

Cross-reactivity analysis of milk proteins from different goat breeds with cow's milk allergens using a proteomic approach

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ABSTRACT

Introduction: Goat's milk thought to be a good substitute for cow's milk protein allergic (CMPA) individuals. However, there is growing evidence that their proteins have cross-reactivities with cow's milk allergens. This study aimed to profile and compare milk proteins from different goat breeds that have cross-reactivity to cow's milk allergens.

Methodology: Proteomics was used to compare protein extracts of skim milk from Saanen, Jamnapari, and Toggenburg. Cow's milk was used as a control. IgE-immunoblotting and mass spectrometry were used to compare and identify proteins that cross-reacted with serum IgE from CMPA patients (n = 10).

Results: The analysis of IgE-reactive proteins revealed that the protein spots identified with high confidence were proteins homologous to common cow's milk allergens such as α -S1-casein (α S1-CN), β -casein (β -CN), κ -casein (κ -CN), and beta-lactoglobulin (β -LG). Jamnapari's milk proteins were found to cross-react with four major milk allergens: α -S1-CN, β -CN, κ -CN, and β -LG. Saanen goat's milk proteins, on the other hand, cross-reacted with two major milk allergens, α -S1-CN and β -LG, whereas Toggenburg goat's milk proteins only react with one of the major milk allergens, κ -CN.

Conclusion: These findings may help in the development of hypoallergenic goat milk through cross-breeding strategies of goat breeds with lower allergenic milk protein contents.

1. Introduction

As the primary source of nutrients for infants, children and also adults, milk has been largely consumed across the world. Milk is an essential food source for humans, and it has been proven to have been a part of the human diet since nearly 6000 years ago (Bleasdale et al., 2021). Milk is regarded as a complete food because of its versatility; it contains both macronutrients and micronutrients (Park and Haenlein, 2010). Cow's milk is commonly used to feed infants who do not have access to breast milk due to its long history of consumption. But in certain cases, cow's milk may be perceived as foreign in the

gastrointestinal system, causing an allergic reaction by the immune system. Milk allergy is more prevalent among infants; as they have an underdeveloped gastrointestinal system, more often in infants as their main source of nutrients comes from milk (Yadav and Naidu, 2015). Although sensitization toward milk allergens among atopic children will subside or go as they age; as their gastrointestinal system developed, the persistence of milk allergy past adolescence has been reported (Skrupak et al., 2007) posing a risk to adult consumers on the consumption of cow's milk. An allergic reaction is usually categorised as IgE-mediated or non-IgE-mediated, its lethality varies depending on the IgE-reactivity of the patient's serum IgE. It can be as simple as rashes or

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sneezing to anaphylaxis which can lead to casualty. IgE produced by the plasma cell upon foreign protein antigen entry played an important role in triggering the allergic reaction; IgE will attempt to bind with the foreign antigen if succeeded will trigger the mast cells to release histamine to initiate an allergic reaction (Sampson et al., 2018; Yu et al., 2016).

This circumstance has compelled people with an allergic reaction toward cow's milk protein to opt for different dairy milk alternatives. Goat, sheep, horse, camel, and donkey milk are among the ruminant milk that has been studied for their hypoallergenic potential (Bernard et al., 1999; Hinz et al., 2012). As an alternative, plant-based milk such as soy milk and nut milk has also been considered. However, in terms of nutrients for infants' postnatal development, mammals milk is a better choice because it naturally contains the essential nutrients for infant development (Vojdani et al., 2018). According to dairy milk product output statistics in Malaysia, dairy milk output seemed to increase steadily from 2013 to 2019, with cows having the highest livestock population, followed by goats. There are only a few large-scale dairy goat farms in Malaysia, as the majority of dairy goat farms are owned by small-scale farmers (Shahudin et al., 2018). Saanen, Jamnapari, Toggenburg, Alpine, and Shami are among the dairy goat breeds raised in Malaysia (Liang and Paengkoum, 2019). These breeds are known to be excellent at producing milk, and farmers practise cross-breeding among these breeds to increase milk yield and quality.

However, due to the general homology between the two species' milk proteins, goat's milk may pose some issues as an alternative to cow's milk for CMPA patients (Bernard et al., 2012). Milk protein from different phylogenetic species usually has the same set of proteins; casein and whey. The cross-reactivity of milk proteins from different species of ruminants has been documented (Bellioni-busnco et al., 1999; El-Agamy et al., 2009; Goodman et al., 2007). However, there have been cases of goat's milk allergy in patients who have never had an allergic reaction to cow's milk, and vice versa (Ah-Leung et al., 2006; Bellioni-busnco et al., 1999; Goh et al., 2019). According to a study on the cross-reactivity of casein fraction among different ruminant species, IgE responses to ewe's and goat's milk were weaker than those to cow's and buffalo's milk, implying that some CMPA patients may have the tolerance to ewe's and goat's milk (Clark and Mora García, 2017). Furthermore, previous research on the genetic differences between goat breeds has been shown to affect the yield and chemical composition of the goat's milk (Selvaggi et al., 2014; Taiwo Idowu and Olufunke Adewumi, 2017). Rather than dismissing goat's milk entirely, its hypoallergenic potential could be further evaluated using proteomic methods.

Allergenomic analysis remains a potent tool for the rapid and comprehensive identification and characterisation of allergens in specific sources, as well as for further direct comparison with other methods. Protein analytical techniques such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoassay techniques such as enzyme-linked immunosorbent assay (ELISA) and Western blotting have been used for allergen detection (Carrera et al., 2020; Sharma et al., 2017). Bottom-up proteomics studies subject a complex protein mixture to several processes such as SDS-PAGE separation and proteolytic cleavage before peptide products are analysed using mass spectrometry (MS). For allergen detection, 2DE separates proteins based on their molecular weight and isoelectric point, resulting in a pattern of protein dispersion. Western blotting can be used to further analyse the specific IgE reactive protein spots produced by SDS-PAGE. MS analysis, such as matrix-assisted laser desorption/ionisation time-of-flight tandem mass spectrometry (MALDI-TOF/TOF MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS), can be used to identify the target protein. The integration of 2DE, 2D-immunoblotting, and MS analysis has been widely used in allergen detection due to its reliability when dealing with food proteins, which can have a wide range of isoforms and are susceptible to post-translational modification and structural changes during storage or processing (Carrera et al., 2018; Villa et al., 2018).

In this study, we aimed to perform a comparative mapping and identification of allergenic proteins in milk from different dairy goat breeds that cross-reacted with cow's milk allergens utilising 2DE paired with IgE-immunoblotting, followed by protein identification via MALDI TOF/TOF MS/MS. Differences in the 2DE protein separation profiles of goat's milk from different breeds; Saanen, Jamnapari and Toggenburg, were analysed on 2DE before proceeding with the IgE-immunoblotting; to determine the goat's milk protein that cross-reacted with cow's milk allergen. Although phylogenetically related ruminant milk proteins are known to be homologous, the genetic variance between the species or breed has shown to be affecting their milk composition and IgE-binding epitope, which should be further investigated.

