

Effect of thermal processing of cow and buffalo milk on the allergenic response to caseins and whey proteins in mice

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Abstract

BACKGROUND: Heat treatment is the most common method for reducing pathogen load, but it remains controversial in reducing the incidence of hyperimmune reactions. The aim of this study was to compare the allergenicity of caseins (CSN) and whey proteins (WP) of thermally processed cow and buffalo milk in a mouse model. Swiss albino mice were sensitised by intraperitoneal injections (administered in three doses at weekly intervals) of CSN or WP from cow or buffalo milk for the evaluation of humoral response and splenocyte stimulation index.

RESULTS: After 3 weeks of intraperitoneal stimulation of mice with milk proteins, the sterilised milk protein group displayed significantly lowered ($P \leq 0.05$) serum IgG and IgE levels, while considerably increased cow milk protein-specific responses (IgE) were shown by proteins of pasteurised milk compared with those of raw milk. The stimulation index of splenocytes induced by CSN or WP of boiled and sterilised milk was also lower ($P \leq 0.05$) than that of raw milk of both cow and buffalo.

CONCLUSION: The experiment showed that boiling and sterilisation of cow and buffalo milk clearly affect the allergenicity by decreasing the humoral and cell-mediated responses in mice. All results indicated that CSN and WP of sterilised milk are less allergenic than those of raw milk in mice.

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Keywords: thermal processing; allergenicity; caseins; whey proteins; pasteurisation; sterilisation

INTRODUCTION

Thermal processing of milk is an essential step of milk production adopted by the dairy industry. Heat treatment of milk aims to extend the shelf life and improve the quality of this complex biological fluid by reducing the microbial load and thus minimising the risk of food poisoning.¹ Cow milk contains numerous proteins, many of which have been shown to be capable of sensitising susceptible individuals.²

Heat treatment is the most common method for reducing pathogen load, but it remains controversial in regard to reducing the incidence of hyperimmune reactions.³ On the one hand, heat treatments lead to the loss of tertiary protein structures; on the other hand, they result in the formation of aggregates or new stable structures in complex food matrices as a consequence of chemical and physical reactions.⁴ These irreversible changes in milk protein structure depend on the processing conditions. Thus thermal processing may alter food in a manner that may mask or unmask antigenic epitopes, thereby reducing or enhancing their recognition.

Neonates are more prone to IgE-mediated allergenic sensitisation than adults owing to their immature gut environment and stronger humoral (Th2) arm of the immune system.⁵ Exposing neonates unknowingly to altered protein structure due to thermal processing may cause their sensitisation during early years of life and the development of fatal autoimmune diseases in later years. Since the incidence of allergic populations is increasing worldwide in urban society,⁶ it is imperative to revisit traditional

methods of milk processing with new technological interventions. For this reason, we attempted to compare the impact of thermal processing of milk on the allergenicity of whole caseins and whey proteins of cow and buffalo milk.

MATERIALS AND METHODS

Mice

Three- to four-week-old male Swiss albino mice were maintained in the Small Animal House of the National Dairy Research Institute, Karnal, India. The animals were housed in polypropylene cages at room temperature under sterilised conditions and supplied with a special milk-free diet. All procedures were approved by the Institutional Animal Ethical Committee.

Thermal processing of milk samples

Fresh cow and buffalo raw milk samples were collected from the Cattle Yard of the National Dairy Research Institute, Karnal,

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India. Pasteurisation of milk was done at 74 °C for 15 s. The raw milk was heated till boiling to get boiled milk. Sterilisation of milk was performed at 120 °C for 20 min in glass bottles in the laboratory.

Preparation of caseins and whey proteins

The skimmed milk was acidified to pH 4.6 by slowly adding 3 mol L⁻¹ HCl.⁷ The precipitated caseins were washed with distilled water to remove all traces of acids. The supernatant containing whey proteins was dialysed using a 10 kDa cut-off membrane to remove all traces of lactose and other mineral components. The total concentration of whey proteins was determined by the method of Lowry *et al.*⁸ and aliquots were stored at -80 °C until use.

