Accepted Manuscript

Lactobacilli hydrolysis of cows' milk proteins abrogates their humoral immunoreactivity in patients with immune-mediated diseases

Goran Vukotic, Ivana Matic, Jelena Begovic, Irina Besu, Milan Kojic, Jelena Djokic, Zorica Juranic, Ivana Strahinic

PII: S0958-6946(16)30230-8

DOI: 10.1016/j.idairyj.2016.07.009

Reference: INDA 4051

To appear in: *International Dairy Journal*

Received Date: 30 October 2015

Revised Date: 18 July 2016

Accepted Date: 21 July 2016

Please cite this article as: Vukotic, G., Matic, I., Begovic, J., Besu, I., Kojic, M., Djokic, J., Juranic, Z., Strahinic, I., Lactobacilli hydrolysis of cows' milk proteins abrogates their humoral immunoreactivity in patients with immune-mediated diseases, *International Dairy Journal* (2016), doi: 10.1016/j.idairyj.2016.07.009.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Lactobacilli hydrolysis of cows' milk proteins abrogates their humoral immunoreactivity in patients with immune-mediated diseases

Goran Vukotic [a,b,†], Ivana Matic [c,†], Jelena Begovic [a], Irina Besu [b], Milan Kojic [a], Jelena Djokic [a], Zorica Juranic [c], Ivana Strahinic [a,*]

[a] *Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444a, Belgrade, Serbia*

[b] *Faculty of Biology, University of Belgrade, Studentski trg 16, Belgrade, Serbia*

[c] *Institute of Oncology and Radiology of Serbia, Pasterova 14, Belgrade, Serbia*

*Corresponding author. Tel.:

E-mail address: strahi@imgge.bg.ac.rs (I. Strahinic)

[†] These authors contributed equally to this work.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

ABSTRACT

The level of humoral immunoreactivity to total cows' milk proteins (TCMP) in sera of patients suffering from recurrent oral ulcerations, gastrointestinal diseases or haematological malignancies was investigated. TCMP were also hydrolysed with two different species of lactobacilli and dramatic changes in the levels of specific IgG and IgE were found with statistically significant decreases in the levels of specific antibodies in sera from all patient groups. The levels below cut-off values of IgG specific for TCMP hydrolysates were detected in sera from all patients, while values of IgE for hydrolysates obtained with *Lactobacillus helveticus* BGRA43 and *Lactobacillus zeae* LMG17315were below cut-off in 85% and 97% of patients, respectively. Competitive ELISA confirmed the specificity of antibodies for immunogenic TCMP epitopes, demonstrating that lactobacilli hydrolyse TCMP by degrading immunogenic epitopes, and could therefore be used in processing of milk proteins to obtain products suitable for patients with altered immune response on TCMP.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

1. Introduction

Lactobacilli are a major and diverse group of lactic acid bacteria, irreplaceable in the production of an array of fermented milk products that have been manufactured for centuries worldwide. Numerous studies have been conducted to understand the processes underlying bacterial fermentation of milk and to ascertain the ways and mechanisms by which lactobacilli can contribute to human health. It has been determined that these bacteria can promote human health through various mechanisms, such as modulation of immune system, affecting pathogen exclusion, or influencing different microbial/host products (Oelschlaeger, 2010).

One of the most important features of lactobacilli used in dairy industry is their proteolytic system, responsible for digestion and utilisation of milk proteins. Bacterial proteolysis of milk proteins may lead to generation of various bioactive peptides with immunomodulatory, antihypertensive, antioxidative, antimicrobial and other health-promoting properties (Hayes, Stanton, Fitzgerald, & Ross, 2007; Wakai & Yamamoto, 2012). In addition, it was recently discovered that proteinases of some probiotic lactobacilli have anti-inflammatory activity (Hörmannsperger, von Schillde, & Haller, 2013; von Schillde et al., 2012). Positive clinical effects of lactobacilli supplementation were shown in patients with allergic and inflammatory skin disorders (Hacini-Rachinel et al., 2009), different gastrointestinal disorders (Verna & Lucak, 2010), chronic inflammation-associated sickness behaviour (D'Mello et al., 2015), and other inflammation-related diseases. These findings put lactobacilli in the centre of future development of therapies for numerous inflammation-related diseases.

One of the immune-related diseases that significantly affects the quality of life of people from both developed and developing countries is food allergies, with prevalence of 5% in adults

ACCEPTED MANUSCRIPT

and 8% in preschool children (Sicherer & Sampson, 2014). Considering that milk is one of the most exploited and consumed food products throughout the world, frequency and severity of milk allergies are medical issues of great importance (Schreier & Wright, 2014; Warren, Jhaveri, Warrier, Smith, & Gupta, 2013). While various milk processing technologies have been examined for reducing the allergenicity of different cows' milk proteins (CMP) (Ehn, Ekstrand, Bengtsson, & Ahlstedt, 2004; Peyron, Mouécoucou, Frémont, Sanchez, & Gontard, 2006), several studies have demonstrated favourable effects of milk fermentation by different strains of lactic acid bacteria (Bu, Luo, Zhang, & Chen, 2010; El-Ghaish et al., 2011; Kleber, Weyrich, & Hinrichs, 2006; Pescuma et al., 2015; Shi et al., 2014).

Alongside patients with diagnosed milk allergies, it has been shown that patients suffering from gastrointestinal disorders can possess excessive immunoreactivity towards cows' milk proteins, as well as to other food proteins, like gliadin, a main component of gluten (Besu et al., 2009). Similar results were obtained for patients with multiple myelomas and non-Hodgkin lymphomas (Juranic et al., 2008, 2009). Moreover, this reactivity can play a role in the aetiology of recurrent aphthous ulcerations, one of the most common but poorly understood oral diseases. The cows' milk free diet in these patients resulted in decrease in the levels of antibodies specific for CMP and remission of clinical symptoms (Besu et al., 2009, 2013). In addition, CMP-free diet in one patient with monoclonal gammopathy (immunological disorder that could be the precursor of multiple myeloma, or lymphoma) led to disappearance of the "M" component (monoclonal IgG (λ) immunoglobulins). This was associated with the drop-off in the levels of anti-CMP IgG (from 892 AU mL^{-1} to 0 AU mL^{-1}) and anti-CMP IgA antibodies (from 240 AU mL^{-1} to 0 AU mL^{-1}), as reported by Juranic et al. (2009). These results indicate the potential

benefit of using non-immunogenic products consisting of hydrolysed CMP in nutrition of these patients.