2. Materials and methods

2.1. Materials

Acrylamide, N,N'-methylenebis acrylamide, sterile-filtered chicken serum (Cat. # C5405), bovine serum albumin fraction V (Cat. # 10735078001) trifluoroacetic acid (TFA), ammonium bicarbonate, and Ponceau S were purchased from Sigma-Aldrich (St. Louis, MO, USA). IPG buffer, pH 4–7, 13 cm IPG strip, iodoacetamide (IAA), Immobiline Dry-strip cover fluid, urea, thiourea, and Amersham ECL Western Blotting Reagent were purchased from GE Healthcare, Chicago, IL, USA. 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1,4-dithiothreitol (DTT), chloroform, sodium dodecyl sulphate (SDS), tetramethylethylenediamine (TEMED), glycine, 0.2 µm polyvinylidene fluoride (PVDF) membrane and ImmunoCAP milk specific IgE test kits (including UniCAP milk allergen f2) were purchased from Thermo Fisher Scientific, Uppsala, Sweden. Coomassie Blue R-250, Bromophenol Blue, Tween-20, acetic acid, and acetonitrile (ACN) were purchased from Merck, St. Louis, MO, USA. Precision Plus Protein Dual Color Standard (Cat. # 1610374), Protein Assay Dye Reagent (Bradford) (Cat. # 5000006), BLUtra prestained protein ladder (Genedirex, Inc, Taiwan), ammonium persulfate, BSA Protein Standard 2 (Bovine serum albumin) (Cat. # 5000007) were purchased from Biorad Laboratories, Hercules, CA, USA. Methanol and ethanol were purchased from HMBG Chemicals, Hamburg, Germany. Sodium sulphite, sodium chloride, potassium dihydrogen phosphate, disodium phosphate, and sodium hydroxide were purchased from R&M Chemicals, Subang, Malaysia. Hydrochloric acid, 37% was purchased from JT Baker, Radnor, PA, USA. Tris, tris-HCL was purchased from Biobasic, Markham, Canada. Millipore® ZipTip with 0.6 µL C18 resin (Cat. # ZTC18S096) was purchased from Millipore, Bedford, MA, USA. Glycerol was purchased from Chemiz, Selangor, Malaysia. Trypsin/LYS-C Mix was purchased from Promega, Madison, WI, USA. Mouse monoclonal anti-human IgE Fc-HRP (B3102E8) used in the IgE-immunoblotting was purchased from Southern Biotech, Birmingham, AL, USA. Rabbit polyclonal anti-beta lactoglobulin antibody (ab112893) and goat polyclonal anti-rabbit IgG Fc-HRP (ab97200) were purchased from Abcam, Cambridge, UK.

2.2. Milk sample preparation

Milk samples (n = 6) from three different goat breeds; Saanen, Jamnapari and Toggenburg, were collected from a commercial goat farm in Kluang, Johor, Malaysia. Midstream milk samples were collected on the farm site during their early morning milking session for analysis and the same samples were also used for a different study by our research group (Salleh et al., 2019). Cow's milk was collected as control from six cattle of Friesian Sahiwal from the University Agricultural Park, UPM, Serdang, Selangor, Malaysia. Upon collection, milk samples were pooled, aliquoted into a 2 mL tube, and snap-frozen in the dry ice-ethanol slurry before being stored at – 80 °C. Before further analysis, pooled milk samples were skimmed according to Vincent et al. (2016). Thawed frozen milk samples were centrifuged at 4600 rpm for 30 min at 4 °C. Skimmed milk was recovered from between the top fat layer and

bottom pellet for protein extraction.

2.3. Protein extraction & Bradford protein assay

Total milk protein extraction was performed using the urea-based buffer dilution method (Mansor et al., 2020). The protein concentration of each protein extract and skim milk sample was determined using the Bradford protein assay. The protein standard curve was created by making seven dilutions of Bovine Serum Albumin protein standard ranging from 0.125 to 2 mg/mL. Then, into a 96-well plate, 10 μ L of each protein standard and protein extract sample was dispensed, followed by 200 μ L of Bradford reagent. The protein sample and Bradford reagent mixture were incubated at room temperature for 5 min before their absorbance was measured at 595 nm using a Biotek Synergy 2 microplate reader. The standard curves were derived as a simple linear regression model with R squared to be at least 0.99 and equation $y = mx + c$, where m is the slope and c is the crossing point at the y-axis. The protein concentration (mg/mL) corresponding to the absorbance reading at 595 nm obtained from each sample dilution was calculated by using this equation. Protein concentration determination was carried out in triplicate.

2.4. CMPA patients' sera collection

Consented paediatric subjects ($n = 15$), who were ranging from one to 15 years of age and referred to the Paediatric Department of Hospital Serdang for allergic symptoms such as eczema, vomiting, and rashes, were interviewed for their clinical allergy history. Subjects with a clear history of CMPA were selected for phlebotomy, where patients' blood was collected for further CMPA-specific serum IgE (sIgE) analysis. A non-CMPA allergic subject was selected as a negative control. Subjects' blood collection was performed under the approval of the Medical Research and Ethics Committee (MREC) (NMRR ID: NMRR-18-1312-42480). Patients' sera were collected by centrifuging the blood at 3000 rpm at 22 °C then stored at -80 °C until further analysis.

2.5. ImmunoCAP

Each patient's cow's milk protein-specific serum IgE was quantified using ImmunoCAP 100 milk-specific IgE system of a complete milk allergen f2 (Thermo Fisher Scientific/Phadia, Uppsala, Sweden) at the Laboratory of Cell Immunology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, according to the manufacturer guidelines. Results were expressed as kilo arbitrary unit per litre (kUA/L). A standard cut-off value was used to interpret the readout value; those with a result of 0–0.34 kUA/L were not considered sensitised to the allergen in question, while those with a result of higher than 0.35 kUA/L were considered sensitised. The severity level of sensitisation were further categorised into classes, where class 0 (absent) is 0.10–0.35 kUA/L, class 1 (low) is 0.36–0.7 kUA/L, class 2 (increased) is 0.7–3.5 kUA/L, class 3 (significantly increased) is 3.5–17.5 kUA/L, class 4 (high) is 17.5–50 kUA/L, class 5 (very high) is 50–100 kUA/L, and class 6 (extremely high) is over 100 kUA/L (Ibrahim and Abdel-Latif, 2017).

2.6. One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1DE)

Each protein extract was subjected to 1DE to assess its quality. A volume of protein extract equivalent to 20 μ g of protein was taken from each extract and diluted with sample loading buffer [0.5 M Tris-HCl, pH 6.8, 10% SDS, Glycerol, and Bromophenol Blue] to a final volume of 10 μ L. The sample mixture was then heated in a 95 °C water bath for 5 min. With a Mini-Protein gel electrophoresis system, the sample mixture was put through a 5% stacking gel followed by a 12% resolving gel at a constant voltage of 90 V. Biorad Precision Plus Protein Dual Color Standards were used to estimate the molecular weight of each protein

band produced. Each gel was stained using the Coomassie Brilliant Blue (CBB) staining protocol after the gel run was completed once the blue indicator line reached the end of the gel cassette. Gels were fixed for 1 h in a fixing solution [30% methanol, 10% acetic acid in ddH₂O] before being incubated in CBB staining solution [0.2% Coomassie brilliant blue, R-250 in 30% methanol, 10% acetic acid, 60% ddH₂O (1:9)] for 3 h on a shaker at ambient temperature before being destained in destaining solution [40% methanol and 7% acetic acid in H₂O] for 40 min.

2.7. Two-dimensional polyacrylamide gel electrophoresis (2DE)

Each protein extract sample was subjected to two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2DE). The 13 cm pH 4–7 IPG strip was rehydrated in a rehydration buffer for 18–20 h at 18–20 °C in the presence of 400 μ g of protein sample. Isoelectric focusing (IEF) was performed on a rehydrated IPG strip using an Ettan IPGphor 2 IEF system at 500 V/4 h, 1000 V/1 h, 5000 V/1 h, 8000 V/1 h, and 29,000 V/1 h at 20 °C. IPG strips were equilibrated for 15 min in equilibration solutions (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, a trace amount of bromophenol blue and 10 mg/mL DTT).

