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ARTICLE

Bovine Milk Caseins and Transglutaminase-Treated Cereal Prolamins Are Differentially Recognized by IgA of Celiac Disease Patients According to Their Age

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The prevalence of celiac disease (CD) has increased worldwide, which could be related to some dietary proteins in infant regimens and/or new food processes, affecting CD-predisposed infants and older children or adults differentially. IgA reactivity to human and bovine caseins, as well as yogurt caseins and prolamins from wheat or maize breads, microbial transglutaminase (mTG)-treated or not, was evaluated in three patient groups: G1, <2 years old; G2, ~3 years old; and G3 >8 years old. Human caseins were not recognized by IgA, whereas IgA reactivity of G2 and G3 was higher to bovine milk caseins. Immunoreactivity of G1 to yogurt caseins was lower and comparable to controls, with no effects due to mTG treatment. However, mTG treatment increased reactivity of G3 to wheat and maize prolamins. IgA immunoreactivity of CD patients to caseins and mTG-treated or not prolamins was age-dependent, which could reflect a differential manifestation of the effects of such proteins on the intestinal barrier.

KEYWORDS: Celiac disease; age-related disease; IgA reactivity; caseins; prolamins

INTRODUCTION

Celiac disease (CD) is an enteropathy triggered by dietary proteins of wheat gluten and related cereals, which has increased to an estimated worldwide prevalence of 1−2% (*1*). Among the causes for the increase in the incidence of CD could be the use of infant formula feeding instead of breastfeeding and the early introduction of cereals in the diet, which have been related to the earlier onset of CD (*2*). Additionally, in recent decades, cereal food technology has changed to fast processes by which proteins are not degraded during manufacture, which could initiate or exacerbate CD in predisposed individuals (*3*). Another change related to CD (*4*, *5*) is the increasing industrial use of microbial transglutaminase (mTG) for improving functional properties of dairy and bakery products (*6*).

CD is characterized by the presence of antibodies against gluten peptides, especially after deamidation by the tissue transglutaminase (tTG), which is also the autoantigen (*7*). Therefore, it was not rare that immunoreactivity of IgA from CD patients' to gluten proteins increased after mTG treatment (*4*, *5*). In addition, some other dietary proteins, such as milk caseins and maize zeins, induced in a contact probe an inflammatory reaction in the CD mucosa of 50% of the patients (*8*) and were recognized by IgA antibodies from other CD patients (*5*, *9*). Possibly such dietary proteins are promoting the induction of inflammation as an early step that allows gliadins to cross the intestinal barrier in CD-predisposed individuals, and it might initiate the cascade of autoimmune reactions (*10*).

Although CD onset can appear at any age, there are some differences in the immune responses among infants and older children or adults. In young children, the cellular immune response is against amino acid sequences, which are not substrates for tTG, whereas in older children and adults, deamidation of the sequences by tTG increases the response (*11*). In a previous study (*5*), we found that reactivity of serum IgA from a 16-year-old celiac patient to gliadins increased after treatment with mTG, whereas the IgA reactivity of a 2.9-year-old patient was the same against gliadins, whether it was mTG-treated or not.

There are also age-related differences in CD manifestations. In children under 2 years old, CD is characterized by diarrhea and abdominal distension, whereas abdominal pain is more common in children older than 2 years old (*12*). Atypical features (e.g., affecting other organ systems) occur in patients with later onset of the disease (*13*). Additionally, D'Amico et al. (*14*) found that the onset of CD symptoms was mainly in the first to second year for nonbreastfed children, whereas it was in the second to third year for exclusively breastfed children. Therefore, we hypothesized that reactivity of serum IgA from CD patients, which is a manifestation of the immune

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response to dietary proteins, could be age-related with differences among groups under 2 years, 3 years old, and older.

The aim of this study was to evaluate the serum IgA reactivity from three different age groups of CD patients (under 2 years, around 3 years, and older than 8 years old) to caseins extracted from human milk, bovine milk, and yogurt mTG-treated or not, as well as prolamins from wheat or gluten-free breads, before and after mTG treatment.

