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Hydrolysate of β-lactoglobulin by *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 656 suppresses the immunoreactivity of β-lactoglobulin as revealed by in vivo assays

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ABSTRACT

Cows' milk allergy, common in young infants, is often solved by the age of five; however, it can persist in some adults. The in vivo immunomodulatory potential of β-lactoglobulin (BLG) hydrolysates obtained with *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL656 (H656) was studied. Sera from mice gavaged with H656 showed lower specific BLG-IgE values than those of allergic mice. Secretion of IL-10, INF-γ and IL-6 was increased and that of IL-4 decreased when allergic spleen cells were stimulated with H656. IL-4 secretion was significantly reduced and concentrations of IL-10, IL-17A and IL-6 were increased when H656 was orally administrated. Highest expression of IL-4 and IL-12 was observed for allergic mice and H656 gavaged mice, respectively. H656 immunisation had a positive effect on intestinal mucosa. Overall, H656 displayed an immunomodulating effect, balancing the allergic Th2 response by stimulating Th1 type cytokine secretion. The hydrolysate might promote oral tolerance towards BLG in infant foods.

1. Introduction

Food allergy is an immune system reaction that occurs when eating certain foods. Even a low amount of the allergen can trigger signs and symptoms such as hives or swollen airways; moreover, food allergy can cause anaphylaxis in some people (Hochwallner, Schulmeister, Swoboda, Spitzauer, & Valenta, 2014). Food allergy affects 6–8 percent of children under three years of age and up to 3 percent of adults. On the other hand, food intolerance produces digestive problems by multiple pathways that can be attributed to enzyme defects and to irritant and toxic reactions (Osborn & Sinn, 2003).

Cows' milk is consumed worldwide especially by children; the use of milk formulae is commonly recommended for children who cannot be breast-fed (Hochwallner et al., 2017). One of the differences between human and cow milk is the presence of β-lactoglobulin (BLG) in the latter. This protein is relatively resistant to digestive enzymes; as a consequence different alternatives have been trialled to digest this protein and decrease its immune reaction. Although there are multiple infant formulae with different degrees of protein hydrolysis, not all of them can prevent allergy symptoms or induce tolerance to milk proteins (Hochwallner et al., 2017).

One of the alternatives to the known methods of producing hydrolysed milk formulae could be the use of the lactic acid bacteria (LAB) proteolytic system to hydrolyse BLG. Some genera of LAB and bifidobacteria have been investigated for their ability to alleviate asthma and food allergy symptoms (Liu et al., 2017). These bacteria are commonly used in the food industry and are generally accepted as safe. Previous results of our group showed that some LAB strains were able to degrade BLG, albeit to different extents (Pescuma, Hébert, Mozzi, & Font de Valdez, 2007, 2008, 2010; Pescuma et al., 2009, 2011, 2015). Furthermore, we demonstrated that *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 656 degrades BLG into middle size and small peptides with molecular mass ranging between 544.07 and 2895.67 as determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS; Pescuma et al., 2009). The peptides identified corresponded mainly to the carboxy-terminal part of BLG, although some peptides were detected for the middle and amino terminal side. Interestingly, this strain could hydrolyse BLG in its main epitopes (V41–K60; Y102–R124; L149–I162). Moreover, in vitro assays showed that *L. delbrueckii* subsp. *bulgaricus* CRL 656 BLG hydrolysates (H656) displayed lower (32%) human IgE binding capacity than those observed for native and denatured BLG (Pescuma et al., 2009). These results demonstrated that H656 could reduce BLG allergenicity by partial hydrolysis probably due to a combined effect of protein degradation and modifications by the bacterial proteinase (Bernasconi, Fritsché, & Corthésy, 2006).

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On the other hand, it has been claimed that partially hydrolysed infant formulae retain the IgE antibody epitopes and promote tolerogenic potential. This kind of formula has been recommended in infants with risk of allergy (Adel-Patient et al., 2012). Oral tolerance to food is an immune process that depends on several factors such as genetic background, age, environmental conditions and composition of the intestinal microbiota, as well as the route and frequency of antigen administration (Adel-Patient et al., 2011). In this work, we aimed to evaluate the immunomodulatory potential of BLG hydrolysate obtained with *L. delbrueckii* subsp. *bulgaricus* CRL 656 using allergic mice, and also to determine its tolerogenic effect.

2. Materials and methods

2.1. Preparation of BLG and BLG-hydrolysates

In this work the following were used: (i) heat treated BLG (80 °C, 30 min, ICN, Biolabs, Eschwege, Germany), (ii) the commercial BLG hydrolysate BioZate® (Davisco International Inc., Eden Prairie, MN, USA), and (iii) BLG hydrolysate obtained by *L. delbrueckii* subsp. *bulgaricus* CRL 656 (H656) as described previously (Pescuma et al., 2011) and subsequently lyophilised.