Since there is no consensus about the impact of an individual milk protein or a particular structure to development of humoral immune response against CMP (Gaudin et al., 2008; Pelaez-Lorenzo, Diez-Masa, Vasallo, & de Frutos, 2010; Wal, 2004), it is prudent to consider that all milk proteins may contribute to immune response. Considering that our previous results pointed to possible association of humoral immune response against CMP with different non-allergic, immune-related diseases, the aim of this study was to investigate whether lactobacilli hydrolysis of total cows' milk proteins (TCMP) can abrogate elevated humoral immunity to TCMP in these patients. For this purpose, sera from patients suffering from recurrent oral ulcerations, different gastrointestinal disorders and haematological malignancies were tested for changes in TCMP-immunoreactivity after treatment of cows' milk with preselected strains of lactobacilli. Considering that substrate specificity of proteinases differs greatly among lactobacilli species, ten different potential probiotic strains were selected for analysis of their proteolytic activity. Two strains that showed the best proteolytic potential, *Lactobacillus helveticus* BGRA43 and *Lactobacillus zeae* LMG17315, were further used for TCMP hydrolysis.

2. Methods

2.1. Patients

Sera were collected from three groups of patients (20 in each group) as well as from healthy volunteers. The first group of 20 patients had recurrent oral ulcerations (ROU), the

ACCEPTED MANUSCRIPT

second group of 20 patients had different gastrointestinal disorders [GD; 12 patients with coeliac disease, 4 patients with ulcerative colitis, 2 patients with inflammatory bowel disease (IBD) and 2 patients with Chron's disease], and the third group of 20 patients had different haematological malignancies (HM) before oncological therapy [11 patients with non-Hodgkin lymphoma (NHL), 4 patients with multiple myeloma and 5 patients with plasmacytoma]. The control group for determining reference cut-off values for bacterial antigens consisted of 33 healthy control subjects.

To confirm elevated humoral immunity against TCMP, the levels of IgG and IgE antibodies specific for TCMP were tested in sera of patients from all groups, as both isotypes of antibodies have been shown to play function in anaphylactic reaction (Johnston, Chien, & Bryce, 2014). The reference cut-off values for anti-TCMP IgG (39 AU mL^{-1}) and anti-TCMP IgE antibodies (10 AU mL^{-1}) were determined by analysing sera samples of 50 healthy control subjects (Besu et al., 2009; Juranic et al., 2009). Analyses of humoral immunity to cows' milk proteins in patients' sera were approved by the Ethics Committee of the Institute of Oncology and Radiology of Serbia and Ethics Committee of the Clinical Centre of Serbia. Written informed consent was obtained from each patient.

2.2. Bacterial strains, media and culture conditions

Six different lactobacilli strains (*Lb. helveticus* BGRA43, *Lactobacillus rhamnosus* BGT10, *Lactobacillus plantarum* BGPV2-45a, *Lb. plantarum* BGBUK2-5, *Lb. plantarum* BGGA-8, *Lb. plantarum* BGHO10) from our laboratory collection (Laboratory for Molecular Microbiology, Institute of Molecular Genetics and Genetic Engineering, University of Belgrade),

as well as four different lactobacilli strains from international collections (*Lactobacillus casei* ATCC393 from American Type Culture Collection and *Lb. zeae* LMG17315, *Lb. plantarum* LMG9208 and *Lb. plantarum* LMG18024 from Belgian Co-ordinated Collections of Micro-organisms) were screened for proteolytic activity against TCMP. Bacterial cells were generally grown and maintained in MRS broth (Merck, GmbH, Darmstadt, Germany) at 30 °C. *Lb. helveticus* BGRA43 was grown and maintained under anaerobic conditions in anaerobic jars using Anaerocult A (Merck) at 37 °C. Stocks of cultures were kept at -80 °C in MRS broth containing 15% (v/v) glycerol. Agar plates were prepared by adding 1.5% (w/v) agar (Torlak, Belgrade, Serbia) to MRS broth. For the test of proteolytic activity, cells were grown at appropriate temperature on milk-citrate agar (MCA) plates with 4.4% reconstituted non-fat skim milk, 0.5% glucose, 0.8% trisodium citrate, 0.1% yeast extract and 1.5% agar (w/v).

2.3. Proteinase activity assay

Proteolytic activity of the *Lactobacillus* strains was assayed as described previously (Kojic, Fira, Banina, & Topisirovic, 1991), with the following modification: as a substrate in reactions, milk powder (Mlekara Subotica AD, Subotica, Serbia) was used. Milk powder was dissolved in 100 mM sodium phosphate buffer at concentration of 20 mg mL^{-1} (w/v), which resulted in a final protein concentration of 5 mg mL^{-1}. The cell suspension dissolved in the same buffer and containing 10^9 cells mL^{-1} was mixed with milk powder solution at a 1:1 (v/v) ratio and incubated for 3 h at 37 °C. Controls for bacterial antigens (RA and Z antigen solutions) were obtained by incubating cell suspensions for 3 h at 37 °C, pelleting the cells and collecting the

supernatants. Quantification of protein bands on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was done using ImageJ.

2.4. Determination of the levels of IgG and IgE specific for undigested TCMP and for two TCMP hydrolysates in patients' sera by enzyme-linked immunosorbent assay

Determinations of the levels of IgG and IgE specific for undigested TCMP and two TCMP hydrolysates (TCMP-RA and TCMP-Z obtained after digestion with *Lb. helveticus* BGRA43 and *Lb. zeae* LMG17315, respectively) were done by enzyme-linked immunosorbent assay (ELISA) tests, as described previously (Besu et al., 2009; Juranic et al., 2009). In addition, levels of antibodies to bacterial antigens possibly present in the working solutions (RA and Z antigen solutions) were also determined.

Briefly, 100 µL of antigen solutions (TCMP, TCMP-RA hydrolysate, TCMP-Z hydrolysate, RA and Z antigen solutions) in bicarbonate buffer, pH 9.5, were added to polystyrene 96-well microtitre ELISA plates at a concentration of 10 µg mL^{-1} (F96 MaxiSorp Nunc-Immuno Plate, Waltham, MA, USA). Wells were incubated at 4 °C for 24 h enabling antigens to bind to polystyrene surface. The next day coated wells were washed and free sites on the polystyrene surface were blocked with 1% bovine serum albumin (BSA; Sigma Aldrich, St Louis, MO, USA) by incubating for 60 min at room temperature. After washing and aspiration, 50 µL of sera samples (dilution 1/100) were added into appropriate wells and incubated for 60 min at room temperature with subsequent aspiration and washing. Horseradish peroxidase-labelled secondary antibodies were used for detection of primary antibodies bound to antigen [sheep anti-human IgG (binding site) and goat anti-human IgE (Sigma Aldrich)]. Appropriate

wells were filled with 50 µL of secondary antibody solution and plates were incubated for 60 min at room temperature and then washed five times. One hundred microlitres of substrate solution 3,3',5,5'-tetramethylbenzidine (TMB; INEP, Zemun, Serbia) was added to the wells and after short incubation (5–10 min), the enzyme reaction was terminated by addition of sulphuric acid (50 µL). The optical density (OD) of the developed colour was measured at 450 nm using a microplate reader (Multiskan EX; Thermo Scientific, Waltham, MA, USA).

