Barro Losada Francisco (Orcid ID: 0000-0002-7652-229X)

Tritordeum Breads are well Tolerated with Preference over Gluten-Free Breads in Non-Celiac Wheat-Sensitive (NCWS) Patients and its Consumption Induce Changes in Gut Bacteria

Susana Sánchez-León[a†], Carmen Haro[b†], Myriam Villatoro[a], Luis Vaquero[c], Isabel Comino[d], Ana B. González-Amigo[c], Santiago Vivas[c], Jorge Pastor[e], Carolina Sousa[d], Blanca B. Landa[b] and Francisco Barro[a*]

[a] Departamento de Mejora Genética Vegetal, Instituto de Agricultura Sostenible (IAS-CSIC), Córdoba, Spain

[b] Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible (IAS-CSIC), Córdoba, Spain

[c] Departamento de Gastroenterología, Hospital Universitario de León, Instituto de Biomedicina, Universidad de León, León, Spain

[d] Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Sevilla, Sevilla, Spain

[e] Novapan, S.L., La Puebla de Alfinden, Zaragoza, Spain

[†]These two authors contributed equally to this work.

[*]Corresponding author (fbarro@ias.csic.es)

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Abstract

BACKGROUND: The ingestion of wheat and other cereals are related to several gut disorders. The specific components responsible for NCWS may include gluten and other compounds. Tritordeum is a new cereal derived from crossing durum wheat with a wild barley species, which differs from bread wheat in its gluten composition. In the present work, we examined the response of NCWS patients to tritordeum bread Gastrointestinal symptoms as well as tritordeum acceptability, Gluten Immunogenic Peptides excretion, and the composition and structure of the intestinal microbiota were evaluated.

RESULTS: Gastrointestinal symptoms of the subjects showed no significant change between the gluten-free bread and the tritordeum bread. Participating subjects rated tritordeum bread higher than the gluten-free bread. Analysis of the bacterial gut microbiota indicated that tritordeum consumption does not alter the global structure and composition of the intestinal microbiota, and only a few changes in some butyrate-producing bacteria were observed.

CONCLUSIONS: All the results derived from acceptability, biochemical and microbiological tests suggest that tritordeum may be tolerated by a sub-set of NCWS sufferers who do not require strict exclusion of gluten from their diet.

Keywords: Non-Celiac Gluten Sensitivity, Tritordeum, Gluten Immunogenic Peptides, Gluten Free Diet, Gut Microbiota.

Introduction

The cereals, and in particular wheat, have a central role in human diet and are important sources of complex carbohydrate, protein and dietary fiber as well as minerals, vitamins and phyto-active compounds (1). They have highly valued organoleptic properties and are functionally versatile allowing them to be use in a great number of food applications. Although in general the cereals are very safe foodstuffs consumed routinely by billions of people, they are implicated in some food allergies and dietary intolerances, mainly related to their protein composition and in particular to gluten proteins present in cereals such as wheat, barley and rye. Gluten-related disorders include wheat allergy (WA) an allergic reaction associated with a spectrum of different proteins, including gliadins and amylase trypsin inhibitors (ATIs) (2); celiac disease (CD), an immune-mediated enteropathy induced by exposure to dietary gluten and non-celiac gluten sensitivity (NCWS) a disease related to the ingestion of gluten-containing foods in persons who do not suffer from WA or CD (3, 4). The specific components responsible for NCWS have still to be confirmed and may include gluten proteins, ATIs, fermentable mono- and polysaccharides and polyols (FODMAPS) or other factors (4), but with an estimated frequency of 6 – 13% the number of persons affected by NCWS is significantly higher than WA (around 1-2%) or CD (around 1%) (3-6). Although there is evidence that not all NCWS sufferers need to exclude gluten completely from their diet as they may have different levels of tolerance (7), many follow a gluten-free diet (GFD), although this may have negative nutritional implications as GFDs containing more fat and less carbohydrate than recommended and tend to be poorer in several important dietary components such as folic acid, calcium, iron and vitamin B6, B12 and D (8, 9). In addition a strict GFD may have negative effects on gut microbiota (10-12). In fact, a microbial dysbiosis in NCWS causes gut inflammation, abdominal pain, metabolic problems, neuroinflammation, gut-brain axis dysfunction, intestinal barrier defects and inflammatory responses to gluten, among others (13, 14). In addition, Uhde et al. (15) demonstrated the existence of an increased intestinal