Immunisation protocol

Groups of seven mice were sensitised three times at weekly intervals by intraperitoneal (i.p.) injection of 20 µg of whole caseins (CSN) or whey proteins (WP) obtained from raw and different thermally processed milk samples, using 2% Al(OH)₃ as adjuvant in 200 µL of phosphate-buffered saline (PBS). Negative control mice were given adjuvant in PBS, while positive control mice were sensitised with the same dose of ovalbumin. One week after the last injection, mice were bled and each mouse's serum was separated by centrifugation at 2000 g for 10 min. Sera were collected and stored at -20 °C until analysis.

Measurement of total IgE and IgG levels

Total IgE and total IgG in serum were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Koma Biotech, Seoul, Japan) according to the manufacturer's instructions. In brief, total IgE and IgG levels in serum were determined using quantitative sandwich ELISAs. To detect these antibodies, plates were coated with 1 µg mL⁻¹ goat anti-mouse IgE or IgG. Serial dilutions of serum were added, followed by 100 µL of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgE or IgG. Plates were allowed to develop with 3,3',5,5'-tetramethyldiamine benzidine (TMB) substrate and the reaction was finally stopped with 100 µL of 2 mol L⁻¹ H₂SO₄. Plates were read at 450 nm against 630 nm as reference wavelength.

ELISA for milk protein-specific serum antibodies

Whole CSN- and WP-specific IgE antibodies were detected by ELISA. Plastic microtitre plates (Nunc, Copenhagen, Denmark) were coated with 100 µg mL⁻¹ protein (CSN or WP) in PBS by overnight incubation at 4 °C. Plates were blocked by incubation for a further 1 h at 37 °C with either 1.0% bovine serum albumin (BSA) (Sigma Chemical Co., St Louis, MO, USA) in PBS (for plates containing CSN) or 1.0% fish gelatine (for plates containing WP). Mouse serum samples diluted 1:5 (v/v) in blocking buffer were added to wells and incubated for 1 h at 37 °C. It was followed by further incubation for 1 h at 37 °C with peroxidase-labelled goat anti-mouse IgE diluted 1:10 000 (v/v) in blocking buffer. Enzyme substrate TMB containing 0.03% H₂O₂ was added to each well and the reaction was stopped after 15 min by the addition of 100 µL of 2 mol L⁻¹ H₂SO₄. Plates were washed with PBS containing 0.05% Tween 20. Substrate conversion was measured as optical density (OD) at 450 nm using an automated ELISA reader (Microscan EC Co. India Pvt Ltd, Hyderabad, India). The antibody concentration in each sample was expressed as OD per 100 µL.

Lymphocyte proliferation assay

Aseptically collected spleen tissues were gently teased with sterile needles and forceps to release splenocytes into RPMI 1640 medium. 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 × *g* for 5 min at 4 °C. The suspension was incubated with erythrocyte lysis buffer (0.17 mol L⁻¹ Tris-HCl, 0.16 mol L⁻¹ NH₄Cl, pH 7.2) for 1 min and washed twice by centrifuging as above with RPMI 1640 medium. Cell viability was checked using trypan blue (0.4% solution). Viable splenocytes (10⁷ cells mL⁻¹) were finally cultured in heat-inactivated 10% foetal calf serum-enriched RPMI 1640 medium with or without 50 µg mL⁻¹ milk CSN or WP and incubated at 37 °C in a humidified atmosphere of 5% CO₂ for 48 h. Then 10 µL of 5 mg mL⁻¹ 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for 4 h at 37 °C. Acidified isopropanol (100 µL of 0.1 mol L⁻¹ 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.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Analysis of variance was performed using GraphPad PRISM Version 5.0 (La Jolla, CA, USA). Differences between groups were determined by applying Student's *t* test. Significance of differences was set at *P* ≤ 0.05.

RESULTS AND DISCUSSION

The commonly used heat treatments for milk during processing, such as pasteurisation, preheating and sterilisation, cause denaturation of the whey proteins. Heating destroys most conformational epitopes by unfolding native proteins. Refolding may allow the reconstruction of some native conformational epitopes, while 'residual structure' may either create new ones or retain some of the originals. Troublesome allergenic epitopes are mainly linear, because these are more likely to survive denaturation in the gut as well as heating. Thus heating can increase or decrease allergenicity depending on the protein involved.