The absorbance of the appropriate blank was always subtracted from the absorbance of the corresponding sample. The levels of serum IgG and IgE antibodies were presented in arbitrary units (AU) mL^{-1}. Human sera with the highest anti-TCMP IgG and IgE levels were used for the calibration.

Intra-assay coefficient of variation was not higher than 4%, while inter-assay coefficient of variation was not higher than 3%.

For determination of the immunoreactivity related only to cows' milk epitopes, the level of antibodies in each serum sample incubated with RA or Z antigen solutions was always subtracted from the level of antibodies in the corresponding serum sample incubated with TCMP-RA or TCMP-Z hydrolysates. Reference cut-off values for RA and Z antigens were determined by analysing the sera of 33 healthy control subjects.

2.5. Examination of antibody specificity by competitive ELISA

Competitive ELISA was performed to confirm the specificity of detected anti-TCMP antibodies. The sera of five patients with ROU or GD (coeliac disease patients) who had the

ACCEPTED MANUSCRIPT

highest levels of antibodies specific for antigenic determinants of unhydrolysed TCMP were chosen for these analyses. For each competitive ELISA test the pool of five sera was made.

To each well of 96-well microtitre ELISA plate 100 µL of unhydrolysed TCMP solution (10 µg mL^{-1}; dissolved in bicarbonate buffer, pH 9.5) was added. Wells were incubated at 4 °C for 24 h. After washing and blocking of wells with BSA, 50 µL of each examined solution (100^{-1} dilution of 5 pooled sera with added solutions of TCMP, or TCMP-RA hydrolysate or TCMP-Z hydrolysate (final concentrations: 0, 1, 10, 100, 500 and 1000 µg mL^{-1}, made in 1% BSA/TTBS) were added to appropriate wells. After 60 min incubation at 37 °C, the primary antibodies bound to TCMP immobilised on the polystyrene surface of the well were detected using HRP-labelled secondary antibodies and TMB, as described previously.

2.6. Statistical analysis

Friedman Test with Dunn's Post Test was used for statistical analysis of differences between the levels of IgG or IgE against TCMP, TCMP-RA and TCMP-Z in patients' sera. Only the patients with the levels of anti-TCMP antibodies above cut-off values were statistically analysed. Statistical package GraphPad Prism 5 was used for statistical analysis and preparation of graphs.

3. Results

3.1. Hydrolysis of milk proteins

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

The proteolytic potential of ten selected lactobacilli strains was assessed following the hydrolysis of TCMP (Fig. 1). Strains *Lb. zeae* LMG17315 (lane 3) and *Lb. helveticus* BGRA43 (lane 10) demonstrated the best proteolytic activity, judging from SDS-PAGE patterns of TCMP degradation, and these two strains were used for further investigation. As can be seen from the gel, all casein fractions were completely hydrolysed with both LMG17315 and BGRA43. In addition, BGRA43 almost completely (over 80%) hydrolysed whey proteins, while their hydrolysis with LMG17315 was partial, as bands of β-lactoglobulin and α-lactalbumin were still detectable after the reaction. ImageJ quantification indicated that LMG17315 hydrolysed about 70% of α-lactalbumin and 60% of β-lactoglobulin (data not shown). It should be noted that three distinct bands of higher molecular masses visible in lane 10 are bacterial proteins, most probably cell surface proteins, originating from BGRA43 (Banina et al., 1998; Fira et al., 2001).

3.2. Immunoreactivity to TCMP and TCMP hydrolysates

Fifty-two of 60 patients (87%) analysed in this study had elevated serum levels of IgG and/or IgE antibodies to TCMP. Each patient with elevated level of IgG specific for TCMP (16 ROU patients, 9 HM patients and 11 GD patients) also had elevated level of anti-TCMP IgE. In addition, 3 ROU patients, 6 HM patients and 7 GD patients had elevated levels of IgE specific for TCMP, while the levels of anti-TCMP IgG were below the cut-off value in their sera.

When considering the patients with above cut-off levels of anti-TCMP IgG and/or IgE, all of them had lower IgG and IgE levels specific for both examined hydrolysates (Fig. 2). The levels of IgG specific for TCMP hydrolysates were below the cut-off value in sera of all patients (Fig. 2A, C, E). Similarly, the levels of IgE specific for TCMP hydrolysates were significantly

ACCEPTED MANUSCRIPT

lower in all patients, compared with the levels of anti-TCMP antibodies (Fig. 2B, D, F). Values below the cut-off of IgE specific for RA-hydrolysates were detected in 85% of patients, while 97% of patients had the same for Z-hydrolysates.

3.3. Immunoreactivity to bacterial antigens

In parallel, we examined the patients' sera for the levels of IgG and IgE specific for the bacterial cell surface proteins or other antigens possibly present in the working solutions (RA and Z antigen solutions). As expected, the results indicated the presence of antibodies against bacterial antigens in the examined groups of patients, as well as in healthy control subjects. Frequencies of healthy control subjects and patients with elevated levels of antibodies to RA and Z antigen solutions are displayed in Table 1. All patients groups showed higher reactivity to Z antigen solution.

To confirm that levels of IgG and IgE detected in experiments with TCMP hydrolysates are specific for immunogenic epitopes in unhydrolysed TCMP, we performed the competitive ELISA tests using pool of five sera of ROU patients (Fig. 3) and pool of five sera of patients with coeliac disease (data not shown). The sera used in competitive ELISA tests were from the patients who showed the highest immunoreactivity to TCMP. The levels of detected anti-TCMP IgG and IgE antibodies were decreasing proportionally as the concentration of free undigested TCMP was increasing, while this effect was not observed with addition of free TCMP-RA or free TCMP-Z hydrolysates (Fig. 3).