permeability in subjects with non-celiac/non-allergic gluten/wheat sensitivity (NCG/WS). Although changes in intestinal microbiota had been associated to gluten free diet (16), those may also exist in NCWS patients but this has not been proven yet (17). It is known that bacterial species mainly belonging to Firmicutes phylum, perform important immunological, structural and metabolic functions in the host, among which, it should be noted its involvement in the preservation of the integrity of the intestinal barrier through the production of short-chain fatty acids (SCFA) (18), so that increased intestinal permeability in subjects with NCG/WS described by Uhde et al. (15) could indicate a possible key pathogenic role of the intestinal microbiota in these patients.

In this context, it is of interest to explore whether NCWS sufferers may safely consume cereal foods modified by techniques such as sourdough fermentation which can reduce levels of gluten proteins and FODMAPS (19, 20) or made using alternative gluten sources which may be better tolerated as they have lower levels of symptom-inducing components (21). In a previous study, the present authors showed that tritordeum, a new cereal crop species derived from crossing durum wheat with a wild barley species (22, 23) differs from bread wheat in its gluten composition, in particular with respect to gliadin composition, showing significantly lower levels of gluten immunogenic peptides (GIP) implicated in CD and possibly also in NCWS. In addition, it was seen that healthy subjects who consumed a tritordeum diet during one week showed a significant reduction of the excretion of GIP in feces by comparison with a bread wheat diet (21). The present study follows on from the previous work, with the aim of examining the response of patients diagnosed as NCWS to tritordeum breads, to test the hypothesis that the reduced GIP levels in tritordeum may allow some NCWS patients to tolerate it in their diet. Additionally, the study analyses the effect of its consumption on the intestinal microbiota of these patients, due to the recent idea, that changes in the intestinal microbiota composition could contribute to the etiology and pathogenesis of NCWS (13).

MATERIALS AND METHODS

Subjects

The study population was obtained from a gastroenterology outpatient clinic at the University Hospital of León specialized in gluten-related disorders mainly celiac disease and NCWS. Patients suffering NCWS were selected from our database with the following inclusion criteria: (i) fulfill the Salerno criteria for NCWS diagnosis (24), (ii) diagnosed at least 6 months previously to the study, (iii) following a strict gluten-free diet with complete symptom remission, (iv) not suffer from any other chronic disease or taking long-term medication. A total of 30 patients were contacted, 12 did not fulfill inclusion criteria and 6 refuse to participate in the study (Fig. 1). Finally, the subjects for the diet intervention were twelve adult volunteers (9 / 12 female) of age 31-57 years. All of them showed negative serology for celiac disease (tissue-transglutaminase IgA antibodies) and the duodenal biopsy results presented normal duodenal villi architecture. HLA-DQ2+ was present in five of the subjects, whilst the remaining seven showed a different HLA-DQ2 or DQ8 haplotype. Of the twelve subjects who started the study, two withdrew voluntarily during the first days of the diet intervention (during the Basal, GFD phase), as they were not able to attend to deliver stool samples (the two were female with HLA-DQ2 or DQ8 not present). Their withdrawal was not associated with any change in gastro-intestinal symptoms or any other physiological reason. The remaining ten subjects followed the two phases of the study, completed the clinical and sensory questionnaires and provided the required stool samples. They were instructed to communicate any medication needed to take during the study period. All study participants provided informed consent, and the study design was approved by the Ethics Review Board of the Hospital of León, Spain (Approval Number 1626).

Study design

The study was performed in two phases ("Basal" and "Tritordeum"), each of which had duration of seven days. At the beginning of the study, subjects were given a hand-out explaining the background / objectives of the diet intervention and giving instructions for the handling and consumption of the bread samples and the collection and delivery of stool samples.

Basal phase: consumption of the GFD normally eaten by the subject including the consumption of the gluten-free bread habitually consumed by each individual. They were instructed to eat an amount of their gluten-free bread between 100-150 g daily. At the end of the phase, Clinical Questionnaires based on Gastrointestinal Symptom Rating Scale (GSRS) and Sensory Questionnaires (Table S1) on palatability and acceptability of the bread consumed (21) were completed and stool samples (Basal phase samples) were collected.