After IEF before being transferred on 12% polyacrylamide gel and sealed with agarose sealing gel. Gel electrophoresis was performed using the Ruby SE 600 electrophoresis system for 15 min at 20 mA/gel, followed by 4 h at 40 mA/gel. Gels were then fixed in fixing solution [40% methanol and 10% acetic acid in H₂O] for overnight fixing at room temperature on a shaker before being stained with CBB staining solution [0.2% Coomassie brilliant blue, R-250 in 30% methanol, 10% acetic acid, 60% ddH₂O (1:9)] for at least 6 h and destained for another 1–2 h in destaining solution [40% methanol and 7% acetic acid] (Al-Obaidi et al., 2017).

2.8. Image acquisition and statistical analysis

One and Two-dimensional gels were examined using a Biorad GS800 calibrated densitometer (Biorad Laboratories, Hercules, CA, USA) with a resolution of 600 dpi and a pixel depth of 32 bits. The images of the 1DE gels were reviewed and analysed with Biorad ImageLab software (Biorad Laboratories, Hercules, CA, USA), whereas the images of the 2DE gels were inspected and analysed with Progenesis SameSpot software version 3.1 v (Nonlinear Dynamics Ltd, Durham, NC, USA). The 2DE gels were subjected to automated single stain analysis, in which the normalised volume of each spot was generated automatically and their differential abundance was determined using one-way ANOVA. The total number of spots for each gel was computed after manual verification of each spot's presence. Protein spots that are significantly different ($p < 0.05$) and have > 1.5 -fold changes were labelled. The power analysis was then carried out to confirm the significant expression differences between the duplicates and the number of replicates ($n = 3$) required to attain the desired 80% power threshold.

2.9. IgE-immunoblotting

Following 1DE or 2DE, polyacrylamide gels were electroblotted on 0.2 μ m Polyvinylidene fluoride (PVDF) membrane at 24 V for 30 min using the Biorad Transblot Semi-Dry transfer system (Biorad Laboratories, Hercules, CA, USA). Each blotted membrane was stained with Ponceau S solution (Sigma Aldrich, St Louis, Missouri, USA) before immunoblotting to confirm the completion of protein transfer. Immunoblotting with each patient's sera was performed on a one-dimensional immunoblot for the first screening. All sera were pooled and further analysed by two-dimensional immunoblots. Blotted membrane was blocked with 3% chicken serum (Sigma Aldrich, St. Louis, MO, USA) in PBST and then incubated with pooled patient's sera diluted 1:9 in blocking buffer overnight at 4 °C. The membrane was washed with PBST for 3 cycles of 10 min washing before incubating with horseradish

peroxidase-conjugated mouse anti-human-IgE antibody (Southern Biotech, Birmingham, AL, USA) diluted 1:10,000 in 15 mL blocking buffer for 3 h at room temperature. After 5 cycles of 10 min washing in PBST and another 2 cycles of 10 min washing in PBS, the immunoblot was developed with Amersham Enhanced Chemiluminescent (ECL) Western blotting reagent GE Healthcare (GE Healthcare, Chicago, IL, USA) using Biorad Chemidoc MP imaging system (Biorad Laboratories, Hercules, CA, USA) and alternately ImageQuant™ LAS 500 (GE Healthcare, Chicago, IL, USA). On both imaging systems, exposure times were primarily set as automatic. In case of failed imaging on the automatic setting, exposure times were set to 1 min, 3 min, 5 min, and 10 min, and then the best image developed was chosen.

2.10. Image analysis and IgE-reactive spot detection on 2-D immunoblots

Coomassie-stained gels were digitized using Biorad GS-800 calibrated densitometer (Biorad Laboratories, Hercules, CA, USA) with 32-bit pixel depth and 600 dpi resolution. Acquired 2DE gels images and ECL-developed blots images were compared on Progenesis SameSpot software version 3.1 v (Nonlinear Dynamic Ltd., Durham, NC, USA). Through alignment adjustment between 2DE gel images and ECL developed immunoblot image, the protein spots on the immunoblotted membrane, matching spots on both 2DE gel images and immunoblotted membrane images were determined and cross-reactive protein spots between the breeds were compared on the software through the single stain analysis.

2.11. In-gel protein digestion

Selected spots from 2DE gels were excised and stored in a 2 mL tube. Gel plaque was washed in ddH₂O for 10 min, twice. Gel plaque was further washed and vortexed in 100 mM ammonium bicarbonate, (NH₄)HCO₃ for 10 min before all liquid was discarded from the tubes. Wash solution [50% ACN in 100 mM (NH₄)HCO₃] was then added to the tube to completely remove the dye on the gel plaques. Each washing was performed with agitation for 5 min and repeat until the dye was completely removed. Gel plaque was then reduced in reduction solution [100 mM DTT in 100 mM (NH₄)HCO₃] in a 60 °C water bath for 30 min. Reduced samples were then added with alkylation solution (55 mM IAA in 100 mM (NH₄)HCO₃) and incubated for 20 min in the dark. Alkylated samples were then washed in washing solution [50% ACN in 100 mM (NH₄)HCO₃] twice before 100% ACN was added and incubated for 15 min at room temperature. All sample gel plaque was then dried in the ScanSpeed MiniVac Evaporator (Saur, Reutlingen, Germany) for 1 h. Overnight trypsin In-gel digestion was performed in a 37 °C water bath with a gel sample added with 7 µg/µL trypsin solutions completely covering the gel plaque in the tubes. The digested peptides were retrieved from the gel by collecting the supernatant produced after mixing and spinning the digested gel plaque with 100% ACN for 15 mins then drying the supernatant using ScanSpeed MiniVac Evaporator (Saur, Reutlingen, Germany) for 1 h. Dried peptides were stored at – 80 °C until further spectrometry analysis.

2.12. Protein identification by MALDI-TOF/TOF mass spectrometry

Peptide samples were reconstituted with 5µL 0.1% TFA in 30% ACN then cleaned-up using Zip-Tips C18 (Millipore, Bedford, MA, USA). Matrix solution [0.7 mg/mL α-Cyano-4-hydroxycinnamic acid (HCCA) in 84.92% ACN, 0.1% TFA, 14.98% ddH₂O] was prepared in advance and stored in – 80 °C until mass spectrometry analysis. On a clean MALDI target plate, 0.5 µL of standard and positive control, BSA was pipetted followed by 1 µL of sample mixture and 0.5 µL matrix solution. All droplets were left to dry before being analysed on Ultraflexxtreme MALDI-TOF/TOF mass spectrometer (Bruker, Bremen, Germany). Peptide Calibration Standard II (Bruker no. 222570) were used. The spectra were then analysed by using MASCOT search engine version 3.5 (Matrix

Science, Boston, MA, USA) against taxonomy mammal entries against Swissprot (containing 553231 sequences, Mammalia 66512 sequences) and NCBI (131178434 sequences, Mammalia 4185157 sequences) databases to identify the proteins of interest. The parameters used for database searches were as follows: The enzyme was set as trypsin with one missed cleavage allowed, with a fragment mass tolerance of 1.0 Da, a peptide mass tolerance of 200 ppm, methionine oxidation as a variable modification, carbamidomethylation as a fixed modification, and mass values as monoisotopic (Usuldin et al., 2017). Identified protein were considered positive if the MASCOT score value greater than 38 with at least a single peptide match. Due to low abundance of protein not all spots of interest could be identified (Hlavackova et al., 2019).

3. Results & discussion

3.1. CMPA patients' sera profiles

The IgE-reactivity of sera from 15 CMPA subjects and a control subject towards cow's milk allergens was measured by performing ImmunoCAP analysis. The clinical attributes and cow's milk specific IgE levels of the CMPA patients were tabulated in Table 1. From Table 1, except for CMPA patient 1 and the control subject, 14 CMPA patients have elevated sIgE against cow's milk allergens at levels above class 1 (≥0.36 kUA/L).

