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Comparative evaluation of allergic sensitization to milk proteins of cow, buffalo and goat

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ABSTRACT

Milk hypersensitivity due to adverse reactions to milk proteins is among the major public concern of food allergies. In present investigation, comparative assessment of humoral and cell mediated immune responses in mice associated with allergenicity of major milk proteins (casein and β -lactoglobulin) of cow, buffalo and goat were carried out. Caseins of all three species of dairy animals appeared to be less immunogenic and allergenic than their corresponding β -lactoglobulins. Cow milk proteins (caseins and β -lactoglobulin) were observed to increase protein specific IgE sensitization as well as lymphocyte proliferation index significantly ($P < 0.01$) as compared to buffalo and goat milk proteins. On the other hand, buffalo and goat milk proteins similar to cow's proteins raised remarkably higher ($P < 0.01$) immunogenic response in terms of IgG levels than the negative control group. The results obtained were correlated with B and T cell epitopes of milk proteins cited in literature after multiple alignment *in silico*. Thus present study suggests cow milk proteins (β -lactoglobulin and casein) are highly allergenic among the milk proteins of three species of dairy animals.

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1. Introduction

Milk is considered nature's most perfect food as it contains almost all the substances essential for human nutrition. Consumption of dairy products is also associated with beneficial health effects beyond its pure nutritional value (Silanikove et al., 2009, 2010). However, cow's milk is also among the most common causes of food allergy in the early years of life. Approximately 1–2% of the adult population and up to 8% of children below the age of three (Helm and Burks, 2000) suffers from cow's milk hypersensitivity. It is an adverse immunological response to cow's milk proteins affecting the cutaneous, intestinal or respiratory systems. Clinical symptoms involve immediate or delayed reactions either due to IgE dependent or cell mediated

type IV hypersensitivity. Early infancy is a critical period for the development of immunological memory, wherein the T helper (Th) balance converts from the Th2-skewed immunity to Th1 cell-type responses under the influence of genetic and environmental factors. Studies suggest that postnatal maturation of the immune system in atopic individuals is attenuated, and atopic infants show an imbalance in Th1/Th2 immune responses by excessive IgE responses (Holt and Jones, 2000). A number of regulatory T-cells appear to be involved in regulating Th responses, and defects in regulatory T-cell responses have been linked to allergic diseases (Stock et al., 2006; Taylor et al., 2006; Wing and Sakaguchi, 2006). The action of allergens is based on their recognition by IgE (or possibly IgG) through linear or conformational epitopes (B cell epitopes). So far, no general structural characteristic of allergens have been discovered: linear epitopes differ in the amino acid number and sequence and an exchange of a single amino acid may lead to disappearance of IgE binding, and further there is no general conformational pattern discernible. Since there

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are no medical treatments currently available for curing food allergies, the best way to prevent unintended exposure to a food allergen is the complete avoidance of the offending food. For various reasons such avoidance may not always be possible, and in certain instances, impossible. Although the gross composition of cow's, buffalo's and goat's milk is very similar, slight differences in protein fractions result in changes in immunological and physico-chemical properties. Human milk proteins have many significant differences in their amino acid composition compared to the milk proteins of animals. Children with a high-risk of atopy or diagnosed cow milk allergy sometimes receive food substitutes. This can include other milk source proteins such as formulae containing goat milk with altered proportion of casein content and/or soy milk, cow milk hydrolysates with destroyed allergenic epitopes or amino-acid based formulae (Walker-Smith, 2003; El-Agamy, 2007; Silanikove et al., 2010) to reduce allergic reactions. It is therefore interesting to consider the adequacy of those alternatives by comparing their biophysical and allergenic properties to cow milk. Similarly the true prevalence of cow and goat milk allergy is not exactly known and is surrounded by controversy. Moreover, no relevant scientific data regarding allergenic sensitization with buffalo milk is available. So the present study has been carried out to compare the allergenic sensitization to major milk proteins (caseins and β -lactoglobulin) of cow, buffalo and goat.

2. Materials and methods

2.1. Mice

Three to four-week-old male Swiss albino mice were maintained in the Small Animal House of the National Dairy Research Institute (N.D.R.I.), India. The animals were housed in plastic polypropylene cages and kept at room temperature in a sterilized condition and were placed on a special milk-free diet. All procedures were approved by the Institutional Animal Ethical Committee.

2.2. Milk sample collection

Whole cow milk was procured from Experimental Dairy Plant (EDP), National Dairy Research Institute, Karnal. Buffalo and goat milks were procured from cattle yard, N.D.R.I., Karnal.

2.3. Isolation of milk proteins

2.3.1. Total caseins (CSN)

Separation of CSN was carried out by isoelectric precipitation method. In brief, collected cow, buffalo and goat milk were centrifuged at $5000 \times g$ for 20 min at 4°C , to obtain skim milk. Immediately after collection, skim milk was warmed up to 40°C , pH was adjusted to 4.6 in case of cow and buffalo milk and 4.1 for goat milk respectively with 1 N HCl followed by stirring for 30 min. The resultant precipitate was separated by filtration through four layers of cheese cloth and washed three to four times with distilled water, until the pH came to 7.0.

2.3.2. β -Lactoglobulin (β -LG)

Separation of β -LG was carried out by the method described earlier Alomirah and Alli (2004). Briefly, whey solution collected after the removal of precipitated CSN was clarified from fine CSN particles by centrifugation at $5000 \times g$ for 15 min at 4°C . The whey proteins except β -LG were precipitated by the addition of 7% NaCl at pH 2.0 after 20 min of incubation at room temperature. β -LG remained in supernatant after the removal of precipitated whey proteins by centrifugation at $10,000 \times g$, 15 min, at 4°C . The precipitation process was repeated thrice to remove all traces of other whey proteins from β -LG solution. Sugar and salts were

removed from this preparation by dialysis using 8 kDa cutoff cellulose ester membrane at 4°C for 24 h. β -LG preparation was concentrated by lyophilization at -40°C for 48 h.

2.4. SDS-PAGE of fractionated milk proteins for purity analysis

The concentration of proteins in various samples was estimated by the method of Lowry et al. (1951). The purity of fractionated milk proteins (CSN and β -LG) were checked by SDS-PAGE using 15% resolving gel of polyacrylamide by the method of (Laemmli, 1970) loading equal concentration ($20 \mu\text{g}/\text{well}$) of fractionated milk protein in each well.