H656 was obtained by hydrolysis of BLG with non-proliferating cells of *L. delbrueckii* subsp. *bulgaricus* CRL 656. Briefly, *L. delbrueckii* subsp. *bulgaricus* CRL 656 was grown in a chemically defined medium (Hebert, Raya, & De Giori, 2000), containing (in grams per litre): glucose 10; KH_2PO_4, 3; K_2HPO_4, 3; sodium acetate, 5; $MgSO_4$ $7H_2O$, ammonium citrate dibasic 1.0; 0.2; L-alanine, 0.1; L-arginine, 0.1; L-asparagine, 0.2; L-aspartic acid, 0.2; L-cysteine, 0.2; L-glutamine, 0.2; L-glutamic acid, 0.2; glycine, 0.1; L-histidine, 0.1; L-isoleucine,0.1; L-leucine, 0.1; L-lysine, 0.1; L-methionine, 0.1; L-phenylalanine, 0.1; L-proline, 0.1; L-serine, 0.1; L-threonine, 0.1; L-tryptophan, 0.1; L-tyrosine, 0.1; L-valine, 0.1; uracil, 0.01; guanine, 0.01; adenine, 0.01; xanthine, 0.01; nicotinic acid, 0.001; calcium pantothenate, 0.001; pyridoxal, 0.002; riboflavin, 0.001; orotic acid, 0.05; folic acid, 0.001; vitamin B12, 0.001; thiamine, 0.001; biotin, 0.01; *p*-aminobenzoic acid, 0,01; Tween 80, 1.0. Cells were harvested by centrifugation (10,000 × *g*, 4 °C) at the exponential growth phase (OD_{560} = 0.65), washed three times with saline solution to eliminate the culture medium, concentrated in 100 mM sodium phosphate (pH 7.0) to a final OD_{560} of 10 and kept at 37 °C for 30 min for depletion of remaining medium components. BLG was dissolved in the same buffer at a concentration of 3 mg mL^{-1} and heat treated (80 °C, 30 min). Non-proliferating cells were incubated in a cell: protein ratio of 2:1 (v/v) for 24 h at 37 °C. After the incubation period samples were centrifuged (10,000 × *g*, 4 °C) and filtered (0.22 μm paper filters) to eliminate *L. delbrueckii subsp. bulgaricus* CRL 656 cells.

BLG and BioZate® were dissolved in the same buffer used to obtain H656 (sodium phosphate buffer, 100 mM, pH 7.0) while lyophilised H656 was dissolved in distilled water. Then, these proteins were sterilised by filtration with 0.22 μm paper filters (Sartorius, Stedim Biotech, Gottingen, Germany) to avoid contamination.

2.2. Mice

BALB/c mice (3–4 week-old females) coming from the second generation of mice fed with the milk protein-free diet AIN93-G (Dyets, Inc 2508 Easton Avenue Bethlehem, PA, USA) were housed in cages under autoclaved bedding and provided with sterile water.

All animal protocols were approved by the Animal Protection Committee of CERELA and followed the latest recommendations of the Federation of European Laboratory Animal Science Associations. All experiments comply with the current animal laws of Argentina.

2.3. Protein administration protocols

Three protein administration protocols modified from that of Adel-Patient et al. (2011) were applied (Fig. 1); in all cases, groups of seven female BALB/c mice were used. Protocol 1 was as follows: mice were administrated with sodium phosphate buffer (0.15 mL) or 2.4 mg of BLG, separately, on days 1, 2, 3, and 8, 9 and 10 by gavage (0.15 mL) using an animal feeding needle. All animals were further sensitised on day 14 by intraperitoneal injection of 5 μg of BLG using Alhydrogel (2%, w/v, InvivoGen, San Diego, CA, USA) as adjuvant, and sacrificed on day 31. Control mice were injected intraperitoneally with the adjuvant only. To test for a tolerogenic response to H656 and Biozate®, these protein hydrolysates instead of BLG were administrated orally using the same protocol as described before (Protocol 2, Fig. 1). A third protocol was used to study the effect of oral administration of BLG on elicitation of allergic reaction (Protocol 3, Fig. 1). In this last protocol, animals were submitted to the same treatment as described previously but received a second intraperitoneal BLG injection on day 28, were orally challenged with 10 mg of BLG in 0.25 mL phosphate buffer on day 35 and then immediately sacrificed (within 35 min). A group receiving only phosphate buffer by gavage, which was not sensitised with BLG, was used as control (naïve mice). Sacrificed mice were previously anaesthetised with an intraperitoneal injection of 3.0 mL (per kg body weight) of ketamine (10%, v/v): xylacin (2%, v/v) and bled by cardiac puncture. Blood was transferred into tubes without anticoagulant incubated at 37 °C for 1 h and centrifuged (1000 × *g* for 5 min); serum was removed and stored at −70 °C until use. Spleens and small intestines were removed under sterile conditions and pooled within groups.

2.4. Measurement of specific IgE-BLG binding capacity

Specific IgE-BLG binding activity was measured using an IgE mouse kit (Mouse IgE Ready-Set-Go, Affymetrix, eBioscience, Campus Vienna Biocenter 2, Vienna, Austria) with the following modifications: plates were coated with BLG (5 μg mL^{-1}) in coating buffer in the wells where sera were to be tested or coated with capture antibody for calibration curve and blanks. Plates were sealed and overnight incubated at 4 °C. Then, plates were washed, blocked with blocking buffer, and washed again as specified in the kit. One hundred microlitres of the assay buffer A and 100 μL of diluted (1/50) mice sera from each mice group were pooled and placed in the wells coated with BLG; for the calibration curve 100 μL of serial dilutions of the standard were placed in the corresponding wells, and 200 μL of the assay buffer A was placed in the blank wells. Plates were sealed and incubated overnight at 30 °C; then, they were washed and the procedure was continued as specified by the manufacturer. Three measurements of two independent assays for each mice group were done; mean values and standard deviations are shown (Table 1).