Tritordeum phase: Continuation of the Basal GFD, but with the substitution of the Basal (gluten-free) bread by tritordeum bread during seven days. Subjects were instructed to consume a minimum of 100 g and a maximum of 150 g of tritordeum bread daily, by the consumption of four slices of bread, giving a daily intake of ca. 5 - 7.5 g of gluten, based on the gluten content of the same tritordeum breads previously reported (21). At the end of this phase, the Clinical and Sensory Questionnaires were completed and stool samples (Tritordeum phase samples) were collected.

Preparation and provision of test breads

The ingredients for the tritordeum breads provided to subjects in the Tritordeum phase were: flour, water, sourdough, salt, and baker's yeast. Breads were made using tritordeum sourdough, prepared 24 h before bread-making, by mixing tritordeum flour,

water and sourdough starter culture (composed of flour, water, lactic acid bacteria and sourdough yeasts). The mixture was allowed to stand at room temperature for 3 h before storage at 5 ºC until use. For bread-making, the main dough got 150g Kg of tritordeum sourdough, and mixed with additional tritordeum flour, water, salt and baker yeast to form the final dough. This dough rested for 90 min at room temperature (22-24ºC). After resting bulk dough was divided into pieces, which were placed into metal loaf molds and allowed to ferment until they achieved 2/3 of the mold volume (approximately 90 min). Loaves were baked at 210 ºC - for 45 min. The acidity values of the tritordeum sourdough and tritordeum bread are the following; Tritordeum Sourdough: TTA 13, pH 4.05, Tritordeum Bread: pH 4.9

After cooling, loaves were cut into slices and frozen in portions. The bread was stored at the Hospital of León, where the subjects received the bread for the Tritordeum phase. The breads were supplied frozen to test subjects for defrosting immediately before consumption.

Evaluation of gastrointestinal symptoms

Gastrointestinal symptoms were reported by participating subjects using the GSRS questionnaire, a validated, self-administered questionnaire that includes 15 questions, which assess gastrointestinal symptoms using a 7-point Likert scale in five domains: Indigestion, Diarrhea, Constipation, Abdominal Pain and Reflux. The severity of symptoms reported in the GSRS increases with increasing score.

Evaluation of acceptability of breads

The Sensory Questionnaire required the subjects to score the bread consumed during the Basal and Tritordeum phases under five attributes: Appearance; Aroma; Crumb Texture;

Taste; and Overall Acceptability, using a numerical scale of 1 – 9, each number corresponding to a description of the level of liking / dislike of the bread.

Collection of stool samples

Subjects were instructed to collect a 2-4 g stool sample into a sealed container after recording their food intake for seven days. Specimens were delivered within the first two hours after deposition and were stored at -80 ºC until processing. All samples were identified and labelled with a randomized numeric code.

Quantification of GIP in stool samples

In the study a total of 20 stool samples were analyzed from 10 subjects. The concentration of GIP in stools was measured by sandwich ELISA (iVYDAL In Vitro Diagnostics® iVYLISA GIP-S kit, Biomedal S.L., Seville, Spain) following the manufacturer's guidelines (21). Briefly, stool samples were mixed with 9 ml Universal Gluten Extraction Solution (UGES) per gram of stool then incubated at 50ºC for 60 min with gentle agitation to release the GIP from the stool matrix. After extraction, samples were diluted 1:10 with dilution solution and ELISA was performed using the provided G12 coated microtiter plate, standards and positive and negative controls. Each sample was run in duplicate and at least two different aliquots of each sample were tested on different days.

Sequencing and Bioinformatics Analysis of the gut microbiota

For each DNA of 36 fecal samples (9 patients per two phase of study and two DNA repetitions for each phase), the V1-V2 hypervariable regions of the bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the universal bacterial primers 8F

(AGAGTTTGATCMTGGCTCAG) and 357R (CTGCTGCCTYCCGTA). In addition, 8nt index and Illumina adapter sequences were added following manufacturer's instructions. The PCR experimental conditions and single-end sequencing procedures were the same as in a previous study (25). The raw NGS data from this study can be found at GenBank database under accession number PRJNA577543.