2.5. Experiment design and sample collection

Six groups of six mice each were sensitized thrice at weekly intervals by intraperitoneal (i.p.) injection of $20 \mu\text{g}/\text{animal}$ of whole CSN or β -LG obtained from cow, buffalo and goat milk using 2% of $\text{Al}(\text{OH})_3$ as adjuvant in PBS. Negative control group mice were only given adjuvant in PBS while positive control mice were sensitized with same dose of ovalbumin. One week after the last injection, mice were sacrificed and blood was obtained by cardiac puncture and sera were separated by centrifugation at $1500 \times g$ for 10 min. Sera collected were stored at -20°C until further analysis. Spleen was collected after opening the peritoneal cavity and transferred to 15 ml of sterile centrifuge tube containing 2 ml of RPMI-1640 media. It was washed and made free from connective tissue and adipose tissue.

2.6. Measurement of total IgE and IgG levels

Total IgE and IgG in serum were determined by using ELISA kit (Koma Biotech, Seoul, Japan). Operating procedures were strictly followed according to the manufacturer's instructions. In brief, total IgE and total IgG levels from serum were determined using quantitative sandwich ELISAs. To detect these antibodies, plates were coated with $1 \mu\text{g ml}^{-1}$ of goat anti-mouse IgE or IgG, respectively. Serial dilutions of serum were added and then followed by the addition of $100 \mu\text{l}$ of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgE or IgG. Plates were allowed to develop with the TMB substrate (3,3',5,5'-tetramethyl diamine benzidine containing 0.03% H_2O_2) and reaction was finally stopped with $100 \mu\text{l}$ of 2 M H_2SO_4 . Plates were read at 450 nm against 630 nm as reference wavelength.

2.7. Measurement of milk protein-specific serum IgE titers

Whole CSN and β -LG specific IgE antibodies were detected using enzyme-linked immunosorbent assay (ELISA). Plastic microtiter plates (Nunc, Copenhagen, Denmark) were coated with $100 \mu\text{g}/\text{ml}$ of protein (CSN or β -LG) in PBS by overnight incubation at 4°C . The plates were blocked by incubation for a further 1 h at 37°C with 1% BSA (Sigma Chemical Co., St. Louis, MO) in PBS (for plates containing CSN) or 1% fish gelatin (for the plates containing WP). Mouse serum samples diluted two times serially in PBS were added to wells and incubated for 1 h at 37°C followed by washing with PBS containing 0.05% Tween 20 and further incubation for 1 h at 37°C with peroxidase-labeled goat anti-mouse IgE diluted 1:10,000 in PBS. Enzyme substrate TMB was added to each well and the reaction stopped after 15 min by the addition of $100 \mu\text{l}$ of 2 M H_2SO_4 . Substrate conversion was measured as optical density at 450 nm using an automated ELISA reader. Antibody titer was determined by dilution ($\log 2$) till absorbance reached 0.150 O.D value.

2.8. Lymphocyte proliferation index (LPI)

Aseptically collected spleen tissues were gently teased with sterile needles and forceps to release splenocytes into the RPMI-1640 media. Tissue suspensions were allowed to stand for 2 min to sediment large tissue clumps. The upper portion containing splenocytes was collected and centrifuged at $1000 \times g$ for 5 min at 4°C . Suspension was incubated with erythrocyte lysis buffer (0.17 M Tris-HCl and 0.16 M NH_4Cl , pH 7.2) for 1 min and washed twice by centrifuging as above with RPMI-1640 medium. Cell viability was checked by trypan blue (0.4% solution). Viable splenocytes (10^7 cells/ml) were finally cultured in heat inactivated 10% fetal calf serum enriched RPMI-1640 medium supplemented with mitogens (LPS ($50 \mu\text{g ml}^{-1}$) or Con A ($25 \mu\text{g ml}^{-1}$) or $50 \mu\text{g ml}^{-1}$ isolated milk proteins (β -LG or CSN) of cow, buffalo and goat, respectively and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 48 h. Then, $10 \mu\text{l}$

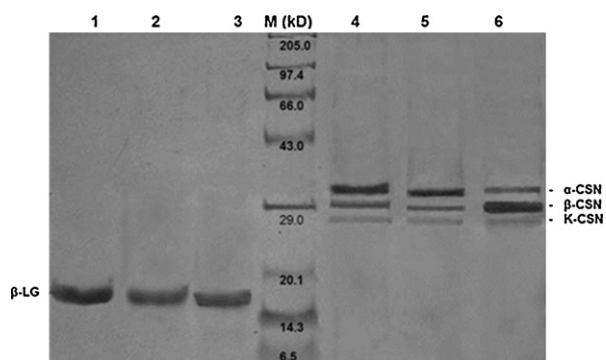


Fig. 1. SDS-PAGE of fractionated milk proteins. Lane 1: Cow β -LG. Lane 2: Buffalo: β -LG. Lane 3: Goat β -LG. M: M.W. marker. Lane 4: Cow CSN. Lane 5: Buffalo CSN. Lane 6: Goat CSN.

of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg ml^{-1}) was added in each well and incubated for 4 h at 37°C . Acidified isopropanol ($100 \mu\text{l}$ of 0.1 N HCl in anhydrous isopropanol) was added and mixed thoroughly to dissolve the dark blue crystals of formazan. Formazan quantification was performed using an ELISA plate reader with 540 nm test and 630 nm reference wavelengths.

2.9. Statistical analysis and multiple sequence alignment

The experimental data were analyzed for significant difference of cow, buffalo and goat milk by the analysis of variance (ANOVA). The CLUSTALW at European Bioinformatics Institute [<http://www.ebi.ac.uk/Tools/clustalw>] was used for generating a multiple sequence alignment [MSA] of CSN (α S1, S2 and β -CSN) and β -LG protein sequences from three different animal species.

3. Results

3.1. SDS-PAGE

SDS-PAGE profile of fractionated milk proteins (CSN and β -LG) is shown in Fig. 1 depicting their purity. Densitometry analysis of this profile also clearly showed that cow and buffalo milk had high amount of α -CSN (45.4 and 44.8%) followed by β -CSN (35.2 and 35.8%) and κ -CSN (19.4 and 19.2%) respectively. On the other hand, goat milk appeared to have more concentration of β -CSN (60.8%) than α -CSN (25.4%) and κ -CSN (13.8%) respectively though same concentrations of total casein protein ($20 \mu\text{g/well}$) were loaded on the gel.

