ORIGINAL PAPERS

Influence of infant feeding on the excretion of gluten immunopeptides in feces

Cristóbal Coronel-Rodríguez[1], Libia Quero-Acosta[2], Federico Argüelles-Martín[3] and M. Cinta Guisado-Rasco[1]

[1]Centro de Salud "Amante Laffón". Distrito Sanitario de AP. Sevilla, Spain. [2]Clínica Santa Isabel. Sevilla, Spain. [3]Hospital Universitario Virgen Macarena. Sevilla, Spain

Received: 12/03/2018 · **Accepted:** 30/07/2018

Correspondence: Cristóbal Coronel-Rodríguez. Centro de Salud "Amante Laffón". Pza. San Martín de Porres, 19. 41010 Sevilla, Spain. **e-mail:** cristobalcoronel@gmail.com

ABSTRACT

Introduction: the secretion of antigens from the diet into breast milk has been extensively documented. The transfer of gliadin could be critical for the development of an immune response.

Objectives: to investigate the presence of immunogenic gluten peptides in the feces of infants fed with different diets.

Material and methods: a blind, prospective, controlled, collaborative study was performed in three hospitals, between September 2016 and January 2017. The study protocol was approved by the Ethics Committee of the hospitals in Seville prior to starting the study.

Results: the cohort was divided into three groups of 30 infants: an experimental group (average age 9.2 ± 2.8 weeks) with exclusive breastfeeding, a control group 1 (average age 10.3 ± 3.3 weeks) exclusively fed with onset formula and a control group 2 (average age 56 ± 3.7 weeks) with infants that consumed gluten on a regular basis. The peptide 33-mer of gliadin was negative in all feces samples from both the experimental and control group 1. With regard to control group 2, the peptide 33-mer of gliadin was negative in 23% of cases (seven children). There was no difference in the amount of gluten ingested by these children compared to those who excreted the 33-mer peptide.

Conclusions: the failure to detect gluten in the feces of infants that were exclusively breastfed indicates that it is probably below the limits of detection. Healthy children who consume gluten may not excrete it in feces.

Key words: Food hypersensitivity. Breast-feeding. Complementary feeding. Introduction. Gluten. Infant.

INTRODUCTION

Several elements affect the development of intestinal immune tolerance throughout life. At birth, the intestine is still immature from a functional point of view and thus requires growth and maturation factors contained within breast milk (BM). Their main function, among others, is to preserve the integrity of the intestinal lamina propria and protect it from infections which could alter the permeability of the intestine. This immunoregulator role of BM (1) defines a period in which the presence of food allergens in the intestine favors the development of tolerance against these food stuff ("immunologic window") (2,3). Nevertheless, there is no conclusive evidence of its pre-emptive effect.

Recent studies claim that BM does not have a significant effect on the development of celiac disease (CD), regardless of whether the infant is exclusively breastfed, or if gluten is introduced whilst breastfeeding. However, none of these studies have been evaluated in infants older than three years of age (4,5). Despite the hypothesis that suggests the existence of a window of immunological tolerance in the intestine, a cohort study of neonates at risk for CD disputes this idea. The introduction of small amounts of gluten into the diet of neonates aged 16 to 24 weeks old did not reduce the risk of developing CD at the age of three years (5) compared to the placebo group.

CD is a multifactorial disease and two risk factors have been clearly identified: an external factor (gluten) and a genetic factor (the HLA-DQ2). Both are crucial and necessary for disease development. The fact that genetically predisposed individuals do not always develop CD begs the question of whether other environmental factors are involved (REO virus?) (6,7), which may prevent the tolerance to gluten peptides.

Previous presentation of advancement of results in the XXIV Congreso of the Sociedad Española de Gastroenterología, Hepatología y Nutrición Pediátrica. Donostia/San Sebastián 25-27/5/2017.