2.5. Cytokine determination using spleen cell culture

Spleens from Protocols 1 or 2 animals were pooled together within the same group, cut into small pieces using a scalpel, and passed through cellular sieves (BD Falcon, Bedford, MA, USA). The obtained cells were suspended in 7 mL RPMI-1640 medium (Sigma) supplemented with 10% (w/v) foetal calf serum, 2 mM L-glutamine, 100 U penicillin 100 mg^{-1} streptomycin, and 0.02 mM β-mercaptoethanol. Cells were centrifuged (1000 × *g*, 5 min) and erythrocytes were removed by adding 2 mL 0.87% (w/v) NH_4Cl at 37 °C for 10 min. Cells were washed and counted in Malassez chamber using 0.4% (v/v) Trypan blue. Flat-bottomed 24-well plates were loaded with 2 mL of a 10^6 cells mL^{-1} suspension. After the cells being fixed

overnight, the culture medium was replaced by fresh RPMI containing BLG, H656 or BioZate® (500 μg mL^{-1}) for cell re-stimulation; the same amount of phosphate buffer present in the samples was added in the control. To induce cell proliferation, 1 μg mL^{-1} concavalin A (Sigma) was added to all wells. Plates were incubated at 37 °C under 5% CO_2 for 72 h. Cell proliferation was measured using a MTT kit (Promega, Madison, USA) following the manufactures instructions. Cytokines (IL-4, IL-10, IL-6, IL-17A and INFγ) in cell supernatants were measured using a Becton Dickinson (BD) Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (BD, San Jose, CA, USA) using a (BD Accuri Flow Cytometer), and data were analysed using the software FCAP Array V1.0.1 (BD). Three measurements of two independent assays for each mice group and ex vivo stimulating condition were done; mean values and standard deviations are shown (Figs. 2 and 3).

2.6. Spleen cytokine expression by Real Time-PCR

Spleens from Protocol 3 mice groups were removed, separately, placed in RNAlater stabilisation solution according to manufacturer's instructions (Thermo Fisher Scientific, Argentina) and stored at −80 °C until use. Spleen tissues were homogenised in TRIzol LS reagent (Ambion, Life Technologies, Argentina) in a Mini-Bead Beater-8 cell disrupter (BioSpec Products Inc., Bartlsville, OK, USA) at maximum speed with 5 cycles of 40 s each, with intervals of 1 min on ice among cycles, and total RNA was extracted according to manufacturer's instructions. RNA concentration was measured using a Qubit® 2.0 fluorometer (Invitrogen™, Life Technologies Co., Carlsbad, CA, USA) using Qubit® HS RNA Assay Kit (Molecular Probes™, Life Technologies Co.). cDNA synthesis was performed using Superscript III kit (Invitrogen). Real Time-PCR was performed in an iQ™5 Multicolour Real-Time PCR Detection System iCycler (Bio-Rad Laboratories Inc., Hercules, CA, USA); gene expression of mouse IL-10, IL-4, IL-12, TNF-α, INF-γ, and actin were examined using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc.). PCR amplification was performed in duplicate and water was used to replace cDNA in each run as negative control. The amplification program consisted in 3 amplification steps plus a melting curve as follows: 95 °C for 4 min, amplification of 40 cycles at 95 °C for 15 s, 50 °C for 30 s and 72 °C 30 s, followed by 1 cycle of 95 °C for 1 min, 55 °C for 1 min and 55 °C- 95 °C with 0.5 °C change in temperature.

The primers used for each cytokine were the following: IL-10 (forward 5′-CGG GAA GAC AAT AAC TG-3′; reverse 5′CAT TTC CGA TAA GGC TTG G-3′), IL-4 (forward 5′-TCG GCA TTT TGA ACG AGG TC-3′; reverse 5′-GAA AAG CCC GAA AGA GTC TC-3′), IL-12 (forward 5′-CGT GCT CAT GGC TGG TGC AAA G-3′; reverse 5′-CTT CAT CTG CAA GTT CTT GGG C-3′), TNF-α (forward 5′-ATG AGC ACA GAA AGC ATG ATC-′3; reverse 5′-TAC AGG CTT GTC ACT CGA ATT-3′) and INF-γ (forward 5′-AAC GCT ACA CAC TGC ATC TTG G-3′; reverse 5′-GAC TTC AAA GAG TCT GAG G-3′). The primers for the normalising gene β-actin were: forward 5′-CGTGAAAAGATGACCCAGATCA-3′; reverse 5′-CACAGCCTGGATGGCTACGTA-3′). Two independent qPCR assays were performed for each condition. The relative expression of the cytokine genes in different conditions were estimated according to the $2^{-\Delta\Delta CT}$ method (Schmittgen & Livak, 2008). Mice not exposed to BLG were used as control. Values reported are the fold changes between each condition and the control and were normalised against the β−actin gene expression.

2.7. Intestine histology

The small intestine of mice belonging to all groups was removed and washed with saline solution (0.15 M NaCl) and placed in formaldehyde 10% (v/v). Tissues were prepared for histological evaluation; serial paraffin sections of 4 mm were made and stained with haematoxylin−eosin for microscopy examination.

2.8. Statistics

All data are expressed as mean values and standard deviations and they were analysed by one-way analysis of variance (ANOVA) and Tukey's post comparison test using MINITAB 16 Statistical Software (Minitab, State College, PA, USA). The experiment was repeated twice with 7 animals per sample in each trial. Differences were accepted as significant when $p < 0.05$.

3. Results

3.1. Specific IgE-BLG binding capacity

The specific IgE-BLG-values for the allergic group (those receiving only buffer by gavage; Protocol 1, Fig. 1) after one intraperitoneal BLG injection (day 14) were 17.4 and 7.4 times higher than those of mice immunised with H656 and BioZate® (Protocol 2), respectively; as expected, no specific IgE-BLG binding activity was detected in non-sensitised mice and low binding values were obtained for the tolerant mice (3.24 and 15.98 ng mL^{-1} for Protocols 1 and 3, respectively) (Table 1). After the second BLG intraperitoneal injection on day 28 and oral challenge (Protocol 3), the IgE-BLG values increased in all tested groups although to different extents, suggesting an increase in the allergenic response after the second challenge with BLG. Interestingly, a lower value of IgE-BLG (90.21 ± 0.15 ng mL^{-1}) was detected when administering H656 orally as compared with BioZate® (158.83 ± 3.47 ng mL^{-1}).

