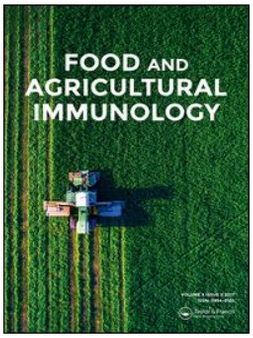

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The absence of immunoreactivity to donkey's milk in patients with recurrent aphthous ulcers and immunoreactivity to cow's milk

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ABSTRACT
Despite the numerous benefits of milk constituents for human health a considerable number of the general population follow a milk-restricted diet due to clinically confirmed or self-assessed adverse reactions to cow's milk consumption. Recurrent aphthous ulcers (RAU) are currently one of the most common oral disorders, with a worldwide distribution and insufficiently defined etiology, which, among other factors, implies the immunological reaction to food proteins. The aim of this study was to determine the immune-reactivity to donkey's milk proteins in patients with RAU and compare it to the reactivity towards the proteins from cow's and goat's milks, in a set of simultaneous experiments. Levels of serum IgA, IgG and IgE antibodies to the same quantity of the examined antigens were determined by enzyme-linked immunosorbent assay. The results indicate that patients with RAU with increased immunity to cow's milk proteins could consider the use of donkey's milk as the best protein source.

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KEYWORDS
Donkey's milk proteins; immunoreactivity; recurrent aphthous ulcers; IgA; IgG; IgE

Introduction

Milk has been used worldwide for centuries, as the first food introduced after birth and consumed through the whole life. Due to its composition rich in proteins, fats and micronutrients it has been considered one of the most natural and highly nutritive parts of an optimal diet. Beyond being a rich source of nutrients essential for normal growth and development, emerging scientific data provide evidence of both preventive and therapeutic effects of milk and its constituents. The bioactive compounds of milk, milk-based products and milk-derived constituents include proteins and peptides, prebiotic carbohydrates, probiotic bacteria and lipids, with beneficial effects supported by vitamins and minerals present. Recent scientific data have shown antihypertensive, antimicrobial, antithrombic, immunomodulatory, opioid, mineral binding effects of bioactive milk-derived peptides as the proposed mechanism of their beneficial effects on growth and development,

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gastrointestinal health, immunity, the cardiovascular system, cognitive function that rationalize their role in health promotion and the prevention of chronic diseases (Nagpal et al., 2012).

Despite the numerous benefits of milk constituents for human health a considerable number of the general population follow a milk-restricted diet due to clinically confirmed or self-assessed adverse reactions to milk consumption, primarily to cow's milk as the most frequently consumed. The broad term of adverse reactions to milk covers immunologically based reactions – milk allergies (both IgE and non-IgE mediated), and non-immunological reactions, that is, milk intolerance (Taylor, Truelove, & Wright, 1964). Disturbances of the gastrointestinal tract associated with food allergies often include oral manifestations such are aphthous oral ulcers, glossitis or stomatitis (Shakeri et al., 2009). Additionally, inflammatory bowel diseases, both Cronh's disease and ulcerative colitis, could be manifested as aphthous-like ulceration (Jurge, Kuffer, Scully, & Porter, 2006) and at the same time are shown to be associated with increased levels of antibodies to milk proteins in circulation (Lerner, Rossi, Park, Albini, & Lebenthal, 1989). These findings rationalize the assessment of milk allergies as part of differential diagnosis of the diseases of oral and gastrointestinal mucosa, the role of food proteins in pathogenesis of the diseases and the rationale of restricted diets as part of therapy. At the same time, if they can be considered safe the consumption of milk proteins from other animal species, especially from those phylogenetically distant from cows and with lower shown cross-reactivity (Restani et al., 1999), can provide both nutritional and health benefits and compensate the deprivation of cow's milk in the diet of vulnerable population.

