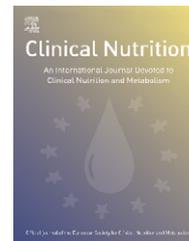




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ORIGINAL ARTICLE

# Celiac disease: *In vitro* and *in vivo* safety and palatability of wheat-free sorghum food products <sup>☆</sup>

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## KEYWORDS

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Palatability;  
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## Summary

**Background & aims:** Celiac disease is a condition in which genetically predisposed people have an autoimmune reaction to gluten proteins found in all wheat types and closely related cereals such as barley and rye. This reaction causes the formation of autoantibodies and the destruction of the villi in the small intestine, which results in malabsorption of nutrients and other gluten-induced autoimmune diseases. Sorghum is a cereal grain with potential to be developed into an important crop for human food products. The flour produced from white sorghum hybrids is light in color and has a bland, neutral taste that does not impart unusual colors or flavors to food products. These attributes make it desirable for use in wheat-free food products. While sorghum is considered as a safe food for celiac patients, primarily due to its relationship to maize, no direct testing has been conducted on its safety for gluten intolerance. Therefore studies are needed to assess its safety and tolerability in celiac patients. Thus the aim of the present study was to assess safety and tolerability of sorghum flour products in adult celiac disease patients, utilizing an *in vitro* and *in vivo* challenge.

**Results:** Sorghum protein digests did not elicit any morphometric or immunomediated alteration of duodenal explants from celiac patients. Patients fed daily for 5 days with sorghum-derived food product did not experience gastrointestinal or non-gastrointestinal

<sup>☆</sup>Names are necessary to report factually on available data; however, the USDA neither guarantees the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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symptoms and the level of anti-transglutaminase antibodies was unmodified at the end of the 5-days challenge.

**Conclusions:** Sorghum-derived products did not show toxicity for celiac patients in both *in vitro* and *in vivo* challenge. Therefore sorghum can be considered safe for people with celiac disease.

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## Introduction

Celiac disease (CD) is one of the most common genetically determined diseases. CD is a condition in which in genetically predisposed people suffer a reaction to gluten proteins found in all *Triticum* species and closely related cereals such as barley and rye.<sup>1</sup> Gluten ingestion in celiac patients causes a variety of gastrointestinal and non-gastrointestinal symptoms and biochemical abnormalities<sup>2,3</sup> which ameliorate after gluten withdrawal. Diagnosis is often made in adulthood and in patients with a long personal history of disease can run misdiagnosed for years.<sup>4</sup> The epidemiological pattern of the disease has dramatically changed in the last few years as a result of the widespread use of highly sensitive and specific serological tests, especially anti-endomysial (EMA) and anti-tissue transglutaminase antibodies (anti-tTG).<sup>5,6</sup> The highest reported prevalence is in Northern Europe and in countries of European ancestry with the overall prevalence in the general population as high as 1%.<sup>2,6,7</sup> Estimates place the number of persons with CD in the US at roughly 3 million.<sup>2,6</sup> The only treatment for CD is lifelong avoidance of gluten proteins. Thus all forms of wheat (*Triticum* sp.) have to be avoided, including durum wheat, spelt wheat, kamut, einkorn, and triticale as well as rye, barley, and possibly oat products.<sup>1,8</sup> Changing to such a restrictive diet in infancy and adulthood may be a difficult experience for many people. High quality gluten-free foods, inexpensive and easily available, are required to make the gluten-free diet as acceptable as possible.

Sorghum [*Sorghum bicolor* (L.) Moench] is a drought-resistant and heat tolerant cereal grain that grows in semi-arid conditions. While sorghum has traditionally been used primarily as animal feed in Western countries, nearly 40% of the world sorghum production is used for human food in Africa, and India.<sup>9,10</sup>

Sorghum is considered a safe food for celiac patients, because it is more closely related to maize than to wheat, rye and barley.<sup>1</sup> Sorghum might, therefore, provide a good basis for gluten-free breads and other baked products such as pasta, cookies, snacks although no direct testing has been conducted on its safety for celiac patients. Significant research has been conducted on sorghum-wheat composite foods and sorghum can be added to wheat flour to produce acceptable breads and other foods.<sup>11–15</sup> However, such composite foods could not be consumed by persons with CD.