3.2. Humoral response

3.2.1. IgE mediated sensitization

Total serum IgE measured in negative control group was observed to be $133.95 \pm 24.04 \text{ ng/ml}$ (Fig. 2A). There was a remarkable increase ($P < 0.01$) of 277.90% of total IgE level in positive control group (mice injected with ovalbumin). The β -LG test groups had shown considerable increase ($P < 0.01$) of 208.40%, 110.49% and 271.65% of total serum IgE when sensitized with cow, buffalo and goat milk proteins, respectively as compared to negative control. Likewise, cow CSN exhibited considerable increase of 142.43% ($P < 0.01$) in total IgE whereas it increased non significantly by 66.26 and 17.76% on sensitization with buffalo and goat CSN respectively than control. Milk protein

specific IgE titers are shown in Fig. 2B. In case of protein specific titers of IgE, the animals of positive control showed the maximum titer that was remarkably higher ($P \leq 0.01$) than milk protein sensitized groups. Among milk protein specific IgE, cow milk proteins (CSN and β -LG) and goat β -LG sensitized groups exhibited remarkably higher ($P \leq 0.01$) values while buffalo milk proteins (CSN and β -LG) and goat CSN sensitized groups had comparatively reduced titer values.

3.2.2. IgG mediated sensitization

Total IgG in mice on sensitization with milk proteins (CSN and β -LG) are shown in Fig. 2C. There was a 128.24% increase in total IgG level in positive control (mice injected with ovalbumin) as compared to negative control. After immunization with β -LG of cow, buffalo and goat caused total IgG level to increase 149.78%, 158.35% and 130.40%, respectively which was significantly higher ($P < 0.01$) from the negative control. Likewise, a significant ($P < 0.01$) increase of 75.63%, 51.62% and 72.03% in total IgG was observed in cow, buffalo and goat CSN sensitized groups, respectively as compared to negative control. Moreover, the levels of total IgG in CSN sensitized groups were considerably less than β -LG sensitized groups.

3.3. Cell mediated response

3.3.1. In the presence of milk proteins (CSN and β -LG)

Addition of isolated milk proteins (CSN and β -LG) of buffalo and goat in the cultured splenocytes of negative control animals did not bring any statistical changes ($P \leq 0.05$) in the LPI but addition of cow milk proteins (CSN and β -LG) increased LPI significantly ($P \leq 0.01$) indicating their strong mitogenic property similar to positive control (ovalbumin) (Fig. 3A). Likewise, addition of cow proteins (CSN and β -LG) and goat β -LG in splenocytes of pre-sensitized animals with the respective protein also showed considerably ($P < 0.05$) higher LPI as compared to negative control, buffalo milk proteins (CSN and β -LG) and goat CSN (Fig. 3B).

3.3.2. In the presence of mitogens

Addition of LPS in cultured splenocytes obtained from milk protein sensitized mice considerably ($P < 0.01$) increased the LPI by 68.1, 49.57 and 51.78% in the cow, buffalo and goat β -LG sensitized groups respectively as compared to negative control group (Fig. 3C). Likewise, CSN sensitized groups exhibited LPI increase by 50.91, 23.68 and 17.04% as compared to negative control. On the other hand addition of ConA significantly ($P < 0.01$) increased LPI of cow and buffalo β -LG sensitized animals as compared to nonsignificant changes in goat β -LG and CSN of all the three species of dairy animals (Fig. 3D).

3.4. In silico analysis of identical sequences and antigenic epitopes

160 amino acids of β -LG, 224 amino acids of β -CN, 214 amino acids of α S1 CSN and 223 amino acids α S2 CSN in cow, buffalo and goat share identical sequence alignments (Fig. 4). Multiple sequence alignments were obtained using the software ClustalW. Similarity and

