity when comparing ryegrass to protein members of the Triticeae tribe ATIs, but further analysis, supplemented by genomic/transcriptomic sequencing efforts, would be needed to ascertain the extent of protein sequence homology. Principally, the ATIs targeted herein from ryegrass cultivars may not necessarily have the same immunoinflammatory properties as the wheat ATIs that are implicated in GRD. ATIs often differ in secondary structure, i.e., the number and position of intrachain disulphide bonds, and may contain different arrangements of α-helices that can influence the ability to activate the innate immune response (47).

CONCLUSION

Ryegrass is a common field contaminant with dense seeds and herbicide-resistant properties. These factors render ryegrass with the potential to enter the supply chain and be inadvertently consumed by the general population. This study provides evidence that gluten-like peptides are present in perennial ryegrass, thereby highlighting the potential risk of unintentional consumption of gluten through the cross-contamination of traditional cereal grains. Indeed, this food safety risk is underscored by a lack of knowledge around the potential for the industry standard ELISA assessment to produce inaccurate information. If these new gluten protein species trigger a Coeliac response but fail to produce an ELISA signal, then a food

safety issue may remain unchecked. Conversely, should ryegrass produce no Coeliac response but a strong ELISA signal, then foods may not meet safety requirements in error, thereby presenting an unnecessary challenge for food manufacturers. Future studies are warranted to explore the immunogenic potential of these new gluten-like proteins to determine their presence in nontraditional cereal grains, determine their natural variation, and deploy methods that can be used to differentiate ryegrass from wheat.

Through targeted measurement of prolamin super-family proteins, a suite of peptides were identified that showed consistent abundance across ryegrass cultivars but were not detected in wheat. These peptides could potentially be used in an assay for detecting ryegrass contamination in food products and differentiating ryegrass from wheat contamination in other cereal grains or processed foods. The methodology developed herein could also be applied to determine the extent of ryegrass presence in commodity grain or after processing into food ingredients. There are however no studies that have reported on whether the gluten-like proteins from ryegrass can trigger CD or other GRDs. Nevertheless, one study revealed cross-reactivity between ryegrass pollen and wheat endosperm proteins (50). Future investigation is required to measure the immune reactive potential of ryegrass and continue this important body of work that now spans over 90 years. Importantly, further studies are also required to enhance the genomic resources available for ryegrass so that species-specific proteins are readily identifiable rather than relying on sequence variation from related taxa to identify peptides and proteins. A combination of clinical studies supplemented by analytical workflows to understand the risk associated with agricultural co-mingling and dietary exposure are needed to ensure food safety and avoid the inadvertent failure of the GF-diet.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession numbers can be found below: https://doi.org/10.25919/8ehe-yr54.

AUTHOR CONTRIBUTIONS

SE-C, JB, MC, and CH designed the study. SS and AA carried out sample preparation and extraction. SE-C and JB carried out acquisition, analysis, interpretation of the data and created the scripts for data curation, and visualisation. AJ provided guidance for building the database, data analysis, data visualisation, and data interpretation. SE-C, JB, AJ, CH, and MC drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Proteome Analysis and Epitope Mapping in a Commercial Reduced-Gluten Wheat Product

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Gluten related disorders, such as coeliac disease, wheat allergy and baker's asthma are triggered by proteins present in food products made from wheat and related cereal species. The only treatment of these medical illnesses is a strict gluten-free diet; however, gluten-free products that are currently available in the market can have lower nutritional quality and are more expensive than traditional gluten containing cereal products. These constraints have led to the development of gluten-free or gluten-reduced ingredients. In this vein, a non-GMO wheat flour that purports to contain "65% less allergenic gluten" was recently brought to market. The present study aims to understand the alteration of the proteome profile of this wheat flour material. Liquid chromatography-mass spectrometry was used to investigate the proteome profile of the novel wheat flour, which was contrasted to a wheat flour control. Using both trypsin and chymotrypsin digests and a combined database search, 564 unique proteins were identified with 99% confidence. These proteins and the specific peptides used to identify them were mapped to the wheat genome to reveal the associated chromosomal regions in the novel wheat flour and the mixed wheat control. Of note, several ω- and γ-gliadins, and low-molecular weight glutenins mapping to the short arm of chromosome 1, as well as α-gliadins from the chromosome 6 short arm were absent or expressed at lower levels in the novel wheat variety. In contrast, the high-molecular weight glutenins and α-amylase/trypsin inhibitors were notably more abundant in this variety. A targeted quantitation experiment was developed using multiple reaction monitoring assays to quantify 359 tryptic and chymotryptic peptides from gluten and related allergenic proteins revealing a 33% decrease of gluten protein content in the novel wheat flour sample in comparison to mixed wheat control. However, additional mapping of known allergenic epitopes showed the presence of 53% higher allergenic peptides. Overall, the current study highlights the importance of proteomic analyses especially when complemented by sequence analysis and epitope mapping for monitoring immunostimulatory proteins.

Keywords: gluten, wheat, celiac disease, allergy, food safety, proteomics, mass spectrometry

INTRODUCTION

Wheat products account for some 20% of dietary calories and protein ingested globally (1). However, in susceptible people wheat proteins can elicit a range of health disorders including coeliac disease (CD), wheat allergy (WA), and non-coeliac wheat sensitivity (NCWS). The allergenic wheat proteins that cause these adverse immune reactions have been mapped to specific genes by The International Wheat Genome Sequencing Consortium (IWGSC) and collaborators (2, 3). CD is caused when dietary gluten reaches the small intestine of genetically predisposed individuals and stimulates an autoimmune response leading to localized damage and subsequent symptoms (4). WA, as well as baker's asthma (BA) and wheat-dependent exercise induced anaphylaxis (WDEIA), involve an IgE-mediated immune response to wheat proteins that are either ingested as food or occur via skin contact or inhalation. While these disorders can be triggered by gluten proteins, BA typically has a non-gluten protein trigger (5, 6). NCWS is diagnosed when symptoms develop in response to cereal grain consumption, but serological testing is negative for both an autoimmune response and the IgE-mediated allergic response, which contraindicates CD and WA, respectively (4, 7). While the term "non-celiac gluten sensitivity" has a history of use (8), NCWS better reflects the non-gluten wheat proteins (9) or non-protein wheat components like FODMAPs (10) that elicit similar symptoms (7). CD is estimated to afflict ∼0.7–1.4% of the global population (11, 12), WA some 0.33–0.75% in adults (13–15), and NCWS being more variable but with prevalence estimated between 0.5 and 13% (16, 17). While gluten proteins are established antigens to those with CD and also contribute to various allergies, non-gluten wheat proteins are potential allergens and antigens capable of causing WA, BA, NCWS, as well as CD (3, 6, 18).

The only effective treatment for these wheat-related immune disorders is the exclusion of wheat and related crop species from the diet. This adds to demand for "gluten free" foodstuffs that resemble traditional wheat products, however wheat is replaced by substitute ingredients that contribute starch but without gluten or other cereal proteins. The absence of gluten proteins, however, can affect the consistency, texture, or taste of gluten-free products, and the substitute ingredients typically come at a higher cost and require recipe alterations (19).

Several approaches have aimed at reducing the gluten content while retaining the health benefits of whole grains or maintaining the unique functionality of cereal grains. One approach is to use ethyl methanesulfonate (EMS) mutagenesis to produce random mutations in genetic material by nucleotide substitution. EMS is often used as the technology base for "Targeting Induced Local Lesions in Genomes" (TILLING) which has proven effective at targeting key wheat enzymes to improve starch composition (20). It has been applied to wheat gluten genes (21), but is challenged by the sheer number of potential allergens and the fact that even low levels of expressed gliadins can elicit CD (22). In barley, ultra-low gluten levels (<5 mg/kg) were achieved in the variety Kebari® by using traditional breeding techniques to combine mutagenesis-derived barley varieties with decreased hordein content and composition (23, 24). Efforts to develop a low-allergen wheat variety have targeted genes that either exhibit a large immune response directly (25), or that conduct epigenetic regulation of downstream gluten protein genes (26), and have also made use of natural null-allele variants (27), CRISPR-Cas9 (28), and RNAi (29). A common phenotype is that downregulation of one or a subset of gluten protein encoding genes is accompanied by the compensatory upregulation of alternate storage proteins (30–34), with a change in technological properties (35). However, technology to characterize gluten in wheat products is the subject of ongoing research (36), as is the targeted removal of CD reactive epitopes from wheat (37).

Recently, a reduced gluten product was released that claims to be a non-GMO wheat variety which contains "*65% less allergenic gluten than traditional flour.*" It is clarified that the product is "*developed for those with sensitive stomachs who don't have gluten or wheat allergies, but who want to reduce the amount of gluten in their diets*" (38) and was developed using wheat prolamin box binding factor (PBF) mutants (21, US patents 9,150,839, 10,412,909, and 10,750,690). There are no reports of the proteome and overall characteristics of proteins present in the reduced gluten flour in comparison to commercial wheat varieties.

Wheat has more than 800 genes with potentially allergenic domains, and some 356 genes encoding reference food allergens are included in the "IWGSC v1.0 reference allergen map" (3). This includes gliadins (including α-, β-, γ-, and ω-subtypes) and glutenins (including low molecular weight (LMW) glutenins and high molecular weight (HMW) glutenin subunits), as well as avenin-like proteins (ALPs), α-amylase/trypsin inhibitors (ATIs), and lipid transfer proteins (LTPs) (3). The gluten proteins contain specific epitopes that are deamidated, recognized, and presented by MHC-II antigen presenting cells in the gastrointestinal tract, in doing so initiating the autoimmune response that characterizes CD (39). The canonical gluten proteins, the gliadins and glutenins, together make up some 80% of the protein content in the wheat endosperm, and the most potent contributors to CD toxicity are the chromosome 6D α-gliadins and chromosome 1D ω-gliadins (ω 1,2 sub-type), followed by the LMW glutenins and γ-gliadin (40). It is therefore important to precisely characterize protein groups and epitopes when quantifying "allergenic gluten" in new products. This present study aimed to understand the alterations to the proteome in this reduced gluten wheat product using LC-MS/MS in comparison to a mixed wheat control.

MATERIALS AND METHODS

Sample Preparation

GoodWheat™ (GW) white bread wheat flour was purchased directly from Arcadia Bioscience (Davis, CA, USA). Replicates of GW and of a mixed-wheat (MW) control flour sample were weighed out in quadruplicate. The MW control consisted of equal parts of flour from wheat cultivars: Alsen, Xiayan, Pastor, Westonia, Baxter, Chara, Yitpi, AC Barrie, and Volcania; selected to represent the diversity of wheat used in commercial production. Gluten proteins were specifically enriched from the wheat using an isopropanol/dithiothreitol (IPA/DTT) solvent as described previously (41). Flour (50 mg) was weighed into a 1.5 mL micro-tube and 500 μL (10 μL/mg) of 55% IPA/2% DTT

was added with vortex mixing until the flour was thoroughly combined with the solvent. The tubes were then sonicated for 5 min at room temperature and incubated in a thermomixer (400 rpm, 30 min, 50°C). The tubes were centrifuged for 15 min at 20,800 ×g. The solutions were centrifuged for 15 min at 20,800 ×g. Protein extracts (100 μL) were added to 10 kDa molecular weight cut off filters (Merck, Bayswater, Australia). The protein on the filter was washed twice with a buffer consisting of 8 M urea in 0.1 M Tris-HCl (pH 8.5) with centrifugation for 15 min at 20,800 ×g. Iodoacetamide (25 mM; 100μL) prepared in 8 M urea and 100 mM Tris-HCl was added to the filters for cysteine alkylation with incubation in the dark for 20 min prior to centrifugation for 10 min at 20,800 ×g. The buffer was exchanged with 100 mM ammonium bicarbonate (pH 8.0) by two consecutive wash/centrifugation steps. The filters were transferred to fresh collection tubes and digestion enzyme, either trypsin or chymotrypsin (Promega, NSW, Australia), was prepared as 10 μg/mL in 100 mM ammonium bicarbonate, 50 mM calcium chloride and 200 μL was added to each filter with incubation for 16 h at 37°C. The filters were centrifuged for 15 min at 20,800 ×g. The filters were washed with 200 μL of 100 mM ammonium bicarbonate, and the combined filtrates were subsequently lyophilized.