Recurrent aphthous ulcers (RAU) are currently one of the most common oral disorders, with a worldwide distribution and insufficiently defined etiology (Scully, 2006), which among other factors implies the immunological reaction to food proteins. The association between immunity to cow's milk proteins (CMP) and oral diseases was described previously (Besu et al., 2009; Taylor et al., 1964; Thomas, Ferguson, Mclennan, & Mason, 1973). We have reported that subjects with RAU, without any gastrointestinal or other systemic disease or abnormalities other than recurrent aphthous ulcerations, had increased humoral (IgA and/or IgG and/or IgE) immunity to CMP (Besu et al., 2009; Besu, Jankovic, Konic-Ristic, Raskovic, et al., 2013). Successful therapy depends on completely eliminating CMP from the patient's diet. However, this implies the restriction of all beneficial effects of milk proteins. Ideally, the replacement food should be hypo- or anallergenic, as well as non-cross-reactive with cow's milk.

Donkey's milk, the milk from a specie phylogenetically distant to cow's, has recently attracted attention for its nutritional and functional properties. Based on its physicochemical properties such as pH value and viscosity, and the content of proteins, sugars and lipids, donkey's milk appeared to be the most similar to human milk (El-Hatmi et al., 2015). Regarding the composition of proteins, donkey's milk is characterized by the low content of casein, especially of αs_2-casein, and relatively high content of whey protein, with a specific structure of β-lactoglobulin (Gubic et al., 2015; Guo et al., 2007; Tesse, Paglialunga, Braccio, & Armenio, 2009). In animal studies donkey and human milk consumption induced the reduction of pro-inflammatory stimuli, increase in antioxidant defense and the beneficial effects on gut microbiota and their metabolism, while none of the effects were shown in animals treated with cow's milk (Lionetti et al., 2012; Trinchese et al., 2015) The observed effects are partly attributed to the specific composition of

lipids and their functional properties (Chiofalo, Dugo, Bonaccorsi, & Mondello, 2011). However, it is considered hypoallergenic and, thus, compared to soy-protein-based formulas, extensively hydrolysed formulas and amino-acid formulas, it has been proposed as the optimal alternative to cow's milk in the population of children with cow's milk protein allergy (Monti et al., 2007; Vincenzetti et al., 2014).

The aim of this study was to determine the immune-reactivity to donkey's milk proteins (DMP) in patients with RAU and compare it to the reactivity towards CMP and the proteins from goat's milk, in a set of simultaneous *in vitro* experiments.

Material and methods

Milk samples

The samples of donkey`s milk were donated from the Special Nature Reserve 'Zasavica', Serbia, the specialized farm of Balkan donkeys as an autochthonous breed (Kugler, Grunenfelder, & Broxham, 2008). Milk samples were fresh composite samples obtained from animals in different periods of lactation. The contents of individual protein fractions of the composite sample of donkey milk from the Reserve Zasavica in different periods of lactation are reported to be: 1.38–1.89 g/L of αs_1-casein, up to 0.11 g/L of αs_2-casein, 0.1–0.55 g/L of β-casein, 1.57–2.73 g/L of α-lactalbumin, 0.26–0.20 g/L β-lactoglobulin. It also contains 2.97–2.49 g/L lysozyme and 0.01–0.25 g/L of lactoferrin (Gubic et al., 2015). Goat's milk used was the composite sample of three fresh samples of milk obtained from the local open market and combined in equal volume ratio. Both donkey's and goat's milk samples were defatted by centrifugation on the day of collection, aliquoted and immediately stored at −20°C until analysis. Commercial skimmed cow's pasteurized milk powder (ICN Biomedicals, Inc. Cosa Mesa, CA.) was used for testing immunoreactivity towards cow's milk.

Studied population

Sera from 50 subjects (19 males and 31 females; average age ± SD, 39.4 ± 19.39 years) with RAU were used in the study. The study protocol was approved by the Ethical Committee of the Faculty of Stomatology, University of Belgrade and all subjects enrolled in the study gave written informed consent. They all were Caucasians and Serbian inhabitants. The clinical examination and the blood sampling were performed at the Clinics of Periodontology and Oral Medicine, Faculty of Stomatology, University of Belgrade, between 2007 and 2009.