Several types of wheat-free food products have been made from sorghum including: breads,<sup>14,16,17</sup> parboiled sorghum,<sup>18</sup> sorghum tortillas,<sup>19</sup> snack foods,<sup>20,21</sup> cookies<sup>22,23</sup> and flatbreads.<sup>23,24</sup> These studies have demonstrated that sorghum can be used to produce high quality human food

products. Recently, in the US sorghum hybrids that produce white grain from a tan plant (often called “food grade” sorghum) are being used for the production of wheat-free foods for persons with CD. The development of white food grade sorghum lines means that white, neutral tasting flour can be produced from sorghum. These flours are useful in food products because they do not impart unusual colors or strong flavors and may be desired over corn flours for these reasons.<sup>25</sup> The present study was planned to evaluate the impact of pure sorghum food products on celiac patients with the aim to promote sorghum cultivation and flour production and use as valuable food for humans in Western countries which traditionally use sorghum mostly for animal feed.

## Materials and methods

### Sorghum and gliadin peptic-tryptic digests

Purified kafirins were isolated from the sorghum hybrid NC+371 by selective precipitation after extraction in 70% ethanol containing sodium metabisulfite as described in Bean et al.<sup>26</sup> Peptic-tryptic (PT) digests of sorghum proteins were prepared as described in Maiuri et al.<sup>27</sup> from purified sorghum kafirins. Digests were monitored by RP-HPLC to insure complete digestion. PT digest from bread wheat was prepared as previously described.<sup>28</sup>

### Sorghum food production

Sorghum bread was produced as described in Schober et al.<sup>17</sup> with the addition of olive oil and cookies as described in Badi and Hosenev.<sup>22</sup> All sorghum food products were made from commercially available white food grade sorghum flour (Twin Valley Mills, Ruskin, NE, USA).

### *In vitro* protocol

#### Patients

Duodenal multiple endoscopic biopsies were performed for diagnostic purposes in eight patients with active CD (mean age 25.4 years, range 21–30) and in four non-CD control (patients affected by esophagitis or functional gastrointestinal disorders). Informed consent was requested from all patients before these procedures. All specimens were washed in 0.15M sodium chloride and examined with a dissecting microscope. One specimen from each patient was oriented and embedded in OCT compound (Tissue Tek, Miles Laboratories, Elkhart, IN, USA), snap frozen in isopentane cooled in liquid nitrogen, stored at  $-70^{\circ}\text{C}$  until cryosection-

ing into 5 µm sections that were stained with hematoxylin and used for diagnosis. The other samples were cultured *in vitro* as described below.

### ***In vitro* organ culture of biopsies of CD and controls**

Immediately after removal, biopsies were cut under stereomicroscopic observation into several fragments of a similar size and weight. Mucosal samples were placed on a stainless steel mesh positioned over the central well of an organ culture dish with the epithelium of the biopsy facing up and the well was then filled with culture medium at 37 °C so as just to reach the cut surface of the biopsies. In this way the surface, which is normally exposed to the luminal content, is fed by capillary action and retains its normal polarity, thus allowing an appropriate physiological model for study. The *ex vivo* challenge took place as previously described<sup>28</sup> using culture media consisting of 10 ml of culture medium consisting of Trowell's T8 medium (6.5 ml), NCTC 135 medium (2 ml), fetal calf serum (1.5 ml), penicillin 50 000 IU and streptomycin 5000 IU. Duodenal biopsies were cultured *in vitro* for 24 h as previously reported<sup>28,29</sup> in the presence of PT digests from wheat, or sorghum (at the final concentration of 1 mg/ml of culture medium) or only culture medium.

At the end of incubation samples were frozen, stored in liquid nitrogen and subsequently prepared for histology and immunofluorescence.

