

Good tolerance to goat's milk in patients with recurrent aphthous ulcers with increased immunoreactivity to cow's milk proteins

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BACKGROUND: Recurrent aphthous ulcers (RAU) represent a very common, but poorly understood mucosal disorder. The connection between immunity to cow's milk proteins (CMP) and oral diseases was noted earlier. The goal of this study was to determine the prevalence of the increased levels of serum antibodies to goat's milk proteins (GMP), by enzyme-linked immunosorbent assay (ELISA) test, in subjects who have RAU and proven increased immunity to CMP.

METHODS: Fifty subjects with RAU (36 with proven increased immunity to CMP and 14 without this increased immunity) were included in this research. Levels of serum IgA, IgG, and IgE antibodies to the same quantity of the examined antigens were determined by ELISA. The statistical analysis of data was performed by Wilcoxon rank-sum test and Mann-Whitney test.

RESULTS: The levels of serum antifresh cow's milk IgA, IgG, and IgE antibodies were significantly higher than the levels of serum antifresh goat's milk, in subjects with RAU with proven increased immunoreactivity to CMP ($P = 0.0003$; $P < 0.0001$; $P < 0.0001$).

CONCLUSIONS: These results indicate that patients with RAU with increased immunity to CMP could consider the use of goat's milk as the alternative protein source.

and women of all ages, races, and geographic regions. It is estimated that at least one in five individuals has at least once been afflicted with aphthous ulcers (1, 2). The connection between immunity to cow's milk proteins (CMP) and oral diseases was noted by Taylor et al. (3), Thomas et al. (4), and Besu et al. (5).

Besu et al. (5) found that 36 of 50 subjects with RAU, who did not have any gastrointestinal or other systemic disease or abnormalities (they just often had recurrent oral ulcerations), had increased humoral (IgA and/or IgG and/or IgE) immunity to CMP. Cow's milk contains several proteins, which can be generally classified in two major groups: caseins and whey proteins (6). Results from work of Besu et al. (7) showed that the levels of anticasein CMP IgA, IgG, and IgE antibodies were significantly higher and more frequently found in subjects with RAU in comparison with healthy controls. This evidence of caseins immunogenicity is in accordance with several independent studies which have demonstrated that the casein fraction has an important antigenic potential (8–10), and it is proven that caseins maintain the capability of binding IgEs even after strong denaturing processes (9, 10).

Elevated humoral immunity to whey proteins was also (but not so frequently) found in these subjects with RAU (7). Because of the absence of β -lactoglobulin in human milk, this protein has been considered the most important cow's milk allergen for a long time (11, 12). It appears to be one of the major cow's milk allergens (13). It is relatively resistant to enzymatic degradation, and immunogenic protein has been, moreover, detected in the blood circulation after ingestion (14). It is often thought that thermal processing should decrease allergenicity (15) and indeed antigenicity of β -lactoglobulin can be partially eliminated by technological treatments (16), although some authors showed that β -lactoglobulin heated at 50°C in the presence of lactose acquired a 100-fold increase in skin reactivity (17). Thermal processing (or even storage in the presence of oxygen) can induce formation of new antigens with simultaneous loss of

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Introduction

Recurrent aphthous ulcers (RAU) represent a very common, but poorly understood mucosal disorder. They occur in men

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epitopes, usually conformational, present in non-treated proteins. These changes could increase, decrease, or could have no overall effects on allergenicity, with the important role of treatment type, food matrix but also the immunologic state of the host (18).

Because goat's milk is similar to cow's milk but with evident reduction in α -casein fraction (6), the goals of this study were the following: (i) To test subjects with RAU with proven increased humoral (IgA and/or IgG and/or IgE) immunity to CMP (RAU+) to goat's milk proteins (GMP), (ii) Comparing the results of testing to GMP for two groups of subjects with RAU (RAU with increased immunoreactivity to CMP (RAU+) and RAU without increased immunoreactivity to CMP (RAU-), (iii) Elucidating the role of technological processing (heat treatments) on decreasing of immunogenicity of milk proteins (cow's and goat's milk).