ACCEPTED MANUSCRIPT

4. Discussion

Numerous studies focused on peptides released during fermentation of milk by *Lactobacillus helveticus* strains, and revealed their antihypertensive, immunomodulatory and even antitumor properties (LeBlanc, Matar, Valdéz, LeBlanc, & Perdigon, 2002; Vinderola, Matar, & Perdigón, 2007; Wakai & Yamamoto, 2012). The antimicrobial, technological and probiotic potential of *Lb. helveticus* BGRA43, as well as its proteolytic system, has been previously characterised in detail by our group (Strahinic et al., 2013a,b). BGRA43 possesses large extracellular proteinase PrtH, which is capable of hydrolysis of all casein fractions, as well as degradation of one of three major allergenic epitopes of β-lactoglobulin (Lozo et al., 2011; Strahinic et al., 2013b). It has been established that fermentation of milk by BGRA43 releases peptides that express significant anti-inflammatory properties on the Nd-THP-1 monocyte cell line due to down-regulation of tumour necrosis factor-α or interleukin-6 production (Tompa et al., 2011). On the other hand, the proteolytic system of *Lb. zeae* LMG17315 is only partially characterised, but it is known that this strain possesses proteinases that differ greatly from the one present in BGRA43 (Kim et al., 2011; Vukotić et al., 2016).

In this study we investigated the ability of proteinases of these two lactobacilli to hydrolyse TCMP for which elevated levels of specific IgE and IgG were previously determined in ROU, HM and GD patients (Besu et al., 2009, 2013; Juranic et al., 2008, 2009). We showed that the levels of antibodies specific for TCMP hydrolysates obtained in this way were below cut-off values for the majority of tested patients indicating that the most of IgG and IgE specific epitopes were destroyed by bacterial hydrolysis.

The most remarkable difference was observed with the levels of IgG, given that no patient expressed above cut-off levels of the antibodies either against TCMP-RA or TCMP-Z hydrolysate. The role of IgG immunoglobulins in cows' milk hypersensitivity is quite controversial. Anthoni, Savilahti, Rautelin, and Kolho (2009) reported the existence of association between levels of milk proteins specific IgG antibodies and gastrointestinal symptoms in adults. The IgG4 immunoglobulins seems to have an immunopathological role in the non-IgE-mediated cows' milk allergy with delayed gastrointestinal symptoms (Sletten, Halvorsen, Egaas, & Halstensen, 2006). In addition, even though the IgE pathway of anaphylaxis has been well characterised, the existence of an IgG pathway is controversial in humans (Khodoun, Strait, Armstrong, Yanase, & Finkelman, 2011). However, the cases of anaphylaxis developed after repeated infusion of large amounts of dextran (Hedin et al., 1980), von Willebrand's factor (Bergamaschini et al., 1995), or therapeutic IgG mAbs (Klastersky, 2006) that have been characterised by the presence of detectable IgG, but not IgE Abs, to the infused compound may be the most likely candidates for human IgG-mediated anaphylaxis.

Considering the importance of IgG highlighted in these publications, achievement of a below cut-off decrease of IgG to TCMP in all patients after bacterial hydrolysis highlights the possible benefit of using milk products fermented with *Lb. helveticus* BGRA43 or *Lb. zeae* LMG17315 in nutrition of these patients.

Taking into account the differences in the levels of specific antibodies in sera of all patients before and after the hydrolysis, the drops of specific IgE levels are perhaps the most important, since the largest frequency of patients included in our study had elevated level of IgE specific for TCMP. Following the hydrolyses, the frequency of patients with elevated level of IgE specific for TCMP hydrolysates was drastically lower. Moreover, 18 patients who had titre

of IgE specific for undigested TCMP several hundred times above the cut-off value showed normal levels of IgE specific to products of BGRA43 hydrolysis, while 13 of those 18 patients showed normal levels of IgE specific for TCMP-Z hydrolysate. Comparing the differences in serum levels of IgE against two different hydrolysates, it could be concluded that hydrolysis with LMG17315 demonstrated better effect, although BGRA43 had similar impact for the majority of the patients. This is interesting since the efficacy of BGRA43 hydrolysis was higher, given that about 30% α-lactalbumin and 40% of β-lactoglobulin remained intact in TCMP-Z hydrolysate. This could mean that α-lactalbumin and β-lactoglobulin, which have been for long time considered the most important milk allergens (Fiocchi et al., 2010), are not source of majority of immunoreactive epitopes for patients tested in this study, and that caseins are critically involved in their immunoreactivity to TCMP.

Although the hydrolysates obtained were not characterised in detail, the results proved the concept of the ability proteinases to destroy immunogenic epitopes in TCMP, which was unambiguously corroborated with the results of competitive ELISA. It should be mentioned, however, that LAB proteinases (or CEPs), responsible for the observed digestion, are not capable of producing di- and tri-peptides, nor free amino acids. In addition, it is known that CEP hydrolysis of only β-casein may lead to generation of more than a hundred different oligopeptides (Juillard et al., 1995). Further determination of diversity and immunogenicity of these epitopes as well as the cleavage sites targeted by analysed lactobacilli proteinases could deepen our understanding of mechanism of the reactions addressed and will be the subject of further studies.

Hydrolysis of immunogenic milk epitopes, demonstrated in our study, presents an important feature of lactobacilli, significant for both application and fundamental research. Our

ACCEPTED MANUSCRIPT

results show that BGRA43 and LMG17315 could be applied in the production of fermented milk products or extensively hydrolysed milk formulas, which would be largely free from epitopes shown here to be recognised by the immune response and to thus present potential health problems, at least for the patient groups analysed in our study.

However, as our study demonstrated, the efficacy of bacteria to destroy immunogenic determinants present in TCMP, the antigenicity of TCMP-RA and TCMP-Z hydrolysates has to be analysed in vivo, as these newly formed products might possess new, previously unexpressed, epitopes that could potentially induce immunogenic reaction after consumption.

Comparative studies of the various hydrolysed formulas present on the market indicated that not all formulas have the same protective benefit in prevention and treatment of atopic diseases in infants and children (Greer, Sicherer, & Burks, 2008). Thus, formulation of novel non-immunogenic foods is expected to increase in the next decades, which is particularly important for infants and children for whom ingestion of milk and milk products is necessary, but who are at the same time most affected with food-associated immune reaction (Sicherer & Sampson, 2014).

Nevertheless, the presence of antibodies to analysed bacterial antigens in rare patients' sera suggests the need for screening for the antibodies specific for bacterial antigen to evaluate the efficacy and safeness of potential use of hydrolysed TCMP. Further screening of other lactobacilli strains may also provide hydrolysates without these unwanted properties and this will also be the subject of further investigations. In addition, proteolytic enzyme preparations from the lactobacilli analysed may be applied as well, bearing in mind that they are more cost-effective and easier to obtain than commercially used ones.

ACCEPTED MANUSCRIPT

Although our research was focussed on patients suffering from different immune-related diseases, it is reasonable to suspect that individuals and children with diagnosed allergies on cows' milk or atopic diseases would also have positive outcome using TCMP hydrolysates obtained with given bacterial enzymes. Since it is known that lactobacilli may prevent and be used in treatment of various allergic diseases (Kim et al., 2014; Pohjavuori et al., 2004; Rosenfeldt et al., 2003), it is reasonable to speculate that fermented milk products can be formulated to contain both live, therapeutic bacteria and bioactive non-immunogenic peptides, generated through the activity of their proteinases.