Discovery Proteomics

The digested samples were reconstituted in 100 μL of 1% formic acid and the peptides (1 μL) were chromatographically separated on an Ekspert nanoLC415 (Eksigent, Dublin, CA, USA) coupled to a TripleTOF 6600MS (SCIEX, Redwood City, CA, USA). The peptides were desalted for 5 min on a ChromXP C18 (3 μm, 120 Å, 10 × 0.3 mm) trap column at a flow rate of 10 μL/min of 0.1% formic acid and separated on a ChromXP C18 (3 μm, 120 Å, 150 × 0.3 mm) column at a flow rate of 5 μL/min. The solvents used were (A) 5% DMSO, 0.1% formic acid, 94.9% water and (B) 5% DMSO, 0.1% formic acid, 90% acetonitrile, 4.9% water. A linear gradient from 3 to 25% solvent B over 68 min was employed followed by 25–35% B over 5 min, an increase to 80% B over 2 min, a 2 min hold at 90% B, return to 3% B over 1 min, and 8 min of re-equilibration. The eluent from the HPLC was directly coupled to the DuoSpray source of the TripleTOF 6600 MS. The ionspray voltage was set to 5,500 V; the curtain gas was set to 138 kPa (20 psi), and the ion source gas 1 and 2 (GS1 and GS2) were set to 103 and 138 kPa (15 and 20 psi). The heated interface was set to 150°C. The discovery data files of individual technical replicates of either trypsin or chymotrypsin digested GW and MW samples were searched using ProteinPilot v5.0.3 with Paragon Algorithm (SCIEX) against a FASTA file consisting of Triticeae tribe proteins from UniProt-KB [accessed 02/2021 supplemented with additional translated gene models from the IWGSC RefSeq v1 Assembly (2), as well as those listed on the common Repository of Adventitious Proteins (thegpm.org/crap)]. The FASTA file contained 817,698 protein sequences.

Targeted Proteomics

Reduced and alkylated tryptic and chymotryptic peptides were chromatographically separated on an Exion LC-40AD UHPLC system (SCIEX) and analyzed on a 6,500+ QTRAP mass spectrometer (SCIEX). Data acquisition was achieved using scheduled multiple reaction monitoring (sMRM) scanning experiments using a 60 s detection window for each MRM transition and a 0.3 s cycle time.

To build the MRM method, a FASTA file containing all identified proteins was imported into Skyline (42), all fully tryptic peptides sized between 6 and 30 amino acids were selected, and repeated peptides removed. All fully chymotryptic peptides between 6 and 30 amino acids were selected in independent experiments. Initially, five transitions were selected per peptide in an unscheduled MRM assay and assessed on both GW and MW samples. Those peptides where at least three transitions reproducibly co-eluted at the expected retention time (RT) without interference were then selected for inclusion in scheduled MRM assays. These were divided across several separate transition lists, such that all data was recorded with a 60 s detection window and maximum cycle time of 0.3 s. Precursor ions where three or more transitions had consistent RT, intensity over 1,000 cps, and a signal to noise ratio (S/N) >5 were kept and the three most intense transitions were selected for subsequent quantitative experiments. In this way, a total of 768 tryptic and 175 chymotryptic peptides that were unique to one protein were monitored, as well as 263 tryptic and 109 chymotryptic peptides that were present in more than one protein. Data was collected on four technical replicates of GW and MW. Peptide peak area data was exported from Skyline and analyzed (Graphpad Prism v8).

To quantify the relative abundance of individual gluten protein groups, peak areas of both unique and non-unique peptides were summed. Proteins were mapped to the wheat genome using the tBLASTn function of CLC Main Workbench v20.0.4 (Qiagen, Denmark), and multiple proteins mapping to the same gene were interpreted as different alleles of the same gene. Quantified MRM peptides were then allocated to protein groups according to the proteins in which they were found. Peptides occurring in proteins from multiple groups were labeled Multiple/Mixed. The significance and fold change of these protein groups were graphed using VolcaNoseR software (43).

A high sequence similarity between gluten proteins meant many peptides were observed that were common to multiple gluten proteins, making it impossible quantify all proteins using unique peptides. To overcome this, peptides quantified via MRM were allocated to gluten protein groups that were quantified using unique peptides for GW and MW, revealing the relative abundance of protein groups in these samples. To do this, all peptides quantified were mapped to the wheat genome (2), and using a combination of sequence alignments, the presence of Pfam domains (PF13016, PF03157, PF00234), and manual checking of the matching proteins were allocated to one of the following protein groups: α-gliadins, ALPs, ATIs, γ-gliadin, HMW-GSs, LMW-GSs, and ω-gliadins. Where the proteins had two or more peptides from multiple protein groups they were defined as "mixed." While the ATIs and ALPs are not canonical gluten proteins, several of the ALPs can function as nutrient reservoir proteins, and the ATIs exhibit some allergenicity making them relevant to this investigation.

To quantify protein groups, the monitored peptides were mapped to the identified gluten protein sequences and non-gluten protein families with immune-reactive properties using 100% sequence matching in the Motif search algorithm in CLC Genomic Workbench v21.0.3 (Qiagen, Denmark), and group specific peptides were identified. Quantitative data on all chymotryptic and tryptic peptides were combined, and the abundance of each peptide in each replicate was normalized to the average seen across all replicates from both GW and MW. Graphs were generated in Graphpad Prism v8.

Gene Enrichment Analysis

GO enrichment analysis was performed to test for the downregulation of certain classes of proteins in GW. Those proteins present in both GW and MW were excluded so that only proteins unique to GW or MW were analyzed for enriched GO terms. GW- or MW-specific proteins were then mapped to the wheat genome using CLC Genomic Workbench v21.0.3 (Qiagen, Denmark), and lists of their corresponding wheat gene identifiers were pasted into g:Profiler (biit.cs.ut.ee/gprofiler/gost) for GO overrepresentation analysis.

Epitope Mapping

The peptides identified at 1% FDR in discovery proteomics were searched for known CD related T cell epitopes [Ludvig M (44)], baker's asthma, and wheat allergy related epitopes collected from the Immune Epitope Database and Analysis Resource (www.iedb.org) using the Motif search algorithm in CLC Genomic Workbench v21.0.3 (Qiagen, Aarhus, Denmark). Additionally, peptides recognized by commercial ELISA kits using R5 and G12 monoclonal antibodies were also mapped to the protein and peptide sequences. Hits with 100% sequence identity were kept in the analysis. The peptides monitored in MRM assays were also mapped to the same protein list, and the overlap between CD epitopes and the monitored peptides were determined. Monitored peptides that contained an entire epitope in their sequence were selected and quantified in GW and MW to give a relative measure of potential immune reactivity.

Protein and ELISA Measurement

Protein estimations were performed using a Coomassie dye binding protein assay using Bradford reagent (Sigma-Aldrich, St Louis, USA) following the manufacturer's instructions. Measurements were made at 595 nm using a Varioskan LUX microplate reader (Thermo Scientific, Scoresby, Australia). Bovine serum albumin (BSA) standard was used in the linear range 0.125–1.5 mg/mL.

Diluted wheat extracts were analyzed by sandwich ELISA using the Ridascreen Gliadin (R-Biopharm, Darmstadt, Germany). The analytical protocols provided by the kit manufacturer were strictly followed. Each of the samples was extracted using the extraction Cocktail (R7006/R7016, R-Biopharm) recommended by the manufacturer for optimal gluten extraction and measured on the using Varioskan LUX microplate reader (Thermo Scientific) in duplicate on a single ELISA plate alongside the supplied standards (representing a gluten concentration of 5–80 mg/kg). The results of absorbance readings were analyzed according to the kit manufacturer's instructions using cubic polynomial regression for the standard curve. The data analyses were performed using Microsoft Excel.

TABLE 1 | Numbers of distinct proteins and of gluten and ATI proteins identified at 99% confidence in trypsin and chymotrypsin data.

	Trypsin	Chymotrypsin	Combined
GW total	**285**	**139**	**360**
GW gluten	76	75	126
α-gliadin	6	11	15
ATI	24	16	29
Avenin-like protein	11	5	12
γ-gliadin	11	8	18
HMW-GS	13	9	17
LMW-GS	11	23	32
ω-gliadin	0	3	3
GW non-gluten	209	64	234
MW total	**360**	**151**	**448**
MW gluten	73	93	138
α-gliadin	6	16	19
ATI	27	19	36
Avenin-like protein	9	6	10
γ-gliadin	10	16	23
HMW-GS	9	9	13
LMW-GS	11	23	32
ω-gliadin	1	4	5
MW non-gluten	287	58	310
GW and MW combined	**440**	**179**	**541**

RESULTS

Discovery Proteomics

To identify the proteins in the GW and MW samples, combined database searches were performed on the discovery proteomics datasets. The numbers of distinct proteins identified at 1% global false discovery rate (FDR) excluding identifications against the common contaminants (cRAP database) are summarized in **Table 1**. This information is generated from the reports available at https://doi.org/10.25919/fr8e-k267, processed with the Protein Alignment Template v3.002 beta (SCIEX) and manual curation.

Of the 541 proteins identified in both GW and MW (**Table 1**), more were identified in tryptic digests (440) than chymotryptic digests (179). Greater representation of α- and γ-gliadins and LMW-GS was achieved using chymotryptic digests, while more ATIs, ALP-derived, and non-gluten proteins were identified in tryptic digests (**Table 1**).

Considering trypsin and chymotrypsin data together enables a more complete comparison of the GW and MW proteomes. Together, there were 360 distinct proteins identified in GW and 448 in MW, with an overlap of 267. The higher number in MW reflects the genetic diversity of the multiple wheat varieties that are present. In GW, 126 of identified proteins were gluten-like proteins (35%) and in MW this number was 138 (31%).

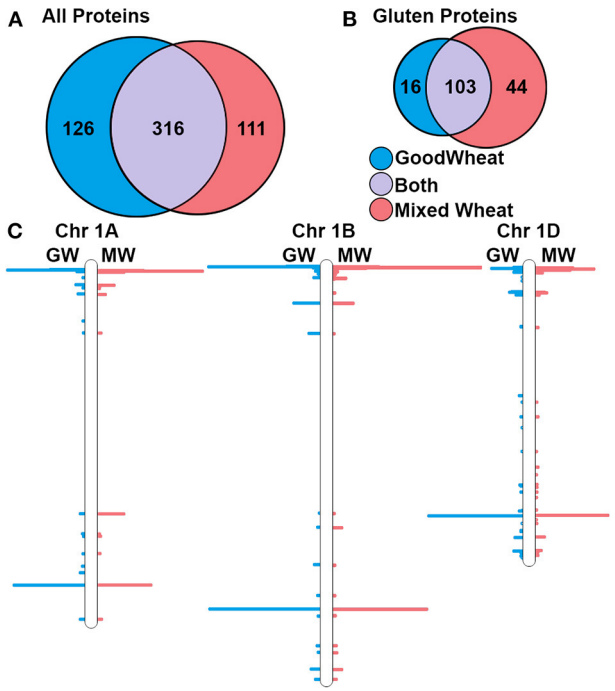

FIGURE 1 | Summary of total and gluten-like proteins identifications at 1% FDR and their corresponding chromosomal locations. **(A)** Venn diagram showing the total number of proteins detected within the GW and MW datasets. **(B)** Detected gluten proteins in MW and GW. **(C)** Locations of genes for detected proteins on wheat chromosomes 1A, 1B, and 1D.

Notably, the GW and MW proteomes share 99 (60%) of the 165 detected gluten proteins. The numbers of proteins identified are compared in **Figures 1A,B**. To identify the chromosomal position of these proteins within the wheat genome protein, sequences were mapped to the IWGSC wheat genome assembly version 1 (2), and the number of peptides observed per 1 million base pairs (Mb) bins was determined. This revealed clusters of detected proteins in all known storage protein gene loci regions of the genome, corresponding to γ- and ω-gliadins, LMW-GS, and HMW-GS (3) on chromosome group 1 and α-gliadins on chromosome group 6. **Figure 1C** shows the location of the peptides detected superimposed on chromosomes 1A, B, and D, respectively. Though non-gluten proteins were also detected across all wheat chromosomes, there were no large-scale chromosome region changes observed in GW and MW, indicating the potential for gene expression of gluten proteins in GW.

Targeted Proteomics

To investigate the quantitative changes across GW and MW wheat samples, LC-MRM-MS-based quantitative assays were developed for all peptides confidently identified in the discovery proteomics experiment (**Figure 2**). A total of 189 tryptic peptides and 170 chymotryptic were targeted. While 84 tryptic and 55 chymotryptic peptides were uniquely present, i.e., in only one protein isoform, many of the peptides monitored by MRM occur in multiple protein isoforms and therefore reflect the relative abundance of more than one protein. While LC-MRM-MS reveals peptide relative abundance, using this information to quantify proteins by combining the constituent peptides is confounded by both the presence of repeated peptides and differential ionization efficiency of various peptides. We therefore categorized peptides into groups that reflect the abundance of major allergen types and did not quantify specific proteins. This revealed the fold-change and significance of tryptic (**Figure 2A**) and chymotryptic peptides (**Figure 2B**) peptides between GW and MW. HMW-GS and ATI peptides tend to be higher in GW than MW, and many tryptic "non-gluten" peptides are higher in GW. Similarly, many LMW-GS, ALP, and α-gliadin peptides are lower in GW than MW.