Total serum IgE levels in mice of the negative control group were 124.36 ± 25.48 ng mL⁻¹, which increased markedly (*P* ≤ 0.001) to 617.23 ± 50.32 ng mL⁻¹ (+396.33%) after 21 days of i.p. injections of ovalbumin in the positive control group (Figs 1A and 1B). Total IgE levels in mice sensitised with cow or buffalo milk proteins (CSN and WP) ranged between 253.38 ± 18.4 and 437.36 ± 11.9 ng mL⁻¹ irrespective of thermal treatment, which were considerably lower (*P* ≤ 0.05) than those of the positive control but markedly higher (*P* ≤ 0.05) than those of the negative control.

Thermal modification involves mainly conformational changes, which may be associated with chemical modifications of the molecule, such as scrambling of disulfide bonds.⁹ These alterations may have an effect on allergenicity by either enhancing or decreasing digestibility and by hiding, destroying or disclosing antigenic epitopes. Since B-cell epitopes are mostly conformational and dependent on the three-dimensional structure of the antigen, while T-cell epitopes are linear, it is probable that denaturation would primarily alter the B-cell reactivity pattern. Reversibility of the thermal modification of β-lactoglobulin (β-LG) is related to temperature, with high-temperature treatment inducing more permanent changes.¹⁰ In the present study, high-temperature

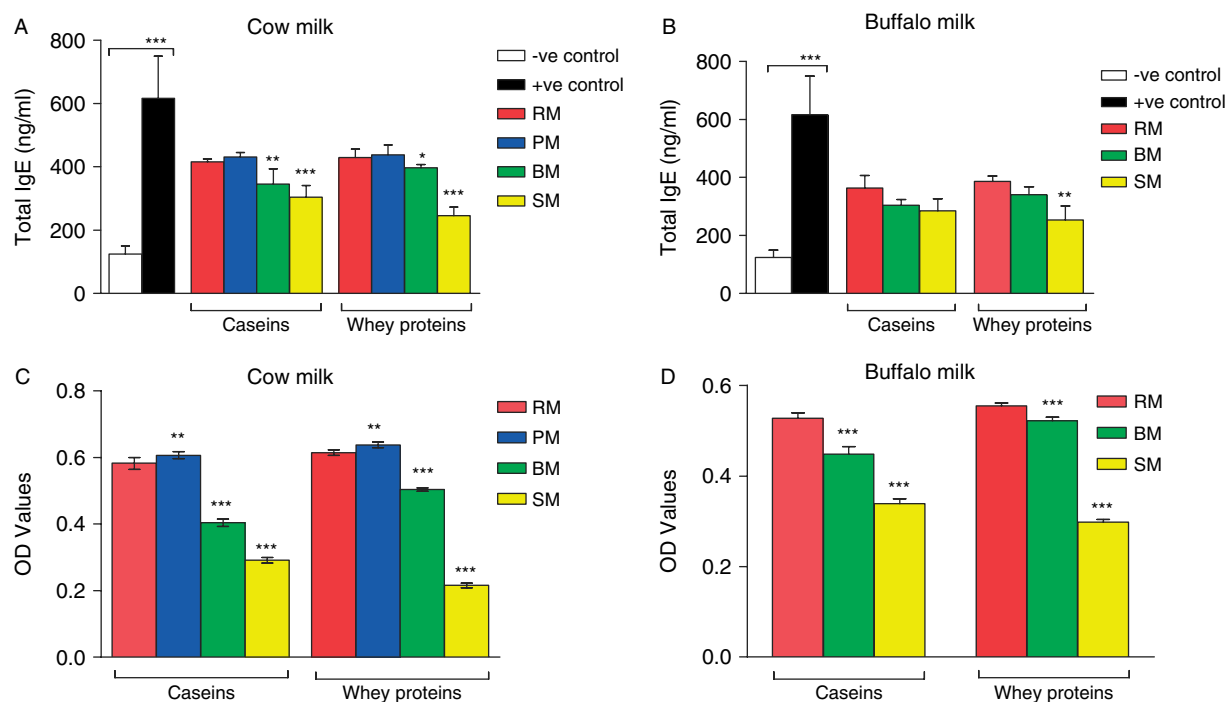


Figure 1. (A, B) Total IgE levels and (C, D) milk protein-specific IgE levels in serum collected from mice sensitised with caseins and whey proteins of processed milk samples: RM, raw milk; PM, pasteurised milk; BM, boiled milk; SM, sterilised milk. Data are average of seven mice per group; error bars indicate SD. ** $P < 0.01$; *** $P < 0.001$.