5. Conclusions

Taking all the results together, we can conclude that with the hydrolysis of TCMP by BGRA43 and LMG17315, drastic differences in the levels of specific IgE and IgG can be observed in sera of most patients from all three investigated groups. Our findings demonstrate that both applied lactobacilli hydrolyse total cows' milk proteins through degradation of the majority of their immunogenic epitopes, even though they possess different enzymes responsible for the digestion.

Acknowledgement

This work was supported financially by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Projects ON173019 and ON175011).

ACCEPTED MANUSCRIPT

We own gratitude to Dr Ljiljana Janković, Dr Radmilo Krstić, Dr Dragan Popović, Dr Njegica Jojić, Dr Slobodan Ristić, Dr Dušan Ristić and Dr Nenad Milanović for providing the patients' sera samples used in this study. In addition, the authors would like to thank Tatjana Petrović for her excellent technical assistance.

References

Anthoni, S., Savilahti, E., Rautelin, H., & Kolho, K.-L. (2009). Milk protein IgG and IgA: the association with milk-induced gastrointestinal symptoms in adults. *World Journal of Gastroenterology*, *15*, 4915–4918.

Banina, A., Vukasinovic, M., Brankovic, S., Fira, D., Kojic, M., & Topisirovic, L. (1998). Characterization of natural isolate *Lactobacillus acidophilus* BGRA43 useful for acidophilus milk production. *Journal of Applied Microbiology*, *84*, 593–599.

Bergamaschini, L., Mannucci, P. M., Federici, A. B., Coppola, R., Guzzoni, S., & Agostoni, A. (1995). Posttransfusion anaphylactic reactions in a patient with severe von Willebrand disease: role of complement and alloantibodies to von Willebrand factor. *The Journal of Laboratory and Clinical Medicine*, *125*, 348–355.

Besu, I., Jankovic, L., Konic-Ristic, A., Raskovic, S., Besu, V., Djuric, M., et al. (2013). The role of specific cow's milk proteins in the etiology of recurrent aphthous ulcers. *Journal of Oral Pathology and Medicine 42*, 82–88.

Besu, I., Jankovic, L., Magdu, I. U., Konic-Ristic, A., Raskovic, S., & Juranic, Z. (2009). Humoral immunity to cow's milk proteins and gliadin within the etiology of recurrent aphthous ulcers? *Oral Diseases*, *15*, 560–564.

ACCEPTED MANUSCRIPT

401 Bu, G., Luo, Y., Zhang, Y., & Chen, F. (2010). Effects of fermentation by lactic acid bacteria on
402 the antigenicity of bovine whey proteins. *Journal of the Science of Food and Agriculture*,
403 *90*, 2015–2020.
404 D'Mello, C., Ronaghan, N., Zaheer, R., Dicay, M., Le, T., MacNaughton, W. K., et al. (2015).
405 Probiotics improve inflammation-associated sickness behavior by altering
406 communication between the peripheral immune system and the brain. *Journal of*
407 *Neuroscience*, *35*, 10821–10830.
408 Ehn, B.-M., Ekstrand, B., Bengtsson, U., & Ahlstedt, S. (2004). Modification of IgE binding
409 during heat processing of the cow's milk allergen beta-lactoglobulin. *Journal of*
410 *Agricultural and Food Chemistry*, *52*, 1398–1403.
411 El-Ghaish, S., Rabesona, H., Choiset, Y., Sitohy, M., Haertlé, T., & Chobert, J.-M. (2011).
412 Proteolysis by *Lactobacillus fermentum* IFO3956 isolated from Egyptian milk products
413 decreases immuno-reactivity of α_{S1}-casein. *Journal of Dairy Research*, *78*, 203–210.
414 Fiocchi, A., Brozek, J., Schünemann, H., Bahna, S. L., von Berg, A., Beyer, K., et al. (2010).
415 World Allergy Organization (WAO) diagnosis and rationale for action against cow's milk
416 allergy (DRACMA) guidelines. *World Allergy Organization Journal*, *3*, 157–161.
417 Fira, D., Kojic, M., Banina, A., Spasojevic, I., Strahinic, I., & Topisirovic, L. (2001).
418 Characterization of cell envelope-associated proteinases of thermophilic lactobacilli.
419 *Journal of Applied Microbiology*, *90*, 123–130.
420 Gaudin, J.-C., Rabesona, H., Choiset, Y., Yeretssian, G., Chobert, J.-M., Sakanyan, V., et al.
421 (2008). Assessment of the immunoglobulin E-mediated immune response to milk-
422 specific proteins in allergic patients using microarrays. *Clinical and Experimental*
423 *Allergy*, *38*, 686–693.

ACCEPTED MANUSCRIPT

Greer, F. R., Sicherer, S. H., & Burks, A. W. (2008). Effects of early nutritional interventions on the development of atopic disease in infants and children: the role of maternal dietary restriction, breastfeeding, timing of introduction of complementary foods, and hydrolyzed formulas. *Pediatrics*, *121*, 183–191.

Hacini-Rachinel, F., Gheit, H., Le Luduec, J.-B., Dif, F., Nancey, S., & Kaiserlian, D. (2009). Oral probiotic control skin inflammation by acting on both effector and regulatory T cells. *PloS One*, *4*, e4903.

Hayes, M., Stanton, C., Fitzgerald, G. F., & Ross, R. P. (2007). Putting microbes to work: dairy fermentation, cell factories and bioactive peptides. Part II: bioactive peptide functions. *Biotechnology Journal*, *2*, 435–449.

Hedin, H., Richter, W., Messmer, K., Renck, H., Ljungström, K. G., & Laubenthal, H. (1980). Incidence, pathomechanism and prevention of dextran-induced anaphylactoid / anaphylactic reactions in man. *Developments in Biological Standardization*, *48*, 179–189.

Hörmannsperger, G., von Schillde, M.-A., & Haller, D. (2013). Lactocepin as a protective microbial structure in the context of IBD. *Gut Microbes*, *4*, 152–157.

Johnston, L. K., Chien, K. B., & Bryce, P. J. (2014). The immunology of food allergy. *Journal of Immunology*, *192*, 2529–2534.

Juillard, V., Laan, H., Kunji, E. R., Jeronimus-Stratingh, C. M., Bruins, A. P., & Konings, W. N. (1995). The extracellular PI-type proteinase of *Lactococcus lactis* hydrolyzes beta-casein into more than one hundred different oligopeptides. *Journal of Bacteriology*, *177*, 3472–3478.