MATERIALS AND METHODS

Sera and Patients. Thirty patients with typical symptoms of CD or family history of CD and positive serological tests to gliadins and transglutaminase were followed at a Mexican children's hospital (Hospital Infantil del Estado de Sonora). After dietary gluten withdrawal, there were clinical and serological improvements, confirming CD. Sera obtained from 12 of them and from 2 other adults assisting in a private medical consult (taken before gluten withdrawal) were included in this study after informed consent. Additionally, the hospital ethics committee approved the study. Symptoms of CD patients were obtained from medical records.

A direct enzyme-linked immunosorbent assay (ELISA) was carried out to evaluate IgA antigliadins and antitransglutaminase (anti-TG), according to the method of Berti et al. (*9*). Briefly, microplates were coated with 100 μL of 5 μg/mL gliadins (isolated from wheat grains) or transglutaminase in coating buffer (100 mM $NaHCO_3$, pH 9.6) overnight. After three washes with PBST (15 mM KH_2PO_4, 150 mM NaCl, pH 7.4, containing 0.2% Tween 20), the plates were blocked with 3% gelatin in PBST for 30 min at room temperature. After three washes, the plates were incubated overnight with human serum samples (diluted 1:100) in PBST containing 0.1% gelatin (PBSTG). The plates were washed three times and incubated with HRP-conjugated anti-human IgA antibodies in PBSTG (1:2000 dilution). After three washes, HRP activity was developed with 3,3′,5,5′-tetramethylbenzidine. The reaction was stopped with 1 M H_2SO_4, and the absorbance was read at 450 nm (Microplate Reader, Bio-Rad, Hercules, CA).

The cutoff value was defined as the mean + 2 SD of the absorbance (optical density) values of six negative sera of healthy donors. The serum reactivity of IgA antigliadin or anti-TG of each serum was expressed as an index value, that is, the optical density of the test serum divided by the cutoff value. Index values of 1.0 and above were considered to be positive (*15*); low titers were considered to be from 1 to 5; modest, from >5 to 10; high titers, from >10 to 20; and very high titers, >20.

Serum patients were pooled into three groups by age (G1, under 2 years old; G2, around 3 years old; and G3, 8 years and older) for immunoreactivity determination and identification of immunoreactive protein fractions. Pools were obtained by adding each serum sample in equal proportion, and a control serum pool was done from healthy children.

Human Milk Samples. For casein extraction, human milk was collected from nine healthy women and pooled in equal amounts.

Food Sample Preparation. Gluten-free (GF) bread and wheat bread (WB) were prepared as previously described (*5*). GF dough was based mainly on rice and maize flour. WB was made according to method 10-09 of the AACC (*16*). During mixing, 300 ppm mTG (Ajinomoto, activa TG) was added to GF and WB formulas (GF + TG and WB + TG, respectively) to obtain the mTG-modified products. Bread controls without mTG treatment were prepared similarly and called GF−TG and WB−TG. All of the breads were frozen or freeze-dried until analyses.

Yogurt samples were provided by A. Gonzalez-Cordova (Dairy Products Laboratory, Centro de Investigación en Alimentación y Desarrollo, A. C.). Yogurt samples were prepared as follows: *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were added (43 °C until pH 4.4 was reached) to bovine milk to obtain control yogurt (Y−TG). To obtain mTG-treated yogurt (Y + TG), 90 ppm mTG (Ajinomoto, activa TG) was added at the time of addition of *L. bulgaricus* and *S. thermophilus* to bovine milk, under the same conditions for Y−TG preparation. Samples were frozen and stored at −20 °C.

Prolamin and Casein Extraction. Extraction of prolamins was carried out as previously described (*5*). Freeze-dried breads were ground, defatted with chloroform (1:5 w/v) by stirring, and filtered twice on filter paper (Whatman no. 1). Defatted samples were extracted two times for 1 h each with 0.5 M NaCl (50 mL) and centrifuged at 2500*g* for 15 min. An additional water extraction was performed in the same way. Precipitates were re-extracted using 70% ethanol (ratio 1:5 w/v) and a longer centrifugation (2500*g*, 30 min).