Each serum was then screened for its capability to bind to cow's milk allergenic proteins by sIgE immunoblotting (Fig. 1). Only 10 patients' sera; patients 3, 6, 7, 8, 9, 10, 11, 12, 13, and 15, were observed to have immunoreactivity to cow's milk proteins. Reactivity of the patient's serum varies; patients 3, 7, 11, 12 showed reactivity to proteins in β-LG region, around 15 kDa area, and caseins region, around 20–25 kDa area. Patients 6, 8 and 15 showed immunoreactivity in the caseins region only, while patients 9, 10 and 13 showed immunoreactivity only in the β-LG region at 18 kDa. Only patients 8 and 13 were IgE-reactive to protein bands in the serum albumin region with low IgE binding.

Table 1

The details of cow's milk allergy patients and their cow's milk-specific IgE levels.

Patient	Gender	Age	Clinical Symptoms	*SPT, mm	sIgE Milk, kUA/L (class)
1	Female	1	skin irritation, sneezing, abdominal cramp	8	0.17 (0)
2	Male	6	skin irritation, repetitive cough, wheezing	-	0.47 (1)
3	Female	4	skin irritation	-	16.7 (3)
4	Female	3	skin irritation	-	3.77 (3)
5	Male	2	skin irritation, mouth itching, vomiting	-	0.36 (1)
6	Male	8	eczema, skin irritation	-	3.34 (3)
7	Male	2	skin irritation, swelling	-	52.3 (5)
8	Male	2	urticarial rash	-	22.9 (4)
9	Female	14	asthma, skin irritation, coughing	-	1.97 (2)
10	Female	3	severe eczema, recurrent skin infection, multiple allergy	-	32.5 (4)
11	Female	6	asthma, rash, allergic rhinitis	-	6.82 (3)
12	Male	3	eczema, urticarial rash	-	33.1 (4)
13	Male	5	anaphylaxis, vomiting, urticarial rash	-	24.6 (4)
14	Male	2	multiple allergy, urticarial rashes, swollen eyes	-	2.51 (2)
15	Male	4	Eczema, multiple allergy	-	32.5 (4)
NC	Male	28	-	-	<0.35 (0)

* SPT, skin prick test; NC, negative control, IgE level classification **class 0**: 0.10–0.35 kUA/L, **class 1**: 0.36–0.7 kUA/L, **class 2**: 0.7–3.5 kUA/L, **class 3**: 3.5–17.5 kUA/L, **class 4**: 17.5–50 kUA/L, **class 5**: 50–100 kUA/L, and **class 6**: > 100 kUA/L

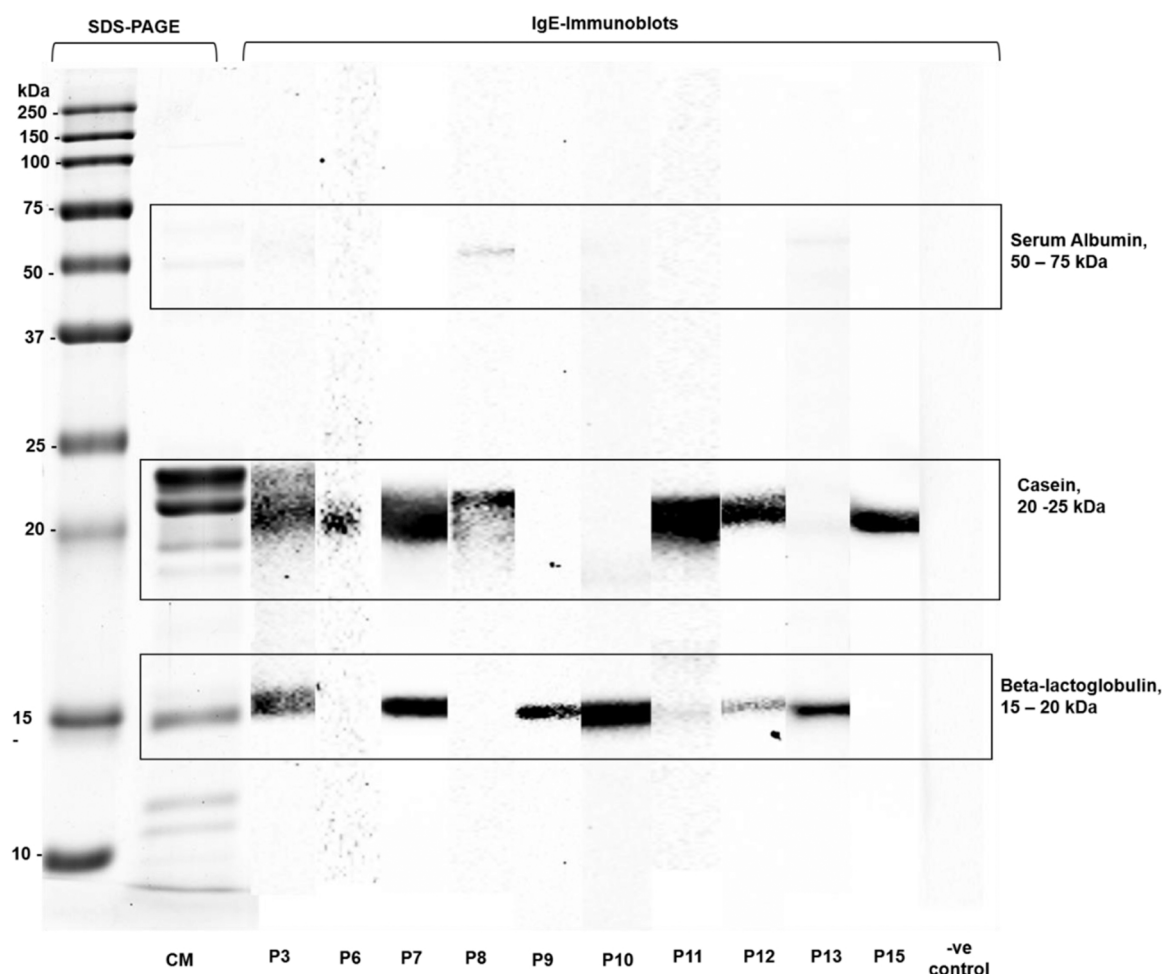


Fig. 1. Individual cow's milk protein allergy patient serum IgE immunoblot to cow's milk protein extract. Under reducing conditions, 12% SDS-PAGE was used to separate protein extracts from cow's milk. On the left, molecular weight protein markers (Precision Plus Protein Dual Color Standard, BioRad, USA) are shown in kilodalton (kDa).

3.2. Comparison of 1D-SDS-PAGE and 1D IgE-immunoblotting

Before IgE-Immunoblotting on 2DE protein blot to identify the cross-reactivity of CMPA patient's sIgE toward goat's milk protein, all reactive sera were pooled and IgE-immunoblotting on 1D protein blot was performed. As shown in Fig. 2, despite the differences in species and breed of goats, all proteins showed cross-reactivity on 1D protein blot. The intensity of the band produced varies; with cow's milk protein (as a control) with the most intense binding at known major cow's milk allergens, caseins and β -LG regions. Additional densitometric analysis revealed that the molecular weights and intensities of protein bands for each sample lane were different (Fig. 3 and Table 2). Four IgE-reactive protein bands were detected in cow's milk samples: 29.5 kDa, 26.8 kDa, and 21.5 kDa, which correspond to caseins molecular weights, and 15.7 kDa, which corresponds to β -LG. Cow's milk protein bands 1, 2, and 5 had higher band intensities, while band 4 had a lower band intensity. Goat's milk proteins have weaker IgE-reactive band intensities than cow's milk proteins. All three goat's milk demonstrated IgE-reactivity to protein bands 1, 2, and 5, but only Saanen demonstrated IgE-reactivity to protein band 3. Jamnapari milk had the most intense band (band 1 and 2) in the caseins region among the goat's milk. Saanen milk protein has the most intense band in the β -LG region. In comparison to other goat breeds, Toggenburg milk protein immunoblot revealed the least intense bands in both caseins and β -LG regions.