Conflicts of interest: Authors declare not to have any conflicts of interest. This study has been approved by the Ethics Committee of the Hospital Virgen Macarena and Hospital Virgen del Rocío in Seville. No funding of any kind was received for this study, except for the donation of the analysis kits by Vircell® Laboratories.

Coronel-Rodríguez C, Quero-Acosta L, Argüelles-Martín F, Guisado-Rasco MC. Influence of infant feeding on the excretion of gluten immunopeptides in feces. Rev Esp Enferm Dig 2019;111(2):134-139.

DOI: 10.17235/reed.2018.5559/2018

According to the last ESPGHAN report (8), breastfeeding does not protect against the development of CD but delays its appearance. This was demonstrated by the fact that infants did not suffer from CD while breastfeeding. However, the proportion of individuals that develop CD at different ages is the same regardless of whether they were fed with breast milk or not (7,8).

Gluten is a protein of low nutritional value that is commonly used in bread production. Its protein chains are very elastic and allow the gases produced by the yeast to inflate them, leading to the rising of the dough (9). Gliadins are gluten fractions that are soluble in alcohol and contain most of the toxic components that affect celiac patients. They are rich in glutamine and proline, and the digestion of these in the gastrointestinal tract is more difficult than for other peptides (10).

In vitro experiments have shown that the digestion of gliadin derives an alfa-gliadin peptide. This peptide is composed of 33 amino acids (33-mer) and it is resistant to gastric and pancreatic proteases, as well as to brush border enzymes of the intestinal lining. This 33-mer gliadin peptide seems to be the most immunogenic of all the currently known peptides. This 33-mer has six epitopes that partly overlap and it is resistant to enzymatic degradation by intestinal and pancreatic enzymes (7,10-12). The average life span of the 33-mer peptide is longer than 20 hours, leading to the belief that it could act as an antigen and therefore could stimulate T-cell proliferation. This would induce a toxic phenomenon in genetically predisposed individuals (7). Other *in vitro* experiments have shown that T lymphocytes isolated from the intestinal mucosa from untreated celiac patients contain a peptide similar to the 33-mel alpha-gliadine (7).

How these partially digested peptides get through the intestinal barrier is still unknown. However, it is hypothesized that it could be due to early infections, which could also influence the disease pathogenesis by increasing permeability (7). Furthermore, it may also be mediated by the action of zonulin, a protein that carries intracellular signals that open tight junctions (13). Gliadin has the ability to induce the liberation of zonulin, with an increase in intestinal permeability and cytokine production (14). Once the peptides get through the barrier epithelium, the 33-mer fragment would act as a substrate for the transglutaminase 2 (TG2) and TG2 would deaminate the peptide. On the other hand, the action gliadin-TG2 (15) on susceptible individuals leads to the development of antiendomysial and antitransglutaminase antibodies, which are the most sensitive and specific tools for diagnosis up to now.

The 33-mer fragment modified by the TG2 is an efficient stimulator of T CD4 lymphocytes, which can only recognize gluten peptides when the HLA-DQ2 and HLA-DQ8 heterodimers are present (10). The stimulation of lymphocytes leads to the immune cascade, which results in the inflammatory response and harm to the celiac mucosa (16). Gluten-reactive T-cells have a Th0/Th1 phenotype and usually release the pro-inflammatory cytokine IFN-γ.

The presence of gluten proteins in breast milk has been reported in previous studies (17). The average level of gliadin in BM is 178 ng/ml (range 5-2,000 ng/ml) and the average level of gliadin epitopes that elicit T-cells are 132 ng/ml for Glia 9 and average levels of Glia 1 are 404 ng/ml (18). Gliadin that was given to mothers with a non-gluten diet appeared in their BM between two to four hours after the gliadin intake (19).