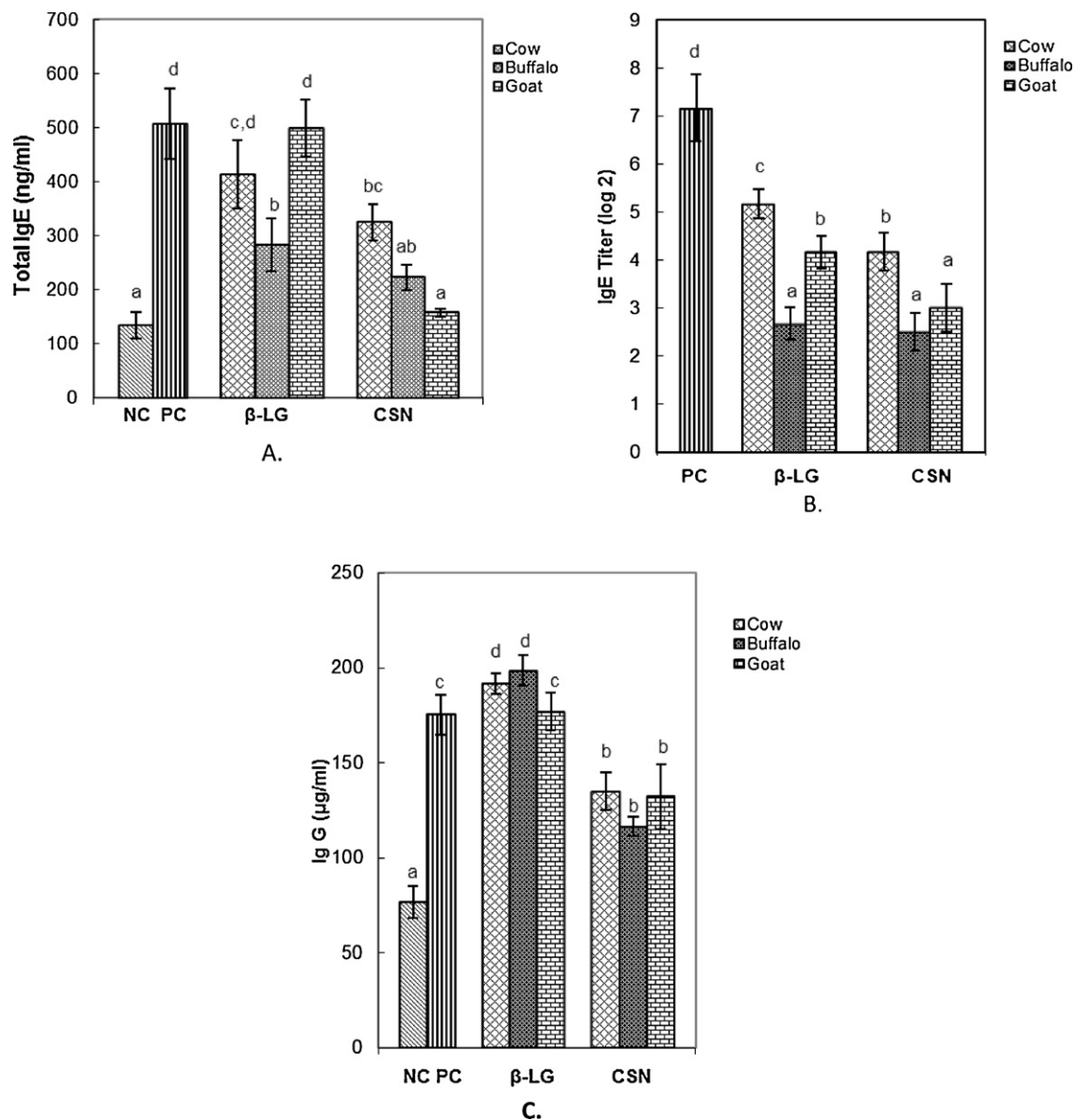


Fig. 2. Humoral response to sensitization with NC (negative control: adjuvant only), PC (positive control: ova-albumin) and respective milk proteins (β-LG and CSN) of cow, buffalo and goat. a,b,c,d values with different letters are significantly different ($P < 0.01$). A. Total serum IgE in experimental groups. B. Sensitizing proteins specific serum IgE. C. Total serum IgG in experimental groups.

difference in amino acid sequences at B-cell epitopes (conformational/linear epitopes) and T-cell epitopes (linear epitopes) obtained from literature (Jarvinen et al., 2001; Inoue et al., 2001; Chatchatee et al., 2001a) in β-LG, β-CN, αS1CSN, αS2 CSN respectively are shown in yellow and gray shades (Fig. 4). β-LG sequence showed difference at four B-cell epitopes (19–34 LVTQTMKGLDIQKVA; 65–78 KPTPEGDLLEILLQK; 145–162 EVDDEALEKFDKALKALP and 159–170 KALPMHIRLSFN) and two overlapping T cell epitopes at position 19–39 and 160–180. In case of β-CN, cow differs in amino acid sequence of one B (70–85 TQSLVYPFGPIPNLSL) and one T cell (98–107 VVPPFLQPEV) IgE binding epitope as compared to buffalo while it differs from goat amino acid sequence at five

B (16–31 RELEELNVPGEIVESL, 70–85 TQSLVYPFGPIPNLSL, 98–107 VVPPFLQPEV, 150–159 LPLPLQSWM, 200–223 MPIQAFLLYQEPVLGPVRGPFPII) and four overlapping T (16–31, 72–81, 98–107, 150–159) cell epitopes. Likewise, amino acids at B cell epitopes (47–60 SKENLCSTFCKEVV, 133–144 VPITPTLNREQL, 159–174 STEVFTKKTLEETEEK, 181–204 KKISQRYQKFALPQYLKTVYQHQQ and 207–216 KPWIQPKTKV) of αS2-CN of cow (Busse et al., 2002) are quite different from buffalo and goat. Buffalo αS2-CN at three (47–60, 159–174, 181–204) of the B cell epitopes do not even resemble with either of the goat and cow. A reasonable amount of similarity and differences of αS1-CN in nine IgE binding B cell 32–51 NENLLRFFVAPFPEVFGKEK, 54–63 ELKSDIGSES, 84–93 EEIVPNSVEQ,