### **Immunolocalizations on tissue sections**

Five µm frozen tissue sections of biopsy samples belonging to each patient and control before and after *in vitro* cultures were fixed in acetone for 10 min then individually incubated for 2 h at room temperature with primary antibodies. We have already reported that in celiac patients *in vitro* challenge with a PT digest from bread wheat specifically induces expression of markers of immunological activation after 24 h of culture. The sections were incubated with the following antibodies: CD3 (mouse Ig, polyclonal Dako 1:100), IL2 receptor (CD25) (mouse Ig, Dako 1:30), and COX2 (Dako 1:100). The antigen expression and distribution was visualized by indirect immunofluorescence as previously described<sup>27,28</sup>. Double immunofluorescence was performed for detection of IL2 receptor+ T cells. The sections were simultaneously incubated with anti-CD25 mouse Ig and rabbit polyclonal anti-CD3 Ig (1:50, Dako). The experiments were performed as previously described.<sup>27</sup>

### **Statistical analysis**

Samples belonging to each patient and cultured with sorghum digest were compared to those challenged with medium as well as with those cultured in the presence of wheat or maize digests using tests for paired samples (Wilcoxon's test for paired samples). The effect of sorghum protein digests on intestinal mucosa of the celiac patients was compared to that observed in controls using unpaired tests (Student's *t* test for unpaired samples). Statistical analysis was performed using SPSS 11 software.

Due to the small number of patients<sup>2</sup> no statistical tests were used in analyzing the VAS scores from the questionnaire on quality and taste of sorghum food. We have reported the individual scores of a selected number of questions in the Results section.

### ***In vivo* protocol**

Two female celiac patients (L.Z. and A.D.G.) from the hospital staff, both doctors, were asked to consume sorghum products for 5 days. They were both known to be strictly compliant to their gluten-free diet. Before the challenge, a routine haematology and laboratory set of analyses was performed, including analysis for anti-transglutaminase antibodies (2.3 and 3.4 UL respectively, normal range 1–5). Sorghum food products, prepared by a trained chef who is an expert in the production of gluten-free food products and who had access to industrial equipment of a medium size gluten-free food industry, were administered daily *per os* in two celiac patients after informed consent. The chef was asked to prepare three different products: bread, cookies and small cakes. Patients were asked to consume bread and cookies in the quantity of 150 g per day for 5 days. Patients were asked to answer a questionnaire (shown in Appendix) on palatability and quality of food (visual analogue scales) and a 20-item questionnaire on gastrointestinal and non-gastrointestinal symptoms occurring during the 5 days challenge and after an additional 7 days (day 12). Serum was analyzed for the presence of anti-tissue-transglutaminase antibodies<sup>30</sup> before the challenge and at day 5 and 15.

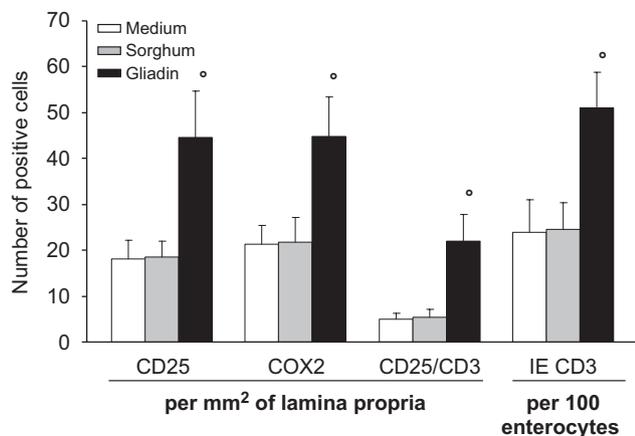
In the case of adverse effects, as revealed by the presence of symptoms or by serological tests, the ingestion of sorghum would have been stopped.

## **Results**

### ***In vitro* protocol**

Indirect immunofluorescence of sections from biopsies of eight celiac patients and four controls challenged with sorghum protein digests showed similar expression of HLA DR, IL2-receptor, COX2, CD3+ IELs and when compared to biopsies cultured with medium alone. In all experiments a gliadin positive control was carried out. For all antigens tested, the antigen expression and distribution increased, as expected, in the biopsies challenged with gliadin alone. Fig. 1 shows the immunohistochemistry data of tissues from the eight celiac patients cultured in presence of sorghum and gliadin digest protein or in their absence (medium only). No differences were noted in the number of positive cells for all inflammatory markers examined among biopsies cultured with or without sorghum. Biopsies from celiac patients cultured in presence of wheat digest showed expected strong increases of all inflammation markers examined when compared to those exposed to sorghum or medium. No significant alteration of antigen expression for the inflammation markers was detected in biopsies from control subjects challenged with gliadin or sorghum digest for 24 h (data not shown for brevity).