ACCEPTED MANUSCRIPT

445 Juranic, Z., Radic, J., Konic-Ristic, A., Jelic, S., Mihaljevic, B., Stankovic, I., et al. (2008).
446 Humoral immunoreactivity to gliadin and to tissue transglutaminase is present in some
447 patients with multiple myeloma. *BMC Immunology*, *9*, Article 22.
448 Juranić, Z. D., Besu, I., Jelić, S., Konić-Ristić, A., Matković, S., Janković, L., et al. (2009). Some
449 patients with NHL possessed immunoreactivity to gliadin and to cow's milk proteins.
450 *International Journal of Hematology*, *90*, 212–216.
451 Khodoun, M. V, Strait, R., Armstrong, L., Yanase, N., & Finkelman, F. D. (2011). Identification
452 of markers that distinguish IgE- from IgG-mediated anaphylaxis. *Proceedings of the*
453 *National Academy of Sciences of the United States of America*, *108*, 12413–12418.
454 Kim, D.-W., Choi, S.-H., Kang, A., Nam, S.-H., Kim, D.-S., Kim, R. N., et al. (2011). Draft
455 genome sequence of *Lactobacillus zeae* KCTC 3804. *Journal of Bacteriology*, *193*, 5023.
456 Kim, S.-O., Ah, Y.-M., Yu, Y. M., Choi, K. H., Shin, W.-G., & Lee, J.-Y. (2014). Effects of
457 probiotics for the treatment of atopic dermatitis: a meta-analysis of randomized controlled
458 trials. *Annals of Allergy, Asthma and Immunology*, *113*, 217–226.
459 Klastersky, J. (2006). Adverse effects of the humanized antibodies used as cancer therapeutics.
460 *Current Opinion in Oncology*, *18*, 316–320.
461 Kleber, N., Weyrich, U., & Hinrichs, J. (2006). Screening for lactic acid bacteria with potential
462 to reduce antigenic response of β-lactoglobulin in bovine skim milk and sweet whey.
463 *Innovative Food Science and Emerging Technologies*, *7*, 233–238.
464 Kojic, M., Fira, D., Banina, A., & Topisirovic, L. (1991). Characterization of the cell wall-Bound
465 proteinase of *Lactobacillus casei* HN14. *Applied and Environmental Microbiology*, *57*,
466 1753–1757.

ACCEPTED MANUSCRIPT

LeBlanc, J. G., Matar, C., Valdéz, J. C., LeBlanc, J., & Perdigon, G. (2002). Immunomodulating effects of peptidic fractions issued from milk fermented with *Lactobacillus helveticus.* *Journal of Dairy Science*, *85*, 2733–2742.

Lozo, J., Strahinic, I., Dalgalarrondo, M., Chobert, J.-M., Haertlé, T., & Topisirovic, L. (2011). Comparative analysis of β-casein proteolysis by PrtP proteinase from *Lactobacillus paracasei* subsp. *paracasei* BGHN14, PrtR proteinase from *Lactobacillus rhamnosus* BGT10 and PrtH proteinase from *Lactobacillus helveticus* BGRA43. *International Dairy Journal*, *21*, 863–868.

Miloradovic, Z. N., Kljajevic, N. V, Jovanovic, S. T., Vucic, T. R., & Macej, O. D. (2015). The effect of heat treatment and skimming on precipitate formation in caprine and bovine milks. *Journal of Dairy Research*, *82*, 22–28.

Oelschlaeger, T. A. (2010). Mechanisms of probiotic actions - A review. *International Journal of Medical Microbiology*, *300*, 57–62.

Pelaez-Lorenzo, C., Diez-Masa, J. C., Vasallo, I., & de Frutos, M. (2010). Development of an optimized ELISA and a sample preparation method for the detection of beta-lactoglobulin traces in baby foods. *Journal of Agricultural and Food Chemistry*, *58*, 1664–1671.

Pescuma, M., Hébert, E. M., Haertlé, T., Chobert, J.-M., Mozzi, F., & Font de Valdez, G. (2015). *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 454 cleaves allergenic peptides of β-lactoglobulin. *Food Chemistry*, *170*, 407–414.

Peyron, S., Mouécoucou, J., Frémont, S., Sanchez, C., & Gontard, N. (2006). Effects of heat treatment and pectin addition on beta-lactoglobulin allergenicity. *Journal of Agricultural and Food Chemistry*, *54*, 5643–5650.

ACCEPTED MANUSCRIPT

Pohjavuori, E., Viljanen, M., Korpela, R., Kuitunen, M., Tiittanen, M., Vaarala, O., & Savilahti, E. (2004). *Lactobacillus* GG effect in increasing IFN-gamma production in infants with cow's milk allergy. *Journal of Allergy and Clinical Immunology*, *114*, 131–136.

Rosenfeldt, V., Benfeldt, E., Nielsen, S. D., Michaelsen, K. F., Jeppesen, D. L., Valerius, N. H., et al. (2003). Effect of probiotic *Lactobacillus* strains in children with atopic dermatitis. *Journal of Allergy and Clinical Immunology*, *111*, 389–395.

Schreier, H. M. C., & Wright, R. J. (2014). Stress and food allergy: mechanistic considerations. *Annals of Allergy, Asthma and Immunology*, *112*, 296–301.

Shi, J., Luo, Y., Xiao, Y., Li, Z., Xu, Q., & Yao, M. (2014). Effects of fermentation by *Lactobacillus casei* on the antigenicity and allergenicity of four bovine milk proteins. *International Dairy Journal*, *35*, 75–80.

Sicherer, S. H., & Sampson, H. A. (2014). Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. *Journal of Allergy and Clinical Immunology*, *133*, 291–307.

Sletten, G. B. G., Halvorsen, R., Egaas, E., & Halstensen, T. S. (2006). Changes in humoral responses to beta-lactoglobulin in tolerant patients suggest a particular role for IgG4 in delayed, non-IgE-mediated cow's milk allergy. *Pediatric Allergy and Immunology*, *17*, 435–443.

Strahinic, I., Lozo, J., Terzic-Vidojevic, A., Fira, D., Kojic, M., Golic, N., et al. (2013a). Technological and probiotic potential of BGRA43 a natural isolate of *Lactobacillus helveticus*. *Frontiers in Microbiology*, *4*, Article 2.