3.2. Cytokine production by splenocyte cells in vitro

3.2.1. Effect of re-stimulation of allergic mice with BLG, H656 and BioZate®

Production of cytokines by mice splenocytes was analysed for control mice (receiving buffer by gavage and one intraperitoneal injection of adjuvant) and those receiving buffer or BLG orally and one intraperitoneal injection of BLG (allergic and tolerant groups, respectively; Fig. 1, Protocol 1). The spleen cells collected were further re-stimulated in vitro with BLG, H656, and BioZate®. Cytokyne production by spleens belonging to different groups is shown in Fig. 2. BLG re-stimulated cells of the allergic group displayed a Th2 profile showing increased IL-4 and IL-10 concentrations values (37.13 and 248.12 pg mL^{-1}, respectively) and lower concentrations of INF-γ (1391.91 pg mL^{-1}) respect to the tolerant group (18.36, 50.37 and 1950.97 pg mL^{-1}, respectively; for BLG ex vivo reactivation; Fig. 2). The IL-17A secretion was higher for the allergic group independently of the type of reactivation applied, while IL-6 secretion by splenocyte cells from all assayed groups was higher when cells were reactivated using H656 (Fig. 2).

When stimulating mice spleen cells with H656, higher concentrations of IL-4 were observed for the control and allergic groups (13.07 and 15.15 pg mL^{-1}, respectively) with respect to tolerant mice (3.54 pg mL^{-1}) (Fig. 2a), although these values were much lower than those when BLG was used (35.33, 37.13 and 18.36 pg mL^{-1} for the control, allergic and tolerant groups, respectively; Fig. 2a). On the contrary, IL-4 values of the allergic group incubated with BioZate® were similar to those when BLG was used for re-stimulation (Fig. 2a). When cells were re-stimulated with H656, INF-γ secretion was much higher in the allergic and control groups (5275.58 and 4413.54 pg mL^{-1}, respectively) than in the tolerant group (2595.38 pg mL^{-1}) (Fig. 2c). Furthermore, in the presence of H656, an increase in the secretion of IL-6, was observed with respect

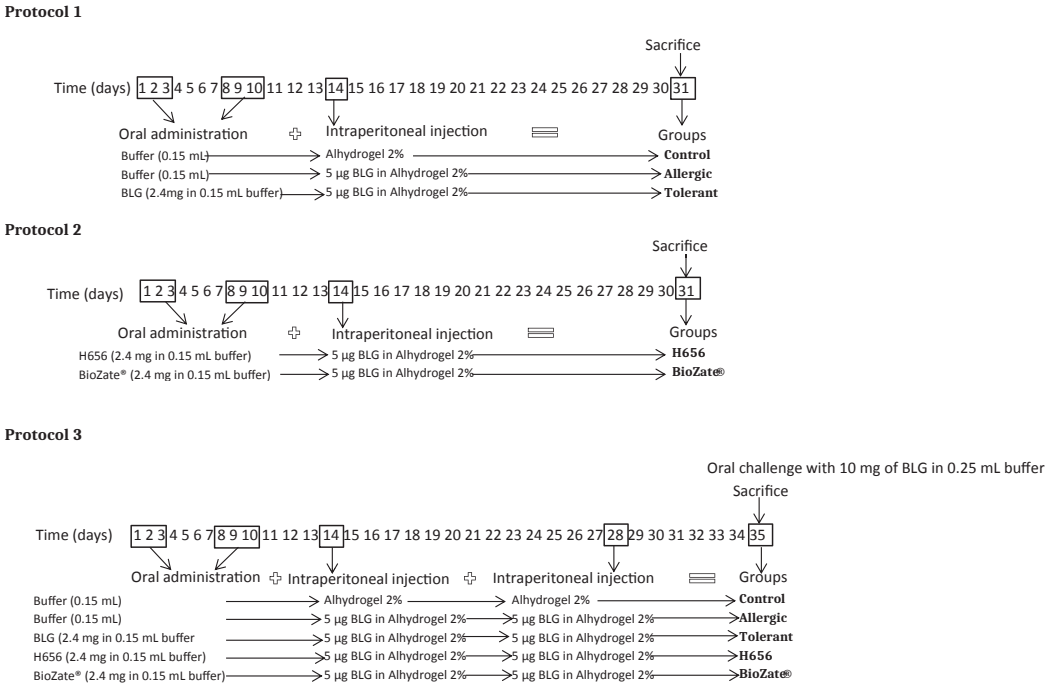

Fig. 1. Mice protein administration protocols.

to incubation with BLG (IL-6, 81.30–91.92%) or BioZate® (IL-6, 86.35–95.50%) for all groups (Fig. 2e).