Figure 2 shows HLA-DR expression by epithelial cells and epithelial phosphorylation qualitative of biopsies control



**Figure 1** The figure shows immunohistochemistry data of tissues sections from intestinal biopsies of celiac patients cultured in presence of sorghum, wheat digest proteins, or in their absence (medium). Data are expressed as number of positive cells normalized per mm<sup>2</sup> lamina propria or per 100 enterocytes (for IEL counts). No differences were noted in the number of positive cells for all the markers examined between sorghum and medium, while, as expected, a strong difference ( $p = 0.001$ , Wilcoxon's test for paired samples) was noted when the biopsies were challenge with gliadin. In the present set of experiments sorghum digest does not elicit the inflammatory response that gliadin does.

(A), exposed to gliadin digest (B), and exposed to sorghum digest (C). No differences were noted in HLA-DR expression in tissue cultures exposed to sorghum when compared to controls, whereas an intense crypt staining was noted in tissue cultured with gliadin.

### **In vivo protocol**

The first day of challenge the patients were given a 20-item clinical questionnaire that did not disclose any particular symptoms and an evaluation scale for the palatability of the sorghum food products. Patients judged cookies and cakes as excellent (VAS scores for item # 1, 2, 8 and 12 above 8 for both patients) whereas bread was rated not as high (item # 1 rated 5–6, respectively). The recipe for bread was therefore changed with the addition of olive oil and a longer fermentation and the new bread was available from the second day. At day five the clinical questionnaire was re-administered with results similar to day one. Blood tests for anti-transglutaminase antibodies were repeated. Palatability of food stored at room temperature was still excellent although, obviously, the bread was dry and the patients toasted it before eating. The VAS rating after 24 h was always above 7 on a scale from 0 to 10 for both cookies and bread. The clinical questionnaire and blood test were repeated after 7 days (day 12 from first food administration) and no difference in symptoms or serum anti-transglutaminase levels were noticed at 7 and again at 15 days after the last intake (2.7 and 3.5 and 2.1 and 3.0 UL, respectively). The patients declared that they would have included sorghum-derived food in their diet if available.