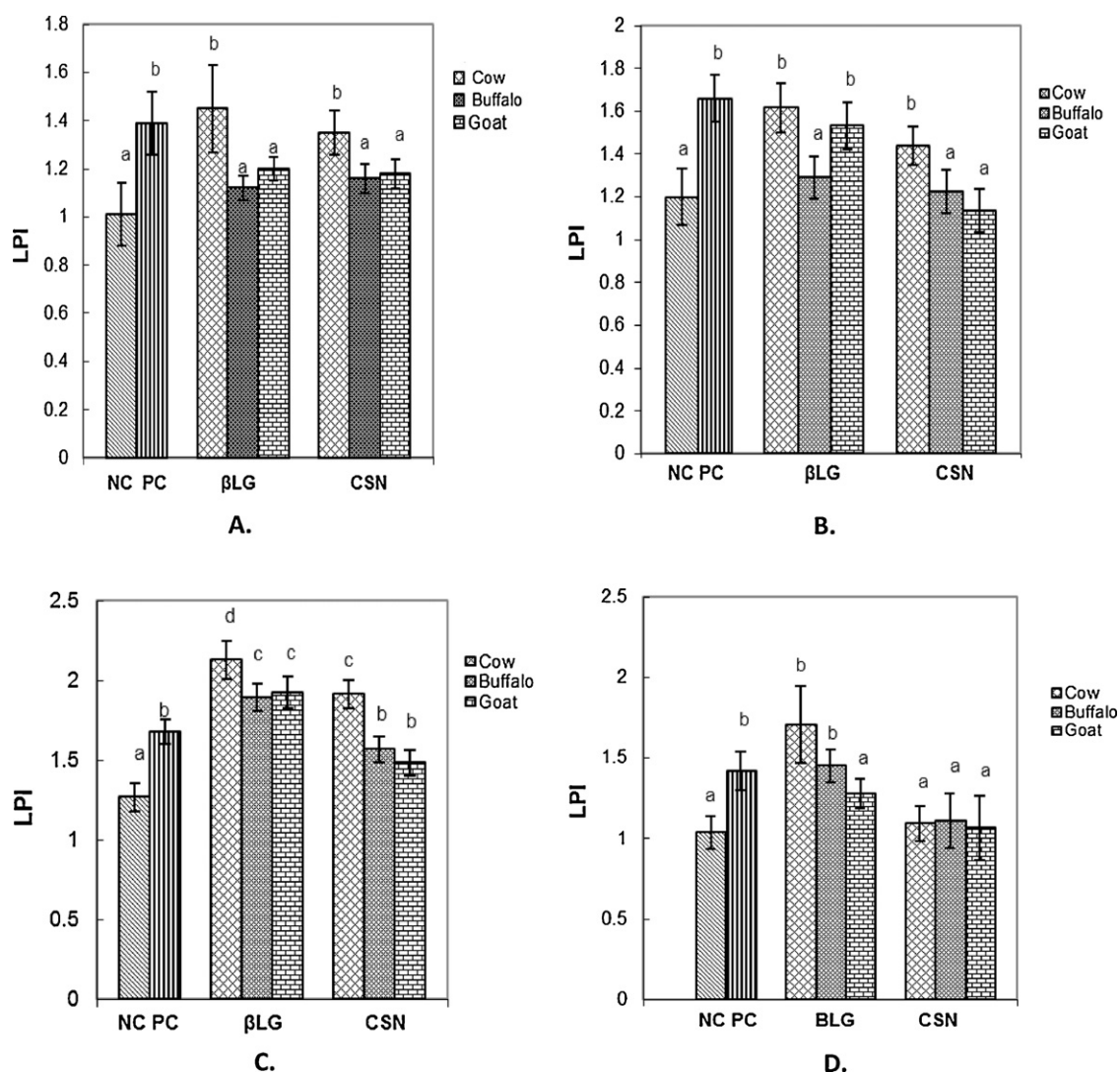


Fig. 3. Lymphocyte proliferation index (LPI) in response to sensitization with NC (negative control: adjuvant only), PC (positive control: ova-albumin) and respective milk proteins (β-LG and CSN) of cow, buffalo and goat. a,b,c,d values with different letters are significantly different ($P < 0.01$). A. Negative control splenocytes in the presence of milk proteins. B. Pre-sensitized splenocytes with respective milk proteins and their presence during culturing. C. Pre-sensitized splenocytes with respective milk proteins and in presence of LPS. D. Pre-sensitized splenocytes with respective milk proteins and in presence of ConA.

98–117 KEDVPSERYLGYLEQLRLK, 124–135 LE IVPN-SAEERL, 138–147 MKEGIHAQK, 154–169 NQELAYFYPEL-FRQFY, 174–189 YPSGAWYYVPLGTQYT, 188–209 YTDAPS-FSDIPNPIGSENSEKT and 10 overlapping T cells epitopes (16–33, 31–50, 46–65, 91–110, 106–125, 121–140, 151–170, 167–184, 196–214, 181–198) of cow with buffalo and goat amino acid sequences were also observed.

4. Discussion

Present investigation has compared hypersensitivity of milk proteins from cow, buffalo and goat. β-LG test groups sensitized with cow, buffalo and goat protein have showed significantly higher ($P < 0.01$) total serum IgE as compared to negative control. However, total IgE were significantly less in goat CSN treated group of animals than the

ovalbumin (positive), β-LG sensitized groups and cow/buffalo CSN sensitized groups. This could be related with present as well as previous findings (Bellioni-Businco et al., 1999; Jenness, 1980; Singh and Singh, 1980) that goat's milk contain only traces of allergenic αCSN protein than β-CN. In case of protein specific titers of IgE, the animals of positive control had the maximum titer which was significantly higher than milk protein sensitized groups. Among β-LG specific IgE titers, cow β-LG sensitized animals exhibited significantly higher values followed by goat and buffalo may be due to the changes in amino acids at four B-cell conformational IgE epitopes and two linear T cell IgE epitopes as predicted by *in silico* analysis (Fig. 4). Further, both bovine variants BLGA and BLGB may not contribute toward different immune hypersensitivity reactions because both amino acids D64G (present in exposed