treatment was used to achieve sterilisation that is quite different from conventional pasteurisation, so its effect on immunogenic properties may not be identical to that of high-temperature treatment (sterilisation). Heating milk to decrease its allergenicity is not a straightforward process; some data in the literature point to a high stability of allergenic epitopes of the whey proteins, with great individual variation.^{11–13} There is no clear evidence in the literature for a significantly decreased clinical effect on allergy after heat treatment. Most studies have been done on the allergenicity of isolated purified individual milk proteins.^{14–16} To our knowledge, this is the first study comparing the allergenicity of whole caseins and whey proteins of cow and buffalo milk with different thermal treatments.

In the case of cow milk CSN-sensitised groups, a non-significant increase in total IgE levels was observed in pasteurised milk, while a considerable decrease ($P \leq 0.05$) was recorded in boiled and sterilised milk. On the other hand, boiling and sterilisation of milk did not bring about any significant decrease in total IgE levels in the case of buffalo milk CSN compared with raw milk. However, the IgE response in buffalo milk protein-sensitised animals was considerably lower than that for cow milk. The effect of milk processing on WP-sensitised animals revealed that cow and buffalo milk sterilisation significantly reduced ($P \leq 0.01$) total IgE levels by 43 and 35% respectively compared with raw milk WP. Our findings suggest that the caseins and whey proteins from raw and thermally processed milk of cow and buffalo provoke different immunological responses. Specific IgE levels decreased significantly in the groups sensitised with caseins or whey proteins of boiled and Sterilised milk, but the effect was more pronounced in the latter (Fig. 2). Contrary to this, CSN and WP of pasteurised cow milk showed significantly higher levels of specific IgE than those of raw cow milk. These inferences of pasteurised milk are supported by a previous report of Host and Samuelsson,¹⁷

who investigated the allergenic potential of raw milk, pasteurised milk (75 °C, 15 s) and pasteurised and homogenised milk in cow milk-allergic children. All samples were positive in skin prick tests (SPT) and double blind, placebo-controlled food challenges (DBPCFC), with a trend to higher allergenicity of pasteurised milk samples. Roth-Walter *et al.*¹⁵ suggested aggregation of β -LG and α -lactalbumin (α -LA) during pasteurisation, causing inhibition of uptake by intestinal epithelial cells *in vitro* and *in vivo*, which redirected uptake to Peyer's patches that promoted significantly higher Th2-associated antibody and cytokine production. Similarly in the present study, the intraperitoneal route of sensitisation may also be eliciting a stronger immune response directly, with probable aggregates of WP produced during pasteurisation on interaction with antigen presenting cells (APCs) present in the peritoneal cavity. It was also reported that heating β -LG exposes so-called hidden epitopes,^{18,19} which may be another explanation for higher IgE responses in mice sensitised with CSN and WP of pasteurised milk. One of the major IgE-binding epitopes on β -LG is situated in the region containing the free SH group in Cys 121.^{20,21} This SH group is known to be exposed after moderate heat treatment²² and oxidised under the high thermal treatment of milk used in this study.

To obtain better visualisation of IgE-mediated allergic sensitisation to milk proteins, ELISA plates were coated with specific CSN and WP obtained from raw and differently processed milk samples. The results (Figs 1C and 1D) revealed that boiling and sterilisation of cow and buffalo milk reduced protein-specific IgE markedly ($P \leq 0.001$). However, sterilisation of cow and buffalo milk appeared to be more effective than boiling, since it reduced casein-specific IgE by 50 and 36% respectively in CSN-sensitised animals and by 65 and 46% respectively in WP-sensitised animals.