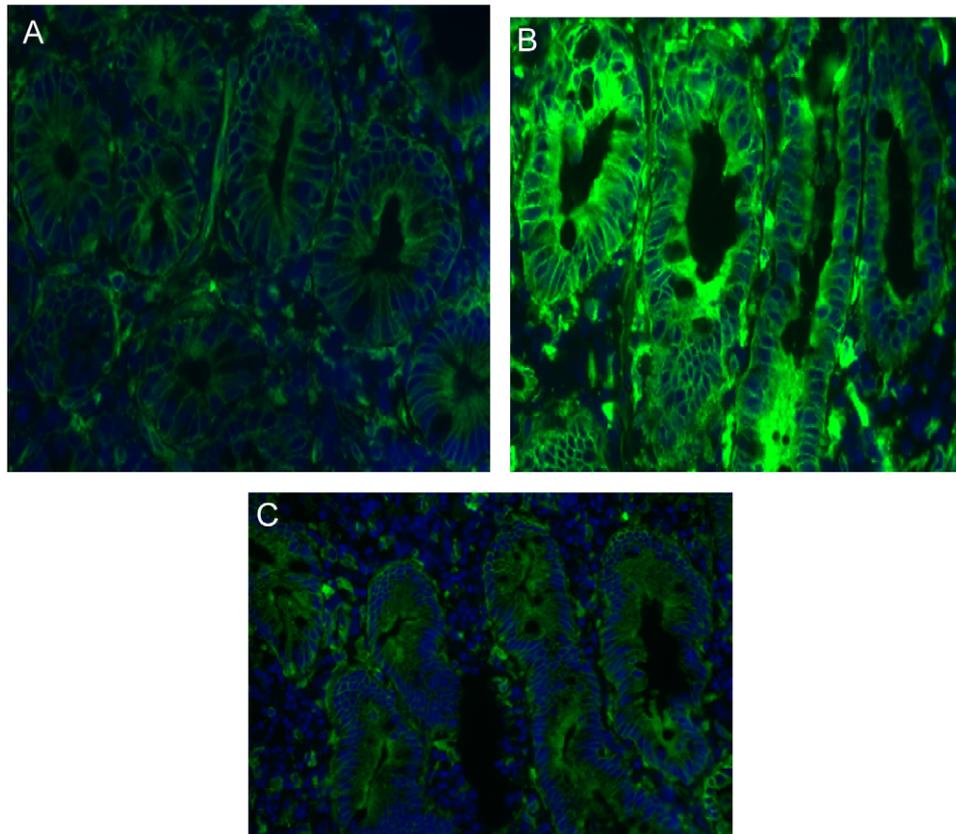
### **Discussion**

According to a number of epidemiological studies on CD prevalence, we expect an exponential increase of the number of CD diagnosis in the very near future.

Therefore, it is important to plan an extensive project of healthcare for CD which includes the training of healthcare and food care professionals but also the availability of large amounts of safe, tasty and inexpensive gluten-free food.

The present paper deals with a study on the safety and palatability of sorghum-derived food. While conducted on only a limited number of individuals for simplicity, this study has shown that sorghum-derived food is safe for gluten intolerant people as demonstrated by *in vitro* and *in vivo* testing. The results are reinforced by the fact that sorghum has long been assumed to be safe due to its relationships to maize, which is known to be safe.<sup>1</sup> The *in vivo* testing was preceded by an *in vitro* culture test in which intestinal biopsies from adult celiac patients were challenged with sorghum digest proteins. No differences were noted between tissues exposed to sorghum and those not exposed. However, gliadin peptides specifically induced (only in celiacs but not in controls) early phosphorylation of protein in epithelial cells.<sup>31</sup> This indicated an early epithelial activation following gliadin challenge; conversely, no epithelial phosphorylation was induced by challenge with the sorghum digest. The epithelial activation precedes the activation of lamina propria mononuclear cells, as revealed by the expression of IL-2 receptor (CD25) by non-T cells, as well as upregulation of COX-2 expression. These events reflect an activation of the innate immune response upon gliadin challenge. The expression of CD25 by CD3+ cells (T cells) is a later event and reflects T cell activation (activation of the adaptive immune response to gliadin). Similarly the expression of HLA-DR by crypt enterocytes is likely a downstream event of IFN-gamma production by activated T cells.<sup>28</sup> Therefore, the parameters we used to test the effects of sorghum in comparison with gliadin reflect the different steps of mucosal activation: activation of the innate immune system, at epithelial and lamina propria level, and activation of the adaptive immune response to gliadin (T cell activation). We can conclude that sorghum was not able to elicit any of the known mucosal response induced by gliadin.

Thereafter, two celiac volunteers were fed for 5 days with sorghum-derived food to gain preliminary information on its palatability. No symptoms or variation of anti-tissue transglutaminase antibodies were noted up to 15 days after exposure. The celiac volunteers reported that sorghum food had excellent palatability. Thus, we hypothesize that sorghum food is safe for gluten intolerant people<sup>32</sup>. The sorghum-derived foods were easily prepared with the sorghum bread and cookies having an excellent palatability as shown by the high VAS scores assigned by both patients answering the questionnaire assessing food quality and taste (Appendix). It should be pointed out that sorghum has been consumed for 1000s of years in parts of Africa and Asia and is currently used in these regions to produce a wide range of high quality food products. Sorghum flour is commercially available in the US and sorghum flour is commonly listed as an ingredient in commercial gluten-free cook books.<sup>33</sup> The sorghum foods also had good keeping quality at room temperature.