The main inclusion criterion was a history of recurrent aphthous ulcerations, and the exclusion of etiological factors that could lead to ulceration of the oral mucosa confirmed by anamnestic data and clinical examination. In brief, all enrolled subjects had no gastrointestinal or other systemic disease or abnormalities, including changes at the skin and other mucosa and they were not using drugs that could cause oral changes. Blood was sampled in the phase of active ulcers, which was confirmed by clinical examination. In 86% (43/50) of subjects, aphthous ulcers were oval in shape with a diameter (2r) smaller than 10 mm while in 14% (7/50) of subjects ulcerations had a diameter bigger than 10 mm.

Sera of these subjects with RAU (who are involved in this research) were tested earlier to CMP, specific CMP, fresh goat's milk (FGM), fresh cow's milk, boiled goat's milk and

boiled cow's milk (Besu et al., 2009, Besu, Jankovic, Konic-Ristic, Damjanovic, et al., 2013, Besu, Jankovic, Konic-Ristic, Raskovic, et al., 2013). In this study we used the same methodology as in prior studies, because we wanted the research to be conducted under the same experimental conditions.

Enzyme-linked immunosorbent assay

Three types of antigens were used: skimmed cow's milk pasteurized powder, FGM and fresh donkey's milk (FDM). Wells of the highabsorbent immunoplate (Nunc^{tm}) were coated with the same quantity of antigen proteins (10 μg). Determination of IgA, IgG and IgE serum's immunoreactivity to CMP, FGM and FDM has been done by home-made enzyme-linked immunosorbent assay tests, using sheep antihuman IgA, IgG (Binding Site, Birmingham, England) and IgE (Sigma Chemicals Co, Saint Louis, MO), with HRP-labelled antibodies as secondary antibodies. Blocker was 1% bovine serum albumin (BSA – Sigma Chemical Co, Saint Louis, MO).

A substrate solution TMB (3,3,5,5-tetramethylbenzidine) (INEP, Zemun, Serbia) was added to the wells and after the incubation for 15 min, the enzyme reaction was terminated by addition of H_2SO_4. The absorbance of developed color was measured using microplate reader (Multiskan EX; ThermoLabsystems, Finland) at 450 nm.

The absorbance of blank (the sample with primary and secondary antibodies but without tested antigens) was subtracted from the absorbance of the tested sample. Obtained values of OD were standardized based on the values obtained for the sera of selected patients with high immunoreactivity to the same type of milk, which were used in each assay as the positive control. Obtained data are expressed as arbitrary units representing the ratio in OD of analysed and control samples.

Statistical analysis

Differences between parameters were evaluated by paired t test and Mann Whitney test.

Statistical package R (2.8.1 (2008-12-22); Copyright (C) 2008; The R Foundation for Statistical Computing; ISBN 3-900051-07-0) was used for data processing. Microsoft Office Excel was used to prepare all graphics.

Results

Humoral immunoreactivity to different types of milk in RAU+ patients

Sera of 36 subjects with RAU, who earlier showed increased immunoreactivity (RAU+) to CMP were tested for CMP again, FGM and FDM. Statistical analysis of the obtained data reveals that the level of anti-CMP IgA immunoreactivity estimated using paired t test was significantly higher than the anti-FGM immunoreactivity in RAU+ subjects ($p = .0028$). The level of anti-FDM IgA immunoreactivity was significantly lower than the anti-CMP immunoreactivity in RAU+ subjects ($p = .0276$). Statistical analyses show that there were no statistically significant differences between data for FGM and FDM (Figure 1(A)).