3.3. Comparison of 2DE gel of milk proteins from different goat breeds by Samespot analysis

To achieve high-resolution protein separation from milk protein extracts, 2DE fingerprints of pooled milk protein extracts from a cow (control) and three dairy goat breeds, Saanen, Jamnapari, and Toggenburg, were obtained (Fig. 4 B and Fig. 5 B, D, and F). In this study, pooled samples were used to cover all possible protein variants from the same breeds of animals. The number of protein spots in cow's milk and milk from Saanen, Jamnapari, and Toggenburg goats varied based on the Samespot analysis (Table 3). Jamnapari goat milk had the most protein spots (267 ± 10.6), followed by Toggenburg goat milk (212 ± 10.6) and Saanen goat milk (199 ± 16.1). The number of milk protein spots in the milk of all three goat breeds is higher than in cow's milk. When milk protein samples from Saanen, Jamnapari, and Toggenburg were compared in triplicate, 109 protein spot volumes were found to be significantly different. Jamnapari had 46 protein spot volumes that were significantly higher, Toggenburg had 44 significantly higher spots, and Saanen had only 17 significantly higher spots. These findings are consistent with the previously reported total protein yields (Mohsin et al., 2019), which are the highest in Jamnapari, followed by Toggenburg and Saanen.

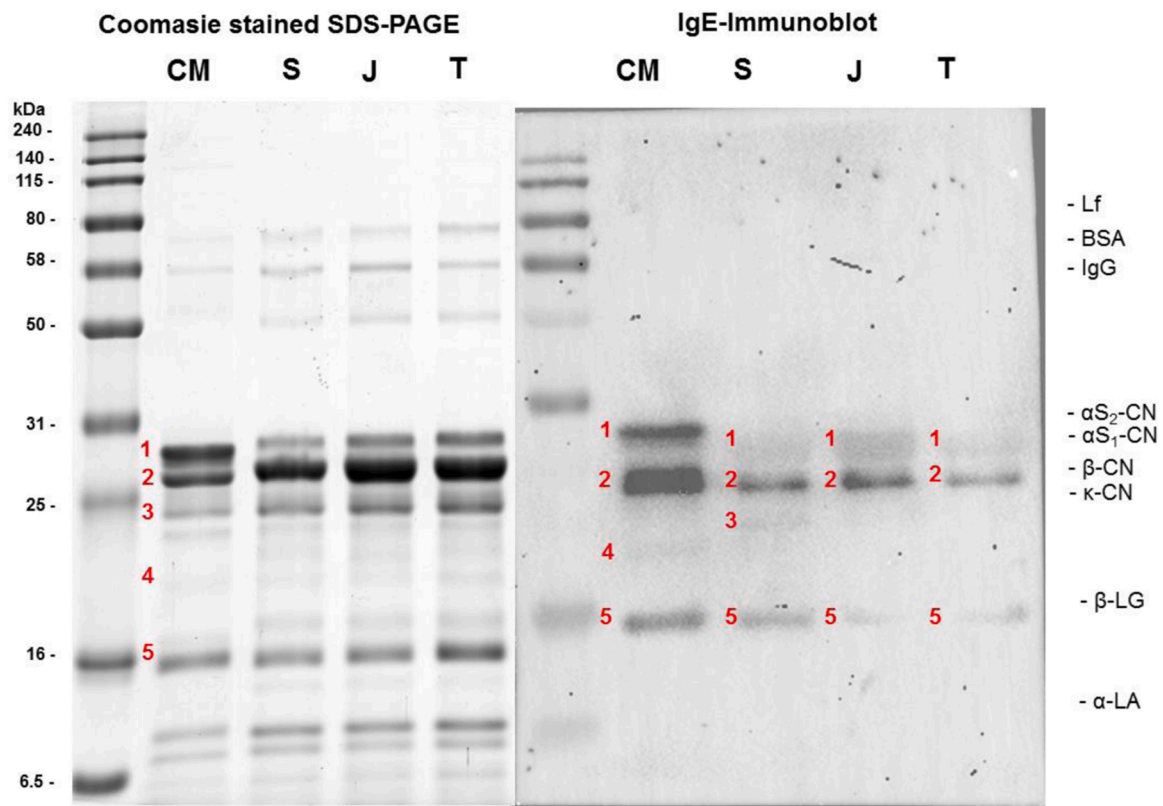


Fig. 2. CBB-stained SDS-PAGE and CMPA-specific serum IgE-immunoblotting of protein extracts from cow's milk (CM), Saanen goat's milk (S), Jamnapari goat's milk (J), and Toggenburg goat's milk (T). IgE-immunoblotting was performed with pooled cow's milk protein allergy patient sera at 1:9 dilution. On the left, molecular weight protein markers (BLUltra prestained protein ladder (Genedirex,inc, Taiwan) are shown in kilodalton (kDa). The protein labels on the right is presumptive protein based on MW (Zenker et al., 2020). IgE -Immunoblot image is a composite image of chemiluminescent imaging of protein blot and colorimetric imaging of the protein marker.

3.4. Allergen identification using 2D-immunoblotting and mass spectrometry

2D IgE-immunoblotting was performed on milk protein blots from three different goat breeds and cow milk as a control. The comparison of CBB-stained and immunostained cow's milk protein blots are shown in Fig. 4. After immunostaining with pooled CMPA patient sera, approximately 20 spots (red and black circles) in cow's milk were found to have serum IgE-reactivity after protein spot analysis with Progenesis SameSpot software. Four protein clusters were observed on the protein blot at around 10–15 kDa for α -LA and β -LG, 15–20 kDa for κ -CN in a near-neutral pH area, and 20–25 kDa for α -S₁-CN in an acidic pH area. Further protein identity confirmation was performed on selected IgE-reactive protein spots (red circles in Fig. 4) and tabulated in Table 4.

Only Two of the protein spots (402 and 385) were identified as milk allergen; Bos d 9 (α S₁-CN) which are officially listed as allergens by the WHO/IUIS (<http://www.allergen.org>)(WHO/IUIS, 2022). Due to some limitation, other IgE-reactive spots have not been identified in this study, but has been previously reported as β -actin (spot 150), κ -CN; Bos d 12 (spot 397,398, 371, 137,196), β -LG; Bos d 5 (spots 278 and 311), and α -LA; Bos d 4 (spot 321) (Anagnostopoulos et al., 2016). Apart from that, a faint train of spots was also found in the Bos d 10 region (α -S₂-CN) (Jensen et al., 2012),

Cross-reactivities of allergenic proteins in goat's milk from Saanen, Jamnapari and Toggenburg breeds were determined by 2D-immunoblotting using pooled CMPA patients sera. Immunoreactive protein spots were excised from 2DE gels and undergone in-gel trypsin digestion before being identified by MALDI-ToF MS/MS-based peptide fingerprinting. Unlike the 1DE protein blot, IgE-immunoblotting on a 2DE protein blot differed significantly in the immunoreactive spots on each

goat breed.