Caseins were extracted from bovine milk (BM), human milk (HM), Y−TG, and Y + TG, according to the method described by Kunz and Lonnerdal (*17*). The acidity of milks and yogurts (20 mL) was adjusted to a pH of 4.3 by adding 1 M HCl, the samples were stirred for 10 min, and $CaCl_2$ was added to the final concentration of 60 mM. Samples were incubated at 4 °C for 1 h. After incubation, the samples were centrifuged (20000*g*, 4 °C, 1 h), and the precipitates were collected.

IgA Immunoreactivity to Prolamins and Caseins. Prolamins and caseins previously extracted were dissolved in 70% ethanol and in 0.9% NaCl, respectively. These solutions were used as coating antigens in the same protein concentration (5 μg/mL) as for serological tests by a direct ELISA as previously described. After microplate blocking, incubation with serum pools from G1, G2, and G3 was done, instead of serum from each individual.

Percentage of immunoreactivity (% immunoreactivity) was calculated as follows: % immunoreactivity = (AbsP/AbsW) × 100, where immunoreactivity is expressed as percentage (*9*), AbsP is the absorbance at 450 nm from prolamin or casein (measured in dilutions 1:100, 1:200, 1:400, and 1:800), and AbsW is the absorbance at 450 nm from gliadins (measured in the same dilutions).

IgA of serum pool to prolamins of mTG-untreated WB was taken as 100% immunoreactivity as reference. Additionally, an internal control of the same sera against the same sample was assayed in each ELISA plate for percent immunoreactivity calculations.

Electrophoresis and Immunoblotting of Caseins. SDS-PAGE was performed on 17% polyacrylamide gels according to the method of Laemmli (*18*). Gels were Coomassie blue-stained or electrotransferred to nitrocellulose membranes by semidry blotting. After transfer, the membranes were blocked for 2 min with 50 mM Tris, 150 mM NaCl, 5 mM NaN_3, pH 7.2, + 2% Tween 20.

Immunodetection of Caseins. Detection of antigen on nitrocellulose membranes containing caseins from HM and BM was carried out according to the method of Calderón de la Barca et al. (*19*). Membranes were incubated overnight with human serum pool diluted 1:50 v/v in TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, 5 mM NaN_3) followed by incubation with a rabbit anti-human IgA (DAKO, Carpinteria, CA) diluted 1:1000 (v/v), and a third incubation with alkaline phosphatase-conjugated goat anti-rabbit antibodies (Bio-Rad) diluted 1:2000. ALP activity was developed using 100 mM Tris, 0.5 mM $MgCl_2$, pH 9.5, with nitroblue tetrazolium chloride and bromo-4-chloro-3-indolyl phosphate−toluidine salt. After color development, the membranes were washed with distilled water and dried.

Statistical Analysis. Immunoreactivity was calculated from duplicates of four dilutions (100, 200, 400, and 800×) tested by ELISA. Mean values were compared by Tukey test using NCSS, version 2001.

RESULTS AND DISCUSSION

Celiac Disease Patient Characteristics. The ages of celiac patients, as well as antigliadins and anti-TG antibody titer indices and symptoms, are presented in **Table 1**. In summary, G1 was under 2 years old (six cases), G2 around 3 years old (three cases), and G3 8 years old and older (five cases). All of the sera had low index values for IgG antigliadin antibodies, but only two cases had low titer for IgA antigliadin antibodies, whereas seven cases presented modest titer, two cases high titer, and three sera very high titer. Anti-TG IgA had low titer for eight sera, modest titer for three sera, high titer for two sera, and very high titer for just one case. Biopsies were taken in just two cases; however, patients with positive titers against gliadins and TG could also be diagnosed as CD patients, if their symptoms improved after a gluten-free diet (*20*). Our patients' clinical and serological indicators improved after gluten withdrawal, confirming CD.