Materials and methods

Studied population

Fifty subjects (19 males and 31 females; 39.4 ± 19.39 (average age $\pm$ SD) with RAU were examined at the Clinics of Periodontology and Oral Medicine, Faculty of Stomatology, University of Belgrade. Subjects with RAU did not have any gastrointestinal or other systemic disease or abnormalities, but they often had recurrent oral ulcerations. All the subjects are Serbian inhabitants, and their sera were collected during the period of 2 years (2007–2009). Ethical permission was obtained from the Ethical Committee of Faculty of Stomatology, University of Belgrade. The experiments were carried out with the comprehension and written consent of each subject.

Sera of these subjects were tested earlier to CMP (5), 36 of them (RAU+) had increased humoral IgA, IgG, or IgE immunoreactivity to CMP, and 14 of them (RAU-) did not have increased humoral immunity to CMP. Later, these subjects were tested to specific cow's milk proteins (SCMP), constituents of cheese or of whey (7). The results of this investigation indicate the strong association between high levels of serum anti-SCMP IgA, IgG, and IgE antibodies, especially to caseins: α -, β -, and κ -casein from cow's milk and clinical manifestations of RAU (7).

Enzyme-linked immunosorbent assay (ELISA)

Five kinds of antigens were used: skimmed cow's milk pasteurized powder (ICN Biomedicals, Inc. Cosa Mesa, CA, USA), fresh goat's milk (FGM), fresh cow's milk (FCM), boiled goat's milk (BGM), and boiled cow's milk (BCM). Boiling procedure included gradient heating up to 100°C. Loss of water induced by heating was compensated subsequently by the addition of distilled water. Total protein content in milk samples was determined based on the nitrogen content according the Kjeldahl method (19). Protein content in boiled milk did not differ compared with the raw milk.

Determination of IgA, IgG, and IgE serum's immunoreactivity to CMP, FGM, FCM, BGM, and BCM has been carried out by homemade ELISA tests.

Individual Steps in ELISA

- 1 Coating: Based on the protein content, analyzed milk samples were dissolved in bicarbonate buffer to the final concentration of 100 μ g/ml. Hundred microliters of antigen solution, providing 10 μ g of protein, were added to the wells of polystyrene microtiter plate (F96 MaxiSorp – NUNC Immuno Plates; Thermo Fisher Scientific Inc., Rockford, IL, USA). Coated wells were incubated at +4°C temperature for 24 h enabling antigens to bind to the polystyrene surface. Next day, solutions containing unbound antigens were removed with aspiration and three consecutive washings with TTBS buffer (0.05% Tween-20 in 20 mM Tris-HCl, 500 mM NaCl, pH 7.5).
- 2 Blocking solution: Free sites on polystyrene surface were blocked with 1% bovine serum albumin (BSA; Sigma Chemical Co, Saint Louis, MO, USA) during 1 h at 37°C. Afterward, blocking solution was aspirated, and wells were washed for five times with TTBS buffer.
- 3 Application of examined sera: 50 μ l of diluted (1:100) subjects' sera were dispensed in appropriate wells and incubated for 1 h at +37°C with subsequent aspiration and washings (four times).
- 4 Application of secondary antibodies: Cetermination of IgA, IgG, and IgE serum's immunoreactivity has been carried out using sheep anti-human IgA, IgG (Binding Site, Birmingham, UK), and IgE (Sigma Chemicals Co), horseradish peroxidase (HRP)-labeled antibodies as secondary antibodies, which bind to the captured human autoantibodies. Plates were incubated for 1 h at +37°C and after that the excess unbound conjugate was removed by washings (five times).
- 5 Development of reaction: A substrate solution TMB (3,3',5,5'-tetramethylbenzidine) (INEP, Zemun, Serbia) was added to the wells (100 μ l) and after the incubation for 15 min, the enzyme reaction was terminated by addition of H₂SO₄ (50 μ l). The optical density (OD) of developed color was measured using microplate reader (Multiskan EX; Thermo Scientific, Waltham, MA, USA) at 450 nm. The absorbance of blank wells (with primary and secondary antibodies but without tested antigens) was always subtracted from the absorbance of tested sample (with antigens). Between-assay variability was corrected by the use of the one control patient's serum (for IgA, IgG, or IgE) and following standardization of obtained OD according to the OD values of control serum. Control serums used for IgA, IgG, or IgE standardization were obtained from subjects based on their high anti-cow's milk immunoreactivity.

Statistical analysis

For testing the differences between parameters, the Wilcoxon rank-sum test and Mann-Whitney test were used.