The gluten peptides are resistant to gastrointestinal digestion (in particular the 33-mer immunotoxic peptide), which ensures that a significant part of these peptides are excreted in feces. This provides a mechanism to detect previous gluten intake (20). Therefore, it is important to prove that BM contains a sufficient amount of gluten to interact with the intestine and is excreted in the feces of exclusively breastfed babies. It is possible that BM is not only immunomodulatory but also performs the antigen presentation function, which is gluten in this case. This leads to breastfed babies reaching a correct gluten tolerance.

Study hypothesis

If BM contains gliadin, gliadin peptides should be found in the feces of breastfed babies. This would mean that BM protects against the development of CD and that it induces intestinal tolerance against gluten due to the fact that it contains small amounts of gluten from the mothers' dietary intake.

MATERIAL AND METHODS

A prospective and single-blinded study from three medical centers was performed between September 2016 and January 2017. The study was approved by the ethic committees of the hospitals Virgen del Rocío and Macarena (Seville, Spain). The study was performed in the Santa Isabel clinic, Amante Lafon health center and Macarena hospital from October to December 2016.

The infant enrolment process was conducted from the beginning of the project and included children who met the inclusion criteria (discarding deferral criteria), until 30 individuals for each study group were obtained.

Study population

Experimental group: 30 healthy exclusively breastfed babies, older than four weeks old and younger than four months old.

Control group 1: 30 healthy babies exclusively fed with first infant formula, older than four weeks old and younger than four months old.

Control group 2: 30 healthy breastfed babies from ten to 14 months old with a daily gluten intake.

Figure 1 shows a summary chart.

Deferral criteria

Breastfed babies who received any nourishment different from BM in the experimental group and control group 1.

Parents who did not sign the informed consent form.