Total serum IgG levels of the negative control group were $273.31 \pm 7.8 \mu\text{g mL}^{-1}$, which increased significantly to 777.2 ± 27.9

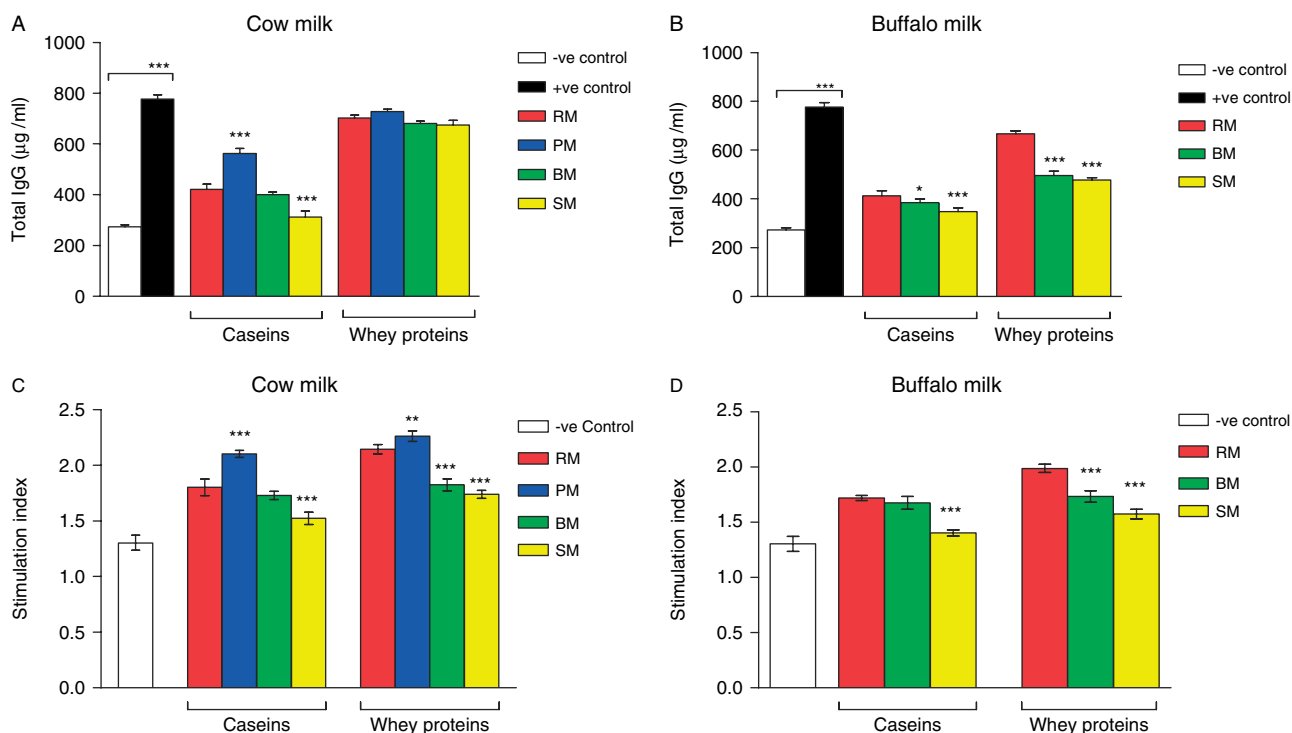


Figure 2. Percentage change in allergenicity of processed milk samples relative to raw milk with respect to milk protein-specific IgE response.