**Figure 2** HLA-DR expression is a specific, gluten-related marker of mucosal inflammation of the intestine of celiac patients exposed to gluten or analogous toxic proteins. Panel A: shows HLA-DR expression in intestinal biopsy cultured with medium (negative control). No staining is noted. Panel B: HLA-DR expression in intestinal biopsy cultured with medium added with gliadin digest (positive control). The picture shows intense crypt staining, sign of mucosal inflammation. Panel C: HLA-DR expression in intestinal biopsy cultured with medium added with sorghum digest. No staining is noted, as in panel A, thus no inflammation is elicited by sorghum digest in the celiac mucosa.

The present study has some limitations such as the number of patients tested and the time of the patients on the sorghum-containing diet, and as such, after the diet we could only assess the presence of antiTTG antibodies in blood and not inflammation at histology for ethical reasons. However, in planning the present study we considered that sorghum does not contain any of the amino acid sequences known to be toxic for celiacs and is closely related to maize, which has been tested and found safe for celiacs. In addition, the two persons testing the sorghum food are two doctors of the hospital staff. They are both aware of the disease and tightly control the gluten content of their diet and one of them develops also herpetiform dermatitis in 2–3 days after consuming gluten. Given the above this study should be considered a pilot study on the safety of sorghum foods and providing evidence to plan an additional study that will include more patients on sorghum-containing diet for a longer period of time, followed by intestinal biopsy to evaluate the possible presence of mucosal inflammation. Such a future study would also evaluate the possible long-term effects of a sorghum-containing diet.

In the present study we did not compare sorghum-derived products with other gluten-free similar products available in commerce. In the questionnaire, however, the two patients rated the bread 7 and 8 on the hedonic scale (question #13,

rating from 0 'did not Like' to 10 'Like very much') and affirmed that they would substitute sorghum bread/cookies to their usual gluten-free similar products.

In Europe sorghum is mainly used as animal feeds and the present study was aimed to a better knowledge of its property for the preparation of gluten-free food will increase its production and utilization. In fact sorghum is utilized in many parts of the world to produce a variety of traditional food products such as: fermented and unfermented flat breads (tortillas), porridge, cooked products, snacks and beverages (opaque beer, very popular in South Africa and clear beer popular in Nigeria).<sup>25</sup> Therefore, a wide variety of different of gluten-free products might derived from a single source. Sorghum is an inexpensive grain and therefore it should be possible to produce sorghum-derived food products that have low costs. Given the results of this study and the similarity of sorghum to maize, it is highly likely that sorghum is a safe food for persons with CD. Additional studies are needed to confirm the long-term safety and acceptability of sorghum-derived food for gluten intolerant people. Sorghum is developing as a value added food for Western Countries and if follow up studies confirm the results in this study, an important food product for celiac populations.

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## Appendix

Questionnaire on sorghum food quality/taste assessment

Please answer all questions (questionnaire is similar for cookies)

1. How would you score the aspect of sorghum bread?

0-----  
-----10

Bad excellent

2. How would you score the taste of bread?

0-----  
-----10

Bad excellent

If you did not like the taste, please specify why.....

3. How would you score the taste of bread top crust?

0-----  
-----10

Bad excellent

If you did not like the taste, please specify why.....

4. How would you score the texture of bread?

0-----  
-----10

Bad excellent

If you did not like the texture, please specify why.....

5. How would you score the aspect of bread after 24 h

0-----  
-----10

Horrible excellent

6. And the taste of bread after 24 h?

0-----  
-----10

Horrible excellent

7. Would you choose to buy this product?

0-----  
-----10

Not at all everyday

8. Would you substitute this product to the ones you usually eat?

0-----  
-----10

Never always

9. Would you recommend this product to friends?

0-----  
-----10

Never yes, definitely

10. Did you have any symptom after the ingestion of the bread under evaluation?

Yes  No  I am not sure

If yes, please specify.....

11. Do you think that increasing the food choice introducing new products is a good thing for celiac people?

Yes  No  I am not sure

please specify why yes/no.....

12. Overall how would you score the quality of sorghum-derived food that you tasted?

0-----  
-----10

Bad excellent

13 How do you like the sorghum bread?

0-----  
-----10

Did not like like very much

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