The normalized peak area for all peptides belonging to each protein group were then summed to compare the overall abundance of each protein group (**Figure 3**). Importantly, GW showed significantly lower abundance of LMW-glutenins, α-gliadins, and γ-gliadins, but showed an increase in HMW-glutenins relative to MW. GW also showed significant decreases in ALPs and increases in ATIs. Changes in net ω-gliadin abundance were not significant. The net change in canonical gluten content can be obtained by adding together the gliadins and glutenins (LMW-GS, HMW-GS, α-, γ-, ω-gliadins), showing that GW has 67% the relative gluten protein abundance as MW (**Figure 3B**).

Gene Set Enrichment Analysis

To understand the enrichment of protein classes within the individual wheat samples, GO enrichment analysis was performed using g:Profiler on those proteins detected with a fold change ≥2 in MW and GW as shown in **Figure 2**. Proteins in MW showed predominant enrichment for nutrient reservoir activity (GO:0045735, **Figure 4A**). GW proteins showed enrichment of several classes of enzyme inhibitor and regulators, as well as enrichment of proteins localizing to the Extracellular Region (GO:0005576) cellular compartment indicating the compensation mechanism for the expression of non-gluten proteins. There was no enrichment of nutrient reservoir activity (**Figure 4B**).

Epitope Mapping

To explore the potential immune reactive nature of proteins detected in GW compared to those in MW, peptides identified in the discovery data that contained full-length immune reactive epitopes were quantified (**Figure 5**). Known immunogenic regions within quantitated MRM peptides are quantified, including HLA-DQ T cell epitopes for CD patients (**Figure 5A**), baker's asthma epitopes (**Figure 5B**), and wheat allergy-related epitopes (**Figure 5C**). It should be noted that these represent a small subset of the known immune reactive epitopes. The discovery analysis results (**Supplementary Table 1**) indicate the presence of additional epitopes that were not quantified with MRM. There were six complete HLA-DQ T cell epitope sequences observed in a total of 25 peptides, nine BA epitopes in 12 peptides, two WA epitopes in 16 peptides, and one WDEIA epitope in one peptide. Overall, HLA-DQ reactive epitopes in

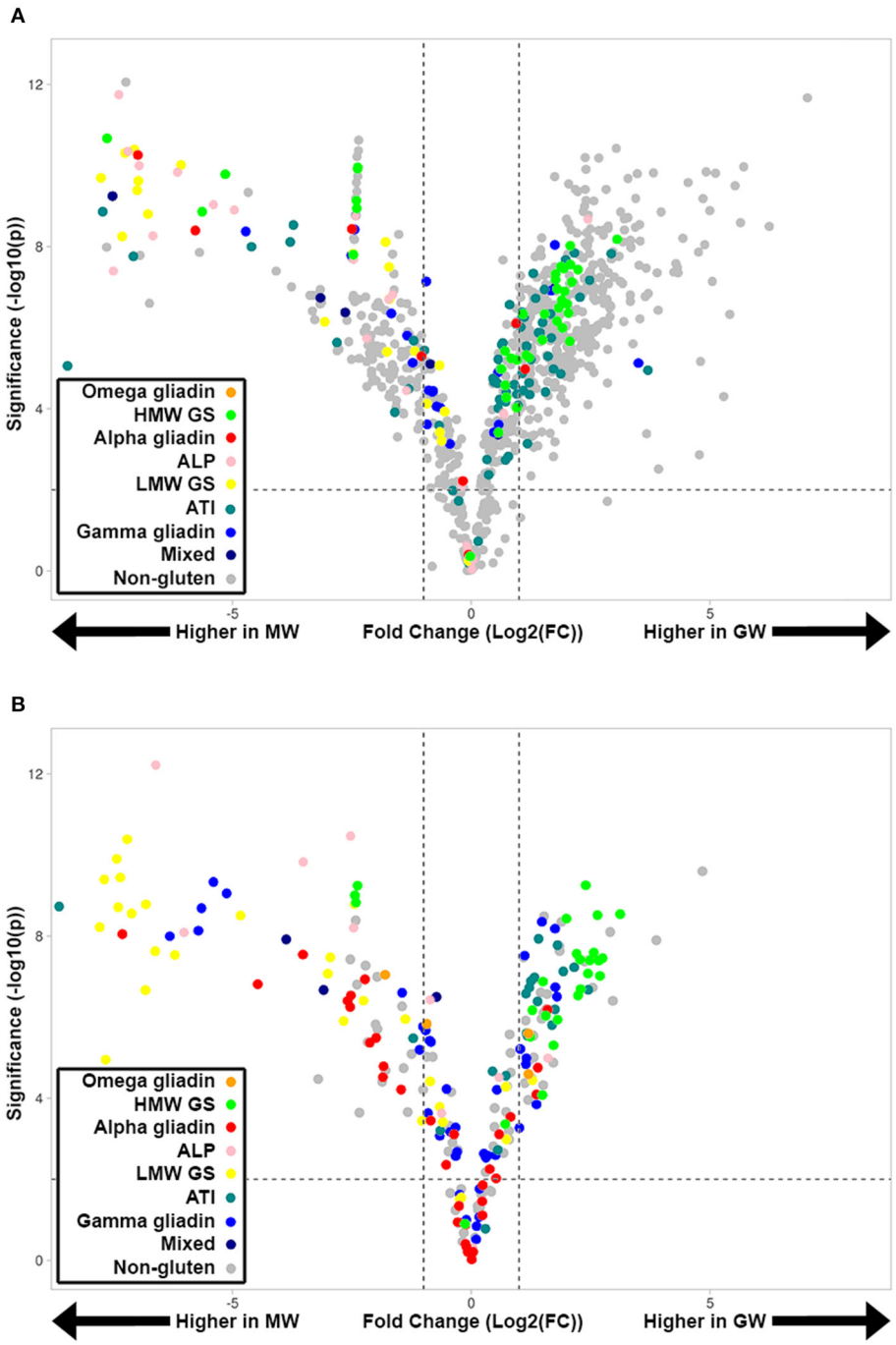

FIGURE 2 | Volcano plots showing quantified tryptic **(A)** and chymotryptic **(B)** peptides in GW and MW samples colored according to gluten group. A fold-change of 2 is indicated by the dashed vertical lines [$Log_2(FC) = \pm 1$]. Peptides above the horizontal dashed line have a significant change in abundance between GW and MW (p-value <0.01 [$-log_{10}(p) > 2$].

GW were present at 67% the relative abundance of MW. BA-reactive epitopes were also more abundant in GW at 180% that of MW.WA-reactive epitopes were also more abundant in GW at 379% the level seen in MW. Only one wheat dependent exercise-induced anaphylaxis epitope was observed, which was notably lower in GW at 17.7% the level seen in MW.

The protein content of MW and GW were evaluated and did not show a significant difference at 0.84 and 0.88 mg/mL, respectively. The gluten content was also evaluated by R5 ELISA and it was interesting to note that GW revealed a 39% higher gluten content than MW, an unexpected result given the overall decrease in gluten peptides detected by LC-MS.

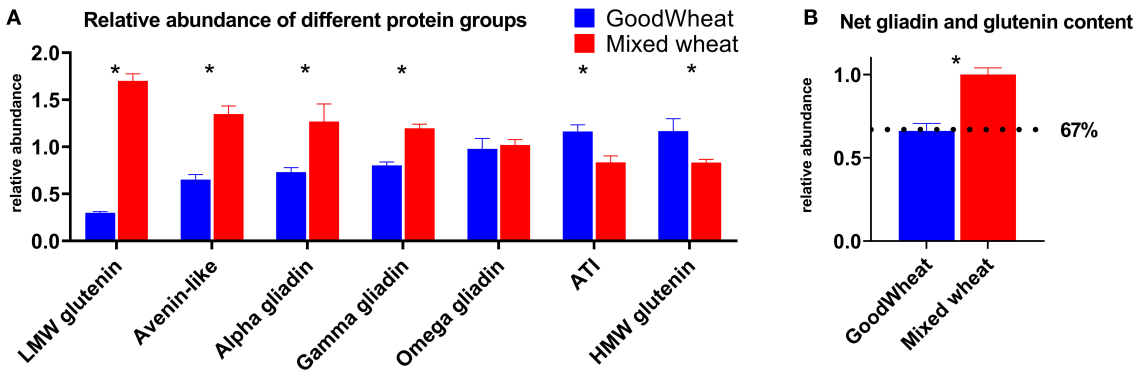

FIGURE 3 | Relative abundance of different gluten or ATI protein groups **(A)**. Quantitation was performed based on all detectable peptides from proteins classified to these groups. Error bars indicate SEM, and significant differences are indicated by asterisk. Adding together the LMW and HMW glutenins, and α-, γ-, ω-gliadins gives the net gliadin and glutenin content **(B)** which equated to GW having an estimated 67% of the gluten content of MW (dotted line).

FIGURE 4 | GO enrichment analysis of proteins showing ≥2-fold increase in: MW **(A)**; or GW **(B)**.

DISCUSSION

The current study used complementary high sensitivity LC-MS techniques to identify gluten proteins and to monitor the relative abundance of gluten and allergenic wheat proteins in a recently developed wheat product (GoodWheat, GW) in comparison to a wheat sample mixed from equal amounts of nine commercial cultivars (Mixed wheat, MW). Peptides from gliadin and glutenin proteins were present in GW at 67% of the abundance of the MW control, indicating an average decrease of 33% (**Figure 3B**). This is complemented by our analysis of intact HLA-DQ reactive epitopes in the monitored peptides which were 67.3% as abundant in GW as MW (**Figure 5A**). While this may reduce but not remove the antigen content of GW, it is accompanied by an increase in peptides known to be related to Baker's asthma and wheat allergy, at 180 and 379%, respectively.

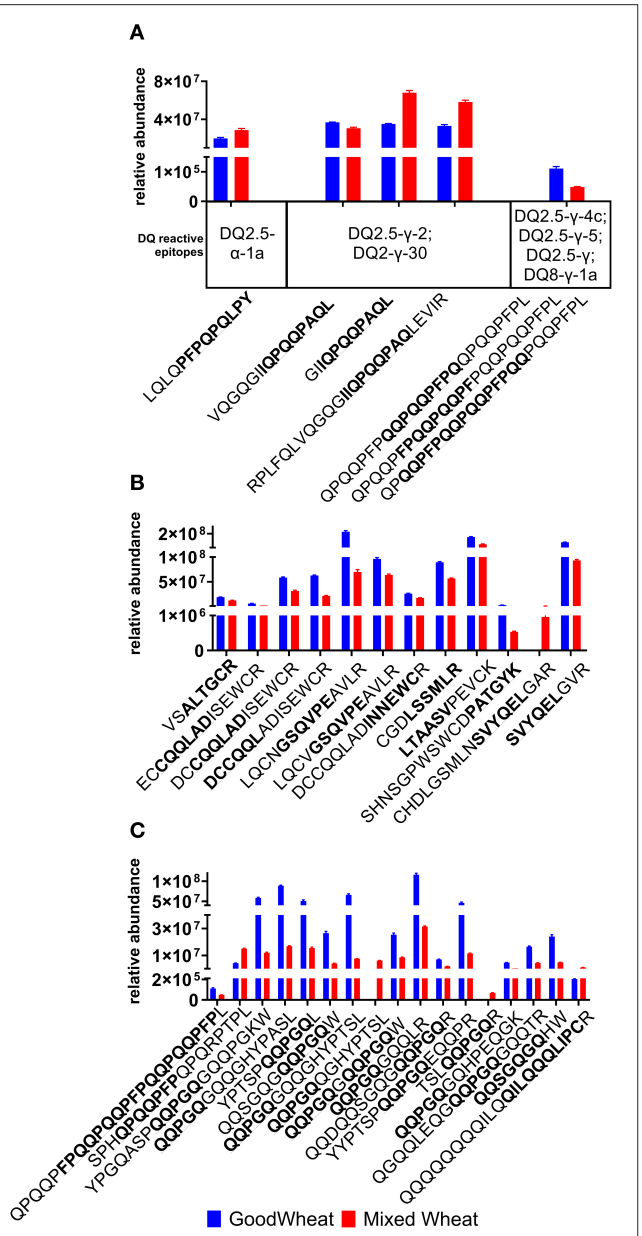

FIGURE 5 | Relative abundance of immune reactive epitopes in peptides quantified in MRM assays, reported as mean and standard error. Epitope sequences are highlighted in bold within the peptide sequence: **(A)** Coeliac disease HLA-DQ reactive epitopes; **(B)** Bakers' asthma; **(C)** Wheat Allergy plus peptide QQQQQQQQILQQILQQQLIPCR which contains the epitope QILQQQLIPC antigenic for wheat-dependent exercise induced anaphylaxis. When calculating the total, duplicate peptides where multiple epitopes were detected were only counted once.