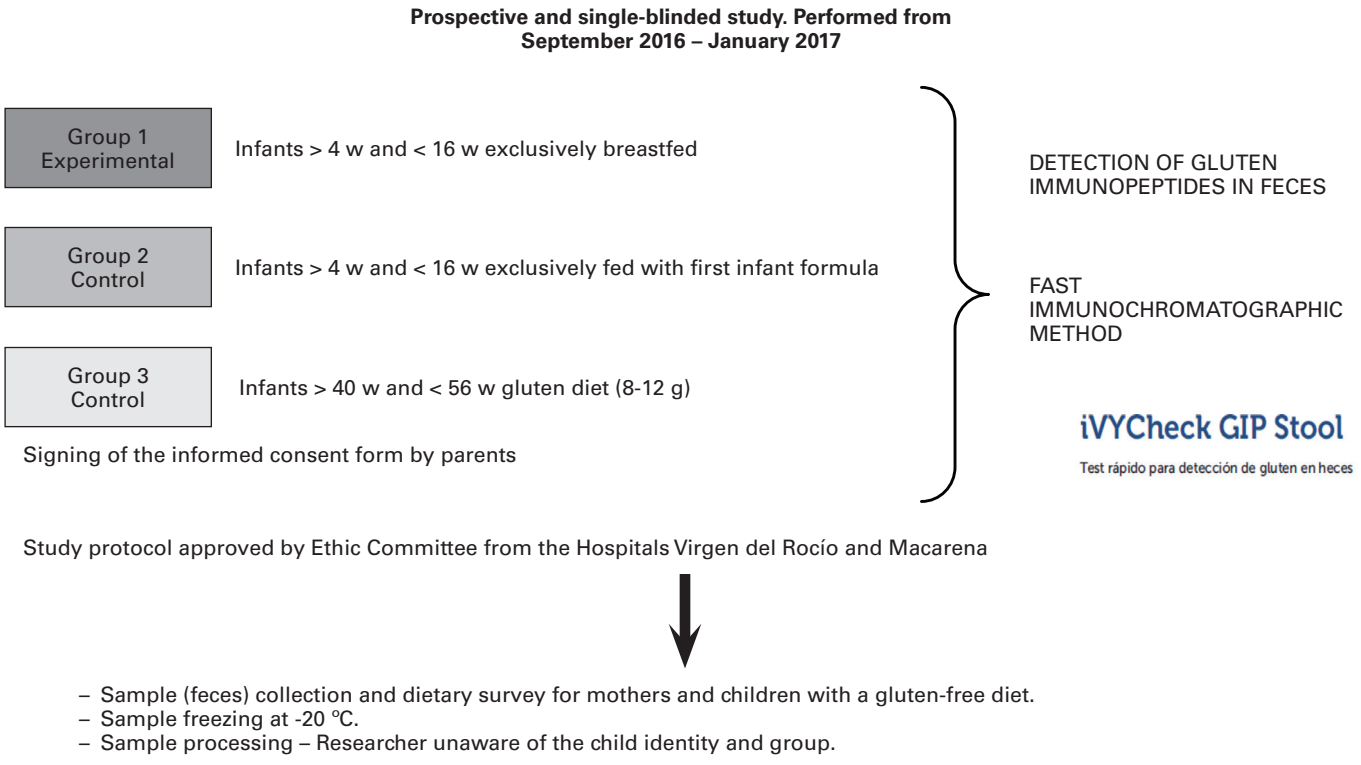

Fig. 1. *Samples, materials and method chart (w: weeks; > 4 w and < 16 w: time-frame from 28 to 112 days).*

Lab materials and method

The samples of recent feces were collected by a relative, according to a standard procedure. A depressor was used to extract the feces from the diaper and were kept in a feces container. Ideally, the samples were not mixed with urine or lotion. The samples were brought to the consulting room and were registered with consecutive numbers, on both the container and the informed consent form. The samples were immediately frozen.

The analysis of the feces was performed per batch; each batch was defrosted simultaneously and contained a different number of samples. Each sample was identified by a number, so that the researcher was unaware of both the baby and the study group (control, group 1 or group 2) of the sample.

The IVYCHECK GIP-Stool® was used to analyze the fecal samples. This kit is based on a fast immunochromatographic test which allows the detection of immunogenic peptides (GIP) in feces. The technique was performed as described by the manufacturer. This immunochromatographic test allows the detection of GIP from dietary intake in feces samples. The first step is the extraction of gluten from a feces sample that is frozen at -20 °C. The sample reacts with the conjugated antibodies (monoclonal antibodies A1 and G12) that are fixed on the strip. This complex moves along the strip by capillarity (Fig. 2). A red colored line appears on the strip result area to indicate a positive result. The absence of a red line indicates a negative test. A control protein is also present to confirm the correct functioning of the test as shown by the blue line (Fig. 3).

The detection limit of the immunochromatographic strip is 0.3 GIP micrograms per gram of feces. This test specifically detects the toxic prolamin fraction of wheat (gliadin), rye (secalin) and barley (hordein) in feces. It also detects oats prolamin (avenin) if the quantity is above the threshold.

Breastfeeding mothers and children from control group 2 (with a daily intake of gluten) performed a short dietary survey with a three-day reminder in order to estimate their daily gluten intake.

RESULTS

A total of 90 children were recruited to the study. Table 1 shows a summary of the results of the three study groups. The experimental group was formed by 30 exclusively breastfed babies with an average age of 9.2 ± 2.8 weeks. Fourteen were male and 16 were female. All were negative for the 33-mer gliadin peptide in feces.

Control group 1 included 30 babies (17 female and 13 females) with an average age of 10.3 ± 3.3 weeks, who were fed exclusively with first infant formula. All had a GIP 33-mer test.

Control group 2 included 30 babies who habitually consumed gluten. This included 15 males and 15 females with an average age of 56 ± 3.7 weeks; 23% (7) did not excrete the GIP 33-mer in feces. No difference was observed between the amount of gluten ingested by these children compared with those that excreted the GIP 33-mer.