The Illumina Miseq Fastq reads obtained were analyzed using the Quantitative Insights into Microbial Ecology 2 (QIIME 2) pipeline (version v2019.7) (26) with default parameters unless otherwise noted. Reads were processed by the DADA2 program using the qiime dada2 denoise-single script (27) which denoises single-end sequences, dereplicates them, and filters chimeras. Open reference operational taxonomic unit (OTU) picking was performed using VSEARCH (28) and the SILVA v132 reference databases at 97% identity, which provides a feature table containing the frequencies of each OTU or taxon per sample (29).

Rarefaction curves of alpha-diversity indexes (Faith_pd, Shannon, Observed OTUS and Good's coverage) and beta diversity (Unweighted and Weighted UniFrac distances) were calculated at an even sampling depth of 10,044 sequences per sample and used to assess differences in microbial diversity between the two phases of the study (https://github.com/qiime2/q2-diversity). Finally, taxonomic and compositional analyses were conducted by using the plugins feature-classifier classify-consensus-vsearch (30) and taxa barplot (https://github.com/qiime2/q2-taxa).

Total fructans

Megazyme Fructan HK enzymatic assay kit (Megazyme, Bray, Ireland) was used for the determination of total fructan contents in flour samples based on standard AACC method 32.32.01.

Protein peptides analysis by Liquid Chromatography–Tandem Mass Spectrometry Analysis

Total protein extraction and trypsin and chymotrypsin digestion of samples were carried out from 2 g of flour as previously reported (21).

Statistical analysis

The ANOVA test was performed to evaluate differences between various samples, followed by the two-tailed Dunnett's post hoc test for multiple mean comparisons. The Student's t test, Wilcoxon test and Friendman test were employed to analyze differences in the same subject among the different phases. The Bonferroni test was applied in the post hoc analysis and *P*-values lower than 0.05 were considered statistically significant.

A non-supervised multivariate hierarchical clustering analysis, using Euclidean distance and the Ward clustering algorithm, and a supervised principal least square-discriminant analysis (PLS-DA) of all bacterial taxa from the different periods of dietary intervention were performed using MetaboAnalyst 4.01 (31).

Differences in alpha diversity indexes were estimated using the non-parametric Kruskal Wallis test and differences for the beta diversity unweighted and weighted UNIFRAC distances were estimated using the PERMANOVA analysis. Finally, the differences in the relative abundance of bacterial taxa between the two phases were tested using the non-parametric Mann-Whitney U test with SPSS Statistics for Windows Version 25.0 (IBM Corp., Armonk, NY). For that only taxa that were present in at least 80% of the samples per each phase were used.

RESULTS

Tritordeum is a new cereal species obtained from crossing durum wheat with a wild barley species (22, 23). It is lacking the D genome and therefore, do not contain the gliadin-related proteins located on it, particularly the α-gliadins harbouring the 33-mer protein fragment (32, 33), the most immunogenic peptide known so far. In previous work, we showed that tritordeum contain lower CD immunogenic peptides than that of bread wheat (21), particularly the α-gliadin peptides. As other grain proteins are related to wheat allergies and NCWS, we have extended the search to other grain components (Supplementary Fig. S1). As showed, ATIs protein peptides are slightly lower in tritordeum in comparison to that of bread wheat. However, other peptides, also belonging to the non-gluten proteins fraction are higher in tritordeum. Fructans content, an oligosaccharide of the FODMAPs, were lower in tritordeum, although not significantly different to that of bread wheat flour (Supplementary Fig. S1). In the present work we carried out a dietary intervention in NCWS patients of tritordeum breads in comparison with the gluten free breads they usually consumed.

Participants recruitment and dietary intervention flow

Of 30 participants recruited from the gastroenterology outpatient clinic at the University Hospital of León, 12 were eligible while 12 do not meet the inclusion criteria and 6 refused to participate (Fig. 1, see material and methods for more details). Finally, two subjects withdrew voluntarily during the first days of the dietary intervention (during the Basal phase) and excluded for the final analysis. The dietary intervention had two phases; Basal and Tritordeum, with a duration of seven days each (Fig. 1). All participants were following a gluten free diet and the only different between the two phases was the bread consumed. Participants were instructed to eat between 100 and 150 grams of the gluten free bread o tritordeum bread

during the Basal or tritordeum phases, respectively. And the end of each phase, GSRS and Sensory questionnaires were completed and stool samples collected.