The overlap between immunogenic DQ epitopes with peptides detected in discovery data, and quantified in MRM is presented in **Supplementary Table 1**. While the use of LC-MRM-MS in this work enabled the quantitation of gluten proteins and highlights its utility in grain protein research with specific reference to gluten, future studies should focus on using complementary extraction buffers to understand more about changes in the GW grain proteome.

In contrast to the MRM analysis that revealed an overall lower gluten content in GW than MW (**Figure 3**), the R5 ELISA estimate of gluten content revealed a gluten content ∼39% higher in GW than MW. The slightly elevated protein content (5%) in GW compared to MW would account for a minority of the observed difference. The elevated ELISA measurement likely reflects an overall increase in the ratio of R5 epitope per unit of protein. The choice of reference material, in particular the ratio of gliadin to glutenin, is known to affect measurements of gluten by ELISA even in simple food matrices (45), and kits that use different primary antibodies will yield different measurements of gluten (46) because of the specificities and sensitivities of the primary antibody (47). Future analyses should investigate the gluten content of GW using alternative ELISA kits or gluten protein quantitation employing fractionation (RP-HPLC or size-exclusion chromatography) protocols.

Important trends were seen in specific gluten protein types (**Figures 2**, **3**), as the LMW-GSs, α-, and γ-gliadins are lower in GW, while the HMW-GSs were significantly more abundant. HMW glutenins contribute more to bread's elastic properties than other gluten proteins due to their relative size and ability to form large polymers (48). Their higher relative abundance in GW indicates that they in part compensate for the lower abundance of gliadins and LMW glutenins (49). Additionally, the level of immune response elucidated by HMW glutenins in CD is significantly lower compared to the α-, γ-, and ω-gliadins and LMW glutenins (40), making their increase less relevant to CD, however it has important implications for WA and BA. Along with ATIs, which were also significantly higher in GW, the higher HMW glutenin content in GW brings more allergenic epitopes related to WA and BA. This is reflected in **Figure 5** as the allergenic epitopes recognized by different B and T cell types are increased by an overall 53.5%.

Interestingly, the ALPs were also present in significantly lower amounts in GW than MW. While named for their resemblance of oat avenins (50), these seed storage proteins share sequence similarity and secondary function with γ-gliadins and LMW glutenins (51). They contribute both to allergenicity (52) and bread dough quality (53), and contain one or two gliadin (PF13016) domains. ALPs also contain CD-related B cell epitopes (3), and their downregulation is important for CD toxicity.

The symmetry of the volcano plot (**Figure 2**) indicates the net decrease in gluten protein and ALP content is accompanied by compensatory expression of other proteins within the grain. GO enrichment analysis of the GW proteome revealed enzyme inhibitors and regulators that are enriched in GW which was also confirmed by the gene set enrichment analysis showing cysteine-rich proteins are overrepresented in the upregulated proteins in GW. Most of these proteins have a defense related function and were upregulated in lieu of proteins with a canonical "nutrient reservoir activity" GO MF annotation (**Figure 4**).

Our proteogenomic analysis indicates that there is no evidence of large-scale chromosome deletions or absence of storage protein gene clusters (**Figures 1B,C**) on chromosome group 1 and 6 in GW. While antibody-based assays or classical

Osborne fractionation were not performed and thus represents a limitation of the present work, gluten proteins were present in both GW and MW and simply expressed at different levels (**Figure 3**). This would suggest the novel GW variety expresses less gluten proteins due to gene regulation at a transcriptional or post-transcriptional level. There are several known mechanisms implicated in seed development and gluten protein expression that may be at play. One is the *LYS3* gene that encodes the transcription factor Prolamin Binding Factor (PBF). PBF is expressed early in seed development, and suppresses seed growth by reducing the expression of developmental and starch metabolism genes (54). Wheat *lys3* mutants have been reported to contain lower levels of gliadins and LMW-GSs (21), which matches our results as shown in **Figure 4**. A barley variety with *lys3a* mutation causing it to not express C-hordein (a class of barley gluten) was used in a breeding program to derive an "ultra-low gluten" barley variety (30, 55), showing it is compatible with selective breeding. These low-gluten PBF mutant lines exhibit increased expression of lysine-rich genes that are otherwise related to developmental processes during germination (30). While it is possible that GW uses *lys3* mechanisms to regulate gluten protein expression, using solely the proteomic information presented in this study we cannot conclusively determine the targeting of *lys3* regulation.

In conclusion, the use of discovery and targeted proteomics-based experiments has enabled the detection and quantitation of gluten and additional allergenic proteins present in the GW and MW samples. This study revealed a 33% decrease in gluten-like proteins in GW and the compensatory expression of non-gluten proteins within MW samples that tend to have enzyme inhibitor or regulator activity GO terms. This study affirms that, as stated by the manufacturer, GW is not compatible with a gluten-free diet. Epitope mapping revealed a reduction in gluten protein-specific epitopes; however, there was an increase in epitopes related to baker's asthma and wheat allergy in GW wheat in comparison to MW. Additionally, the chromosomal level analysis of detected proteins showed no significant differences between GW and MW. Future studies focusing on integrating LC-MS/MS results with clinical measurements would be needed to investigate the nutritional benefits of GW. Overall, the current study exemplifies the use of proteogenomic approaches as a tool to explore the safety and/or health benefits of wheat varieties targeted toward consumers with wheat-related disorders.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://doi.org/10.25919/fr8e-k267.

AUTHOR CONTRIBUTIONS

MN-W: sample preparation, LC-MS data collection, data analysis, and manuscript preparation. AJ and UB: data analysis and manuscript preparation. MC: project concept and design and manuscript preparation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.705822/full#supplementary-material

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Investigation of Protein and Epitope Characteristics of Oats and Its Implications for Celiac Disease

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The use of pure oats (oats cultivated with special care to avoid gluten contamination from wheat, rye, and barley) in the gluten-free diet (GFD) represents important nutritional benefits for the celiac consumer. However, emerging evidence suggests that some oat cultivars may contain wheat gliadin analog polypeptides. Consequently, it is necessary to screen oats in terms of protein and epitope composition to be able to select safe varieties for gluten-free applications. The overall aim of our study is to investigate the variability of oat protein composition directly related to health-related and techno-functional properties. Elements of an oat sample population representing 162 cultivated varieties from 20 countries and the protein composition of resulting samples have been characterized. Size distribution of the total protein extracts has been analyzed by size exclusion-high performance liquid chromatography (SE-HPLC) while the 70% ethanol-extracted proteins were analyzed by RP-HPLC. Protein extracts separated into three main groups of fractions on the SE-HPLC column: polymeric proteins, avenins (both containing three subgroups based on their size), and soluble proteins, representing respectively 68.79–86.60, 8.86–27.72, and 2.89–11.85% of the total protein content. The ratio of polymeric to monomeric proteins varied between 1.37 and 3.73. Seventy-six reversed phase-HPLC-separated peaks have been differentiated from the ethanol extractable proteins of the entire population. Their distribution among the cultivars varied significantly, 6–23 peaks per cultivar. The number of appearances of peaks also showed large variation: one peak has been found in 107 samples, while 15 peaks have been identified, which appeared in less than five cultivars. An estimation method for ranking the avenin-epitope content of the samples has been developed by using MS spectrometric data of collected RP-HPLC peaks and bioinformatics methods. Using ELISA methodology with the R5 antibody, a high number of the investigated samples were found to be contaminated with wheat, barley, or rye.

Keywords: avenin, ELISA, HPLC, epitope prediction, celiac disease, oat

INTRODUCTION

Celiac disease (CD) is an autoimmune disorder triggered by the consumption of gluten proteins of, primarily, wheat, rye, and barley in a part of the population with certain genetic predispositions. The pathological processes induced by gluten in these individuals cause villous atrophy in the small intestines. The disease manifests in a range of symptoms from nutrient malabsorption to reproduction problems. The prevalence of CD is, on average, 1% worldwide, making it one of the most common food-related adverse reactions. Currently, the only way to treat CD is to adhere to a lifelong gluten-free diet (GFD) (1, 2). By omitting staple cereals, a GFD represents a risk of decreased intake of vitamins (predominantly, B group vitamins), important minerals (zinc, magnesium, selenium, and iron), and dietary fiber. The GFD is, generally, also accompanied by an excess intake of proteins, fats and sugars. Thus, the GFD must always be constructed with the help of a trained healthcare professional to aim for nutritional balance (3, 4).

Consumption of oats carries a number of nutritional benefits, including high contents of bioactive compounds such as β-glucans and antioxidants along with vitamin E and avenanthramides, as well as being an important source of proteins, fats, vitamins, minerals, fibers, phenolic acids, flavonoids, sterols, and phytic acid (5–8). Several clinical studies confirm that the soluble fiber β-glucan is strongly related to lowering blood cholesterol (LDL) levels (9–11). It can stimulate the immune system as well and positively affects the functioning of the human intestinal flora. Since oats are one of the best sources of fatty acids among the cereals, especially linoleic acid and low amounts of saturated fat, it plays a great role in reducing the risk of cardiovascular diseases (12, 13). The Food and Drug Administration of the United States of America has allowed a health claim for an association between consumption of diet, which is high in oatmeal, oat bran, or oat flour and has reduced the risk of coronary heart disease (14). This opened the era of novel utilization of oats in human nutrition as a key component in gluten-free diet (GFD) (15, 16) and as oat protein isolates, a cheap and valuable protein source for the food industry (17).

The benefits of both applications of oats as human food sources are directly related to the protein composition of the oats used, producing these food products: the inclusion of oats in the diet of celiac patients has been a controversial issue. Oats are a less likely candidate to trigger CD due to their protein composition. On the other hand, all of the important techno-functional properties of oats are directly related to the ratio of polymeric and monomeric proteins in the sample.

Wheat prolamins are the key players in the formulation of CD, especially their α- and γ-gliadin subunits (18, 19). These proteins contain a number of T cell stimulatory epitopes, mostly in their repetitive regions (20–22). In the case of oats, the main storage proteins are the 11S- and 12S-type globulins that consist approximately 80% of the total protein content. The remaining fractions are water-soluble albumins (14–20%) and the alcohol-soluble prolamins, named avenins (4–14%), depending on the genotype (23).

Oats are, in general, considered to have low CD-triggering potential due to their lower prolamin content, higher digestibility, and lower affinity to MHC (Major Histocompatibility Complex) molecules associated with CD compared with that of wheat prolamins (24).

A range of clinical studies has taken place to investigate the safety of oats in the GFD. Despite inconsistent results, a growing body of evidence concludes that the consumption of oats in moderate amounts (20–25 g/day for children and 50–100 g/day for adults) is safe for most patients with celiac in remission (25–29). A major problem of oat consumption in the celiac context is that gluten contamination from other gluten-containing cereals occurs frequently during conventional agricultural and food-processing practices (30, 31). The problem is being addressed in several countries by developing agricultural and industrial procedures to produce oats free from gluten contamination, referred to as pure oats (32–35). In line with the findings described above, the inclusion of pure oats in the GFD in moderate amounts is recommended by multiple countries, including the EU (36), the U.S. (37), and Canada (38). The legal gluten-free threshold of 20 mg/kg gluten applies to these oat products as well.

Although pure oats are considered to be safe for most patients with celiac, there are a number of studies suggesting that oats may be able to trigger CD on their own, but only affected the minority of the population with celiacs connected to individual sensitivity and the condition of the intestine (39). In a study by Lundin et al. (40), conducting a 12-week oat challenge, 18 out of 19 patients tolerated oats well. However, a single patient developed complete villous atrophy. This patient produced T cells that showed affinity to avenins and were used to identify two avenin epitopes (PYPEQEEPF and PYPEQEQPF) that may have been responsible for triggering villous atrophy. These results were limited to this single patient, but they raised questions about the presence of celiac-related epitopes in oat avenins.

According to the results of Silano et al. (41), laboratory and clinical tests with a large number of patients and a control group proved that differences can occur based on certain oat genotypes and individual sensitivity of patients as well. In the tests, duodenum segments derived from patient and control subjects were examined by fluorescent microscopy after incubation with protein extracts from different oat genotypes. Increased gliadin-induced transglutaminase enzyme production was observed on the segments incubated with protein extracts of wheat and certain oat genotypes. This suggests that not only the contamination of oats with other gluten-containing grains can cause problems, but there are oat cultivars that contain protein sequences that are low risk for patients with celiac. Based on the study of Real et al. (42), there is a great variety of potential immune reactivity of oat cultivars, which can generate a higher or lower degree of immune response in patients with celiac disease.