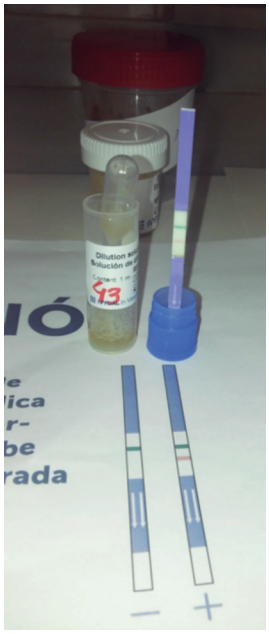

Fig. 2. *Sample result rising by capillarity.*

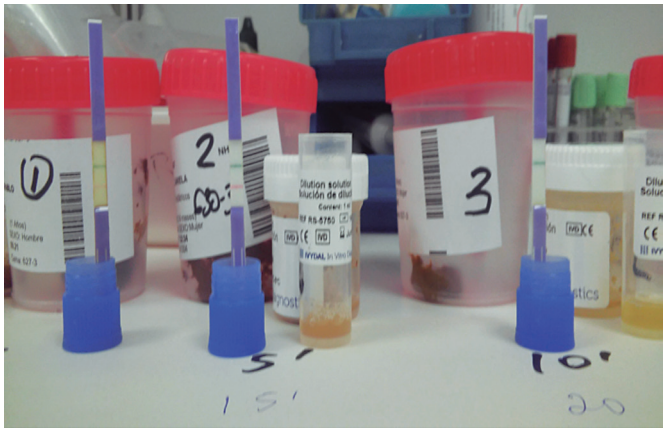

Fig. 3. *Possible results left to right: slightly positive, positive and negative.*

DISCUSSION

Gluten is a protein of low nutritional value that is commonly used for bread production. Its protein chains are very elastic and allow the gases from yeast to inflate them, leading to rising of the dough (9). Gliadins are an alcohol-soluble fraction of gluten and contain most components that are toxic to celiac individuals. Gliadins are rich in glutamine and proline and their digestion in the gastrointestinal tract is more difficult than for other peptides (10).

In vitro experiments have shown that there are still non-digested regions which generate an alfa-gliadin peptide after the digestion of gliadin. This peptide is composed of 33 amino acids (33-mer) and it is resistant to gastric and pancreatic proteases, as well as border enzymes of the intestinal lining. The average life span of the 33-mer peptide is longer than 20 hours and had led to the belief that it could act as an antigen and therefore stimulate T-cell proliferation. This would induce toxic phenomena in genetically susceptible individuals (7). Other *in vitro* experiments have also shown that T lymphocytes isolated from intestinal mucosa from untreated celiac patients contain a peptide similar to the 33-mel alpha-gliadin (12).

Gliadin peptides pass through the epithelium via transcytosis, but this mechanism is still unclear. In active CD, there is an increase in trans-epithelial transport and the processing of these peptides by the epithelium cells is also altered. Thus, toxic peptides (19-mer) and immunogenic peptides (33-mer), both intact and partially broken down, may be able to get through (21). Several studies support this theory and there is a high level of transport from the apical membrane of enterocytes to the basal membrane in celiac patients, via a mechanism dependent on gamma interferon (IFN-γ). Nevertheless, the literature with regard to the production and action of the 33-mer peptide in healthy people intestines is limited.

Nowadays, this 33-mer peptide is used to detect voluntary or inadvertent dietary transgressions, as it can be detected in feces (22). More recently, it has been shown that it can be detected in urine (23) with a higher sensitivity than the classic serological test. In fact, this peptide has recently been included in the catalogue of diagnostic tests in hospital services.