Percentage of Immunoreactivity to Caseins. Percentage of IgA immunoreactivity of sera pools from G1, G2, G3, and healthy control to human and bovine milk caseins is shown in **Figure 1**. Whereas the percentage of immunoreactivity of each serum group to human milk caseins was comparable ($p > 0.05$) to that of the healthy control, immunoreactivity to bovine milk caseins was higher ($p < 0.05$) in sera of CD patients. IgA immunoreactivity of G1 to bovine milk caseins was lower ($p < 0.05$) than that of G2 and G3, with comparable ($p > 0.05$) values between one another. The reason for differences and similarities among groups could be related to the feeding regimens, mainly formula-based, according to the local customs in northwestern Mexico (*21*). In Mexico, currently CD is not diagnosed and treated at the onset of the disease; therefore, patients in group G2 probably had a longer duration of CD at the time of the study than those from G1 and, as result, they were more exposed to cow's milk caseins in addition to gluten proteins.

To our knowledge, there are no other studies that compare IgA immunoreactivity of CD patients' sera to caseins from human and bovine milks. As the onset of CD is delayed in breastfed children (*2*, *14*), it could be important to recommend breastfeeding in cases of high-risk infants because of family history of CD or autoimmune diseases.

Berti et al. (*9*) found 39% IgA immunoreactivity to bovine caseins with respect to 100% immunoreactivity to gliadins in celiac patients, a percentage similar to our data for G3. Furthermore, it was shown that IgA immunoreactivity to digested caseins is lower than to native caseins (*9*). However, some patients with CD on a strict gluten-free diet still experience CD-like symptoms after bovine milk intake that are not due to lactose intolerance (*22*) or to the presence of gluten in the milk (*23*). Therefore, some remaining casein peptides after digestion could be responsible for CD symptoms. Moreover, there is a high homology of some peptides of bovine β-casein to the gluten peptide, with amino acid sequence LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF from α-gliadins (motif position: 57−89) (*24*). Additionally, bovine milk caseins elicited a response in the mucosa of some adult celiac patients similar to that caused by gluten proteins (*8*).

Immunodetection of Caseins. Figure 2 shows the electrophoretic pattern of human and bovine caseins (**Figure 2A**) and their immunodetection on membrane (**Figure 2B**). Lane 2 of **Figure 2A**, containing human milk caseins, presents a heavily stained band of an apparent 29000 mass identified as β-casein. In lane 3, bovine milk caseins present three major and a minor band. The upper ones around 39000 correspond to α-caseins, the faint-stained at 30000 is κ-casein, and the heaviest stained one near 28000 is β-casein. All of the estimated molecular masses from the calibration curve are apparent. Identification was possible because β-casein is the most abundant protein and the only one of the human caseins detected by SDS-PAGE (*25*). Bovine caseins have anomalous migrations on SDS-PAGE, with electrophoretic mobilities lower than their corresponding molecular masses; thus, identification was done by comparison with the patterns of purified caseins run under the same conditions (*26*).

IgA from the G1 serum pool did not recognize human caseins, resulting in a null stain of strip 2, whereas a weak stain is shown in strip 3 for bovine α-caseins (**Figure 2B**). **Figure 2B** also shows that human caseins in strip 2 were not recognized by IgA from G3, but bovine α- and β-caseins were strongly recognized, as they were by IgA from G2 sera (figure not shown). Altogether, the results of the immunoreactivity assays (**Figure 1**) and these of immunodetection on membrane agree.

Percentages of Immunoreactivities to Prolamins. Percentages of immunoreactivities to prolamins extracted with 70% ethanol from different breads are shown in **Table 2** (lines 1−4). One hundred percent of immunoreactivity was given to prolamins (gliadins) of plain wheat bread (WB−TG) for each serum group. There were no differences ($p > 0.05$) in immunoreactivity to prolamins from WB after mTG treatment with respect to this of WB−TG in sera from G1 and G2, whereas it increased 33% for G3. These results agree with data from T-cell response to nondeamidated gluten peptides frequently found in children and, to a lesser extent, in adult CD patients (*11*).

Table 2 also shows that IgA from G1 and G2 presented immunoreactivity to prolamins from gluten-free bread (GF−TG) similar to that obtained by IgA from healthy children (control). However, IgA from G3 patients to GF−TG prolamins had comparable ($p > 0.05$) immunoreactivity to these of WB−TG. Similarly, IgA from G1 and G2 sera reacted to GF + TG prolamins as IgA from control, whereas the IgA from G3 immunoreactivity to GF + TG was increased with respect to GF−TG, from 98 to 195%. It was previously demonstrated that the prolamins responsible for this reactivity are zeins from maize in GF breads (*5*). In addition, these results together with those obtained for IgA immunoreactivity to caseins confirm our hypothesis that the IgA immunoreactivity to dietary proteins in CD is age-related.