Statistical analysis of IgG immunoreactivity to different types of milk in RAU+ patients shows statistically significant differences between data for CMP vs. FGM, CMP vs. FDM, FGM vs. FDM. The level of anti-CMP IgG immunoreactivity, estimated using paired t test

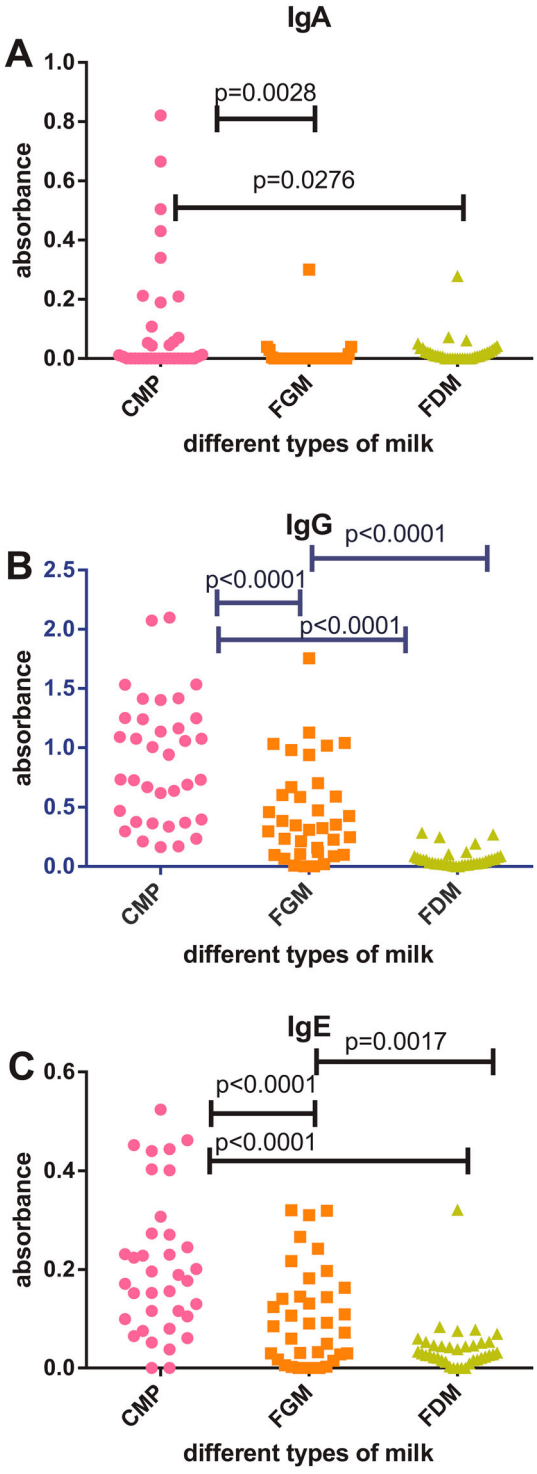

Figure 1. Serum IgA (A), IgG (B), IgE (C) immunoreactivity with different types of milk (CMP, cow's milk proteins; FGM, fresh goat's milk; FDM, fresh donkey's milk) determined for subjects with RAU with proven increased immunoreactivity to CMP (RAU+).

was significantly higher than the anti-FGM and anti-FDM IgG immunoreactivity in RAU+ subjects ($p < .0001$). The level of anti-FDM IgG immunoreactivity was significantly lower than the level of anti-FGM IgG immunoreactivity in RAU+ subjects ($p < .0001$) (Figure 1(B)).

Testing of IgE immunoreactivity to different types of milk in RAU+ patients shows statistically significant differences between data for CMP vs. FGM, data for CMP vs. FDM and data for FGM vs. FDM. The level of anti-CMP IgE immunoreactivity was significantly higher than the levels of anti-FGM IgE immunoreactivity ($p < .0001$) and anti-FDM IgE immunoreactivity ($p < .0001$). The level of anti-FDM IgE immunoreactivity was significantly lower than the level of anti-FGM immunoreactivity in RAU+ subjects ($p = .0017$) (Figure 1(C)).

Humoral immunoreactivity to different types of milk in RAU+ and RAU− subjects

Sera of 36 subjects with RAU, who earlier showed increased immunoreactivity (RAU+) to CMP and sera of 14 subjects with RAU but without increased immunity to CMP (RAU−), were tested to CMP again, and then to FGM and FDM.