β-lactoglobulin	
cow	MKCLLLAL--ALTGGAQALIVTQTMKGLDIQKVA GTWYSLAMAASDIS LLDAQSAPLRVY
buffalo	MKCLLLALGLALACAAQAIIVTQTMKGLDIQKVA GTWYSLAMAASDIS LLDAQSAPLRVY
goat	MKCLLLALGLALACGIAIIVTQTMKGLDIQKVA GTWYSLAMAASDIS LLDAQSAPLRVY ***** *:.*. *:*****:*****
cow	VEELKPTPEGDLLEILLQKWENECQAQKKIIAEKTKIPAVFKIDALNENKVLVDLTDYKKY
buffalo	VEELKPTPEGDLLEILLQKWENECQAQKKIIAEKTKIPAVFKIDALNENKVLVDLTDYKKY
goat	VEELKPTPEGDLLEILLQKWENECQAQKKIIAEKTKIPAVFKIDALNENKVLVDLTDYKKY *****:*****:*****
cow	LLFCMNSAEPEQSLCQCCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQQCHV
buffalo	LLFCMNSAEPEQSLCQCCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQQCHV
goat	LLFCMNSAEPEQSLCQCCLVRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEEQQCHV *****:*****:*****
β-casein	
Cow	MKVLILACLVALALARELEELNVFGEIVESLSSEESITRINKKIEKFQSEEQQTEDEL
Buffalo	MKVLILACLVALALARELEELNVFGEIVESLSSEESITHINKKIEKFQSEEQQMEDEL
goat	MKVLILACLVALAIAREQEELNVVGETVESLSSEESITHINKKIEKFQSEEQQTEDEL *****:*** ***:*****:*****
Cow	QDKIHFFAQTSQSLVYFPFGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSVKKEAMAPK
Buffalo	QDKIHFFAQTSQSLVYFPFGPIPKSLPQNIPPLTQTPVVVPPFLQPEIMGVSKVKEAMAPK
goat	QDKIHFFAQTSQSLVYFPFGPIPNSLPQNILPLTQTPVVVPPFLQPEIMGVSKVKEAMAPK *****:*****:*****
Cow	HKEMPFKYPVEPFTESQSLTLTDVENLHLPLPLLSQSMHQPQLPPTVMFPQSVLSL
Buffalo	HKEMPFKYPVEPFTESQSLTLTDVENLHLPLPLLSQSMHQPQLPPTVMFPQSVLSL
goat	HKEMPFKYPVEPFTESQSLTLTDVEKLHLPLPLVQSMHQPQLPPTVMFPQSVLSL *****:*****:*****
Cow	SQSKVLPVPQKAVPYQORDMPIQAFLLYQEPVLGVPVRGPFPIIV--
Buffalo	SQSKVLPVPQKAVPYQORDMPIQAFLLYQEPVLGVPVRGPFPIIV--
goat	SQPKVLPVPQKVVQ--RDMPIQAFLLYQEPVLGVPVRGPFPIIVV *.*.*****.* *****:*
α S1 casein	
Cow	MKLLILTCLVAVALARPKHPKHQGLPQEVLENENLLRFFVAPFPEVFGKEKVNELSKDIG
Buffalo	MKLLILTCLVAVALARPKQPIKHQGLPQGVLENENLLRFFVAPFPEVFGKEKVNELSTDIG
Goat	MKLLILTCLVAVALARPKHPINHQGLSPEVPNENLLRFVAPFPEVFRKENINELSKDIG *****:***:***. *****:*****
Cow	SESTEDQAMEDIKQMEAESISSSEIIVPNSVEQKHIOKEDVPSEYRLGYLEQLRLKKYK
Buffalo	SESTEDQAMEDIKQMEAESISSSEIIVPISVEQKHIOKEDVPSEYRLGYLEQLRLKKYN
Goat	SESTEDQAMEDAKQMKAGSSSSSEIIVPNSAEQKYIQKEDVPSEYRLGYLEQLRLKKYN *****:***.* *****:*****:*****
Cow	VPQLEIVPNSAEERLHSMKEGIHAQQKEPMIGVNNQELAYFYFELFRQFYQLDAYPSGAWY
Buffalo	VPQLEIVPNSAEERLHSMKEGIHAQQKEPMIGVNNQELAYFYFELFRQFYQLDAYPSGAWY
Goat	VPQLEIVPKSAEEQLHSMKEGNPAHQKQPMIAVNNQELAYFYFELFRQFYQLDAYPSGAWY *****:***:***** *****:*****:*****
Cow	YVPLGTQYTDAPSFSDIPNPIGSENSEKTMPLW
Buffalo	YVPLGTQYTDAPSFSDIPNPIGSENSGKTMPLW
Goat	YVPLGTQYTDAPSFSDIPNPIGSENSGKAAMPLW *:*****:*****:*****
α S2 casein	
Cow	MKFFIFTCLLAVALAKNTMEHVSSEE-SIISQETKYQEKNMALNP SKENLCSTFCKEVY
Buffalo	MKFFIFTCLLAVALAKNTMEHVSSEE-SIISQETKYQEKNMALNP SKENLCSTFCKEVI
goat	MKFFIFTCLLAVALAKHMEHVSSEEPINIFQEIYQEKNMALHPKKEKLTCTSCSEEVY *****:*****:*****
Cow	RNANEEYSIGSSSEESA EVATEEVKITVDDKHYQKALNEINQFYQKFPQYLQYLYQGPI
buffalo	RNANEEYSIGSSSEESA EVATEEVKITVDDKHYQKALNEINQFYQKFPQYLQYLYQGPI
goat	RNANEEYSIRSSSEESA EVAPEEIKITVDDKHYQKALNEINQFYQKFPQYLQYLYQGPI *****:*****:*****
Cow	VLNPWDQVKNRNVPIPTLNREQLSTSEENSKKTVDME STEVFTKTKLTETEEKNRLNFI
Buffalo	VLNPWDQVKNRNVPIPTLNREQLSTSEENSKKTVDME STEVITKTKLTETEDNRLNFI
goat	VLNPWDQVKNRNPPTPTVNREQLSTSEENSKKTIDME STEVFTKTKLTETEEKNRLNFI *****:***:*****:*****:*****:*****
Cow	KKISQRYQKFPALPQYLKTVYQHQKAMKFWIQPKTKV IYVRYL
buffalo	KKISQHYQKFTWQYLKTVYQYQKAMKFWTQPKTNV IYVRYL
goat	KIISQYQKFAWPQYLKTVQHQKAMKRWTPKTNV IYVRYL * ** * *: ***** *:***** *

Fig. 4. Multiple sequence alignment of cow, buffalo and goat milk proteins. Yellow shade: B cell epitopes. Gray shade: T cell epitopes (Jarvinen et al., 2001; Inoue et al., 2001; Chatchatee et al., 2001a; Busse et al., 2002). Blue shade: Place of substitution in A and B forms of β-LG. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

flexible loop) and V118A (buried β -strand) substitutions present outside the B and T cell epitopes responsible for allergic reactions (Fig. 3). Qiu et al. (1999) observed that the former substitution leads to observable conformational changes and is probably responsible for lowered solubility and enhanced oligomerization and gelation propensity of variant A than B while later sequence difference causes minimal structural change to the equilibrium structure but alters the dynamic properties of the molecule and is probably responsible for the lowered thermal stability of variant B by disrupting internal hydrophobic packing. Significantly low titer of specific IgE against goat milk proteins under present investigation supports previous observations of Lara-Villoslada et al. (2004), who reported hypoallergenicity of goat milk versus cow milk while performing studies of atopy on mice. Likewise, goat milk seems to be less allergenic than cow milk on the basis of experiments carried out on guinea pigs, using goat and cow milk and their lactosera (Sanz Ceballos et al., 2009). Moreover, on careful examination of IgE binding epitopes by multiple sequence alignment, two of the T cell epitope (19–34 and 160–180) and one of the B cell epitope (19–34) of β -LG, goat and buffalo resembles. CSN have hardly a clear three dimensional structure so presence of preferentially linear epitopes (Chatchatee et al., 2001a). Moreover, at one of the IgE binding epitopes (98–107) of cow β -CN, the amino acid sequence of buffalo matches with goat instead of cow. In case of alpha S1 and S2 CSN, Spuerger et al. (1997) reported allergenicity of α -CSN from cow, sheep, and goat and showed an increased antibody binding to bovine α -CSN compared to the sheep and goat proteins, but the differences were significant only in the groups of atopic children and of healthy controls. *In silico* studies also revealed considerable identity in chemical properties between goat and sheep but a considerable dissimilarity in cow with goat and sheep CSN proteins that effect secondary structures (Masoodi and Shafi, 2010). Among CSN IgE titer, cow whole CSN exhibited higher titer than buffalo and goat would be explained on the basis of their considerable difference in amino acids in alpha S1 involved in B and T cell epitopes between the three species as evident from study of overlapping synthetic peptides to identify major IgE binding regions of α S1-CN in patients with CMA (Chatchatee et al., 2001b; Cocco et al., 2003) on multiple sequence alignment (Fig. 3). Likewise, amino acids at B cell epitope of α S2-CN of cow (Busse et al., 2002), are quite different from buffalo and goat. Stéphanie et al. (2011) also observed that intraperitoneal injections of β -LG induced significant IgE and IgG1 responses in germ free mice while CSN injections provoked the production of IgG1 toward κ - and α S1-CSN. Likewise, in present study animals used are four weeks old weanlings and so they may also exhibited Th2-polarized antibody responses combined with defective immunosuppressive mechanisms.