Gastro-intestinal symptoms

The results from the completion of the GSRS questionnaires, evaluating the parameters Indigestion, Diarrhea, Constipation, Abdominal Pain and Reflux, at the end of the Basal and Tritordeum phases indicated that the gastrointestinal symptoms of the subjects showed no significant change between the Basal phase (gluten free bread) and the Tritordeum phase (Fig. 2). The global mean value (data for all subjects) during the Basal phase was 36.5 vs 34.1 during the Tritordeum phase (with higher values equating to lower gastrointestinal wellbeing) but these scores were not significantly different (P = 0.414). Examination of the questionnaire results for individual subjects showed that at the individual level there were no significant differences in gastrointestinal health scores (Fig. 2).

The analysis of GIP in stool samples showed that, as expected, there was a low excretion of GIP (mean value 0.6 (0 – 2.9) μg GIP/g feces) by subjects during the Basal phase, although two subjects showed excretion levels above expected possibly due to dietary transgressions (Fig. 3). Higher levels of GIPs were detected in stool samples from the Tritordeum phase, confirming their consumption of gluten. The mean level of GIP detected in stool samples in the Tritordeum phase, was 1.5 μg GIP/g feces, with concentration ranged from 0.5 to 3.2, although this difference was not statistically significant (*P* = 0.114). The results obtained in the Tritordeum phase contrasted with those reported previously for subjects who included standard wheat breads containing gluten in their diet, GIP concentration ranged from 0.30 to 75.68 μg/g feces (21).

Acceptability

Based on the Sensory Questionnaire, participating subjects rated tritordeum bread higher than the Basal (gluten-free) bread they habitually consumed for each of the five attributes scored (Appearance, Aroma, Texture, Taste and Overall Acceptability) and with a significant difference (P = < 0.05) seen with each attribute (Fig. 4). In particular, Texture and Taste were scored highly in tritordeum breads in comparison with gluten-free breads (Tritordeum mean 7.3 *vs* Basal mean 3.6 and Tritordeum mean 7.7 *vs* Basal mean 4.6, respectively). The mean Overall Acceptability score for tritordeum bread was 7.2 *vs* 5.1 for Basal breads (Fig. 4).

Diversity and Changes of the gut microbiota

After processing of reads a total of 361,584 sequences were retained and a sample was lost. The sampling depth selected for rarefication was 10,044. Satisfactory Good´s coverage of the diversity was obtained for all samples since the mean value was > 99.9% for al samples. We did not find any significant differences in bacterial diversity between the Basal phase and the Tritordeum phase, with any of the alpha diversity indexes used (Faith_pd, P = 0.480; Shannon, P = 0.635, and observed OTUS, P = 0.701) at any of the rarefaction depths analyzed (Fig. 5A). In the same way, we did not find any significant differences in the beta diversity between both phases both for Unweighted (P = 0.429) and Weighted (P = 0.461) Unifrac distances. Similarly, no significant differences in both Unweighted and Weighted Unifrac distances were found between both phases for each patient when analyzed independently (P > 0.091; Fig. 5B).

In total, 3 phyla, 5 classes, 5 orders, 9 families and 17 genera, were identified with a mean relative abundance equal to or greater than 0.1% in both phases and with a presence equal to or greater than 65% in all samples for each phase (Supplementary Fig. S2).

We did not find significant ($P > 0.05$) differences in the relative abundance of any taxa at phylum, class, order and family level between Basal and Tritordeum phases. On average, the taxa with the highest abundance both at the Basal phase and the Tritordeum phase were: Firmicutes 89.2% - 90.1% and Actinobacteria 3.7% - 3.2% at the phylum level, Clostridia 69.9% - 72.2%, and Erysipelotridia 12.1% - 10.7% at class level, and Clostridiales 69.9% - 72.2% and Eryselotrichales 12.1% - 10.7% at order level and Lachnospiraceae 36.3% - 38.7% and Ruminococcaceae 26.1% - 23.9% at family level (Supplementary Fig. S2).

Finally, at genus level we found significant differences in the relative abundance of two bacterial genera: a higher relative abundance in *Ruminococcaceae UCG-013* ($P = 0.024$) and a lower relative abundance in *Faecalibacterium* ($P = 0.011$) genera in Basal phase as compared with Tritordeum phase (Fig. 7). These results are in agreement with those observed in the PLS-DA analysis (Fig. 6).