This study, regardless of our prior knowledge that BM contains gliadin (24), proves that feces from breastfed babies do not contain this peptide, or that is it present in very low concentrations (ng/ml order). This peptide would also be further digested in the intestine and would therefore reach such a low concentration level that it would not be detected by the IVYCHECK GIP test. As expected, children fed with infant formula had a negative result, as formula does not

Table 1. *Results*

Group	Breastfed (experimental group)	Formula Control 1	DDG Control 2
n	30	30	30
Average age (weeks ± DS)	9.2 ± 2.8	10.31 ± 3.37	56.2 ± 3.7
Sex	14 males 16 females	17 males 13 females	15 males 15 females
GIP	100% negative	100% negative	7 negatives (23%)

DGI: daily gluten intake; SD: standard deviation; GIP: detection of GIP 33-mer in feces.

contain gluten and the composition is clearly defined and regulated, regardless of their brand. These results do not provide convincing evidence of the preventive effect of breastfeeding. Furthermore, it does not provide any proof that breastfeeding significantly influences the development of CD, regardless of its exclusive or non-exclusive nature (4,5).

With regard to 1-year old children who consume gluten, 23% did not excrete the 33-mer gliadin peptide. This is due to the fact that the digestive enzymes do not produce it from the intake of gluten, or it is produced in such small quantities that it cannot be detected by the test. The short dietary survey by Trinidad Rodríguez et al. (25) allowed us to obtain information from the long-term consumption model. It is even more effective when estimating the usual intake of general food groups. The questionnaire inquired about the number of times per week/month that certain groups of food of 45 different items were consumed, for a total of six days during the following two weeks (three days per week). Therefore, we considered it useful for the present study, as the aim was to estimate the intake of cereals that contain gluten (complex carbohydrates) and they are highly consumed in the Mediterranean diet.

CONCLUSIONS

Current scientific evidence leads us to believe that BM contains gliadin and its peptides and that BM is the ideal carrier for an effective presentation of this antigen to the intestine of a breastfed baby. The fact that the 33-mer-alfa-gliadine peptide was not detected in the feces of exclusively breastfed babies does not discard this theory. However, it probably indicates that this peptide is found at a lower concentration than the detection limit of the test, or that breastfed babies excrete other kinds of gliadin peptides in their feces.

It is noteworthy that healthy children aged one year old, even though they consume gluten proportionally to their size, do not no excrete equivalent quantities of PIG (33-mer gliadin) in feces. Thus, these two interesting results need to be explored in subsequent studies.