Deamidation of glutamine residues by tTG increases the binding affinity of peptides to antigen-presenting cells, creating strong T-cell stimulatory epitopes (*13*). The analogue deamidation by mTG also increases the IgA immunoreactivity to gliadins (*4*, *5*). According to Ciccocioppo et al. (*27*), both innate and adaptive immune responses are involved in CD. However, peptides that stimulate the innate immune system are not deamidated peptides, whereas IgA antibodies against deamidated peptides are generated by the adaptive immune system. Thus, the innate immune recognition could be more important than the adaptive one in children younger than 3 years old, although it might be also related to the age of CD onset.

Percentage of Immunoreactivity to Caseins from Yogurt. Table 2 (lines 5 and 6) shows that mTG treatment of yogurt caseins (Y + TG) did not change immunoreactivity of IgA from any group of celiac patients, with respect to untreated yogurt (Y−TG). An immunoreactivity comparable to the

Table 1. Clinical Description of Age Groups of Celiac Disease Patients

age group	celiac patients	age[a] (years)	IgG antigliadin index value	IgA antigliadin index value	IgA anti-TG index value	symptoms[b]
G1	CP1	0.8	2.6	11.7	4.8	D, AP, F, MA, UW, and positive biopsy
	CP2	1	1.7	8.3	2.7	D, MA, UW
	CP3	1.1	3.5	8.8	3.8	D, UW, F
	CP4	1.7	1.7	7.4	2.1	AP, Cn
	CP5	2	2.2	6.1	5.8	AP, Cn
	CP6	2	4.1	21.4	13.3	D, AP, F
G2	CP7	2.9	3.5	35.7	26.8	D, C, AP, and positive biopsy
	CP8	3	1.4	4.6	2.1	F, UW, AP, A
	CP9	3.3	1.8	2.6	2.0	D, AP, F
G3	CP10	8	1.0	23.3	9.7	D, AP, F, BT, UW
	CP11	13	3.1	6.8	4.5	D, F, AP, MA, SH
	CP12	16	2.8	13.9	8.6	D, MA, UW, SH
	CP13	29	3.4	7.8	15.3	MA, AP
	CP14	32	1.6	8.2	4.0	AP, F

[a] Age at diagnosis. [b] D, diarrhea; AP, abdominal pain; C, cramp; MA, malabsorption; UW, underweight; SH, short height; F, flatulence; A, anemia; Cn, constipation.

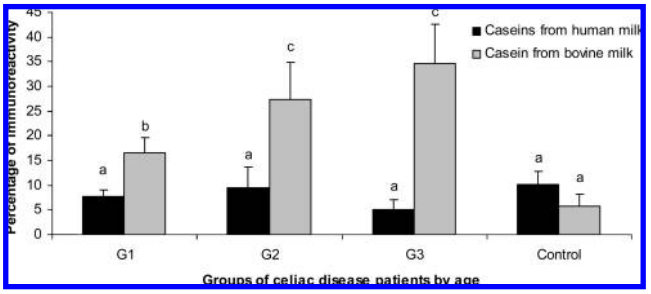

Figure 1. Percentage of IgA immunoreactivity from sera of different CD patient groups to caseins from both human and bovine milks. Different letter indicate significant differences at $p < 0.05$.