3.2.2. Tolerogenic effect of hydrolysates by oral administration

The ability of hydrolysates H656 and BioZate® to induce oral tolerance to BLG was analysed by administrating the hydrolysates to mice by gavage (Protocol 2, Fig. 1). When H656 was administrated orally, IL-4 secretion significantly diminished (98.0–97.7%) when incubating with BLG or BioZate® (Fig. 3a) while increased concentrations values of IL-10 and IL-17A (Fig. 3b,d) were observed when compared with those obtained when BioZate® was orally administrated instead (80.02 and 80.85, respectively; Fig. 3). Re-stimulation of the H656 and BioZate® groups with H656 resulted in an increased concentration of IL-6 compared with re-stimulation with BLG or BioZate®. Moreover, IL-10, IL-17A, IL-6 values were higher ($p = 0.004$, $p = 0.017$, $p = 0.001$, respectively) than those obtained for the allergic group (62.6, 76.9 and 58.1%, respectively; Fig. 2) with BLG re-stimulation. Although, when stimulating cells with BLG, no significant differences were detected for INF-γ secretion between BioZate® (2653.35 pg mL^{-1}) and H656 (3004.23 pg mL^{-1}) groups (Fig. 3c), concentrations of this cytokine were higher than for the allergic group (1391.91 pg mL^{-1}; $p = 0.005$) (Figs. 2 and 3). When BioZate® was given by gavage and cells were re-stimulated with BLG; IL-4, IL-10, IL-17A and IL-6 secretion was similar to the allergic group.

Table 1
Specific IgE-BLG values in treated and non-treated mice sera.[a]

Groups	IgE (ng mL^{-1})	
	Intraperitoneal injection	Oral challenge
Control	ND	ND
Adjuvant	ND	ND
Allergic	447.17 ± 6.04^{d}	724.39 ± 63.85^{c}
Tolerant	3.24 ± 0.2^{a}	15.48 ± 1.74^{a}
H656	25.81 ± 1.67^{b}	90.21 ± 0.15^{a,b}
BioZate®	60.62 ± 0.94^{c}	158.83 ± 3.47^{b}

[a] Mice in the control, adjuvant and allergic groups were gavaged with buffer (0.15 mL), mice in the tolerant group were gavaged with 2.4 mg BLG in 0.15 mL buffer, those in the H656 group were gavaged with 2.4 mg of BLG hydrolysates obtained with *L. delbrueckii* subsp. *bulgaricus* CRL 656 in 0.15 mL buffer and those in the BioZate® group were gavaged with 2.4 mg of BioZate in 0.15 mL buffer. All mice were intraperitoneally injected with 5 μg of BLG except for the adjuvant (2% alhydrogel) and control groups. Oral challenge was done by administration of 10 mg of BLG (Fig. 1). Different superscript letters represent significant differences between groups ($p < 0.05$).

3.3. Cytokine gene expression in treated spleen mice determined by RT-PCR

After the second BLG intraperitoneal injection and oral challenge (Protocol 3, Fig. 1), the cytokine gene expression of spleen cells was determined (Fig. 4). Consistent with the results obtained for cytokine production in cell cultures, RT-PCR also showed the highest expression of IL-4 (2.5–3.7 times) in the allergic group (significant differences among cytokines between mice groups are shown in Fig. 4) as compared with the other groups; no differences on this interleukin expression was observed among the remaining groups. When BioZate® was administrated orally, the highest expression (1.8–2.0×) of the modulating cytokine IL-10 was detected. However, when H656 was used, an increase in IL-12 (1.6–2.7 times) was observed compared with the allergic, tolerant and BioZate® groups. Values of mRNA expression correspond to fold-changes between each group and those obtained for naïve mice.

3.4. Intestine histology

The small intestine of allergic mice showed severe alterations in the gut mucosa and leukocyte infiltration (Fig. 5), being these alterations diminished in tolerant (BLG orally administrated) mice for which intestinal sections showed almost the same morphology to the ones of naïve mice. H656 treated (gavaged) mice showed no leukocyte infiltration while alterations in gut mucosa were detected