Comparing the data for IgA immunoreactivity to CMP, we detect statistically significant differences between results for RAU+ and RAU− subjects (Figure 2(A)). The level of anti-CMP IgA immunoreactivity, estimated using the Mann Whitney test, was significantly lower in RAU − subjects in comparison to RAU+ subjects ($p = .0039$). Statistical analyses show that there were no statistically significant differences between levels of anti-FGM and anti-FDM IgA immunity for these two tested groups of subjects (Figure 2(A)).

Statistical analysis of IgG immunoreactivity to different types of milk in RAU+ and RAU− subjects shows statistically significant differences between levels of IgG immunity to CMP and FGM for these two tested groups of subjects (Figure 2(B)). The levels of anti-CMP and anti-FGM IgG immunoreactivity, estimated using the Mann Whitney test, were significantly lower in RAU− subjects in comparison to RAU+ subjects ($p < .0001$; $p = .0002$). There were no differences between levels of anti-FDM IgG immunity for RAU+ and RAU− subjects.

Comparing the data for IgE immunoreactivity to CMP, FGM and FDM, we detect statistically significant differences between levels of immunoreactivity for the two tested groups of subjects (Figure 2(C)). The levels of anti-CMP, anti-FGM and anti-FDM IgE immunoreactivity, estimated using the Mann Whitney test, were significantly lower in RAU− subjects in comparison to RAU+ subjects ($p < .0001$; $p = .0057$; $p < .0001$), but with the lowest level of anti-FDM IgE antibodies.

Discussion

Immunity to CMP occurs in the patients with RAU (Besu et al., 2009). Due to frequent ulcerations the of the patients with RAU has been changed since they experience pain and suffer. CMP-restricted diet could be considered rational for these patients as a strategy to mitigate the immunological component of the disease. Results from our earlier study show that the exclusion of cow's milk protein from the diet of these subjects with RAU, with increased humoral immunoreactivity to CMP, led to the disappearance of oral changes (Besu et al., 2009). The possibility for RAU patients to safely use milk from other mammalian species has been examined and it was concluded that goat's milk