There was significant increase in total IgG was observed for β -LG and CSN sensitized groups as compared to negative control. Though IgG levels for β -LG groups was significantly more than CSN treated animals. Thus our observations support the previous studies where CSN and β -LG were reported major immunogenic proteins in cow

milk that generate IgE and IgG responses (Gjesing et al., 1986; Docena et al., 1996). Hanane et al. (2009) observed that intraperitoneal sensitization of Balb/c mice with cow β -LG or whey performed in the presence of Alum $\text{Al}(\text{OH})_3$ produced anti- β -LG or anti whey IgG, IgG1, IgG2a and IgE with a high IgG1/IgG2a ratio translating Th2 type response. Prakash et al. (2004) also reported buffalo milk β -CSN fraction have more antigenicity than cow milk as it produced increased levels of CSN specific IgG in immunized animals contrary to present observations where no significant difference was observed in IgG related immunogenicity between these proteins of cow and buffalo. Sanz Ceballos et al. (2009) reported lower levels of specific IgG and IgG1 antibodies against goat milk and lactosera (having 57.4% β -LG) than cow milk and its lactosera in contrast to present investigation where total levels of IgG have shown statistically no major difference between cow and goat β -LG and CSN groups.

Although there is no reliable single laboratory test available for the diagnosis of milk allergy, if an allergic mechanism is suspected, a number of laboratory studies may be useful in delineating specific proteins responsible for these disorders (Hill et al., 1998; Majamaa et al., 1999; Suomalainen et al., 1994). Since specific IgE antibodies do not mediate about half of the reactions following the ingestion of milk (Carr et al., 1990), the study of cellular responses is very important. In late reactions various tests on cellular immunity have been reported but with disparities in their results. The measurement of cellular immune response to CSN and WP of cow's, buffalo's and goat milk protein by lymphocyte proliferative assay could be useful to ascertain whether the animals possess sensitized splenocytes to specific protein. Contradictory reports are available from the previous workers, regarding splenocyte proliferation on addition of purified milk proteins in the cultured splenocytes of naïve animals in presence of mitogens. Under present study only cow milk proteins (CSN and β -LG) have shown enhanced proliferation of splenocytes than buffalo and goat milk proteins. However no report is available to compare present results where splenocyte proliferation was visualized after sensitizing the animals earlier with respective proteins where in present study goat β -LG also responded in similar manner to cow milk proteins. Carr et al. (1990) reported that α -s1 CSN, a major component of whole CSN, could enhance the mitogen-stimulated proliferation of murine splenic cells, when included in *in vitro* cell culture due to its micelle nature. Other investigators (Mahmud et al., 2004; Wong et al., 1998) reported proliferative response of resting and mitogen stimulated splenocytes on the addition of purified whey proteins supports our findings of LPS induced proliferation in CSN and β -LG sensitized groups irrespective of the dairy animal in contrast to the findings of Prioult et al. (2004) and Mercier et al. (2004). More proliferative response observed under present investigations by LPS than ConA because former is a B cell stimulator and later is a T cell stimulator. Intra-peritoneal route of injecting soluble antigens evoke B cells to stimulate humoral arm of immune response than T cell mediated cytotoxic response. The intra-peritoneal route of administering food allergen appeared to be more sensitive and accurate because there

are evidences that exposure via the diet or in drinking water is more likely in rodents to cause immunological hyporesponsiveness than its sensitization (Dearman and Kimber, 2007). Significant increase in LPI observed under present investigation on addition of cow β -LG and CSN in corresponding milk protein sensitized groups also support previous findings of Lara-Villoslada et al. (2004) who got more proliferation in cow milk sensitized groups than negative control and goat milk sensitization.

5. Conclusion

In conclusion, based on the maximum response of total IgG, IgE, specific IgE and lymphocyte proliferation index cow milk protein (β -LG, CSN) exhibited increased humoral and cell-mediated responses than buffalo and goat hence concluded to have more hyperimmune sensitizing property. Although the major proteins of milk from different species of dairy animals have nearly same primary structures but minute substitutions/additions or deletions of amino acids at various B cells and T cell epitopes changes their intrinsic property to generate IgE mediated or cellular hyperimmune responses.