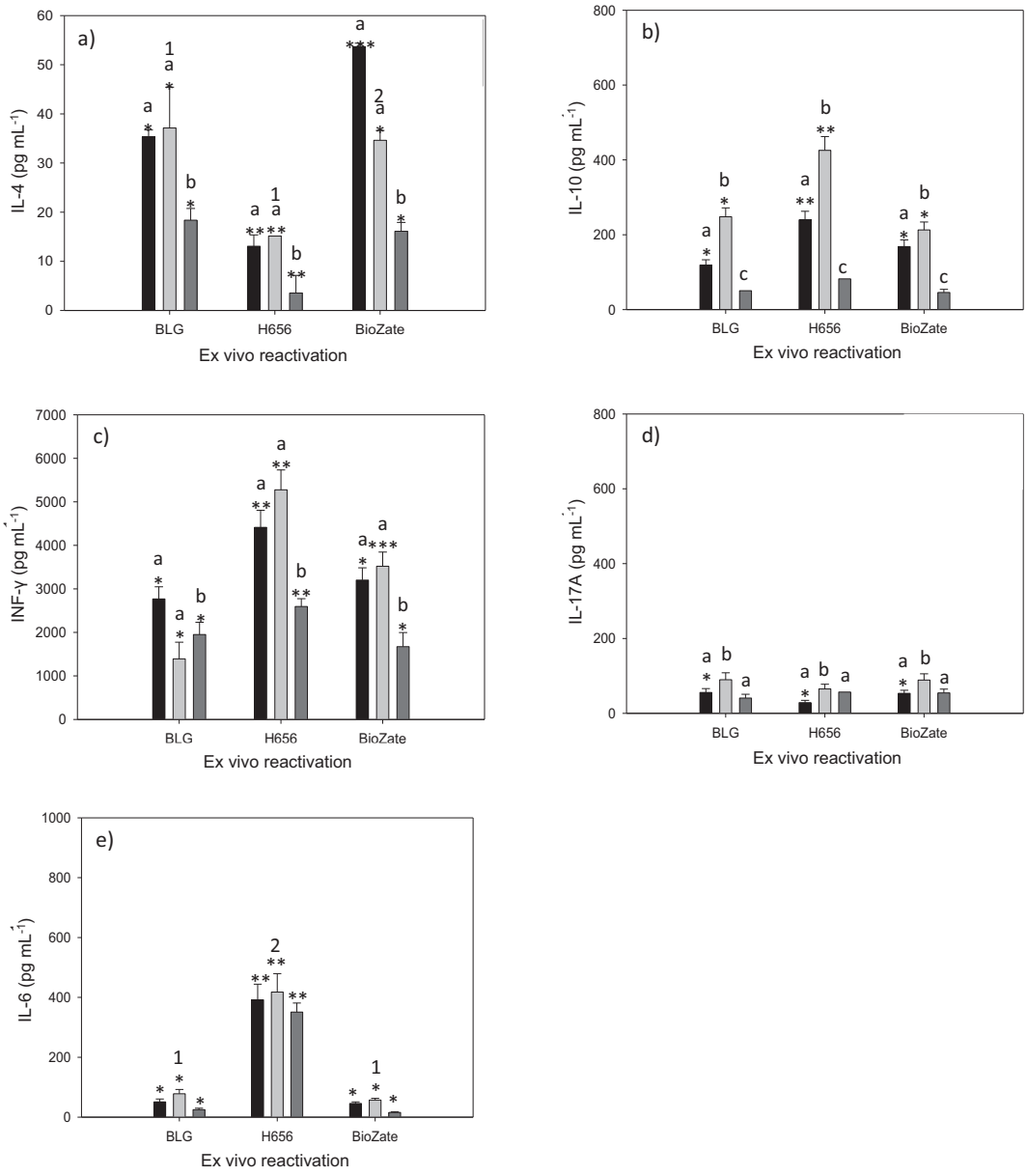

Fig. 2. Cytokine secretion by pooled splenocytes reactivated with BLG, H656 or BioZate®. Splenocytes were isolated from spleens of naïve mice (control, ■), gavaged with buffer and sensitized with BLG (allergic, ■), or gavaged with BLG and sensitized with BLG (tolerant, ■). Different letters in graphs represent significant differences among mice groups (control, allergic and tolerant); numbers represent significant differences among in vivo reactivation treatments (BLG, H656 and BioZate). Finally, asterisks indicate significant differences between in vivo reactivations within the same group.

for the BioZate® group although leukocyte infiltration was lower than in the allergic group.

4. Discussion

Cows' milk allergy mainly affects children younger than 3 years old and although a natural tolerance develops until the age of 5, approximately 1% of adults remain cows' milk allergic usually associated with severe reactions (Schocker, Recke, Kull, Worm, & Jappe, 2017). Some studies suggest an association with respiratory allergy and asthma in adulthood (Schouten et al., 2008). To date, different strategies to solve or alleviate milk allergy have been applied by using probiotics, prebiotics, symbiotics, and protein hydrolysates (Jacquot, Gauthier, Drouin, & Boutin, 2010; Kostadinova et al., 2017; Schouten et al., 2009).

Probiotic bacteria can influence the immune reaction towards allergens; prevention of allergy symptoms may be due to different factors. It has been claimed that probiotics may modulate the host immune response by balancing the Th1/Th2/Th17 profile (Maiga et al., 2017), while commercial milk protein hydrolysates alone or with the addition of probiotics (*Lactobacillus rhamnosus* GG) reduced intestinal permeability and induced oral tolerance preventing allergic symptoms by immunomodulation (Aitoro et al., 2016). In addition, the proteolytic activity of lactobacilli can have a positive effect on milk protein digestibility as some *Lactobacillus* strains can produce bioactive peptides with immune-modulating, antimicrobial, and antihypertensive effects by hydrolysis of food proteins (Bu, Luo, Chen, Liu, & Zhu, 2013). Thus, the production of Th1 and Th2 at systemic level can be enhanced by the consumption of lactic fermented food products. Previously, we found that