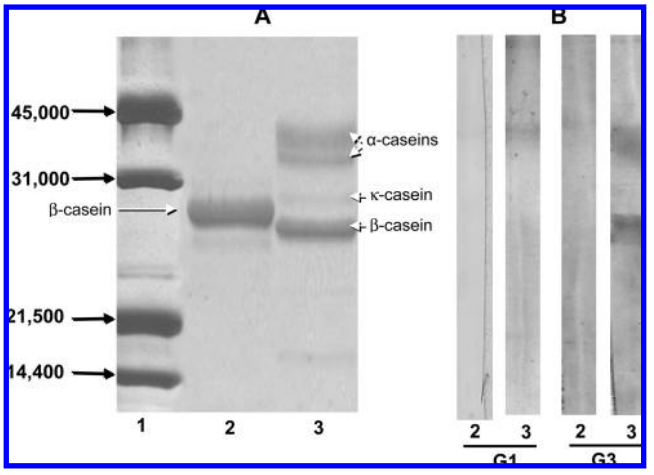

Figure 2. Electrophoretic pattern of human and bovine milk caseins (**A**) and immunodetection in membrane (**B**) using serum IgA of CD patients: lane 1, MW standards; lane 2, human milk caseins; lane 3, bovine milk caseins. (**B**) Blotted caseins in lanes 2 and 3 were immunodetected for IgA using serum pools of G1 or G3 patient groups.

healthy control was detected in Y−TG and Y + TG (around 11%) testing of the serum pool from G1. Serum pools of G2 and G3 groups presented values for immunoreactivity similar to those of yogurt caseins, whether mTG-treated or not, greater ($p < 0.05$) than for G1.

In general, reactivity percentages were lower for caseins extracted from yogurt than for the ones extracted from cow's milk. A possible explanation could be that the aggregation and cross-linking of caseins (mTG-dependent or not) in yogurt reduced the epitopes recognized by CD patients' IgA in caseins from bovine milk.

For deamidation, mTG requires an excess of glutamine residues relative to lysine content in the peptide chains (*28*). Gluten has a high Gln/Lys ratio (*29*), whereas this value is lower for caseins (*30*). Therefore, the probability of cross-linking by mTG instead of deamination is higher for caseins. Additionally, the pH of yogurt favors cross-linking, because deamidation requires pH around 6 (*31*).

There was no recognition of human milk caseins by IgA antibodies of CD patients regardless of the age group, whereas bovine milk α- and β-caseins induced a medium reactivity in the sera of all three age groups tested with higher values for G2 and G3. However, such immunoreactivity decreased in yogurt-extracted caseins to values comparable to those of the healthy controls for G1.

Treatment of yogurt with mTG did not affect the IgA immunoreactivity to its caseins, whereas such treatment increased reactivity to wheat and maize prolamins from WB + TG and GF + TG breads only in group G3. Additionally, serum IgA of groups G1 and G2 did not recognize maize prolamins from gluten-free breads whether mTG-treated or not. It remains an interesting question as to why only IgA of older children and adults reacts against maize prolamins if it is a common ingredient in foods since infancy in Mexico.

In conclusion, bovine milk caseins and prolamins treated or not with mTG were differentially recognized by IgA of CD patients according to their age. This could reflect a differential manifestation of the effects of such dietary proteins on the intestinal mucosa. Possibly, the tested dietary proteins could induce an inflammation as an early step, and this in turn allows gliadins to cross the intestinal barrier, initiating the cascade of autoimmune reactions in CD (*10*). Therefore, because of their proteins, infant feeding formulas and bakery products obtained by current technological processes could be environmental factors responsible for the increase in CD prevalence worldwide.

ABBREVIATIONS USED

CD, celiac disease; mTG, microbial transglutaminase; tTG, tissue transglutaminase; GF, gluten free; WB, wheat bread; GF−TG, mTG-nontreated gluten-free bread; GF + TG, mTG-treated gluten-free bread; WB−TG, mTG-nontreated wheat bread; WB + TG, mTG-treated wheat bread; Y−TG,

Table 2. Percentage of Immunoreactivity of Prolamins and Caseins from Food Products Treated or Not with mTG[a]

source of antigen		percentage of immunoreactivity of G1 (≤2 years old) sera	percentage of immunoreactivity of G2 (~3 years old) sera	percentage of immunoreactivity of G3 (>8 years old) sera
prolamins	WB−TG	100 a	100 a	100 a
	WB+TG	100 ± 10.3 a	100.4 ± 2.4 a	132.9 ± 20.3 d
	GF−TG	10.0 ± 0.2 b	8.7 ± 2.1 b	98.0 ± 4.4 a
	GF+TG	9.8 ± 0.9 b	11.6 ± 5.8 b	195.1 ± 33.0 e
caseins	Y−TG	11.41 ± 3.95 b	27.15 ± 11.52 c	26.44 ± 6.33 c
	Y+TG	11.72 ± 3.98 b	26.10 ± 10.65 c	24.43 ± 5.62 c

[a] Different letters indicate significant difference at $p < 0.05$.

mTG-nontreated yogurt; Y + TG, mTG-treated yogurt; BM, bovine milk; HM, human milk; G1, group of CD patients under 2 years old; G2, group of CD patients around 3 years old; G3, group of CD patients older than 8 years.