Fig. 5 shows the SameSpot analysis of the 2D-immunoblot profile of goat's milk, which revealed 12 IgE-reactive protein spots in Jamnapari milk, and eight IgE-reactive protein spots in Toggenburg milk, and six IgE-reactive protein spots in Saanen milk. Some of these protein spots overlapped between the breeds, as shown in the Venn diagram in Fig. 6. Between Saanen and Jamnapari, five protein spots (1282, 1263, 1239, 2667, and 2661) overlapped. However, only two protein spots overlapped between Jamnapari and Toggenburg (protein spots 1388 and 2658), and no IgE-reactive protein spot was detected between Saanen and Toggenburg. MALDI-TOF tandem mass spectrometry was used to identify these sIgE-reactive protein spots, which are listed in details in Table 5 and simplified in Table 6.

As per Table 5, α -S₁-CN was the most frequently identified IgE-reactive protein in goat milk protein samples, and it was observed in the cluster of spots 1239, 1282, 2664, 1263, and 2667, with pI ranging from 4.15 to 4.49 and MW ranging from 30 to 32 kDa. While goat's milk proteins are not currently officially listed in the WHO/IUIS allergen nomenclature, the caprine α -S₁-CN allergen was listed in the Allergome database as Cap h 9 allergen (www.allergome.org) (Mari et al., 2009). In the milk from Saanen and Jamnapari, the majority of the α -S₁-CN spots were found to have IgE reactivities. These findings were mirrored in their relative protein volumes, which are significantly higher in Saanen and Jamnapari but lower in Toggenburg. β -CN was identified as Cap h 11 in the spot of 1334 and was found to have IgE-reactivity only in Jamnapari milk. It has an experimental pI of 4.66 and MW of 28, which differs slightly from the theoretical pI of 5.26 and MW of 24.8 kDa. Except for one κ -CN spot (1388) that was IgE-reactive in Jamnapari and Toggenburg milk, κ -CN or Cap h 12 was identified in the spots of 1438 and 1392 at pI 4.75–4.88 and MW of 30–32 kDa and mostly observed to

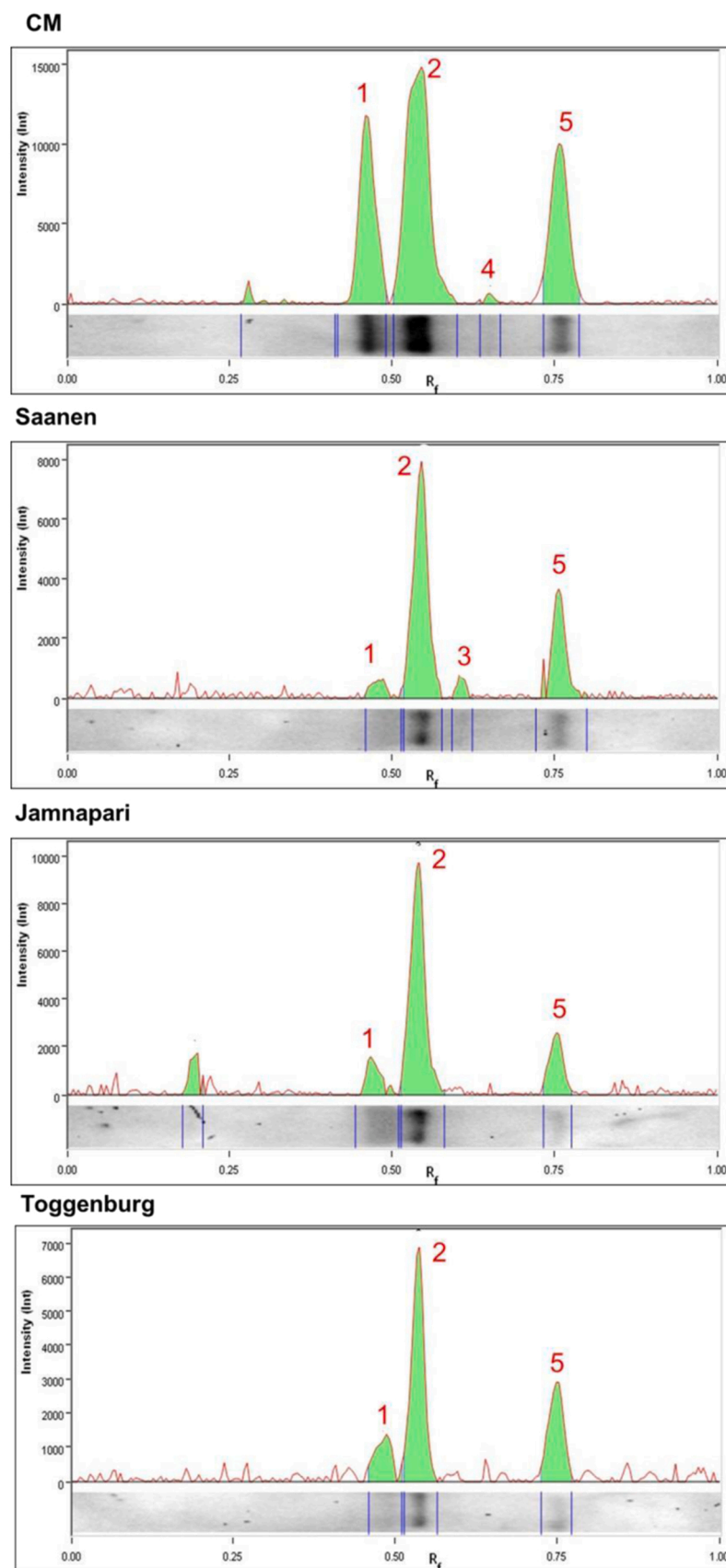


Fig. 3. The densitometry plots of the IgE-reactive protein lane profiles of milk from cows (control), Saenen, Jamnapari, and Toggenburg goats, as analysed using Biorad ImageLab software (Biorad Laboratories, USA).

Table 2

The relative molecular weight (MWr) of the IgE-reactive protein bands and band intensity values from the respective milk samples. CM is cow's milk, S is Saanen, J is Jamnapari, and T is Toggenburg.

Lane	Band No.	Mol. Wt. (KDa)	Adj. Volume (Int)
CM	1	29.5	3586,854
CM	3	26.8	6725,564
CM	4	21.5	108,965
CM	5	15.7	3345,503
S	1	28.8	226,033
S	2	26.6	2486,992
S	3	24.4	158,286
S	5	15.7	1102,045
J	1	29.2	436,082
J	3	26.8	2814,775
J	5	16.0	715,469
T	1	28.7	445,887
T	2	26.9	1661,152
T	5	16.2	866,503

have IgE-reactivities in Jamnapari milk only. β -LG was identified as Cap h 5 in spot 2661, with a pI of 5.53 and a MW of 14 kDa. However, β -LG (spot 2661) was only IgE-reactive in Jamnapari and Saanen milk. Spots with equivalent protein identities but different isoelectric points may suggest that some of these proteins have undergone post-translational modifications or that they are potential isoforms of the proteins (The-sbjerg et al., 2022; Yao and Udenigwe, 2018; Zhu et al., 2005). Despite this finding, the relative volumes of these proteins in these three goat breeds were comparable. However, except α -S₁-CN protein clusters in Saanen and β -CN in Jamnapari, other IgE-reactive protein spots in their respective protein regions in the 2DE maps have relatively weaker spot intensities than cow's milk protein spots. Overall, milk from Toggenburg had the least number of IgE-reactive proteins that cross-reacted with known allergens in cow's milk. Among the identified proteins, β -actin, a protein commonly found in blood serum, may also be present in milk, as evidently shown in earlier studies on the comprehensive proteomics of goat's milk (Cunsolo et al., 2015). Its presence has also been detected in the milk of cows with mastitis, implying that it may be due to the condition of the does' mammary gland (Hogarth et al., 2004).