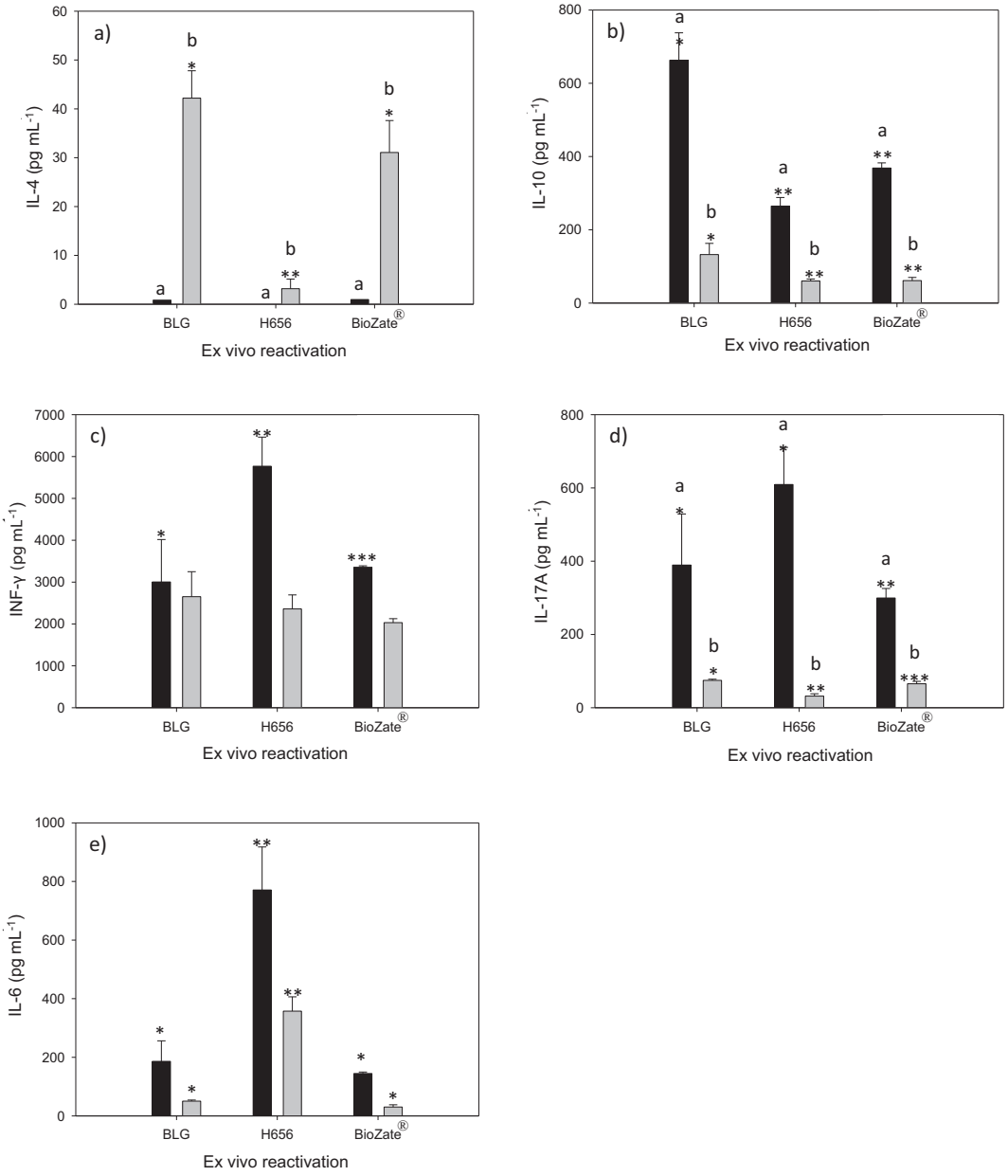

Fig. 3. Cytokine secretion by pooled splenocytes reactivated with BLG, H656 or BioZate®. Mice were previously gavaged with H656 or BioZate® and sensitized with BLG. Different letters in graphs represent significant differences among mice groups (■, control; ■, allergic; ■, tolerant); numbers represent significant differences among in vivo reactivation treatments (BLG, H656 and BioZate). Finally, asterisks indicate significant differences between in vivo reactivations within the same group.

L. delbrueckii subsp. *bulgaricus* CRL 656 could cleave allergenic peptides of BLG and the resulting hydrolysate (H656) was less immuno-reactive than native BLG as revealed by in vitro assays (Pescuma et al., 2009, 2011).

In this work, the potential immunomodulatory and tolerogenic effects of H656 using an allergic mice model was evaluated. The results show that when H656 was administrated orally, the BLG-IgE binding values were considerably lower than those obtained for the allergic group. Similar results were noted by Adele-Patient et al. (2012) for BLG-IgE production when administrating orally cyanogen bromide BLG hydrolysates using a similar murine model. While Prioult, Pecquet, and Fliss (2004, 2005) observed that only the acidic peptide fractions obtained by hydrolysis of BLG by trypsin and the protease of *Bifidobacterium lactis* NCC362 had a reduced IgE binding capacity compared with native BLG, no differences on the IgE binding capacity of commercial fermented milk products compared with native BLG were noted by Ehn, Allmere, Telemo, Bengtsson, and Ekstrand (2005).

It has been reported that for IgE expression and Th2 lymphocyte profile establishment, the production of IL-4 by T cells is required (Chapoval, Dasgupta, Dorsey, & Keegan, 2010). Indeed, the potential allergenicity of a peptide can be indirectly measured through its ability to stimulate IL-4 production by B-cells. Likewise, the high concentration of BLG-IgE in the allergic (buffer gavage) group was in agreement with the expression of the IL-4 gene as determined by RT-PCR and the obtained IL-4 values when BLG re-stimulation of

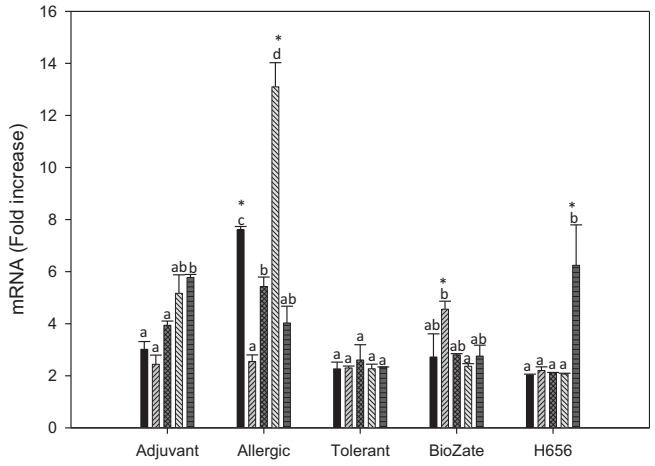

Fig. 4. . Expression of IL-4 (■), IL-10 (▨), INF-γ (▩), TNF-α (▧) and IL-12 (▤) genes by spleen cells from treated mice determined by RT-PCR. Groups of spleens from mice gavaged with buffer and intraperitoneally injected with adjuvant (Adjuvant) and mice gavaged with buffer, BLG, BioZate® and H656 and intraperitoneally injected with BLG were named Allergic, Tolerant, BioZate®, and H656, respectively. Letters indicate significant differences among cytokines within each group. Asterisks indicate significant differences among mice groups for respective cytokines.