$\mu\text{g mL}^{-1}$ after sensitisation with ovalbumin in the positive control group (Figs 3A and 3B). In the cow and buffalo milk protein-sensitised groups, IgG levels were 421 ± 8.1 and $412.55 \pm 8.4 \mu\text{g mL}^{-1}$ respectively in CSN-sensitised animals and 702.17 ± 14.3 and $667.16 \pm 4.71 \mu\text{g mL}^{-1}$ respectively in WP-sensitised animals. Pasteurisation of cow milk increased total IgG levels significantly ($P \leq 0.001$) in CSN-sensitised mice (+34%) and non-significantly in WP-sensitised mice (+4%) compared with those for raw milk. Boiling of cow milk reduced total IgG levels non-significantly (−5%) while sterilisation reduced them markedly ($P \leq 0.001$, −26%) in CSN-sensitised mice. In the case of WP groups, non-significant variations were observed in total IgG levels in response to various thermal treatments of cow milk. On the other hand, mice sensitised with buffalo milk proteins showed significantly reduced ($P \leq 0.05$) total IgG levels in the case of boiled and sterilised milk compared with raw milk. The decreased sensitising capacity of CSN and WP of cow and buffalo milk after boiling and sterilisation are in agreement with previous findings of Kilshaw *et al.*²³ Animals given heated whey, in which β -LG was more severely denatured, failed to produce antibodies to β -LG or α -LA. Cooking clearly diminished the antigenicity of alpha-lactalbumin (ALA) and β -LG, which may explain the fact that cooked milk is better tolerated by many patients than raw milk,²⁴ because a marked reduction in the allergenicity of milk can be observed after boiling (100 °C) for 10 min, while boiling for 2 and 5 min respectively induced no significant change in SPT and dot-immunoblotting experiments.^{25,26} Heating of skimmed milk for 10 min resulted in a decrease of approximately 50 and 66% in IgE binding for α -LA and caseins, while β -LG and serum albumin lost their IgE-binding ability in scored crossed radio-immunoelectrophoresis (CRIE).²⁷ Norgaard *et al.*²⁵ proved the inactivation of β -LG and serum albumin after boiling milk samples for 10 min, while caseins still induced positive reactions in SPT. During heating of cow milk, allergenicity has been shown to be reduced but not completely

eliminated. The results from this study illustrate an important consideration that the allergenicity of WP of both boiled and sterilised milk decreased more effectively compared with that of total CSN. Gjessing *et al.*²⁷ found that caseins have higher heat stability than the whey proteins β -LG and serum albumin. These results are supported by those of Nowak-Węgrzyn *et al.*²⁸ who found that a substantial reduction in allergenicity occurred after heating milk for 10 min at 100 °C.

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 such disorders.^{29–35} Since specific IgE antibodies do not mediate about half of the reactions following the ingestion of milk,³⁶ 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 and buffalo milk proteins by lymphocyte proliferative assay could be useful to ascertain whether animals possess splenocytes sensitised to specific proteins. The results for the stimulation index (SI) in response to CSN and WP obtained from differently processed cow and buffalo milk samples are shown in Figs 3C and 3D. *In vitro* addition of cow and buffalo milk proteins (CSN and WP) increased the SI significantly ($P \leq 0.05$) compared with the negative control. Addition of caseins isolated from thermally processed cow milk in splenocytes from mice pre-sensitised with respective milk proteins increased their SI by 50.25, 75.17, 44.17 and 27.08%, while the increase was 78.83, 88.58, 52.08 and 45.8% with respective cow whey proteins as compared with the control group. Similarly, milk proteins obtained from buffalo increased the SI by 43.33, 39.50 and 16.92% with CSN and 65.67, 44.33 and 31.08% with WP obtained from raw, boiled and sterilised milk respectively compared with the control. On comparative assessment of the SI within the cow casein group of thermally processed and raw