The contradictory preclinical and clinical results and the findings of research aimed at the genetic variability of avenin immunoreactivity (41, 43) suggest that oat varieties are not created equal in terms of their safety in CD. It has important implications for pure oat production and highlights the

importance of screening oat cultivars for the presence of celiac-related avenin epitopes. Fric et al. (27) found that the monoclonal antibody G12 developed for gluten detection (44, 45) cross-reacts with some sequences in avenins, but these peptides were considered irrelevant regarding the presence or absence of the clinically proven toxic internationally agreed celiac epitopes. The researchers suggested it may be a suitable tool for a fast, high-throughput prescreening of oat varieties (46). However, the G12 do not recognize the internationally confirmed oat avenin epitopes (47), but the antibody response is well correlated with the results of T cell proliferation and interferon γ release (46). The results of the clinical studies did not support the *in vitro* measures; the reasons could be that avenins did not contain any proteolytically resistant peptides longer than 10 amino acids, and avenin peptides have low-binding stability on HLA-DQ2.5 (48).

However, to obtain reliable information about the presence of celiac-related epitopes, immunological results should be accompanied by data on protein composition. The current scientific status about the safety of oats does not provide arguments to categorize certain oat cultivars as really harmful regarding CD. LC-MS (liquid chromatography–mass spectrometry) is the most important tool for the identification and quantification of immunoreactive cereal proteins (49). However, the quantification of gluten epitopes with this precise method can still be limited due to the high cereal protein polymorphism and an incomplete gluten database of oat immune responsive proteins (50).

The overall aim of our study is to demonstrate the variability of oat protein composition directly related to health-related and techno-functional properties. In this first report, we summarize our findings related to genetic factors in an international population of different oat cultivars that have been analyzed using a complex relatively fast and cost-effective protein separation methodology, suitable for characterizing large sample populations, and the resulting data have been evaluated, applying published proteomic information. While the data collected in this study on the overall protein composition, including the ratio of polymeric to monomeric oat proteins, can be directly related to functional properties, the results of the detailed analysis of avenin proteins can help breeders to select oat lines with suitable storage protein composition. The application of the same techniques, monitoring the effects of growing conditions on the protein composition of oat as well as the relationships between the protein composition and the techno-functional properties, is in progress and planned to be reported in subsequent publications.

MATERIALS AND METHODS

Plant Material

In this study, 162 oat cultivars and breeding material were analyzed with different genetic backgrounds and places of origin, 37 from Australia, 2 from Belgium, 9 from Canada, 4 from Chile, 5 from China, 1 from England, 1 from Ecuador, 2 from Finland, 4 from Germany, 2 from Holland, 40 from Hungary, two different regions and breeding backgrounds (Szeged and Martonvásár), 2 from Japan, 2 from New Zealand, 2 from Peru, 2 from Poland, 7 from South Africa, 5 from Sweden, 34 from USA, and 1 from Uzbekistan. All of the names of the varieties are coded with the first three letters of the origin plus a running number to comply with proprietary issues and breeding licenses. For easier handling and interpretation of the large dataset, eight subpopulations (R1-R8) were created from all of the analyzed varieties, based on, more or less, the geographic origin of the samples that served as a basis of data evaluation (**Supplementary Table 1**). The oat samples were derived from small plot field growing. After harvest, samples were stored in a dry and cold warehouse. The dehulling was made with Satake grain testing mill TM-05 (Satake Engineering Co. Ltd., Japan), dedicated only to GF grains, and grinding of hulled grains was carried out with a Retsch MM 400 ball mill (Retsch GmbH, Germany) in a gluten-free laboratory environment, which was monitored with the R-Biopharm RIDASCREENRIDA®QUICK Gliadin test stripes (Art. No.: R7003).

Protein Content

The protein content of oat flours was determined by the Dumas method (N × 5.95), an adaptation of the AOAC official method (51) using an automated protein analyzer (LECO FP-528, USA).

Characterizing the Protein Composition of Cultivars by Size Exclusion-High Performance Liquid Chromatography (SE-HPLC)

Size exclusion-high performance liquid chromatography analyses have been carried out with three replicate injections from two replicate extracts. A simplified version of the procedure of Gupta et al. (52) was applied as a one-step extraction. Based on preliminary studies, it was found that more than 95% of the proteins of oats can be extracted by simply vortexing the samples, so in contrast with the observations in the case of wheat, there was no need for a second consecutive extraction step using sonication The size exclusion-high performance liquid chromatography (SE-HPLC) using the procedure of Batey et al. (53) was used as modified by Larroque and Békés (54) with a mixture of two stock buffer solutions: A (12 g of 0.2 M NaH_2PO_4 + 500 ml MQ H_2O) and B (17 g of 0.2 M Na_2HPHO_4 + 500 ml of MQ H_2O). The final SE buffer solution was prepared by mixing 90 ml of solution A + 110 ml of solution B + 600 ml MQ H_2O + 4-g SDS.

Single grains from different samples were placed in 2 ml Eppendorf tubes with a 72-mm-diameter steel ball bearing placed on top of the grain. The tubes were lysed using a Qiagen®TissueLyser II (Qiagen GmbH, Germany) at 27 strokes/s frequency for 7 min. Flour from each tube (10 mg) was weighed in fresh 2 ml Eppendorf tubes, and 1 ml of an SE-HPLC extraction buffer was added to each tube. The tubes were then vortexed, using MO BIO Laboratories, Inc. Vortex-Genie®2 at setting 6 for 30 min. They were subsequently centrifuged for 15 min at 13,000 rpm, using Eppendorf Centrifuge 5424. The supernatant was then aspirated using a 1 ml syringe. The supernatant was then passed through a 0.45 µl filter into an HPLC vial. The vials were placed in an Agilent Technologies 1200 series HPLC instrument and were analyzed using the following

parameters: a Mobile Phase of 50% acetonitrile (ACN), HPLC grade, with 0.1% trifluoroacetic acid (TFA) and 50% water HPLC grade, with 0.1% trifluoroacetic acid (TFA) was used. The SE column (Agilent AdvanceBio Sec 300A, 2.7 μl, 4.6 × 300 mm) was washed for 60 min with 100% water to 100% acetonitrile and stabilized for 1 h before commencing the analysis. The column was used at room temperature, at 120-bar pressure; the injection volume was 10 μl at a flow rate of 0.350 μl/min. The SE-HPLC separation resulted in 10 peaks (P1-P10), polymeric globulin proteins eluted first (P1-P5), avenins in P6 fraction, while the four latest eluted little peaks (P7-P10) (integrated together) contained the soluble non-avenin proteins.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

About 60 mg oat flour was extracted using 70% ethanol and vortexed in a horizontal vortex (Vortex-Genie® 2, MO BIO Laboratories, Inc., USA) at setting 6 for 30 min. Samples were centrifuged for 15 min at 13,000 rpm g using Eppendorf Centrifuge 5,424. The supernatant was aspirated with taking care of the pellet and passed through a 0.45 μl filter into an HPLC glass vial. The samples were prepared in triplicate and were centrifuged for 20 min at 15870 × g. The supernatant was filtered using a 0.45 μm filter. The protein extracts were separated using Agilent 1200 LC Systems (Agilent Technologies, USA) by the method of Larroque et al. (55). About 10 μl of extracts were injected into a C18 reversed-phase ZORBAX 300SB-C18 column (4.6 mm × 150 mm, 5 μm, 300 Å, Agilent Technologies, USA), maintained at 60°C column temperature and at 50-bar column pressure. The applied eluents were 67% ultrapure water (Buffer A1) and 33% acetonitrile (Buffer B1), each containing 0.1% TFA (HPLC grade, Sigma Aldrich). The separation was carried out using a linear gradient from 33 to 80% Buffer B1 over 65 min at a flow rate of 1 ml/min.

RP-HPLC analyses have been carried out with three replicate injections from two replicate extracts.

R-Biopharm RIDASCREEN R5 ELISA Analyses

In order to detect gluten contamination from wheat, rye, or barley, oat samples were analyzed with the R-Biopharm RIDASCREEN® Gliadin assay (catalog number: R7001, R5 monoclonal antibody, sandwich format, LoD: 0.5 mg/kg gliadin or 1 mg/kg gluten, LoQ: 2.5 mg/kg gliadin or 5 mg/kg gluten). Extraction and the ELISA procedure were carried out in line with the kit instructions, adapted to local laboratory equipment. Briefly, 1 g of oat flour samples was weighed in 50 ml Falcon tubes. About 10 ml Cocktail solution (R-Biopharm, catalog number: R7016) was pipetted to each sample under a chemical hood. After vortexing, the samples were incubated at 50°C for 40 min in a shaking water bath (OLS Aqua Pro, Grant Instruments, United Kingdom). After cooling the samples to room temperature, 30 ml 80 V/V% ethanol was added to the samples, followed by 1 h of shaking on a table-top shaker (1,500 rpm, Vibrax VXR basic, IKA Werke, Germany). The samples then were centrifuged for 10 min at 2,500 × g at room temperature (LISA, AFI, France). Supernatants were diluted 1:12.5 with the sample diluent solution provided to the kit (the concentrate was pre-diluted prior to use according to the kit manual). About 150 μl of kit standards and samples were loaded to a transfer plate in duplicate. Finally, 100 μl of each sample and standard was transferred to the ELISA plate with a multichannel pipette. The plate was incubated for 30 min at room temperature and then was washed with the pre-diluted wash buffer provided for the assay in line with the kit instructions (ELx50 automatic plate washer, BioTek, USA). Then, 100 μl of the pre-diluted conjugate was added to all wells followed by 30 min of incubation at room temperature. After washing, 50 μl substrate and 50 μl chromogen were added to all wells, and the plate was incubated for 30 min at room temperature covered by aluminum foil. Finally, 100 μl of stop solution was added to all the wells, and absorbance values were obtained at 450 nm using a plate spectrophotometer (iMark, BioRad, USA). Data were analyzed with the Microplate Manager 6 software (BioRad, USA) using the cubic spline fit to create a standard curve. The results were the subject of further calculations to obtain the reporting unit of mg/kg gluten as per the kit instructions.

Prediction of Avenin-Epitope Levels

The immunodominant T cell epitopes of oat DQ2.5-ave-1a (PYPEQEEPF), DQ2.5-ave-1b (PYPEQEQPF) (56, 57), DQ2.5-ave-1c (PYPEQEQPI) (48), and DQ2.5-ave-2 (PYPEQQPF) were predicted, and the epitope containing avenin levels in different oat varieties was calculated based on the study by Tanner et al. (58). Sollid et al. (47) determined the celiac disease–relevant, internationally agreed T cell epitopes recognized by $CD4^+$T cells, namely, DQ2.5-ave-1a, DQ2.5-ave-1b, and DQ2.5-ave-1c. The study of Tanner even included the DQ2.5-ave-2 that contained only the minority of the investigated oat varieties, and the prediction was made based on it.

Briefly, Tanner et al. carried out RP-HPLC analysis from an Australian oat variety (cv. Wandering). The representative RP- HPLC chromatogram of the purified oat protein sample contained 18 well-defined RP peaks. RP-HPLC fractions were collected from the purified avenin sample and using MALDI-TOF-MS, and LC-MS/MS analysis of the chymotrypsin digested samples was carried out for protein identification. RP-HPLC analysis in this study has been carried out using the identical protocol in the same laboratory by the same operators as reported by Tanner et al. (58), resulting in matched elution profiles of avenin peaks with the published data and those derived from this study. The mass spectrometric information on the avenin peaks eluted at certain retention times the work of Tanner has been adopted to characterize the corresponding RP-HPLC peaks in our study. The individual and cumulative amounts of avenin proteins containing the four oat avenin T cell epitopes have been determined by selecting and summing the peak intensities based on the retention times of the peaks, expressed in [mg/100 g avenin] units using the average molecular mass of avenin proteins as 29 kDa (43) and with the molecular mass values of the four avenin epitopes, calculated from their amino acid composition and, finally, converted to [mg/100 g sample] units by multiplying

the mg/100 g avenin values by the SE-HPLC-based avenin content and by the protein content of the samples.