Statistical package R (20) was used for data processing. Microsoft Office Excel (Microsoft Corporation, Redmond, WA, USA) 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 skimmed cow's milk pasteurized powder (CMP) were tested to CMP again and then to FGM, FCM, BGM, and BCM. Statistical analysis of obtained data reveals that the level of anti-FCM IgA immunoreactivity estimated using Wilcoxon rank-sum test was significantly higher than the anti-FGM immunoreactivity in RAU+ subjects ($P = 0.0003$). Statistical analysis shows that it was not statistically significant differences between data for FGM and BGM and data for FCM and BCM (Fig. 1A).

Data for IgA immunoreactivity to different tested types of milk were generally lower for all patients than data for IgG or IgE immunoreactivity (causing the overlapping of dots in the chart).

Statistical analysis of IgG immunoreactivity to different types of milk in RAU+ patients shows statistically significant differences between data for FCM versus. FGM, FCM versus. BCM, FGM versus. BGM. The level of anti-FCM IgG immunoreactivity estimated using Wilcoxon rank-sum test was significantly higher than the anti-FGM immunoreactivity in RAU+ subjects ($P < 0.0001$), the level of anti-BCM IgG immunoreactivity was significantly higher than the level of anti-FCM immunoreactivity in RAU+ subjects ($P = 0.0166$), and the level of anti-BGM IgG immunoreactivity was significantly higher than the level of anti-FGM immunoreactivity in RAU+ subjects ($P = 0.0052$) (Fig. 1B).

Testing of IgE immunoreactivity to different types of milk in RAU+ patients shows statistically significant differences between data for FCM versus. FGM and data for FCM versus. BCM. The level of anti-FCM IgE immunoreactivity was significantly higher than the level of anti-FGM IgE immunoreactivity ($P < 0.0001$). The level of anti-BCM IgE immunoreactivity was significantly higher than the level of anti-FCM immunoreactivity in RAU+ subjects ($P < 0.0001$) (Fig. 1C).

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 FCM.

Comparing the data for IgA immunoreactivity with CMP, FGM, and FCM, we detect statistically significant differences between results for RAU+ and RAU- subjects (Fig 2A). The levels of anti-CMP and anti-FCM IgA immunoreactivity, estimated using Mann–Whitney test, were significantly higher in RAU+ subjects in comparison with RAU- subjects ($P = 0.0039$; $P = 0.0061$). Statistical analysis shows that it was not statistically significant differences between levels of anti-FGM IgA immunity for these two tested groups of subjects (Fig. 2A).

Statistical analysis of IgG immunoreactivity to different types of milk in RAU+ and RAU- subjects shows statistically significant differences between levels of IgG immunity