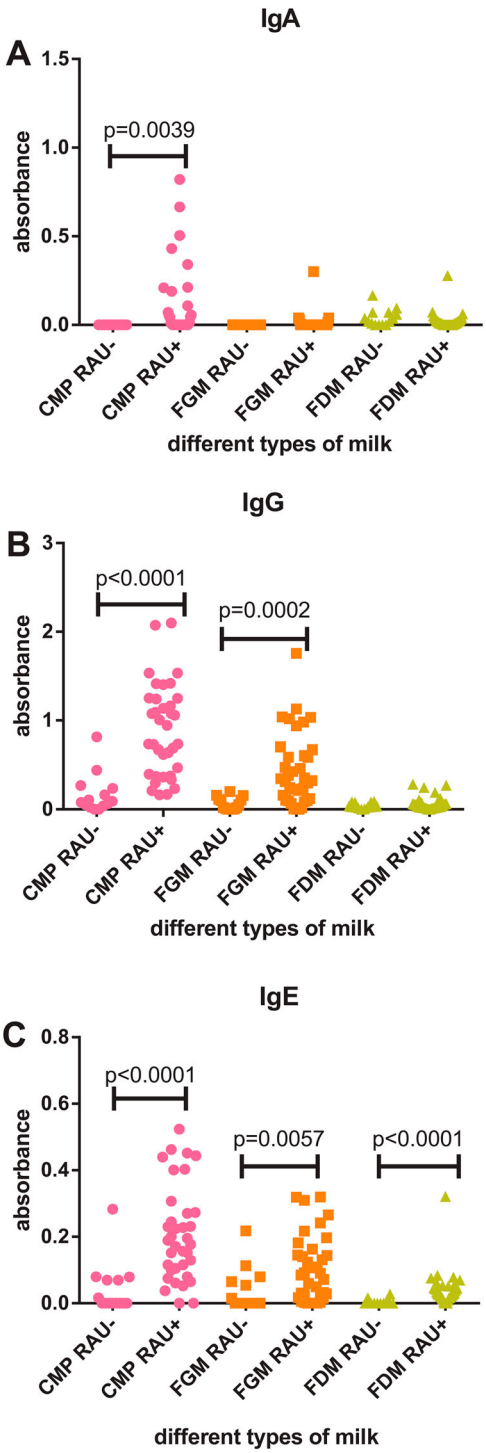

Figure 2. Serum IgA (A), IgG (B), IgE (C) immunoreactivity with different types of milk (CMP, cow's milk proteins; FGM, fresh goat's milk; FDM, fresh donkey's milk) determined for subjects with RAU with proven increased immunoreactivity to CMP (RAU+) and subjects with RAU without increased immunoreactivity to CMP (RAU−).

could be a replacement for most of the population although some of the RAU patients tested have shown increased level of antibodies to GMP, especially IgG and IgE isotypes (Besu et al., 2013). It was shown by the immunization of Guinea pigs with fresh cow and goat milk that cow milk possesses a greater sensitization capacity than goat milk than even in other mammalian species (Ceballos, Sampelayo, Extremera, & Osorio, 2010).

In search for optimal source of milk proteins for patients with RAU with increased immunoreactivity to CMP, we investigated immunoreactivity of RAU patients' sera to DMP.

The main nutritional and bioactive component of milk is proteins, which at the same time represent the predominant factors causing milk protein allergies. Milk proteins are divided into casein complexes and whey protein fractions. Depending of the species, the ratio between casein and whey proteins is different. The content of casein in cow milk is 2.46–2.80 g/100 g (Guo et al., 2007; Zicarelli, 2004); goat milk – 2.81 g/100 g (Leitner et al., 2004), while donkey and human milk are characterized by a low content of casein fraction 0.64–1.03 g/100 g and 0.32–0.42 g/100 g, respectively (Gubic et al., 2015; Guo et al., 2007). Guo et al. (2007) reported that the content of whey proteins in human milk is in the range of 0.68–0.83 g/100 g; in cow milk 0.55–0.70; and 0.49–0.80 in donkey milk. Donkey milk has the most comparable protein composition with human milk and the ratio between casein and whey proteins (Gubic et al., 2015).

The data obtained in this study could be, at least partly, explained by the lower amount of αS1-casein in donkey's milk which was previously hypothesized to underlie tolerance of goat's milk by some children allergic to CMP, as reported previously (Ellis, Short, & Heiner, 1991; Webber, Graham-Brown, Hutchinson, & Burns, 1989).

Lara-Villoslada, Olivares, and Xaus (2005) explained that the lower allergenicity of goat milk compared to cow milk is due to the fact that a lower share of αs1-casein reduces the sensitivity to another allergen protein, namely, β-lactoglobulin. According to Cunsolo et al. (2009), considerable differences might be found between the primary structure of donkey and bovine as1-casein, which could be related to the already demonstrated low allergenic properties of donkey milk and could contribute to better tolerance of donkey milk.

The study of Monti et al. (2007, 2012) found donkey milk to be a valid feeding solution in a selected population of children with cow's milk protein allergy, for whom soy-protein-based formulas, extensively hydrolysed formulas and amino-acid formulas could not be used to replace cow's milk and who, because of their concomitant multiple food allergies, required a substitute food that was palatable and tolerated, as well as being nutritionally valid.

Conclusion

Our study found FDM to be a valid feeding solution in a selected population of patients with RAU with immunoreactivity to CMP.

Additionally we have shown that there are no statistically significant differences between IgA immunoreactivity against FGM and FDM in RAU subjects who have increased immunoreactivity to CMP. This implies that patients with this type of IgA immunoreactivity could safely consume any goat's or donkey's milk. Finally, data presented that aligned with our previous data indicate that nutritional concealing is of great importance in the therapy of RAU, and that personalized nutrition should include restrictions only if it is supported with the assessment of immunological phenotype. After exploration of the impact of milk from different animal species on patients with

RAU with proven immunoreactivity to CMP, it was concluded that for every patient with this problem it would be good to do (on the basis of its results) an individual nutrition programme and select the best choice.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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