Using proteomics data of Tanner in such a way is based on the assumption that their data, which are based on the detailed study on a single cultivar (cv. Wandering), is representative for oat cultivars in general. The approach to the prediction of epitopes from RP-HPLC data is strictly reliable when these data would be supported and confirmed by amino acid sequence data, demonstrating (at least in a representative number of cultivars), the actual presence and amounts of intact avenin epitope sequences in the distinguished HPLC peaks. With the lack of such data, the predicted epitope levels can be interpreted as the measure of the possible variation of epitope contents in the cultivars in the sample population rather than the exact epitope levels in the individual samples.

The cumulative amounts of the presumably immune reactive avenin proteins per variety were determined and expressed as a percentage of the sample mass by combining the peak data of RP- and SE-HPLC separation and protein content of the samples.

Statistical Analyses

In the cases of both SE- and RP-HPLC analyses, mean values, standard deviation, and coefficient of variation have been calculated based on the six replicate data derived from the three replicate injections of two replicate extracts. The calculations have been carried out using MS Excel functions. Sample groups have been characterized by the variation of the above-mentioned mean values of different protein compositional data. To avoid any possible confusion, different notations are used for describing the variation among the replicate measurements of a given sample (mean, stdev, and cv) and the variation among the means of different measurements in a group of samples (mean, stdev, and cv).

In case of parameters derived more than one, standard deviations were calculated based on the Gaussian error propagation law (59) from the means and standard deviation values ($\boldsymbol{\sigma}$) from the individual parameters: in case of the cumulative amount of epitopes, the geometrical mean of the four standard deviations were used while the following equation was used for the determination of the standard deviation of the avenin levels in mg/100 g samples unit:

$$\boldsymbol{\sigma}_{\text{amg/100 g sample}} = 10^{-4} * \mathbf{mean}_{\text{protein}*} \left[(\boldsymbol{\sigma}_{\text{avenin}})^2 * \right.$$
$$\left. * (\mathbf{mean}_{\text{cum.epitop}}) + (\boldsymbol{\sigma}_{\text{cum.epitop}})^2 * (\mathbf{mean}_{\text{avenin}})^2 \right]^{0.5}$$

RP-HPLC profiles of the samples have been compared using pattern recognition techniques. The PATMATCH software (60) has been used for matching the chromatograms and identifying the corresponding peaks based on their elution time. Variation of retention times of peaks observed among replicate analyses and the minimum differences between the mean values of individual peaks have been determined and used to match the corresponding peaks from different samples. Similarity matrices using the presence and absence of peaks with the same elution time (S%) or with relative amounts of these individual peaks (S'%) have been constructed, also applying the PATMATCH software (60):

$$\mathbf{S_{A,B}}\% = 100^* \left(\frac{2^* n_{A,B}}{n_A + n_B} \right)$$
$$\mathbf{S'_{A,B}}\% = 100^* \left(\frac{2^* \sum_{n_{A,B}}^{i=1} e_i}{n_A + n_B} \right)$$

where $\mathbf{n}_\mathrm{A}$ and $\mathbf{n}_\mathrm{B}$ are the number of peaks in samples A and B, $\mathbf{n}_{\mathrm{A,B}}$ is the number of peaks with identical elution times in samples A and B, $\mathbf{e}_\mathrm{i}$ is a weighting factor describing the relative intensity of peaks with identical elution time. Cluster analysis was carried out applying the similarity matrices with the Morpheus R package (https://software.broadinstitute.org/morpheus/).

ANOVA test and multiple comparisons of mean values based on the least significant difference (LSD) by Student *t*-test were carried out as implemented in the NCSS 2021 Statistical Software (2021), (NCSS, LLC. Kaysville, Utah, USA, ncss.com/software/ncss).

RESULTS

Protein composition of the oat flour samples has been characterized on two levels: distribution of the total protein content after size-based separation was determined with SE-HPLC, followed by the RP-HPLC-based determination of the qualitative and quantitative composition of the avenin fraction.

SE-HPLC Analyses

More than 99% of the total amount of oat flour proteins has been extracted in the first step of the extraction procedure of Gupta et al. (52), without applying sonication. Comparison of samples has been carried out, therefore, using this simplified one-step procedure.

Three main protein groups have been detected based on the SE-HPLC separation (**Figure 1**). The polymeric protein fraction consisting of five well-defined peaks (P1–P5) with retention times of 5.2, 6.4, 7.4, 7.9, 8.3 min, respectively. The next main group is the avenin-type proteins, labeled P6 in **Figure 1** (retention time: 9.6 min), while the third group, containing a rather complex mix of the monomer globulin proteins (P7–P10), eluted in the region of 10–12 min. The elution profile of the 70% ethanol extract is also shown in **Figure 1**, clearly indicating that the ethanol-soluble proteins are eluted as one single peak (P6), analyzing the total protein extract.

The reproducibility of the peak intensity measurements has been monitored by calculating the mean, stdev, and cv values for each peak from their six replicate analysis data (**Supplementary Table 2**). Based on the averages of cv values calculated from the data of the 6 replicates among the 162 samples, the overall errors for the polymeric, avenin, and non-avenin monomeric protein group measurements are 5.018, 6.016, and 7.145%, respectively.

The distribution of the proteins among the three main groups and inside of the polymeric fraction shows a well-defined trend all around the 162 samples. The polymeric fraction represents about three-quarters of the total protein content (Mean: 73.14%,

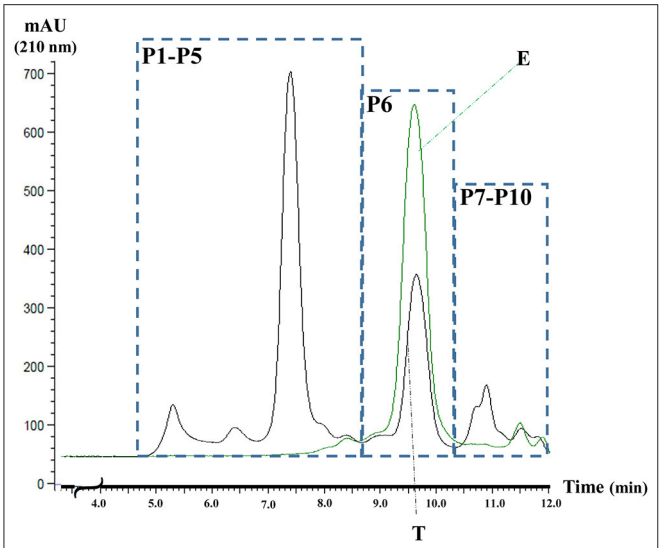

FIGURE 1 | The typical SE-HPLC profile of the total oat protein extract (T) and 70% ethanol extract of oat flour (E). AU, absorbance units at 210 nm, P1-P5-polymer fraction, P6-avenins, and P7-P10-monomer globulins.

min: 63.29%, max: 86.60%); the amount of the avenin fraction is varied between 8.86 and 27.72% (mean: 19.38%), while the amount of the monomeric globulin fraction is between 2.89 and 11.85% (mean: 7.29). In each sample, the relative amounts of the five subfractions of the polymeric proteins show a P1< P2 > P3 >> P4 ⋙ P5 trend.

Comparing the relative distribution of the proteins in the different geographic regions (R1-R8), it was found (**Table 1**) that the total amount of polymeric proteins and its distribution among the five subfractions (with the exception of P2), the amount of monomeric globulin proteins, and the ratio of the polymeric to monomeric globulin proteins, show significant differences among the eight geographic groups.

Compared to the data in the rest of the geographic groups, the highest amount of polymeric proteins (means: 75.10 and 74.65%) and polymeric to monomeric protein ratio (means: 11.89 and 13.47%) were found in the R1 and R7, respectively. The cause of these values derived from significantly higher amounts of P1 fraction found in the R1 and R7 groups (means: 25.65 and 17.35%, respectively.), compensated only partly with the significantly lower values of P3 (6.29 and 8.94%, respectively) in these groups.

Beyond the apparently uniform avenin levels observed at the comparison of mean values in the different geographic groups, some extremely low (AUS05: 8.86%) and extremely high (AUS14: 27.72%) avenin contents were observed, for example, in the R1 sample group. These cultivars could have great potential to be applied to nutrition-related breeding programs.

RP-HPLC Analysis

The RP-HPLC patterns and peak distributions showed great variation in the number and composition of different avenin polypeptides, indicating the extent of genetic and proteomic diversity in this large oat population (**Supplementary Table 3**). In the 162 oat samples, 76 distinct peaks have been matched by the PATMACH software in the 25.75 to 47.25 min elution time interval using a 0.10 min window to identify the corresponding peaks in the different chromatograms. It means that, if the differences in retention times of a particular peak in different samples were lower than 0.10 min, then the peaks have been evaluated as identical peaks. Using this procedure, the number of peaks in a given sample has been determined, indicating a large variation between 6 and 18 peaks (Mean: 10). This variation in the number of separated peaks can be explained by the variability of the resolution of RP-HPLC technique as the function of the amounts of proteins in a peak: the individual peaks in certain cases might contain more than one protein type (as it was shown in the work of Tanner et al. (58), characterizing individual RP-HPLC peaks by using the mass spectrometric methodology.

As it was observed in previous studies (for example, Tanner et al., 2019), most of the avenin polypeptides are eluted in two elution time intervals: 20 peaks have been found in the 25.75-32-min interval and 37 in the 38–47.25-min interval, representing the 45.58 and 48.42% of the total avenin content, respectively.

The number of appearances of a peak with a given retention time in different samples was found to be extremely variable. There are three peaks with the retention times of 25.75, 34.50, and 35.00 min found only in three cultivars, namely in US12, HUN25, and CAN06; 17 peaks have been identified, which appeared in less than 6 samples, while the peak with the retention of 42.39 min was found in 107 samples.

The level of large polymorphism of avenin polypeptides in the sample population investigated in this study is well demonstrated by the S% similarity matrix (not shown) and the cluster analysis diagram (**Supplementary Figure 1**). Based on the dendrogram, six clusters (A to F) can be identified characteristically containing or missing certain peaks indicated in **Table 2** with bold or with italics, respectively. As the color scale of the diagram clearly indicates, similarities among samples in the cluster are significantly larger than those in any other clusters. The list of the clusters for the different samples is indicated in the last column of **Supplementary Table 3**.

Some interesting observations can be made, investigating the distribution of samples in the different clusters based on their origin (**Table 2**). While the samples in R2, R5, and R8 groups are scattered in different clusters, most of the samples in R6 group are together in Cluster C, the ones in R3 either C, D, or E, but not in A or B cluster; 18 from the 40 samples in R7 can be found in Cluster F, and 36 from the 39 samples in R1 are located in Cluster A.

Differences among the avenin composition of the samples are significantly enlarged if the amounts of the different peaks are used in similarity calculation (S'%) instead of the presence/absence-based comparison (S%). Expression levels of avenins with the same retention times in different samples have been found largely not uniform among the peaks.

The reproducibility of the peak intensity measurements has been monitored through the 1,530 peaks found in the whole sample population by calculating the mean, stdev, and cv values for each peak from their six replicate analysis data, resulting

TABLE 1 | Statistical analysis on the variation of the size-based distribution of the total proteins of oats samples among the different geographic regions.