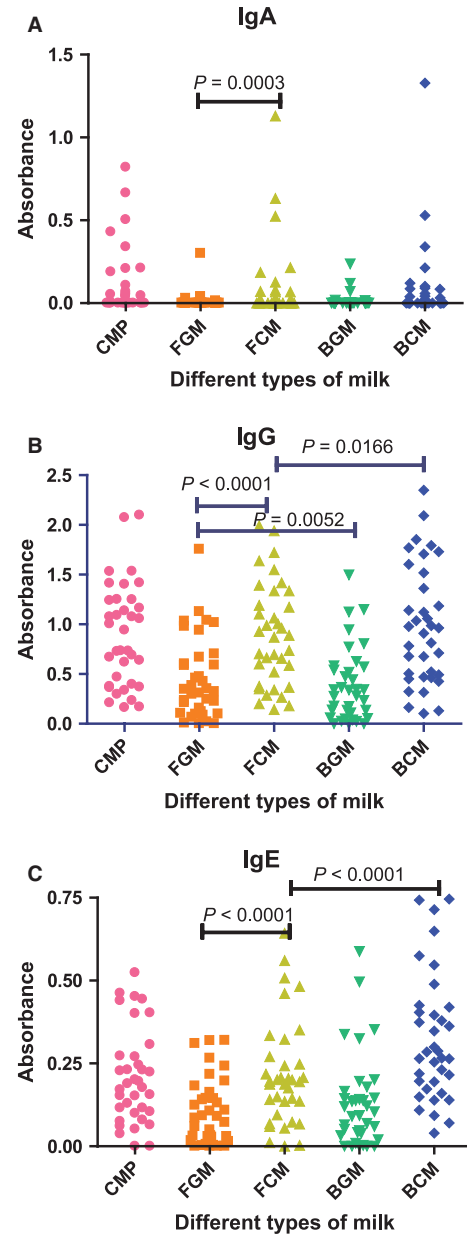


Figure 1 Serum IgA (A), IgG (B), IgE (C) immunoreactivity with different types of milk (CMP, cow's milk proteins; FGM, fresh goat's milk; FCM, fresh cow's milk; BGM, boiled goat's milk; BCM, boiled cow's milk) determined for subjects with recurrent aphthous ulcers (RAU) with proven increased immunoreactivity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

to FCM in comparison with FGM (Fig. 2B). The levels of anti-CMP, anti-FCM, and anti-FGM IgG immunoreactivity, estimated using Mann–Whitney test, were significantly higher in RAU+ subjects in comparison with RAU- subjects ($P < 0.0001$; $P = 0.0002$; $P < 0.0001$).

Comparing the data for IgE immunoreactivity with CMP, FGM, and FCM, we detect statistically significant differences between levels of immunoreactivity for two tested groups of subjects (Fig. 2C). The levels of anti-CMP, anti-FCM, and anti-FGM IgE immunoreactivity, estimated using Mann–Whitney test, were significantly higher in RAU+

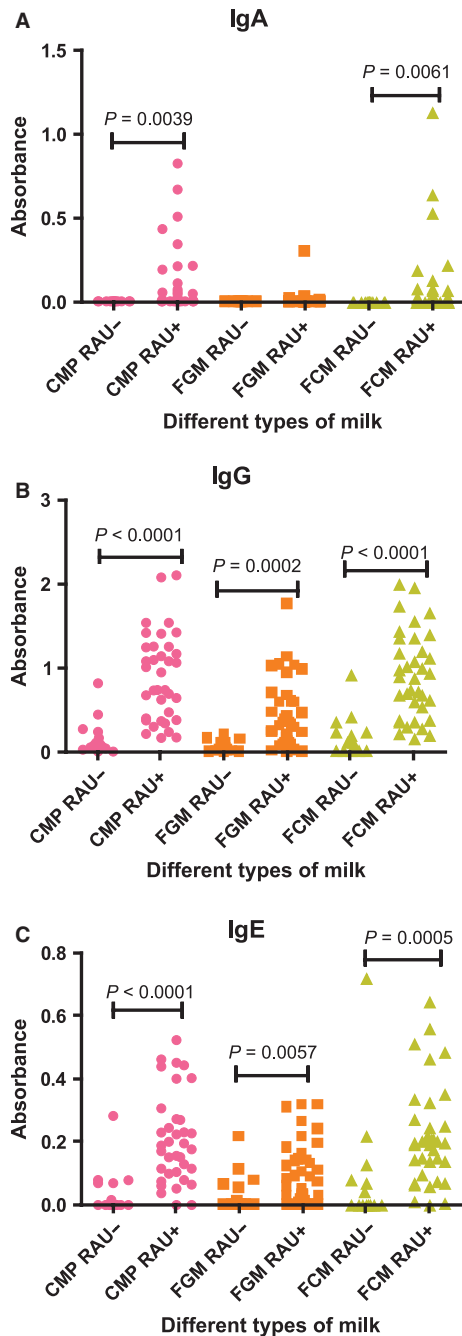


Figure 2 Serum IgA (A), IgG (B), IgE (C) immunoreactivity with different types of milk (CMP, cow's milk proteins; FGM, fresh goat's milk; FCM; fresh cow's milk) determined for subjects with recurrent aphthous ulcers (RAU) with proven increased immunoreactivity to CMP (RAU+) and subjects with RAU without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

subjects in comparison with RAU- subjects ($P < 0.0001$; $P = 0.0057$; $P = 0.0005$).

Discussion

Restani et al. (6) evaluated the cross-reactivity between milk proteins from different animal species. In their study, when the sera of six children highly allergic to cow's milk

were used in the immunoblotting technique, it was possible to show the formation of IgE-antigen complexes with all milk tested apart from camel's milk. Compared with cow's milk, three of six sera in this study, showed a lower reaction to goat's milk. This result could be justified by the lower amount of α -casein and could explain why some allergic children are able to tolerate goat's milk, as reported previously in studies of Ellis et al. (21) and Webber et al. (22). Restani et al. (6) observed that cow's and buffalo's milk present a similar protein composition; ewe's and goat's milk are similar to cow's milk, but with evident reduction in α -casein fraction. Camel's milk shows a different pattern with numerous bands in the area of caseins and the absence of β -lactoglobulin.