Unlike cow's milk protein blots, goat's milk protein blots from each goat breed reveal IgE-binding on different protein regions, with only 7 spots; 1282, 1263, 1239, 2667, 2661, 2658, and 1388 on Jamnapari

milk protein blot correspond to Saanen and Toggenburg milk protein blot. Throughout 1DE, protein samples were reduced in sample buffer in the presence of heat to linearize the protein, resulting in completely separate protein bands on 1D gels, whereas there was no heat-reducing step that linearized protein in the extract during the first-dimensional isoelectric focusing in 2DE. The milk protein antigen-binding site (Lisson et al., 2014, 2013) on the protein may have been affected by conformational differences in protein separated by 1DE and 2DE, preventing IgE from binding to the epitopes of the protein, as documented in previous studies on IgE epitope blocking studies on allergic binding site that concluded low-affinity allergen did not trigger an allergic reaction on blocked antigen site (Zukin et al., 2017). According to previous studies on the effect of breed on milk protein production, the breed of an animal species caused differences not only in chemical composition (Mohsin et al., 2019) but also in IgE-reactive spots on the milk protein blots (Ballabio et al., 2011; Bertelsen et al., 2014; Cebo et al., 2012; Hazebrout et al., 2014).

Toggenburg milk was observed in this study to have low IgE-binding capacity as compared to Saanen and Jamnapari. Low IgE binding to the protein was found to be related to the amount of protein expressed. This finding could be linked to the genetic variability of casein and whey proteins in goats. Even though casein and whey protein alleles in goats are highly polymorphic, Toggenburg was found to have a higher frequency in the weak F allele (0.327) of the α -S₁-CN variants (Torres-Vázquez et al., 2008). In contrast, Saanen has a higher frequency of intermediate E (0.418) and poor F (0.371) alleles (Torres-Vázquez et al., 2008). This could also explain why Jamnapari milk has higher IgE-reactivities to α -s1-CN, as the α -s1-CN variant has a higher frequency in the strong A allele (0.51) (Marini et al., 2011). Higher IgE-reactivities have been linked to stronger alleles of α -S₁-CN variants (Ballabio et al., 2011). The genetic polymorphisms of both β -CN (A1, A2, B) and κ -CN (A, B, E) affected the in vitro digestion pattern of the different purified genetic variants, as previously demonstrated by Lisson et al. (2013) and this was reflected in the IgE-binding epitopes of the digests. Despite having similar relative protein volumes in the three goat breeds (Table 5), some of these sIgE-reactive proteins and allergens have different IgE-binding capacities. This could be because some immunodominant epitopes have genetic polymorphisms in caseins that influence their allergenic potential. Single amino acid substitutions or deletions have previously been shown to cause changes in immunoreactivity in

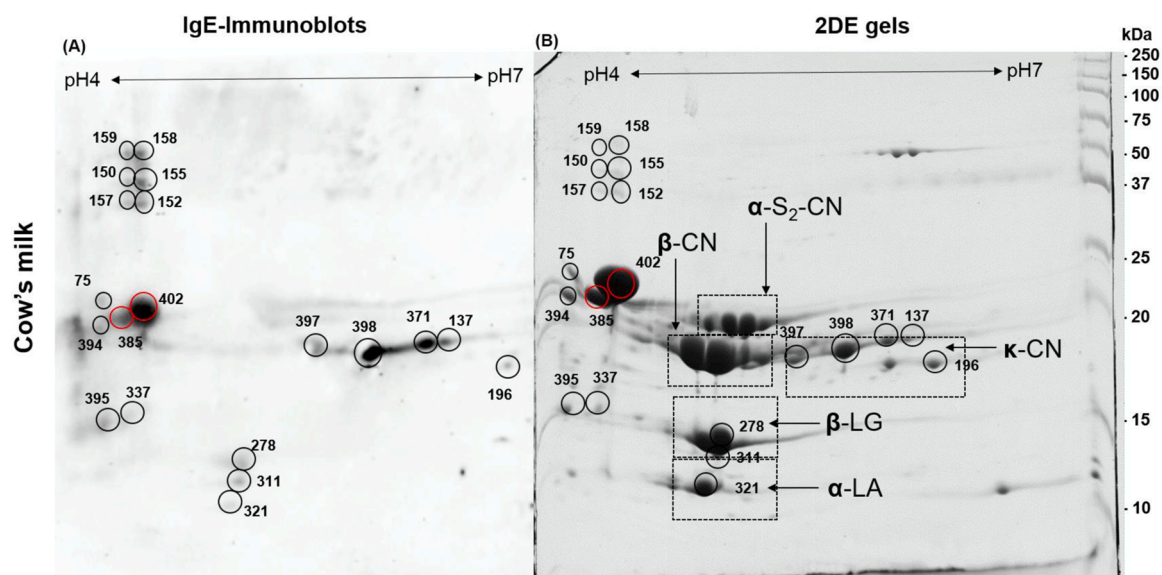


Fig. 4. (A.) IgE-immunoblots using pooled sera of cow's milk allergy patients at 1:9 dilution for extracts from cow's milk as a control and (B.) CBB-stained 2DE gel. The 2DE gel on the right panel facilitates the location and identification of IgE-reactive protein spots (in black and red circles). The identities of protein spots in red circles are listed in Table 4.