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References

- Alomirah, H.F., Alli, I., 2004. Separation and characterization of beta-lactoglobulin and alpha-lactalbumin from whey and whey protein preparations. *Int. Dairy J.* 14, 411–419.
- Bellioni-Businco, B., Paganelli, R., Lucenti, P., 1999. Allergenicity of goat's milk in children with cow's milk allergy. *J. Allergy Clin. Immunol.* 103, 1191–1194.
- Busse, P.J., Jarvinen, K.M., Vila, L., Beyer, K., Sampson, H.A., 2002. Identification of sequential IgE-binding epitopes on bovine alpha(s2)-casein in cow's milk allergic patients. *Int. Arch. Allergy Immunol.* 129, 93–96.
- Carr, R.I., Webster, D., Sadi, D., Williams, H., Walsh, N., 1990. Immunomodulation by opioids from dietary casein. *Ann. N. Y. Acad. Sci.* 574, 374–376.
- Chatchatee, P., Jarvinen, K.M., Bardina, L., Vila, L., Beyer, K., Sampson, H.A., 2001a. Identification of IgE and IgG binding epitopes on beta- and kappa-casein in cow's milk allergic patients. *Clin. Exp. Allergy* 31, 1256–1262.
- Chatchatee, P., Jarvinen, K.M., Bardina, L., Beyer, K., Sampson, H.A., 2001b. Identification of IgE- and IgG-binding epitopes on alpha(s1)-casein: differences in patients with persistent and transient cow's milk allergy. *J. Allergy Clin. Immunol.* 107, 379–383.
- Cocco, R.R., Jarvinen, K.M., Sampson, H.A., Beyer, K., 2003. Mutational analysis of major, sequential IgE-binding epitopes in alpha s1-casein, a major cow's milk allergen. *J. Allergy Clin. Immunol.* 12, 433–437.
- Dearman, R.J., Kimber, I., 2007. A mouse model for food allergy using intraperitoneal sensitization. *Methods* 41, 91–98.
- Docena, G.H., Fernandez, R., Chirido, F.G., Fossati, C.A., 1996. Identification of casein as the major allergenic and antigenic protein of cow's milk. *Allergy* 51, 412–416.
- El-Agamy, E.I., 2007. The challenge of cow milk protein allergy. *Small Rumin. Res.* 68, 64–72.
- Gjesing, B., Osterballe, O., Schwartz, B., Wahn, U., Loewenstein, H., 1986. Allergen-specific IgE antibodies against antigenic components in cow milk and milk substitutes. *Allergy* 41, 51–56.
- Hanane, N., Hanane, N., Hanane, K., Omar, K., Djamel, S., 2009. A model of intestinal anaphylaxis in whey sensitized Balb/c mice. *Am. J. Immunol.* 5, 56–60.
- Helm, R.M., Burks, A.W., 2000. Mechanisms of food allergy. *Curr. Opin. Immunol.* 12, 647–653.
- Hill, D.J., Ball, G., Hosking, C.S., 1998. Clinical manifestations of cows' milk allergy in childhood: associations with in-vitro cellular immune responses. *Clin. Allergy* 18, 469–479.
- Holt, P.G., Jones, C.A., 2000. The development of the immune system during pregnancy & early life. *Allergy* 55, 688–697.
- Inoue, R., Matsushita, S., Kaneko, H., Shinoda, S., Sakaguchi, H., Nishimura, Y., Kondo, N., 2001. Identification of β -lactoglobulin-derived peptides and class II HLA molecules recognized by T cells from patients with milk allergy. *Clin. Exp. Allergy* 31, 1126–1134.
- Jenness, R., 1980. Composition and characteristics of goat milk: review 1968–1979. *J. Dairy Sci.* 63, 1605–1630.
- Jarvinen, K.M., Chatchatee, P., Bardina, L., Beyer, K., Sampson, H.A., 2001. IgE and IgG binding epitopes on α -lactalbumin and β -lactoglobulin in cow's milk allergy. *Int. Arch. Allergy Immunol.* 126, 111–118.
- Laemmli, V.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lara-Villoslada, F., Olivares, M., Jimenez, J., Boza, J., Xaus, J., 2004. Goat milk is less immunogenic than cow milk in a murine model assay of atopy. *J. Pediatr. Gastroenterol. Nutr.* 39, 354–360.
- Lowry, O.H., Rosebrough, N.F., Farr, A.C., Randall, L., 1951. Protein measurement with Folin-phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Mahmud, R., Matn, M.A., Otani, H., 2004. Mitogenic effect of bovine β -lactoglobulin and its proteolytic digests on mouse spleen resting cells. *Pakistan J. Biol. Sci.* 7, 2045–2050.
- Majamaa, H., Moisio, P.H.K., Kautiainen, H., Turjanmaa, K., 1999. Cow's milk allergy: diagnostic accuracy of skin prick and patch tests and specific IgE. *Allergy* 54, 346–351.
- Masoodi, T.A., Shafi, G., 2010. Analysis of casein alpha S1 & S2 proteins from different mammalian species. *Bioinformation* 4, 430–435.
- Mercier, A., Gauthier, S.F., Fliss, I., 2004. Immunomodulating effects of whey proteins and their enzymatic digests. *Int. Dairy J.* 14, 175–183.
- Prakash, E.S., Pushpa, B.P., Jayaprakash, H.M., 2004. Effect of enzymatic hydrolysis on antigenicity of casein fractions of cow and buffalo milk. *J. Food. Sci. Technol.* 41, 390–393.
- Prioult, G., Pecquet, S., Fliss, I., 2004. Stimulation of interleukin-10 production by acidic beta-lactoglobulin-derived peptides hydrolyzed with *Lactobacillus paracasei* NCC2461 peptidases. *Clin. Diagn. Lab. Immunol.* 11, 266–271.
- Qin, B.Y., Bewley, M.C., Creamer, L.K., Baker, E.N., Jameson, G.B., 1999. Functional implications of structural differences between variants A and B of bovine beta-lactoglobulin. *Protein Sci.* 8, 75–83.
- Sanz Ceballos, L., Sanz Sampelayo, M.R., Gil Extremera, F., Rodriguez Osorio, M., 2009. Evaluation of the allergenicity of goat milk, cow milk, and their lactosera in guinea pig model. *J. Dairy. Sci.* 92, 837–846.
- Silanikove, N., Leitner, G., Merin, U., Prosser, C.G., 2010. Recent advances in exploiting goat's milk: quality, safety and production aspects. *Small Rumin. Res.* 89, 110–124.
- Silanikove, N., Shapiro, F., Silanikove, M., Merin, U., Leitner, G., 2009. Hydrogen peroxide-dependent conversion of nitrite to nitrate as a crucial feature of bovine milk catalase. *J. Agric. Food Chem.* 57, 8018–8025.
- Singh, V.B., Singh, S.N., 1980. Milk casein: electrophoretic alpha, beta and gamma fractions from four Indian goat breeds during lactation. *Int. Goat Sheep Res.* 1, 125–131.
- Spuerger, P., Walter, M., Schiltz, E., Deichtmann, K., Forster, J., Mueller, H., 1997. Allergenicity of α -caseins from cow, sheep, and goat. *Allergy* 52, 293–298.
- Stéphanie, M., Bernard, Hervé, L., Przybylski-Nicaise, Corthier, G., Rabot, S., Wal, J.M., Hazebrout, S., 2011. Allergenic and immunogenic potential of cow's milk β -lactoglobulin and caseins evidenced without adjuvant in germ-free mice. *Mol. Nutr. Food Res.* 55, 1700–1707.
- Stock, P., DeKruyff, R.H., Umetsu, D.T., 2006. Inhibition of the allergic response by regulatory T cells. *Curr. Opin. Allergy Clin. Immunol.* 6, 12–16.
- Suomalainen, H., Soppi, E., Isolauri, E., 1994. Lymphocyte response to cow's milk proteins in patients with cow's milk allergy: relationship to antigen exposure. *Pediatr. Allergy Immunol.* 5, 20–26.
- Taylor, A., Verhagen, J., Blaser, K., Akdis, M., Akdis, C.A., 2006. Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells. *Immunology* 117, 433–442.
- Walker-Smith, J., 2003. Hypoallergenic formulas: are they really hypoallergenic? *Ann. Allergy Asthma Immunol.* 90, 112–114.
- Wong, K.F., Middleton, N., Montgomery, M., Dey, M., Carr, R.I., 1998. Immunostimulation of murine spleen cells by materials associated with bovine milk protein fractions. *J. Dairy Sci.* 81, 1825–1832.
- Wing, K., Sakaguchi, S., 2006. Regulatory T cells as potential immunotherapy in allergy. *Curr. Opin. Allergy Clin. Immunol.* 6, 482–488.