REFERENCES

1. Chirico G, Marzollo R, Cortinovis S, et al. Antiinfective properties of human milk. J Nutr 2008;138:S1801-6. DOI: 10.1093/jn/138.9.1801S
2. Sansotta N, Piacentini GL, Mazzei F, et al. Timing of introduction of solid food and risk of allergic disease development: understanding the evidence. Allergol Immunopathol (Madr) 2013;41:337-45. DOI: 10.1016/j.aller.2012.08.012
3. Fernández Rodríguez M, Martín-Crespo Blanco MC. La introducción precoz de alimentos en lactantes con lactancia materna aparentemente no previene la alergia alimentaria. Evid Pediatr 2016;12:49.
4. Vriezinga SL, Auricchio R, Bravi E, et al. Randomized feeding intervention in infants at high risk for celiac disease. N Engl J Med 2014;371:1304-15.
5. SIGENP (Italian Society of Pediatric Gastroenterology, Hepatology, and Nutrition) Working Group on Weaning and CD Risk. Introduction of gluten, HLA status, and the Risk of celiac disease in children. N Engl J Med 2014;371:1295-303.
6. Bouziat R, Hinterleitner R, Brown JJ, et al. Reovirus infection triggers inflammatory responses to dietary antigens and development of celiac disease. Science 2017;356:44-50. DOI: 10.1126/science.aah5298
7. Parada A, Araya M. El gluten. Su historia y efectos en la enfermedad celiaca. Rev Med Chile 2010;138:1319-25. DOI: 10.4067/S0034-98872010001100018
8. Szajewska H, Shamir R, Mearin L, et al. Gluten introduction and the risk of coeliac disease: a position paper by the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition. J Pediatr Gastroenterol Nutr 2016;62(3):507-13. DOI: 10.1097/MPG.0000000000001105
9. Belderok B. Developments in bread-making processes. Plant Foods Hum Nutr 2000;55:1-86. DOI: 10.1023/A:1008199314267
10. Shan L, Molberg O, Parrot I, et al. Structural basis for gluten intolerance in celiac sprue. Science 2002;297:2275-9. DOI: 10.1126/science.1074129
11. Shan L, Qiao SW, Arentz-Hansen H, et al. Identification and analysis of multivalent proteolytically resistant peptides from gluten: implications for celiac sprue. J Proteome Res 2005;4:1732-41. DOI: 10.1021/pr050173t
12. Ortigosa L. Detección de péptidos inmunogénicos de gluten en heces (GIPs): nuevo método de monitorización de la dieta sin gluten. Canarias Pediátrica 2017;41(2):163-6.
13. Fassano AA. Zonulin and its regulation of intestinal barrier function: the biological door to inflammation, autoimmunity, and cancer. Physiol Rev 2011;91(1):151-75. DOI: 10.1152/ physrev.00003.2008
14. Fasano AA. Intestinal zonulin: open sesame! Gut 2001;49:159-62. DOI: 10.1136/gut.49.2.159
15. Anderson RP, Degano P, Godkin AJ, et al. In vivo antigen challenge in celiac disease identifies a single transglutaminase-modified peptide as the dominant agliadin T-cell epitope. Nat Med 2000;6:337-42. DOI: 10.1038/73200
16. Green PH, Jabri B. Coeliac disease. Lancet 2003;362:383-91. DOI: 10.1016/S0140-6736(03)14027-5
17. Troncone R, Scarcella A, Donatiello A, et al. Passage of gliadin into human breast milk. Acta Paediatr Scand 1987;76:453-6. DOI: 10.1111/j.1651-2227.1987.tb10498.x
18. Hopman GD. Gluten intake and gluten-free diet in the Netherlands. Department Pediatry, Leiden University Medical Center (LUMC), Leiden University; 2008. ISBN 978-90-9023381-9. Disponible en: https://openaccess.leidenuniv.nl/handle/1887/13118. Consultado 25-2-2018.
19. Chirdo FG, Rumbo M, Anon MC, et al. Presence of high levels of non-degraded gliadin in breast milk from healthy mothers. Scand J Gastroenterol 1998;33:1186-92. DOI: 10.1080/00365529850172557
20. Comino I, Real A, Vivas S, et al. Monitoring of gluten-free diet compliance in celiac patients by assessment of gliadin 33-mer equivalent epitopes in feces. Am J Clin Nutr 2012;95(3):670-7. DOI: 10.3945/ajcn.111.026708
21. Menard S, Lebreton C, Schumann M, et al. Paracellular versus transcellular intestinal permeability to gliadin peptides in active celiac disease. Am J Pathol 2012;180(2):608-15. DOI: 10.1016/j.ajpath.2011.10.019
22. Comino I, Fernández-Banares F, Esteve M, et al. Fecal gluten peptides reveal limitations of serological tests and food questionnaires for monitoring gluten-free diet in celiac disease patients. Am J Gastroenterol 2016;111(10):1456-65. DOI: 10.1038/ajg.2016.439
23. Moreno ML, Cebolla Á, Muñoz-Suano A, et al. Detection of gluten immunogenic peptides in the urine of patients with coeliac disease reveals transgressions in the gluten-free diet and incomplete mucosal healing. Gut 2017;66(2):250-7. DOI: 10.1136/gutjnl-2015-310148
24. Chirdo FG, Rumbo M, Añón MC, et al. Presence of high levels of non-degraded gliadin in breast milk from healthy mothers. Scand J Gastroenterol 1998;33:1186-92. DOI: 10.1080/00365529850172557
25. Trinidad Rodríguez J, Fernández Ballart G, Cucó Pastor E, et al. Validación de un cuestionario de frecuencia de consumo alimentario corto: reproducibilidad y validez. Nutr Hosp 2008;23(3):242-52.