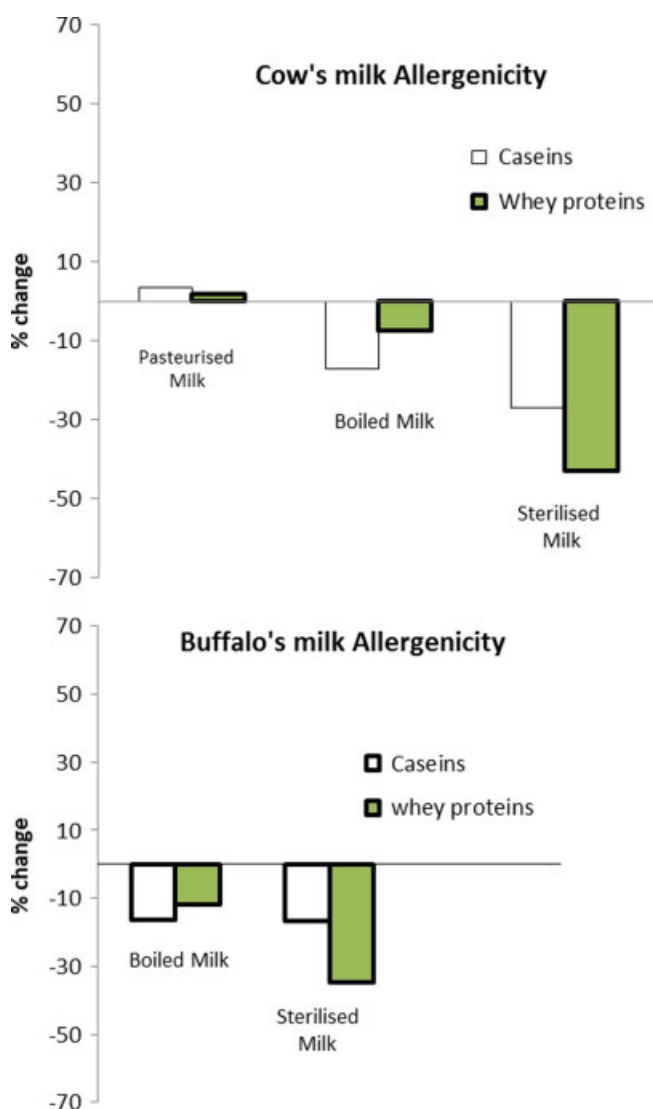


Figure 3. (A, B) Total IgG levels in serum and (C, D) stimulation index in cultured spleen cells collected from mice sensitised with caseins and whey proteins of processed milk samples: RM, raw milk; PM, pasteurised milk; BM, boiled milk; SM, sterilised milk. Data are average of seven mice per group; error bars indicate SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

milk, it was observed that boiling and sterilisation reduced the SI significantly ($P \leq 0.05$) by 4% and ($P \leq 0.001$) 15% respectively, in contrast to pasteurisation where it was increased by 17% as compared with raw milk. Similar observations were recorded in cow WP groups, where SI decreased significantly ($P \leq 0.001$) by 15 and 19% on addition of WP of boiled and sterilised milk respectively, in contrast to pasteurisation. In the case of buffalo milk, the SI with CSN also decreased considerably ($P \leq 0.001$) by 18% on sterilisation compared with raw milk, whereas mice sensitised with WP of boiled and sterilised buffalo milk showed markedly reduced ($P \leq 0.01$) SI by 13 and 21% on their addition in respective splenocytes compared with raw milk.

In the present investigation also, whole CSN and WP of cow and buffalo milk, irrespective of their processing type, enhanced the proliferation of splenocytes as compared with the negative control. Most researchers have shown the enhancement of splenocyte proliferation on addition of purified milk proteins in cultured splenocytes of naïve animals in the presence of

mitogens. However, no report is available for comparison with the present study, where splenocyte proliferation was visualised after previously sensitising the animals with respective proteins obtained from thermal processing of milk. Carr *et al.*³⁶ reported that α_{s1} casein, a major component of whole caseins, could enhance the mitogen-stimulated proliferation of murine splenic cells when included in *in vitro* cell culture owing to its micellar nature. Other investigators^{37,38} reported a proliferative response of resting and mitogen-stimulated splenocytes on addition of purified whey proteins, in contrast to the findings of Prioult *et al.*³⁹ and Mercier *et al.*⁴⁰ In the present study the SI against CSN and WP of boiled and sterilised milk was lower, but it increased on pasteurisation as compared with raw milk. This could be attributed to the higher mitogenic response of milk proteins due to their aggregation during pasteurisation, which may not occur at higher thermal treatment.

CONCLUSIONS

This paper shows the effect of thermal processing of cow and buffalo milk on the allergenic responses of caseins and whey proteins. Thermal treatment, especially in the dairy industry, is a common and cheap method to reduce pathogens, but it remains controversial whether this method affects the allergenicity of milk. The present experiment has shown that boiling and sterilisation of both cow and buffalo milk clearly affect the allergenicity by decreasing the humoral and cell-mediated responses of mice challenged intraperitoneally with caseins and whey proteins. More novel approaches to milk processing involving high-temperature or high-pressure treatment also need to be explored in further studies. In addition, beyond boiling and sterilisation, ultrahigh temperature (UHT) may be an important parameter to reduce the allergenicity of milk proteins. Thus it is necessary to explore the health implications of different milk-processing techniques such as UHT and high-pressure processing on animal models in advance.

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