In our study, we tested sera of 36 subjects with RAU, who earlier showed increased immunoreactivity (RAU+) to CMP, (5) and revealed that the level of anti-FCM IgA, IgG, and IgE immunoreactivity was significantly higher than the anti-FGM immunoreactivity in these patients. That finding is in accordance with results of Restani et al. (6), because ewe's and goat's milk are similar to cow's milk but with evident reduction in α -casein fraction. In our earlier study (7), we point to the strong association between the presence of high levels of serum IgG and IgE antibodies, especially to α - and β - and κ -casein from CMP and clinical manifestations of RAU.

Umpierrez et al. (23) describe the case of a patient who had allergic reactions after eating goat cheese and after touching goat and sheep cheese, but not after consuming cow's milk or cow's milk dairy products. The results of ELISA inhibition in this study suggest that goat casein shows a high degree of cross-reactivity with sheep casein and, to a much lesser extent, with cow casein. This conclusion is also in accordance with our results, because our RAU+ patients, who had increased immunoreactivity to CMP, didn't show increased immunity to goat's milk. We also test RAU- patients to goat's milk, and this testing was a good check to elucidate whether these patients have an hidden increased immunity to goat's milk, because they did not have increased immunoreactivity to cow's milk. Results of this study show that these 14 RAU- subjects were without increased immunity neither the CMP nor the GMP.

Regarding the influence of thermal treatment on the immunoreactivity to different milk samples, the levels of antiboiled cow's and anti-BGM IgG immunoreactivity in RAU+ subjects were significantly higher than the levels of anti-fresh cow's and anti-FGM immunoreactivity. Also, the level of anti-BCM IgE immunoreactivity in RAU+ subjects was significantly higher than the level of anti-FCM.

Although heat treatment of food has numerous advantages mostly targeted to the safety for consumers, its influence on allergenicity is also well recognized. The precise outcomes are still undefined (18) and depend on type of proteins in raw food, type of the treatment, or food matrix. It is shown previously that caseins maintain the capability of binding IgEs even after strong denaturing processes (9, 24). Regarding β -lactoglobulin, it was shown that immunoreactivity against this food protein can be partially eliminated by heating treatments (16). At the same time, heat treatment (at/over 100°C) of proteins within food matrices could induce elevation in immunoreactivity,

targeted probably to the glycated proteins formed in the reaction of amino acids with reducing sugars (Maillard reaction) (17). The difference in IgA, IgG, IgM, and IgE immunoreactivity to raw and processed food, including raw milk and processed milk-containing products, was also shown by Vojdani et al. (2009) (25) implicating also again the role new antigens formed as a products of Maillard reaction (18), together with the hypothetic influence of cross-reactivity of formed antibodies to the advanced glycation end products (AGE) of the proteins within human body and influence of this cross-reactivity in different pathologies.

It is difficult to suggest drinking of unprocessed milk, because it is an individual decision which each person has to decide itself; we only suggest that if somebody uses unprocessed milk, the animal from which the milk is, must be absolutely healthy.

In conclusion, these results indicate that patients with RAU with increased immunity to CMP could consider the use of goat's milk as the alternative protein source. From this point of view, this finding is of great importance, because appropriate diet, but not totally restricted (without cow's milk, but with goat's milk) could be the ideal resolution for these patients. The role of technological processes of milk, such as heat treatment or hydrolysis, on diet regime, indicates the need for further investigations.