spleen cells occurred. In contrast, when the spleen cells of the allergic group were incubated with H656, a significantly lower IL-4 and higher IL-10 and INF-γ secretion respect to BLG incubation was observed. Consistently, the acidic peptides obtained by BLG hydrolysate by trypsin and a protease of a *Bifidobacterium animalis* or *Lactobacillus paracasei* NCC2461 stimulated IL-10 and INF-γ production by spleen cells from BLG-primed mice (Prioult et al., 2004, 2005). However, we observed that when re-stimulating allergic mice spleen cells with H656 an increase in IL-6 release together with a decrease in IL-4 production was observed with respect to stimulation with BLG or BioZate®. In this respect, it has been claimed that IL-6 release can restore Th17 homeostasis in food allergic patients (Johnston, Chien, & Bryce, 2014). Interestingly, in the current study IL-4, IL-10 and IL-6 secretion was different depending on the hydrolysate used (H656 or BioZate®). Hochwallner et al. (2017) reported that different hydrolysed milk formulae differed in their capacity to prevent allergy symptoms and induce pro-inflammatory responses due to diverse peptide composition.

Furthermore, the sensitising capacity of BLG could be reduced by co-administrating this protein with its digests, while no effect on oral tolerance was observed for complete BLG digests (Bøgh, Barkholt, & Madsen, 2013). Adel-Patient et al. (2012) reported that synthetic peptides had different abilities to induce oral tolerance towards BLG; peptides containing intact S-S bridges showed the highest immune modulating potential. In our work, we observed that H656 displayed higher capacity to induce oral tolerance than the commercial BioZate® when administrating both by gavage and stimulating the cells with BLG as a decrease in IL-4 and an increase in IL-10 and IL-17A concentrations were observed (Fig. 3). In support, Frossard, Zimmerli, Rincon Garriz, and Eigenmann (2016) reported that IL-17A secretion is lower in antigen-activated cells from tolerant patients than from allergic ones.

Finally, an oral challenge to BLG was applied to all mice after a second BLG peritoneal injection to analyse cytokine expression and intestine alterations. Results showed an over-expression of IL-4 in the allergic group. Interestingly, when BioZate® was used to immunise the mice, a higher expression of the modulating cytokine IL-10 was observed, while when H656 was orally administrated, higher expression of the IL-12 gene was detected. This latter cytokine regulates Th1 cell differentiation and suppresses Th2 cell proliferation (Berin & Sampson, 2013; Chung, 2001). On the whole, our results suggest that the BLG hydrolysate obtained by *L. delbrueckii* subsp. *bulgaricus* CRL 656 possesses lower immune reactivity than the native protein and that oral administration of H656 may induce oral tolerance to BLG. Due to the fact that BLG is not completely digested, the peptides released by this strain may be

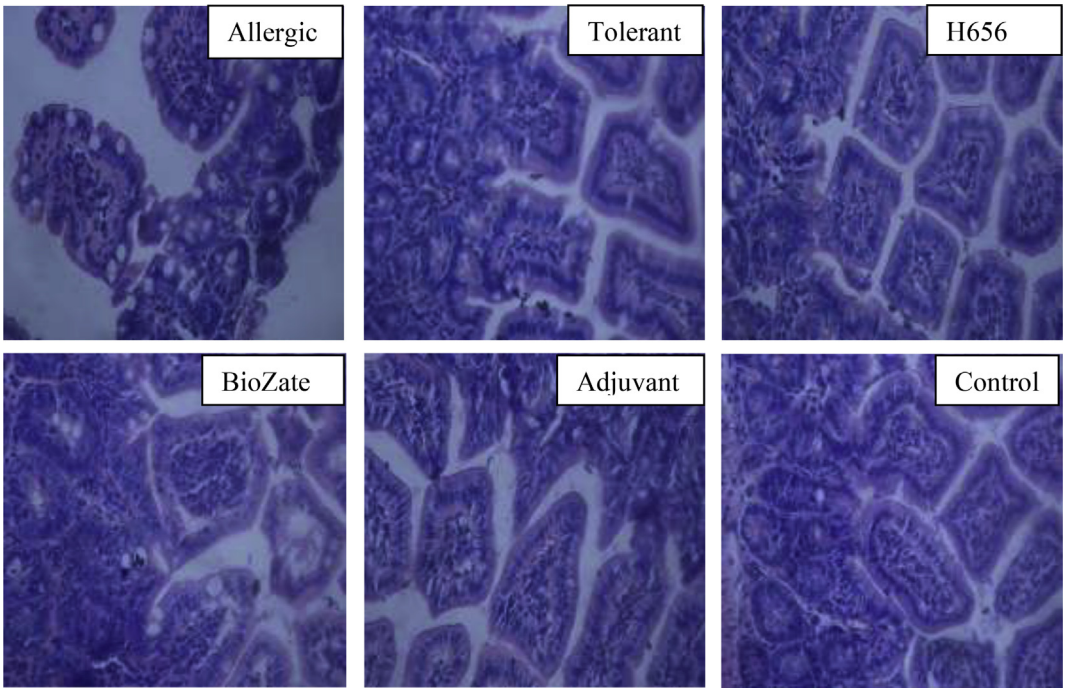

Fig. 5. Histological study of small intestine slices stained with haematoxylin-eosin.

responsible for the immune modulation observed. In support, Bu, Luo, Zhang, and Chen (2010) reported that milk fermentation by LAB could reduce the antigenicity of BLG in skim milk in vitro although proteolysis of BLG was limited.

5. Conclusions

The BLG hydrolysate obtained with *L. delbrueckii* subsp. *bulgaricus* CRL 656 may immunomodulate the allergenic symptoms towards BLG balancing the Th1/Th2/Th17 response by increasing mainly IL-6, IL-10 and INFγ secretion.

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