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LITERATURE CITED

(1) Green, P. H. R.; Cellier, M. D. B. Celiac disease. *New Engl. J. Med*. **2007**, *357*, 1731–1743.
(2) Ivarsson, A.; Hernell, O.; Stenlund, H.; Persson, L. Breast-feeding protects against celiac disease. *Am. J. Clin. Nutr*. **2002**, *75*, 914–921.
(3) Rizzello, C. G.; De Angelis, M.; Di Cagno, R.; Camarca, A.; Silano, M.; Losito, I.; De Vincenzi, M.; De Bari, M. D.; Palmisano, F.; Maurano, F.; Gianfrani, C.; Gobbetti, M. Highly efficient gluten degradation by lactobacilli and fungal proteases during food processing: new perspectives for celiac disease. *Appl. Environ. Microbiol*. **2007**, *73*, 4499–4507.
(4) Berti, C.; Roncoroni, L.; Falini, M. L.; Caramanico, R.; Dolfini, E.; Bardella, M. T.; Elli, L.; Terrani, C.; Forlani, F. Celiac-related properties of chemically and enzymatically modified gluten proteins. *J. Agric. Food Chem*. **2007**, *55*, 2482–2488.
(5) Cabrera-Chavez, F.; Rouzaud-Sández, O.; Sotelo-Cruz, N.; Calderón de la Barca, A. M. Transglutaminase treatment of wheat and maize prolamins of bread increases the serum IgA reactivity of celiac disease patients. *J. Agric. Food Chem*. **2008**, *56*, 1387–1391.
(6) Moore, M. M.; Heinbockel, M.; Dockery, P.; Ulme, H. M.; Arendt, E. K. Network formation in gluten-free bread with application of transglutaminase. *Cereal Chem*. **2006**, *83*, 28–36.
(7) Dieterich, W.; Esslinger, B.; Schuppan, D. Pathomechanisms in celiac disease. *Int. Arch. Allergy Immunol*. **2003**, *132*, 98–102.
(8) Kristjánsson, G.; Venge, P.; Hallgren, R. Mucosal reactivity to cow's milk protein in coeliac disease. *Clin. Exp. Immunol*. **2007**, *147*, 449–455.
(9) Berti, C.; Trovato, C.; Bardella, M. T.; Forlani, F. IgA anti-gliadin antibody immunoreactivity to food proteins. *Food Agric. Immunol*. **2003**, *15*, 217–223.
(10) Clemente, M. G.; De Virgiliis, S.; Kang, J. S.; Macatagney, R.; Musu, M. P.; Di Pierro, M. R.; Drago, S.; Congia, M.; Fasano, A. Early effects of gliadin on enterocyte intracellular signalling involved in intestinal barrier function. *Gut* **2003**, *52*, 218–223.
(11) Vader, L. W.; de Ru, A.; Van Der Wal, Y.; Kooy, Y. M. C.; Benckhuijsen, W.; Mearin, M. L.; Drijfhout, J. W.; Van Veelen, P.; Koning, F. Specificity tissue transglutaminase explains cereal toxicity in celiac disease. *J. Exp. Med*. **2002**, *192*, 643–649.
(12) Demir, H.; Yuce, A.; Kocak, N.; Ozen, H.; Gurakan, F. Celiac disease in Turkish children: presentation of 104 cases. *Pediatr. Int*. **2000**, *42*, 483–487.
(13) Schuppan, D.; Junker, Y. Turning swords into plowshares: transglutaminase to detoxify gluten. *Gastroenterology* **2007**, *133*, 1025–1038.
(14) D'Amico, M. A.; Colmes, J.; Stavropoulos, S. N.; Frederick, M.; Levy, J.; DeFelice, A. R.; Kazlow, P. G.; Green, P. H. R. Presentation of pediatric celiac disease in the United States: prominent effect of breastfeeding. *Clin. Pediatr*. **2005**, *44*, 249–258.
(15) Lagerqvist, C.; Ivarsson, A.; Juto, P.; Persson, L. Å.; Hernell, O. Screening for adult coeliac disease—which serological marker(s) to use? *J. Intern. Med*. **2001**, *250*, 241–248.