The hierarchical clustering analysis showed a trend to group the two dietary phases of each patient in pairs, indicating the maintenance of the structure and composition of the intestinal microbiota of each patient after consumption with tritordeum (Supplementary Fig. S3 and S4). PLS-DA of all bacterial taxa showed a good separation of both dietary phases ($P < 0.01$; Fig. 6A) and ranked 15 bacterial taxa as the most important in projection (VIP scores > 1.5 at $P < 0.05$; Fig. 6B) allowing differentiation of both phases, of which eight taxa are SCFA-producing bacteria.

DISCUSSION

The study presented here follows a previous study on acceptance, digestibility and immunotoxicity of tritordeum, which showed that, in comparison with bread wheat, tritordeum breads were well accepted, had less gluten (49% reduction), lower levels of

immunogenic gliadin epitopes (59% reduction in γ-gliadin epitopes, 77% reduction in α-gliadin epitopes) and that the levels of GIP excreted in stool samples of healthy volunteers were significantly reduced (21). Regarding non gluten proteins and other compounds also related with NCWS, tritordeum flour showed slightly lower content of ATIs protein peptides and comparable content of total fructans than that of bread wheat. The present work extends the previous study by examining the tolerance (gastro-intestinal health) and acceptance of tritordeum breads by patients diagnosed with NCWS who habitually follow a GFD. The impact of tritordeum consumption on the gut microbiota of these patients was also evaluated.

Considering the tolerance of tritordeum breads by participating subjects, the analysis of GSRS questionnaires completed at the end of the study showed that there was no difference in the global mean gastrointestinal health score from the Tritordeum diet and the Basal (gluten-free bread) diet. Also, at the individual level there were no significant differences in gastro-intestinal health scores from the two diet phases. Thus, over the seven-day intervention period at the level of tritordeum bread consumed (100 – 150 g daily, from four slices of bread) all the NCWS patients showed good tolerance, with no deterioration in gastro-intestinal wellbeing, comparable to that of the gluten free bread of their choice.

The measurement of GIP levels in stool samples after each of the two phases of the study showed that, as expected, during the Basal phase subjects had lower GIP excretion, but that GIP excretion was observed during the Tritordeum phase, confirming the consumption of gluten. The levels of GIP detected in stool samples from the Tritordeum phase (mean 1.5 μg GIP/g feces) were similar to the levels detected in the previous study using healthy subjects, which had a mean of 1.2 and a range of 0.16 to 14.87 μg GIP/g feces and in which the subjects consumed the same quantity of tritordeum bread daily (21). However, two individuals showed high levels of GIP (2.5 and 2.9 μg GIP/g feces) excretion during the Basal (gluten-free bread)

phase, indicating gluten exposure (volunteer or not) of the GFD. Values of GIP excretion for these two individuals in the Tritordeum phase were 1.1 and 1.9 µg GIP/g feces, respectively.

Considering the evaluation of acceptance of tritordeum bread in comparison with the gluten-free breads habitually consumed by the subjects, the Sensory Questionnaire showed that tritordeum breads were given significantly higher scores for all five of the acceptance attributes. Taste and texture in tritordeum bread were rated particularly highly in comparison with gluten-free breads and the subjects had a clear overall preference for tritordeum breads, awarding a mean Global Acceptance score of 7.2 for tritordeum versus 5.1 for gluten-free breads.

There is considerable controversy surrounding NCWS, because unlike the other gluten-related disorders CD and WA whose diagnosis is based on objective parameters, supported by accepted biomarkers, the diagnosis of NCWS is based on the presence of intestinal and extra-intestinal symptoms associated with the consumption gluten-containing foods and on negative results for CD and WA biomarkers (24). At the pragmatic level, NCWS sufferers are diagnosed by the exclusion of CD and WA and by the disappearance of symptoms on the adoption of a GFD (34). Further, there is active discussion as to whether NCWS is a disease separate from irritable bowel syndrome (IBS), as several studies suggest that the symptoms overlap and that FODMAPS may play a major role in addition to / rather than gluten (4, 35), although other studies present evidence that gluten-induced reactions are responsible for NCWS symptoms (36, 37) and ATIs also may play a role (4). Some studies suggest that ATIs could trigger the innate immune response in the intestinal monocytes, macrophages and dendritic cells, leading to the release of pro-inflammatory cytokines, providing intestinal inflammation (38, 39). In addition, it has been shown that FODMAPs, particularly fructans, could be the main trigger of NCWS in certain patients (40) , as 24 out of 59 participants in the study had the highest Gastrointestinal Symptom Rating Scale-Irritable Bowel Syndrome (GSRS-IBS) scores after