SE-HPLC	Region	R1	R2	R3	R4	R5	R6	R7	R8	F	p
fraction	*n*	39	11	43	7	7	7	40	8		
P1	mean	25.65	14.74	15.52	15.57	16.30	14.48	17.35	14.01	34.54	0.0000
		(b)	(a)	(a)	(a)	(a)	(a)	(a)	(a)		
	min	14.92	11.30	10.31	14.03	15.14	12.45	11.63	10.48		
	max	39.25	17.30	20.62	18.74	17.35	15.79	23.79	16.38		
	Sd	5.34	1.99	2.76	1.71	0.83	1.08	3.08	2.14		
P2	mean	34.67	35.92	36.65	35.25	35.36	35.67	35.05	35.97	0.43	0.8799
		(a)	(a)	(a)	(a)	(a)	(a)	(a)	(a)		
	min	13.37	31.14	30.70	32.80	32.73	32.85	7.37	32.06		
	max	47.80	39.32	42.16	39.52	39.26	37.12	49.52	39.33		
	Sd	6.01	2.29	2.19	2.50	2.06	1.55	8.92	3.10		
P3	mean	6.29	13.51	12.88	12.43	13.86	10.55	8.94	13.79	12.24	0.0000
		(a)	(b)	(b)	(b)	(b)	(ab)	(a)	(b)		
	min	3.56	11.35	6.43	10.40	12.13	9.43	3.19	10.31		
	max	12.91	16.26	17.39	13.95	15.28	12.19	34.22	18.87		
	Sd	2.32	1.79	2.48	1.39	1.09	1.00	6.81	2.44		
P4	mean	3.77	2.17	2.20	2.23	2.17	2.40	4.81	2.34	4.80	0.0001
		(ab)	(a)	(a)	(a)	(a)	(a)	(b)	(a)		
	min	0.26	1.93	1.61	2.04	1.96	2.22	2.23	1.94		
	max	8.31	2.55	3.00	2.46	2.33	2.58	9.56	2.95		
	Sd	1.33	0.20	0.30	0.18	0.14	0.16	0.46	0.37		
P5	mean	4.73	5.16	5.23	5.29	5.15	5.70	8.50	5.55	6.93	0.0000
		(a)	(a)	(a)	(a)	(a)	(a)	(b)	(a)		
	min	1.94	4.58	3.81	4.84	4.65	5.28	2.93	4.60		
	max	6.61	6.05	7.13	5.85	5.54	6.13	19.99	7.00		
	Sd	1.22	0.48	0.72	0.43	0.33	0.38	0.52	0.87		
	mean	75.10	71.50	72.47	70.77	72.84	68.79	74.65	71.66	4.15	0.0003
Polymers		(c)	(ab)	(b)	(ab)	(b)	(a)	(c)	(ab)		
	min	66.13	68.76	63.29	66.56	70.39	65.52	64.27	67.67		
(P1–P5)	max	84.02	74.35	80.23	73.32	76.19	71.08	86.60	76.84		
	Sd	4.36	1.63	3.52	2.72	1.92	2.01	5.25	3.44		
	mean	18.32	19.84	19.85	20.33	19.78	21.88	18.98	20.09	1.66	0.1240
Avenins		(a)	(a)	(a)	(a)	(a)	(a)	(a)	(a)		
	min	8.86	17.61	14.65	18.61	17.87	20.30	10.08	16.89		
(P6)	max	27.72	23.23	26.62	22.47	21.27	23.56	27.06	24.31		
	Sd	3.97	1.85	2.54	1.64	1.25	1.44	3.79	2.81		
	mean	6.56	8.66	7.67	8.90	7.39	9.32	6.37	8.25	7.21	0.0000
Monomers		(a)	(b)	(ab)	(b)	(ab)	(b)	(a)	(b)		
	min	4.08	6.69	4.28	7.66	5.94	8.60	2.89	5.47		
(P7–P10)	max	9.47	11.85	10.88	10.99	8.98	10.92	10.23	10.35		
	Sd	1.24	1.37	1.65	1.25	1.05	0.90	2.18	1.56		
Polymer	mean	11.89	8.44	10.00	8.10	10.06	7.45	13.47	9.05	6.70	0.0000
to		(bc)	(a)	(b)	(a)	(b)	(a)	(c)	(ab)		
monomer	min	7.35	5.89	6.27	6.05	7.84	6.00	6.41	6.63		
ratio	max	19.39	10.49	18.29	9.33	12.82	8.25	27.82	14.04		
	Sd	2.68	1.27	2.77	1.28	1.69	0.83	5.55	2.32		

P values highlighted in red indicate significant differences among groups. Different letters indicate significantly different mean values based on Student t-test ($p < 0.05$).

in a 7.18% for the average value for the cv values. The r^2 value between elution times and cv values of peak intensities of peaks eluted at a given elution time was found to be 0.0036, while a strong negative correlation was found between the peak intensities, and their reproducibility ($r^2 = 0.7934$): in the 10–15% peak intensity interval, the cv values are smaller than 6%,

TABLE 2 | Characteristic peaks in the six similarity clusters of avenin protein RP-HPLC profiles and the origin-based distribution of oat samples among the clusters.

Cluster	Characteristic peaks						Origin	R1	R2	R3	R4	R5	R6	R7	R8
	Retention time (min)						n	39	11	43	7	8	7	40	7
A	*27.80*	**30.64**	*43.33*	**43.67**			38	36	2						
B	**27.00**	**28.78**	**29.09**	**29.32**	*30.12*	**43.10**	*43.33*	8						8	
C	**29.09**	*29.32*	*30.64*	**40.50**	*43.67*			36		4	15	2	5	6	2
D	**28.78**	*29.09*	**43.33**	*43.67*	*44.58*	**45.66**	**46.33**	26	1	2	10	2		3	4
E	**28.78**	*29.09*	*31.09*	**40.50**	*43.10*	*43.67*		33	1	3	17	2	2	5	1
F	*29.86*	**30.12**	*30.64*	**32.11**	**42.39**	**43.33**	*44.58*	21	1		1			18	

Retention times in bold indicate peaks existing in a cluster, while retention times in italics indicate no peaks in a cluster. Blank fields indicate that, in that certain subpopulation, the characteristic RP profile defined is absent.

while in 6, 7 and 10, 11% in the 5–10 and 10–15% intensity intervals, respectively.

Predicting the Amount of Celiac-Related Oat Epitope-Containing Components

Applying the data provided by Tanner et al. (58) for the composition of avenin fraction of the oat variety cv., the amounts of the celiac-related oat epitope-containing components of the 162 oat samples have been predicted based on their RP-HPLC analysis results.

Six dominant peaks were identified, containing conserved avenin types: peak 3 (R.T. = 28.133 min) in 43 samples, peak 6 (R.T. = 30.465 min) in 80 samples, peak 8 (R.T. = 31.152 min) in 60 samples, peak 15 (R.T. = 44.158 min) in 36 samples, peak 16.2 (R.T. = 44.408 min) in 48 samples, and peak 16.3 (R.T. = 44.914 min) in 79 samples. Peak 3 contained the gliadin-like avenin (L0L6J0), peak 6 contained, also, a gliadin-like avenin (L0L6K1), peak 8 contained an Asat-Prolamin10 protein and a 23539 Da avenin (Q09072), peak 15 contained an avenin-F protein, with an alternative name celiac immunoreactive protein 2 or gamma-avenin-3 (Q09097) and an Asat-Prolamin71 protein, peak 16 contained an avenin (I4EP54), a gliadin-like avenin (L0L6J0), and an Asat-Prolamin15 protein. In the case of peaks 3, 6, and 8, the predominant avenin epitope is the DQ2.5-ave-1a (PYPEQEEPF), in peak 15, the DQ2.5-ave-1b (PYPEQEQPF) and DQ2.5-ave-1c (PYPEQEQPI), while, in peak 16, all the above mentioned three avenin epitopes occurred.

The individual and cumulated amounts of avenin epitopes have been determined by selecting and summing the RP-HPLC data according to their retention time, and then converting the resulting values to epitope contents based on their molecular mass. Finally, these values in [mg/100g total avenin] have been converted to [mg/100 g sample] units. Mean values, standard deviations, and cv values were calculated from the six replicate RP-measurements together with the protein content of the samples and six replicate SE-HPLC data for avenin content in case of the conversion to [mg/100 g sample] unit – (**Supplementary Table 4**).

Satisfactory reproducibility has been observed for the individual and cumulated epitope levels (average cv values calculated for the 162 samples for the DQ2.5-ave1a, DQ2.5-ave11b, and DQ2.5-ave1c epitopes and for their cumulated value: 0.096, 0.067, 0.082. and 0.063, respectively). The cv values for the avenin levels expressed in [mg/100 g sample] units varied between 0.003 and 0.129 with an average of 0.062.

R5 ELISA

For the pure oat line development study, a small population consisting of 32 Australian and 35 Hungarian samples (**Supplementary Table 5**) was selected from the basic population for ELISA testing. Samples were selected to cover a wide range of crude protein content using samples with sufficient available amounts. The presence of potential gluten contamination from other cereals was tested with the R5 ELISA method of R-Biopharm. Based on the results of this test, 19 Australian and 24 Hungarian samples of the investigated oat varieties were uncontaminated, thus, deemed appropriate for the requirements

of pure oat cultivation in terms of purity. Our results confirm that gluten contamination of oats is a serious problem and must be carefully addressed when providing seeds for growing pure oats.

DISCUSSION

The aim of our work was to carry out a high-throughput analytical screening completed with immune analytic measurements to develop a reliable prediction method for estimating the amount of avenin proteins and those that contain celiac-related epitopes. This special prediction method utilizes the combined application of SE- and RP-HPLC separation of the total protein content of the oat flour samples and differentiates the absolute levels of the four main avenin epitopes of the samples, and also provides the celiac-related epitope, containing avenin content in the oat flour (g/100 g).

Most of the oat-related research in the last 10 years concentrated on avenins, debating on their harmfulness in relation to celiac disease. Meanwhile, oats started to be recognized as a healthy and nutritious cereal, containing a high concentration of soluble fiber (β-glucan) and being dense in nutrients. It has physiological benefits like reducing hyperglycemia, hyperinsulinemia, and hypercholesterolemia, and several other benefits are discussed in several reviews like the one by Ibrahim et al. (61).

Interestingly, no application of SE-HPLC on characterizing oat proteins is reported in the critical work of Sunilkumar and Tareke (62), which reviewed the analytical methods for measurement of oat proteins by covering 2,000 works published between 1970 and 2015.

However, the application of size-related analytical techniques like SE-HPLC has a large potential to be used in selecting oat lines for industrial ingredient use (61).

In the scientific literature, there are many useful high-throughput studies on the methods developed to estimate the immunoreactivity of oat avenins and the availability of safe oat varieties for patients with celiac. A combined method using RP-HPLC and electrophoresis of oat avenins has been reported earlier (63), and the utility of the RP-HPLC for the identification of oat varieties has been demonstrated (64). It has also been suggested that RP-HPLC of alcohol-soluble storage protein fractions would be useful for selecting oat varieties with reduced immunogenicity for patients with CD (42). Giménez et al. (65) differentiated 120 oat cultivars from five geographical origins based on RP-HPLC peak profiles of avenins, combined with G12 competitive ELISA. The researchers confirmed that the RP-HPLC technique is useful to establish groups of varieties, differing in degree of storage proteins with low immunoreactivity for patients with CD, but not sufficient to uniquely identify the different varieties of the set (65). Schalk et al. (66) presented well-defined gluten protein fractions and types of wheat, rye, barley, and oat flours using mixtures of four cultivars each to account for the genetic variability between different cultivars, including the most relevant cultivars in Germany 2012.

Souza and co-workers revealed that avenin patterns of the examined oat cultivars are not distributed equally based on the place of origin (67). Previous papers reported the connection between oat prolamins and disease resistance genes. Gimenez et al. (65) pointed out that, according to this correlated variation, environmental and breeding factors caused non-random avenin profile variability. The study aimed to evaluate how variable avenin protein patterns of different oat cultivars are linked with low avenin content. Colgrave et al. (68) developed a high-throughput and sensitive approach to identify the possible source of gluten-like proteins in the view of contamination of GF grain. It reveals that the examined commercial oat flour samples were, in fact, contaminated by trace amounts of wheat.

Based on the results of our study, the high variability of avenin fraction composition and biodiversity of cultivated oat varieties are in agreement with the results of several research groups who are experts of this field.

The key avenin peptides that stimulate the pathogenic gluten-specific T cells in patients with CD *in vivo* have been defined (48, 69). These peptides contain the immunodominant T cell epitopes DQ2.5-ave-1a (PYPEQEEPF), DQ2.5-ave-1b (PYPEQEQPF), DQ2.5-ave-1c (PYPEQEQPI), and DQ2.5-ave-2 (PYPEQQPF) with close sequence homology to barley T cell epitopes immunoreactive in CD such as DQ2.5-hor-3a (PIPEQPQPY) (69). Londono et al. (70) investigated 13 Avena species, and no perfect gluten epitopes were found in avenins; besides this, none of the R5 and G12 antibodies recognition sites were found. The ELISA assay is a widely used method that gives quantified information about the contamination level and traces the possible source of gluten-like proteins in cereal crops. ELISA R5 shows no cross-reactivity to oats and can, therefore, be used to assess wheat, rye, or barley contamination in oats. The study of Comino and co-workers allowed the classification of oat varieties into three groups based on their degree of affinity for the G12 antibody: a highly reactive group is not safe for patients with celiacs; the moderate recognition group is not recommended, and one with no reactivity is a potential celiac safe group (46, 71). However, oat avenin extracts usually have a low G12 antibody response, the G12 reactivity well correlates with the results of T cell proliferation and interferon γ release. A direct correlation of the reactivity with G12 and the immunogenicity of the different prolamins were observed (72). In contrast, a comprehensive study by Londono and co-workers proved (70) that the signals of R5 and G12 should not be interpreted as differences in immunogenicity of oat varieties because of the lack of antibody recognition sites in avenins.

However, some preclinical studies working with cell cultures revealed differences in the immunogenicity of the different oat genotypes (46, 72); the results of the clinical investigations and data with organ culture system did not correlate, and refuted them (73, 74). Based on their results, oats do not display *in vitro* activities related to CD pathogenesis, and the T-cell reactivity could be below the threshold for clinical relevance, and it affects only a minority of patients. Besides this, researchers elaborated on the real CD-toxicity of the oat CD-immunogenic epitopes (48) and concluded that these have high protease

TABLE 3 | Variation of the amounts of celiac-related avenin epitopes among 106 oat samples.