ACCEPTED MANUSCRIPT

510 Strahinic, I., Lukic, J., Terzic-Vidojevic, A., Lozo, J., Kojic, M., & Topisirovic, L. (2013b). Use
511 of *Lactobacillus helveticus* BGRA43 for manufacturing fermented milk products. *Food*
512 *Technology and Biotechnology*, *51*, 257–265.
513 Tompa, G., Laine, A., Pihlanto, A., Korhonen, H., Rogelj, I., & Marnila, P. (2010).
514 Chemiluminescence of non-differentiated THP-1 promonocytes: developing an assay for
515 screening anti-inflammatory milk proteins and peptides. *Luminescence*, *26*, 251–258.
516 Verna, E. C., & Lucak, S. (2010). Use of probiotics in gastrointestinal disorders: what to
517 recommend? *Therapeutic Advances in Gastroenterology*, *3*, 307–319.
518 Vinderola, G., Matar, C., & Perdigón, G. (2007). Milk fermentation products of *L. helveticus*
519 R389 activate calcineurin as a signal to promote gut mucosal immunity. *BMC*
520 *Immunology*, *8*, Article 19.
521 Von Schillde, M.-A., Hörmannsperger, G., Weiher, M., Alpert, C.-A., Hahne, H., Bäuerl, C., et
522 al. (2012). Lactocepin secreted by *Lactobacillus* exerts anti-inflammatory effects by
523 selectively degrading proinflammatory chemokines. *Cell Host and Microbe*, *11*, 387–396.
524 Vukotić, G., Strahinić, I., Begović, J., Lukić, J., Kojić, M., & Fira, D. (2016). Survey on
525 proteolytic activity and diversity of proteinase genes in mesophilic lactobacilli.
526 *Microbiology*, *85*, 33–41.
527 Wakai, T., & Yamamoto, N. (2012). Antihypertensive peptides specific to *Lactobacillus*
528 *helveticus* fermented milk. In R. H. Sammour (Ed.), *Biotechnology - Molecular studies*
529 *and novel applications for improved quality of human life*. Rijeka, Croatia: InTech.
530 Wal, J. -M. (2004). Bovine milk allergenicity. *Annals of Allergy, Asthma and Immunology*, *93*,
531 S2–S11.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

532 Warren, C. M., Jhaveri, S., Warrier, M. R., Smith, B., & Gupta, R. S. (2013). The epidemiology
533 of milk allergy in US children. *Annals of Allergy, Asthma and Immunology*, *110*, 370–
534 374.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Table 1

Frequencies of healthy control subjects and patients with elevated humoral immunity to RA and Z antigen solutions.

Solution	Healthy controls	Patient groups		
		ROU	GD	HM
IgG RA	2/33	2/20	0/20	1/20
IgG Z	1/33	6/20	3/20	4/20
IgE RA	4/33	0/20	0/20	1/20
IgE Z	2/33	6/20	6/20	0/20

ACCEPTED MANUSCRIPT

Figure legends

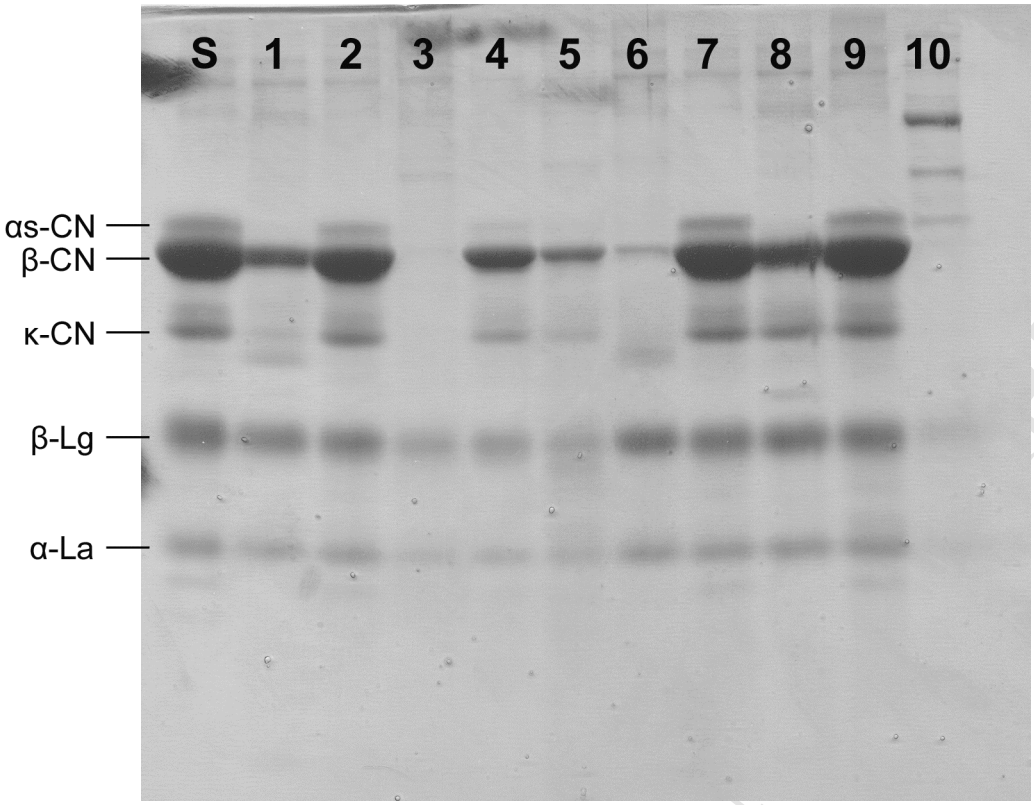
Fig. 1. Hydrolysis pattern of total cows' milk proteins (TCMP) by different lactobacilli strains after 3h of incubation at 37 °C. Lane S, starting substrate (TCMP); lane 1, *Lb. rhamnosus* BGT10; lane 2, *Lb.plantarum* BGHO10; lane 3, *Lb. zeae* LMG17315; lane 4, *Lb. plantarum* BGBUK 2-5; lane 5, *Lb. plantarum* LMG18024; lane 6, *Lb. casei* ATCC393; lane 7, *Lb. plantarum* BGPV2-45a; lane 8, *Lb. plantarum* LMG9208; lane 9, *Lb. plantarum* BGGA-8; lane 10, *Lb. helveticus* BGRA43. Abbreviations are: αs-CN, α-casein; β-CN, β-casein; κ-CN, κ-casein; β-Lg, β-lactoglobulin; α-La, α-lactalbumin. For reference on protein bands see Miloradovic, Kljajevic, Jovanovic, Vucic, & Macej (2015).