consuming fructan. However, 22 out of 59 participants had the highest score after consuming placebo. The mechanism of fructans, or other FODMAPS, in inducing NCWS is not clear. FODMAPs are not digested nor absorbed in the gastrointestinal tract and their rapid fermentation in the intestines, may result in excessive gas production, bloating and pain. Tritordeum contains fructans at comparable levels of bread wheat. In this work, compounds like ATIs or fructans were not analyzed in all the different gluten-free breads consumed by the NCWS participant. However, the fructan content has been determined in a wide range of wheat, rye and gluten-free breads (41). Surprisingly, the fructan content in gluten-free breads was very similar to that of gluten-containing breads. This is possibly due to the enrichment of gluten-free breads with Inulin-type fructans (ITFs) to improve the nutritional quality of gluten-free breads (42). In fact, Morreale et al. (43) reported the fructan content of gluten-free breads enriched with various types of ITFs and show that fructan concentrations were up to 4.59 g / 100 g (wet basis) in some gluten-free breads.

While the exact causal mechanism for NCWS has still to be defined, it appears evident that there exist a very significant number of people who report symptoms when they consume wheat-containing foods, with frequencies estimated between 6 and 13% in different populations (44). These figures are related to a strong increase in the avoidance of wheat and other gluten-containing cereals over the last decade, with estimates up to 10 – 20% of populations in Westernized countries (4). The avoidance of wheat has resulted in an almost exponential increase in the adoption of GFDs (44), although there is increasing recognition that at the nutritional level GFDs tend to be inferior to cereal-containing diets due to increased levels of fats, and reduced levels of complex carbohydrate, protein, vitamins and minerals (8, 44). In addition, there is overwhelming evidence for the positive effects of the consumption of dietary fiber as supplied by wholegrain cereals, and it is more difficult to consume the recommended levels of dietary fibre when observing a GFD (45, 46).

In contrast to CD, there is evidence that some NCWS sufferers may not need to totally exclude cereals from their diets, but may be able to tolerate a reduced gluten intake or gluten sources with reduced levels of GIP, either from the use of alternative cereal flours or by gluten modification via processes such as sourdough fermentation (9, 19). This concept is supported by the results of the present study, in which a group of ten medically-diagnosed NCWS sufferers who follow a GFD and habitually consume gluten-free bread were able to consume 100 – 150g of tritordeum bread daily, giving an estimated daily gluten consumption of 5 – 7.5 g over a period of seven days, without any of them reporting a change in gastro-intestinal health parameters.

Finally, evidence is emerging that GFDs may negatively affect the gut microbiota (10-12). In this sense, our study has not observed global changes in the diversity of the intestinal microbiota between the Basal phase and the Tritordeum phase. However, we have found a significant decrease in the relative abundance of *Ruminococcaceae UCG-013* gnus and a significant increase of *Faecalibacterium* genus after consumption of tritordeum bread. Both genera belong to the *Ruminococcaceae* , a bacterial family that is associated with intestinal health maintenance (47) .

One interesting results was the observed increase in the relative abundance of *Faecalibacterium* genus after seven days of consumption of tritordeum bread. *Faecalibacterium* is one well-known butyrate producing bacteria within the human intestinal gut microbiota (48). A new pathogenic mechanism involved in NCWS has recently been proposed in which butyrate producing bacteria, especially *Faecalibacterium* among others may play a key beneficial role NCGS (15, 49-51). Butyrate constitutes the energy source of the enterocytes, keep the tight junctions, stimulates mucin production avoiding intestinal permeability, and finally it induces the production of antimicrobial peptides (52, 53). Taken together, all these functions of butyrate ultimately result in intestinal homeostasis and

immune tolerance (48, 54). In line with this, our results may suggest that the long-term consumption of tritordeum could be a good alternative to GFD in patients with NCWS, if the increase on *Faecalibacterium* abundance observed after one week of tritordeum bread intake is maintained at long term.