References

1. Ship JA, Chavez EM, Doerr PA, Henson BS, Sarmandi M. Recurrent aphthous stomatitis. *Quintessence Int* 2000; **31**: 95–112.
2. Nolan A, McIntosh WB, Allam BF, Lamey PJ. Recurrent aphthous ulceration: vitamin B1, B2, and B6 status and response to replacement therapy. *J Oral Pathol Med* 1991; **20**: 389–91.
3. Taylor KB, Truelove SC, Wright R. Serologic reactions to gluten and cow's milk proteins in gastrointestinal disease. *Gastroenterology* 1964; **46**: 99–108.
4. Thomas HC, Ferguson A, McLennan JG, Mason DK. Food antibodies in oral disease: a study of serum antibodies to food proteins in aphthous ulceration and other oral diseases. *J Clin Pathol* 1973; **26**: 371–4.
5. Besu I, Jankovic L, Magdu IU, Konic-Ristic A, Raskovic S, Juranic Z. Humoral immunity to cow's milk proteins and gliadin within the etiology of recurrent aphthous ulcers? *Oral Dis* 2009; **15**: 560–4.
6. Restani P, Gaiaschi A, Plebani A, et al. Cross-reactivity between milk proteins from different animal species. *Clin Exp Allergy* 1999; **29**: 997–1004.
7. Besu I, Jankovic Lj, Konic-Ristic A, et al. The role of specific cow's milk proteins in the etiology of recurrent aphthous ulcers. *J Oral Pathol Med* 2013; **42**: 82–8.
8. Savilahti E, Kuitunen M. Allergenicity of cow milk proteins. *J Pediatr* 1992; **121**: S12–20.
9. Restani P, Velona T, Plebani A, et al. Evaluation by SDS-PAGE and immunoblotting of residual antigenicity in hydrolysed protein formulas. *Clin Exp Allergy* 1995; **25**: 651–8.
10. Docena GH, Fernandez R, Chirido FG, Fossati CA. Identification of casein as the major allergenic and antigenic protein of cow's milk. *Allergy* 1996; **51**: 413–6.
11. Goldman AS, Sellars WA, Halpern SR, Anderson DW, Furlow TE, Johnson CH Jr. Milk allergy. II. Skin testing of allergic and normal children with purified milk proteins. *Pediatrics* 1963; **32**: 572–9.
12. Gjesing B, Osterballe O, Schwartz B, Wahn U, Lowenstein H. Allergen-specific IgE antibodies against antigenic components in cow's milk and milk substitutes. *Allergy* 1986; **41**: 51–6.
13. Wal JM. Bovine milk allergenicity. *Ann Allergy Asthma Immunol* 2004; **93**: S211.
14. Husby S, Host A, Teisner B, Svehag SE. Infants and children with cow milk allergy/intolerance. Investigation of the uptake of cow milk protein and activation of the complement system. *Allergy* 1990; **45**: 547–51.
15. Malanin K, Lunberg M, Johansson SGO. Anaphylactic reaction caused by neoallergens in heated pecan nut. *Allergy* 1995; **50**: 988–91.
16. McLaughlan P, Anderson K, Widdowson E, Coombs R. Effect of heat on the anaphylactic sensitizing capacity of cow's milk, goat's milk and various infant formulae fed to guinea pig. *Arch Dis Child* 1981; **56**: 165–71.
17. Bleumink E, Berrens L. Synthetic approaches to the biological activity of beta-lactoglobulin in human allergy to cow's milk. *Nature* 1966; **212**: 541–3.
18. Davis PJ, Smales CM, James DC. How can thermal processing modify the antigenicity of proteins? *Allergy* 2001; **56**: 56–60.
19. Kjeldahl JA. *Anal Chem* 1883; **22**: 366–82.
20. R Development Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2008. ISBN 3-900051-07-0. Available at: <http://www.R-project.org> (accessed on 15 November 2008).
21. Ellis MH, Short J, Heiner DC. Anaphylaxis after ingestion of a recently introduced hydrolysed whey protein formula. *J Pediatr* 1991; **118**: 74–7.
22. Webber SA, Graham-Brown RAC, Hutchinson PE, Burns DA. Dietary manipulation in childhood atopic dermatitis. *Br J Dermatol* 1989; **121**: 91–8.
23. Umpierrez A, Quirce S, Maranon F, et al. Allergy to goat and sheep cheese with good tolerance to cow cheese. *Clin Exp Allergy* 1999; **29**: 1064–8.
24. Høst A. Cow milk allergy in infants. *North Eur Food Dairy J* 1987; **9/10**: 302–4.
25. Vojdani A. Detection of IgE, IgG, IgA and IgM antibodies against raw and processed food antigens. *Nutr Metab (Lond)* 2009; **6**: 22.

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Conflict of interest

None declared.