(16) AACC International. *Approved Methods of the American Association of Cereal Chemists*, 10th ed.; AACC: , 2000; method 10-09.
(17) Kunz, C.; Lonnerdal, B. Human milk proteins: separation of whey protein and their analysis by poliacrilamide gel electrophoresis, fast protein liquid chromatography (FPLC), gel filtration and anion-exchange chromatography. *Am. J. Clin. Nutr*. **1989**, *49*, 464–470.
(18) Laemmli, U. K. Cleavage of structural protein during assembly of the head of bacteriophage T4. *Nature* **1979**, *227*, 680–685.
(19) Calderón de la Barca, A. M.; Yepiz-Plascencia, G. M.; Bøg-Hansen, T. C. Hydrophobic interactions between gliadin and proteins and celiac disease.. *Life Sci*. **1996**, *59*, 1951–1960.
(20) Scoglio, R.; Di Pasquale, G.; Pagano, G.; Lucanto, M. C.; Magazzu, G.; Sferlazzas, C. Is intestinal biopsy always needed for diagnosis of celiac disease? *Am. J. Gastroenterol*. **2003**, *98*, 1325–1331.
(21) Calderón de la Barca, A. M.; Bolaños-Villar, A.; Román-Pérez, R.Protein contents of milk surrogates and their sanity regulation.. *Salud Publica Mex*. 1996, *38*, 268−275.
(22) Nieminen, U.; Kahri, A.; Savilahti, E.; Farkkila, M. A. Duodenal disaccharidase activities in the follow-up of villous atrophy in coelica disease. *Scand. J. Gastroenterol*. **2001**, *36*, 507–510.
(23) Dekking, L.; Koning, F.; Hosek, D.; Ondrak, T. D.; Taylor, S. L.; Schroeder, J. W.; Bauer, M. Intolerance of celiac disease patients to bovine milk is not due to the presence of T-cell stimulatory epitopes of gluten. *Nutrition* **2009**, *25*, 122–123.
(24) Darewicz, M.; Dziuba, J.; Minkiewicz, P. Computational characterization and identification of peptides for in silico detection of potentially celiac-toxic proteins. *Food Sci. Technol. Int*. **2007**, *13*, 125–133.
(25) Green, M. R. Identification of human milk κ-casein on polyacrylamide gels by differential staining with ethyl-stains-all and chymosin sensitivity. *J. Histochem. Cytochem*. **1986**, *34*, 147–150.
(26) Creamer, L. K.; Richardson, T. Anomalous behavior of bovine α_{s1}- and β-caseins on gel electrophoresis in sodium dodecyl sulfate buffers. *Arch. Biochem. Biophys*. **1984**, *234*, 476–486.
(27) Ciccocioppo, R.; Di Sabatino, A.; Corazza, G. R. The immune recognition of gluten in coeliac disease. *Clin. Exp. Immunol*. **2005**, *140*, 408–416.
(28) Jong, G. A. H.; Koppelman, S. J. Transglutaminase catalyzed reactions: impact on food applications. *J. Food Sci*. **2003**, *67*, 2798–2806.

(29) Hoseney, R. C. Cereal proteins. In *Principles of Cereal Science and Technology*; Hoseney, R. C., Ed.; American Association of Cereal Chemistry: , 1986; pp 69−88.
(30) Carles, C.; Huet, J. C.; Ribadeau-Dumas, B. A new strategy for primary structure determination of proteins: application to bovine β-casein. *FEBS Lett*. **1988**, *229*, 265–72.
(31) Ohtsuka, T.; Umezawa, Y.; Nio, N.; Kubota, K. Comparison of deamidation activity of transglutaminases. *J. Food Sci*. **2001**, *66*, 25–29.

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