In addition, according to the PLS-DA analysis, the microbial community of both phases tends to separate associated to differences of important SCFA-producing bacteria. Thus, we have observed that after the consumption of the tritordeum bread, besides *Faecalibacterium* genus, that increases its relative abundance significantly, other SCFA-producing bacteria show a similar trend including [*Ruminococcus*] *gauvreauii* group (55), *Roseburia* (47-49), *Clostridium sensu stricto 1* (56), *Bacteroides* (57), *Lachnoclostridium,* [*Eubacterium*] *xylanophilyum* group (58), and *Lachnospiraceae NK4A136* group (59) (Fig. 6). This data is relevant since the SCFA are involvement in the preservation of the integrity of the intestinal barrier (18). To confirm those trends, future studies are needed increasing the number of patients included in the study as well as increasing the period of consumption of tritordeum bread to confirm its long-term positive effects on the intestinal microbiota by increasing SCFA producing bacteria demonstrating the increase of these metabolites after its consumption.

CONCLUSIONS

The present study shows that a group of ten diagnosed NCWS subjects who follow a GFD were able to consume tritordeum breads for a week without suffering negative effects on gastro-intestinal health. In terms of acceptability, participating subjects showed clear preference for tritordeum breads in comparison with their usual GF bread. Tritordeum bread intake show significantly reduced levels of GIP excretion by comparison with standard wheat bread as reported in a previous study of the group, although it cannot be concluded from the present study that this is the only factor which may have allowed its tolerance by NCWS subjects. Nevertheless, the results suggest that tritordeum may be tolerated by some of NCWS

sufferers who do not require strict exclusion of gluten from their diet and may benefit from its better organoleptic and nutritional properties. In addition, although the global structure and composition of the intestinal microbiota of each patient was stable after tritordeum bread consumption, there was a trend to increase the relative abundance of some SCFA-producing bacteria, which may suggest a potential increase of the intestinal healthy status in these patients.

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FIGURE LEGENDS

Figure 1. Participants recruitment and dietary intervention flow.

Figure 2. Comparison of the Symptom Rating Scale (GSRS) questionnaire for all patients (boxes) and results for individual subjects (each line represent one individual subject).

Figure 3. Comparison of the fecal Gluten Immunogenic Peptides (GIP) content after consumption of gluten-free bread (basal) and tritordeum breads.

Figure 4. Differences in descriptive sensory analysis of the two phases of the dietary intervention. Values of the sensory questionnaire for Overall acceptance, Appearance, Aroma, Texture, and Taste of the sensory questionnaire for the gluten-free bread (basal) and tritordeum bread. Data are the means of ten replications +/- standard deviation.

Figure 5. Alpha and Beta diversity analysis results. (**A**) Alpha rarefaction curves of Faith_pd, Shannon and Observed otus alpha diversity indexes for the Basal and Tritordeum phase at a depth of 10044 sequences per sample. P-value for each alpha diversity index was estimated using Kruskal-Wallis test. (**B**) Principal Coordinate Analysis (PCoA) of the unweighted and weighted UniFrac beta diversity distances between the Basal and Tritordeum phase at a depth of 10044 sequences per sample. *P*-value was estimated using a Permanova test comparing the Basal and Tritordeum phases globally or for each patient independently.

Figure 6. Partial Least Squares - Discriminant Analysis (PLS-DA). (**A**) Partial least square-discriminant analysis (PLS-DA) 2D scores plot of the bacterial taxa of each phase of study. Red: Basal phase; Green: Tritordeum phase. The model was established using two principal components; explained variance is in parentheses. (**B**) Loading importance of bacterial taxa in the first PLS-DA component. Colored boxes (red: Basal phase; green: Tritordeum phase) indicate relative concentrations of the corresponding bacterial taxa in each phase. VIP: Variable Importance in Projection. (*)Short-chain fatty acids (SCFA)-producing bacterial taxa.

Figure 7. Summary taxa of Basal and Tritordeum phases at genus level. Graphs represented show the bar plots with the relative abundance of each taxonomic group in percentage within each phase, Basal and Tritordeum at genus level. Other corresponds to the sum of unassigned taxa with the Silva 132 database and the bacterial taxa present in less 80% of the samples per each phase. Colored asterisks show significantly different taxa with *P*-value < 0.05. The statistically significant differences between each phase were tested by non-parametric Mann–Whitney U test.