Fig. 2. The levels of IgG (A, C and E) and IgE (B, D and F) antibodies against unhydrolysed total cows' milk proteins (TCMP) and after the hydrolysis of TCMP with *Lb. helveticus* BGRA43 (TCMP-RA) and *Lb. zeae* LMG17315 (TCMP-Z). The levels of antibodies specific for TCMP, TCMP-RA and TCMP-Z in sera of all patients with recurrent oral ulcerations (A, B), gastrointestinal disorders (C, D) and haematological malignancies (E, F) were determined by ELISA. Values obtained for each patient were plotted as points on the graph and connected by the line. All measurements were done in duplicate. Friedman test with Dunn's Multiple Comparison Test was used for statistical analysis, *** $p < 0.005$ compared with control

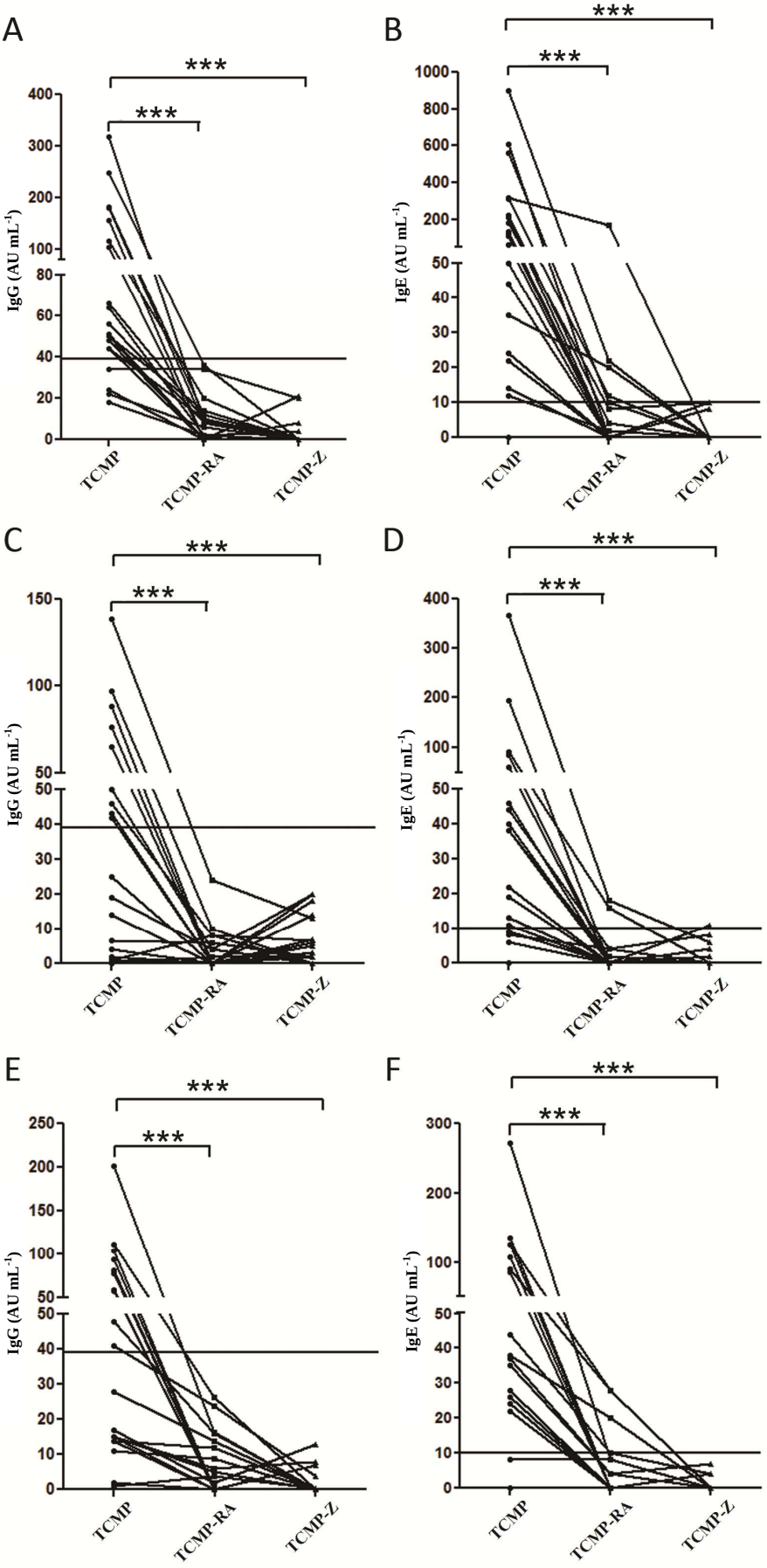
Fig. 3. Competitive ELISA for determination of specificity of (A) IgG and (B) IgE from patients' sera to immunogenic epitopes of unhydrolysed total cows' milk proteins (TCMP). The mixture of sera from five patients with recurrent oral ulcerations was pre-incubated either with unhydrolysed total cows' milk proteins (×) or products from hydrolysis of TCMP

with *Lb. helveticus* BGRA43 (○) or *Lb. zeae* LMG17315 (□) in final concentrations of 0, 1, 10, 100, 500 and 1000 µg mL^{-1} and thereafter incubated in the wells pre-coated with unhydrolysed TCMP (10 µg mL^{-1}). All measurements were done in duplicate, calculating the mean OD450 values.

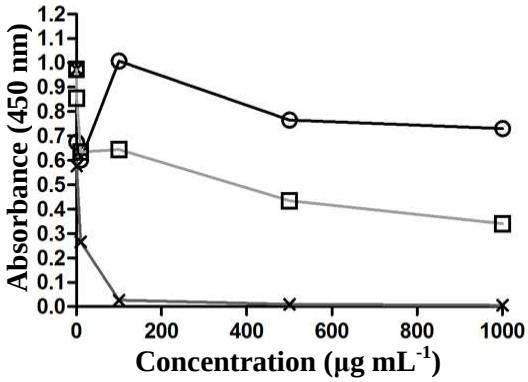
ACCEPTED MANUSCRIPT



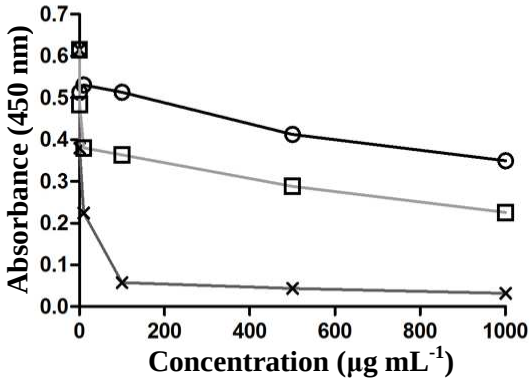
ACCEPTED MANUSCRIPT


A
B
C
D
E
F
***
IgG (AU mL-1)
IgE (AU mL-1)
TCMP
TCMP-RA
TCMP-Z

A



B



ACCEPTED MANUSCRIPT