	DQ2.5- ave-1a	DQ2.5- ave-1b	DQ2.5- ave-1a	DQ2.5- ave2	Cumulative amount of celiac related avenin epitopes	
	mg/100 g avenin					mg/100 g sample
Mean	1501.28	676.72	585.2	18.55	2763.2	84.92
min	0	0	0	0	103.84	2.20
max	3753.64	1651.76	1651.76	39.16	6900.52	270.60
StDev	859.76	419.76	432.52	14.09	1427.8	55.44
C.V.	0.57	0.62	0.74	0.76	0.52	0.65

TABLE 4 | ANOVA comparison on the predicted celiac-related avenin epitope contents of samples in the eight regions of origin.

		mg/100 g avenin								mg/100 g sample	
Group	**n**	**DQ2.5-ave-1a**		**DQ2.5-ave-1b**		**DQ2.5-ave-1c**				**DQ2.5-ave-1a + DQ2.5-ave-1b + DQ2.5-ave-1c + DQ2.5-ave2**	
R1	39	1441.00	(ab)	513.48	(a)	349.36	(a)	2304.28	(a)	47.52	(a)
R2	11	1744.60	(b)	697.84	(a)	552.64	(b)	2995.08	(ab)	96.36	(ab)
R3	43	1769.24	(b)	755.04	(a)	714.56	(bc)	3239.72	(b)	106.04	(ab)
R4	7	1930.72	(b)	841.72	(a)	830.72	(c)	3603.16	(b)	123.64	(b)
R5	7	1216.16	(a)	592.68	(a)	557.04	(b)	2365.88	(a)	82.28	(ab)
R6	7	1522.84	(ab)	722.04	(a)	658.24	(b)	2903.56	(ab)	102.64	(ab)
R7	40	1079.76	(a)	696.96	(a)	614.68	(b)	2392.28	(a)	77.88	(ab)
R8	8	1981.32	(b)	807.40	(a)	680.24	(b)	3468.96	(b)	117.92	(b)
	F-Ratio	3.0997		1.4139		2.8668		2.5627		5.6672	
	p	0.0044		0.2034		0.0077		0.0159		0.0001	

Different letters indicate significantly different mean values based on Student t-test ($p < 0.05$). P values highlighted in red indicate significant differences among groups.

sensitivity (22) and a relatively low HLA-binding capacity (48). Another research group has also demonstrated the sensitivity of avenins to proteolytic enzymes; DQ2.5-ave-1a and DQ2.5-ave-1c were completely digested by pepsin, trypsin, and chymotrypsin. The DQ2.5-ave-1b was proteolyzed by brush border enzymes (mostly by the prolylendopeptidase) (74). The susceptibility of oat avenins to proteolysis corresponds to their low-proline content (an average of 6% in avenins) (74). Both factors, together, significantly reduce the immunoreactivity of avenins and thus of oat-based foods. These findings were confirmed by the study of Hardy and co-workers in a large-scale oat challenge proved that the ingestion of oat is safe for patients with celiac without intestinal damage and serological relapse.

Because pure oat consumption carries a low risk for patients, the researchers declare that the strict control of production systems of pure oat is of utmost importance, and the regular follow-up of the patients with CD is recommended. Based on the R5 R-Biopharm RIDASCREEN® Gliadin assay of the selected subpopulation showed that 35% of the samples were contaminated. This highlights the necessity of improving the pure oat line and developing very sensitive and specific analytical methods for the sake of food safety.

All observations described above were derived from a reasonably large study where the carefully executed experiments were carried out with 2 × 3 replicates. The resulting data have been thoroughly analyzed statistically, taking into consideration the non-trivial characteristics of cumulative and complex parameters, where the actual results were derived from several independent measurements with experimental errors. The reproducibility of the two chromatographic separations, as well as the final cumulative results, seems to be satisfactory with the relative errors being under 12%.

These positive experimental characteristics, however, do not avoid two principal limitations of the prediction method introduced here:

i. The reliability of the predicted information derived from this prediction process strongly depends on the validity of the assumption that the proteomic data (derived from the analysis of one single cultivar) are representative of oat cultivars in general. The predicted epitope levels should be validated by detailed proteomic analysis to avoid this limitation. With the lack of such validation, the predicted epitope levels can be interpreted as the measure of the possible variation of epitope contents in the cultivars in the sample population rather than the exact epitope levels in the individual samples.
ii. Because of the limited resolution of the RP-HPLC separation of avenin proteins, some oat polypeptides co-elute, producing false-positive results. Therefore, the predicted epitope levels have to be interpreted as upper limits.

In this study, large qualitative and quantitative differences have been observed in the avenin composition of the samples investigated: both the individual and cumulative amounts of the four oat avenin epitopes show large variation.

Analyzing the data, the most important observation is that, while certain cultivars do not contain all the four different epitopes, there is no variety among the 106 samples not containing any DQ2.5-ave epitopes.

Data shown in **Table 3** were calculated from the mean values of replicate measurements (**Supplementary Table 4**); the average amount of the DQ2.5-ave-1a epitope in the samples is more than double compared with those of DQ2.5-ave-1b or DQ2.5-ave-1c epitopes (1501.28, 676.72, and 585.20 mg/sample), respectively. The number of cultivars where the presence of the individual epitopes has been demonstrated (**Table 3**) shows the same sequence: 104, 100, 93, and 3. The amount of DQ2.5-ave2 epitope in the three samples (US14, US31, and HUN13) where this epitope is present is marginal (34.75, 9.20, and 11.70 (mg/100 g sample)], respectively. Huge variation in the levels of the individual epitopes has been found, with larger than 0.5 cv values for each epitope class. The cumulative amount of epitope content in the samples varied between 2.20 and 270 mg in the 100 g sample with a strongly asymmetric distribution (**Supplementary Figure 2**), with the maximum number of 46 cultivars (28.40%), containing 26–50 mg/100 g epitopes. Two cultivars have been found with epitope levels of less than 5 mg/100 g (HUN31 and AUS04); these rarely found low levels could be utilized in breeding for healthy oat varieties.

As the results of the large variation of epitope levels in the whole sample population, significant differences among the origin-based subgroups can be observed (**Table 4**) for the amounts of DQ2.5-ave-1a and DQ2.5-ave-1c, but not for DQ2.5-ave-1b. The highest F value (5.6672) was found for the cumulative epitope levels data expressed as [mg/100 g sample] what can be explained by the fact that these values do not only derive from the variation in avenin composition, but they are varied by the total amount of avenin proteins as well as the protein content of the samples. The comparison of mean values, in this case, shows significantly lower levels in the Australian samples (47.52 mg/100 g sample) compared with the South African and South American samples (117.92 and 123.61 mg/100 g samples), respectively.

The celiac-related epitope content of an oat sample is determined by its avenin composition, but the relative expression levels of both avenin- and non-avenin-type polypeptides can overwrite the ranking of the overall epitope levels in the samples, as it is illustrated in **Figure 2**: In the samples in the circled interval of the figure, the epitope levels expressed in mg/100 g avenin protein unit are misleading, underestimating the amount of epitopes taken by the consumed oat.

As it is well established for all cereal crops, including oats, both the protein content and protein composition are highly affected by the growing conditions, including both environmental and agrotechnical factors. Based on an unpublished large project carried out in our laboratory, investigating the alteration of the protein composition of 180 oat cultivars under rainfed and irrigated conditions, protein content of the samples of the same cultivar can be altered by 15 relative percentages while the ratio of polymeric and avenin proteins can vary by 38 relative percent caused by the water availability.

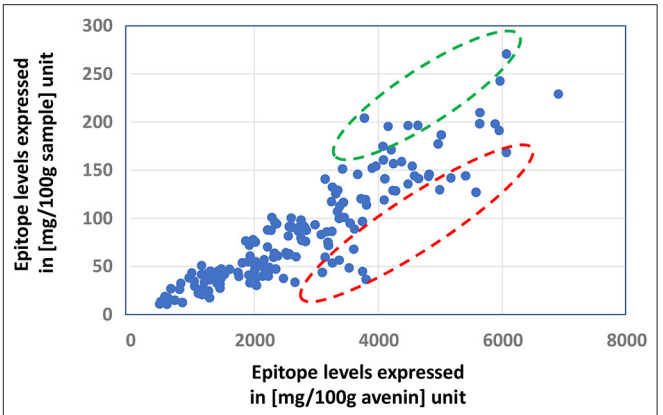

FIGURE 2 | Demonstrating the importance of expression levels of avenin and non-avenin proteins in the ranking of relative celiac epitope amounts of oat samples by the comparison of ranking samples based on the amount of celiac-related epitopes expressed in (mg/100 g avenin) and (mg/100 g sample) units. Relative celiac-related epitope levels in samples in the red circle are largely underestimated by the simple comparisons of the epitope levels in the samples, not taking into account the total protein content and its avenin content. Circled data with red and green indicate under- and overestimated epitope levels using [mg/100 g avenin] units, respectively, not considering the contribution of protein content and avenin content of the sample.

The observation illustrated in **Figure 2** underlines the need for quantitative characterization of the overall protein composition rather than simply concentrating on the avenin composition, estimating the celiac-related epitope content of oat samples.

CONCLUSION

Utilization of oats lines for human consumption requires the use of a reliable methodology of monitoring the presence and quantity of epitope containing components in the samples, and a better understanding of chemical composition and technological properties is needed. Both of these aspects require the active use of quantitative protein analytical techniques for the characterization of the whole spectra of oats proteins, albumins, globulins, prolamins, and glutelins. The application of detailed protein composition data has huge potential both in evaluating oats breeding lines in the pre-breeding selection phase and in monitoring oats-containing products in the food industry.

The combination of SE- and RP-HPLC methodology with active use of available proteomic data seems to be a satisfactory tool for these types of applications. Relating SE-HPLC-related quantitative protein analytical data to functional properties of oat samples like water and oil-binding capacity, emulsifying and foaming properties and even rheological properties of oats-containing doughs are in progress to utilize the data collected in this study.

Despite these valid and serious above mentioned limitations of the prediction method developed in this work, our view is that, with the lack of any other (better) relatively high throughput and

cheap method, what is applicable to large sample populations the method is suitable to be used as a preselection screening tool in oat breeding in its present form already. Ongoing attempts to carry out further individual RP peak proteomic validation studies on different oat varieties, hopefully, will make our prediction method much more accurate in the future.

DATA AVAILABILITY STATEMENT

The original contributions generated for the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

FB, ZBu, GG, and ST designed the study. KÁ, KS, BV, GV, and OV provided samples and sample preparation. FB, GG, CF, ZBu, DR, ZBi, ES, BL, ZS, EM, SP, and ST performed experiments and analyzed data. GG, ZBu, DR, KÁ, ZBi, and FB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.702352/full#supplementary-material

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Conflict of Interest: KÁ, BL, and SP were employed by company Cereal Research Non-Profit Ltd. KS was employed by company First Pest Mill and Bakery Ltd. FB was employed by company FBFD PTY Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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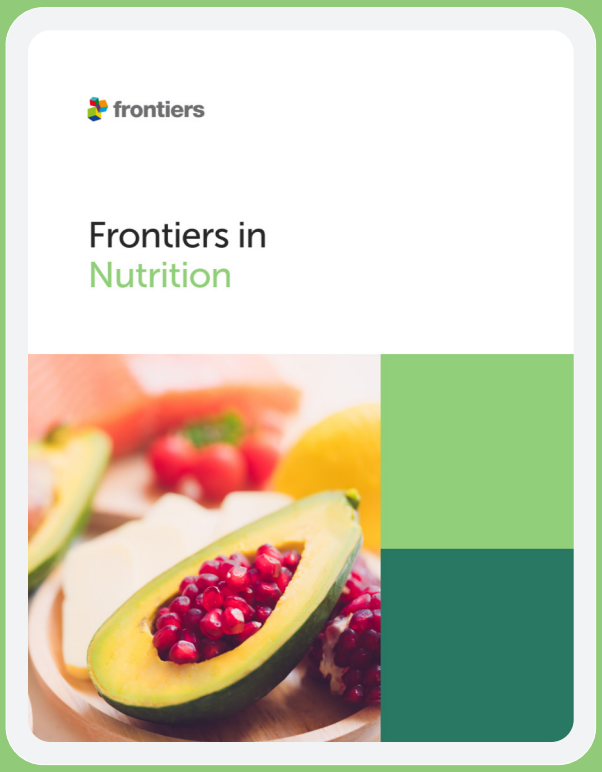

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