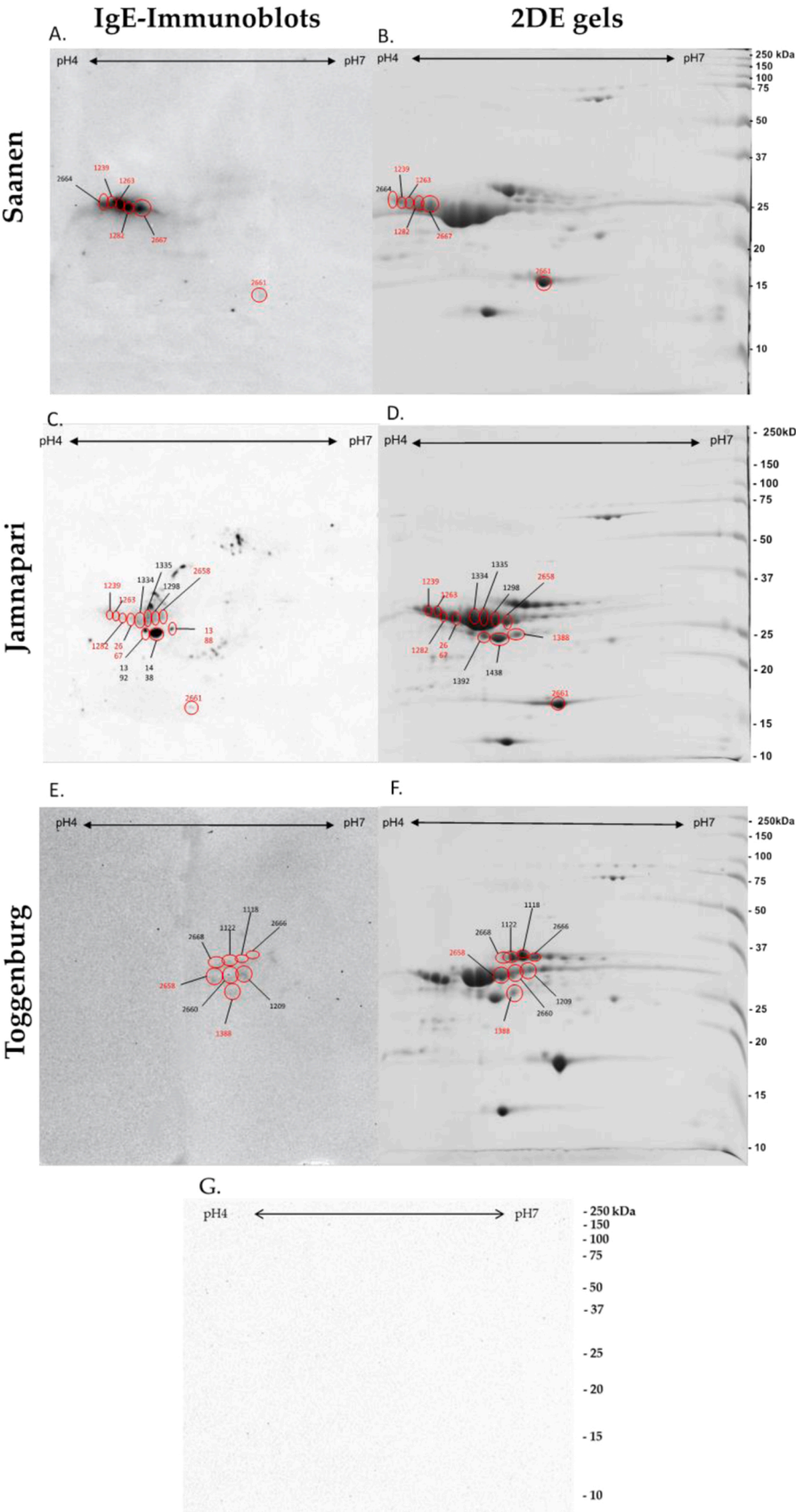


Fig. 5. CBB-stained 2DE gels and IgE-immunoblots using pooled sera of cow's milk protein allergy patients at 1:9 dilution for extracts of goat's milk of Saanen (A, B), Jamnapari (C, D), and Toggenburg (E, F). Pooled milk samples (n = 6 samples/ breed) were focused on IPG strip pf pH4–7 and electrophoresed using 12% polyacrylamide gel. The 2DE gels on the right panel facilitate the location and identification of IgE-reactive protein spots (with black and red labels). The red label indicates IgE-reactive milk protein spots that are unique to respective goat breeds. The identities of protein spots in red circles are listed in [Table 5](#). (G) is the negative control using sera from a non-CMPA subject.

Table 3
Total number of detectable spots from 2DE gels from cow’s milk and goat’s milk from three breeds that were used in this study. Standard deviation generated from 3 gels replicate for each sample.

Milk sample	Number of spots
Friesian-Sahiwal (cow)	145 ± 14.5
Saanen	199 ± 16.1
Jamnapari	267 ± 10.6
Toggenburg	212 ± 15.6

some α -S₁-CN and β -CN epitopes with the tested sera, resulting in a decrease or increase in immunoreactivity (Lisson et al., 2014). However, this remains a speculation and future work should consider validating genetic variance in individuals or similar breeds. In this study, we used pooled milk samples from each breed to ensure that the samples covered

all possible variants. This is because polymorphisms can change the amino acid sequence and affect their isoelectric point as well as post-translational modifications that may result in a distinct horizontal train of spots and a marginal shift in isoelectric point and cause distinct separation of isoforms on 2DE gels (Jensen et al., 2012).

In this study, weaker IgE-reactivities of proteins spots were detected in Toggenburg milk 1DE and 2 DE proteins blot, as shown in Figs. 2 and 5. There is a chance the CMPA patient sera used in this study were already sensitised to these proteins. Although higher IgE binding has been associated with higher allergenicity of the milk proteins and persistent allergy (Savilahti et al., 2010), it should be noted that IgE-reactivity does not imply that the proteins are allergens. It should also be noted that the proteins used in immunoblotting were denatured, which means that some of the milk allergen’s conformational epitopes may have been lost, and some hidden linear epitopes within the allergen or potential allergen may now be more accessible for binding (Walczyk

Table 4
Protein identification of cow’s milk protein spots detected in 2DE IgE-immunoblot by MALDI-TOF/TOF MS.

ID	Protein	Isoallergen	Taxonomy	Accession no.	Score	Matches	Peptide Sequence
402	α -S ₁ -CN, partial	Bos d 9	<i>Bos taurus</i>	159793209	160	2	PIKHQGLPQE VLNENLLRFF VAPFPEVFGK EKVNELSKDI GSESTEDQAM EDIKQMEAES ISSSEEIVPN SVEQKHIQKE DVPSERYLGY LEQLRLKKY KVPQLEIVPN SAEERLHSMK EGIHAAQKEP MIGVNQELAY FYPELFRQFY QLDAYPSGAW YYVPLGTQYT DAPSFSDIPN PIGSENSEKT TMPLW
385	α -S ₁ -CN	Bos d 9	<i>Bubalus bubalis</i>	70905149	97	1	MKLLILTCLV AVALARPKQP IKHQGLPQGV LLENLLRFFV APFPEVFGKE KVNELSTDIG SESTEDQAME DIKQMEAESI SSSEEIVPIS VEQKHIQKED VPSERYLGYL EQLLRLKKYN VPQLEIVPNL AEEQLHSMKE GIHAAQKEPM IGVNQLAYF YPQLFRQFYQ LDAYPSGAWY YVPLGTQYPD APSFSDIPNP IGSENSEKTT MPLW



Fig. 6. Venn diagram of IgE-reactive spots on goat’s milk proteins from three different breeds.

Table 5
Characteristics of IgE-reactive milk protein spots from Saanen, Jamnapari, and Toggenburg 2DE profile identified via MALDI-TOF/TOF MS/MS.

Spot ID	Protein	Allergen	GI no./ Accession no.	Score	Theoretical pI	Experimental pI	Mass (Da)	MW (kDa)	Matches	Peptide sequence	sequence coverage / %	Function	Average normalised volumes		
													Saanen	Toggenburg	Jamnapari
1239* a	α-S ₁ -CN	Cap h 9	954 / P18626	114	5.32	4.23	24,289.59	32	1	R.FVVAPFPEVFR.K	5	Important role in the capacity of milk to transport calcium phosphate.	6.73 ± 0.064	6.57 ± 0.150	6.46 ± 0.110
1282* a	α-S ₁ -CN	Cap h 9	954 / P18626	101	5.32	4.39	24,289.59	30	1	R.FVVAPFPEVFR.K	5	Important role in the capacity of milk to transport calcium phosphate.	7.17 ± 0.200	7.11 ± 0.083	7.03 ± 0.039
2664* a	α-S ₁ -CN	Cap h 9	954 / P18626	83	5.32	4.15	24,289.59	32	1	R.FVVAPFPEVFR.K	5	Important role in the capacity of milk to transport calcium phosphate.	6.6 ± 0.170	6.52 ± 0.350	6.37 ± 0.210
1263* a	α-S ₁ -CN	Cap h 9	954 / P18626	80	5.32	4.34	24,289.59	31	1	R.FVVAPFPEVFR.K	5	Important role in the capacity of milk to transport calcium phosphate.	7.02 ± 0.170	6.88 ± 0.110	6.77 ± 0.027
2667* a	α-S ₁ -CN	Cap h 9	954 / P18626	77	5.32	4.49	24,289.59	30	1	R.FVVAPFPEVFR.K	5	Important role in the capacity of milk to transport calcium phosphate.	7.21 ± 0.099	7.22 ± 0.130	7.06 ± 0.035
2661* a	β-LG	Cap h 5	968 / P67976	77	5.44	5.53	19,921.40	14	2	K.VAGTWYSLAMAASDISLLDAQSAPLR.V K.VAGTWYSLAMAASDISLLDAQSAPLR.V	28	Major component of whey, probably involved in the transport of retinol	7.5 ± 0.091	7.65 ± 0.120	7.52 ± 0.049
1334* a	β-CN	Cap h 11	164115 / P33048	74	5.26	4.66	24,865.09	28	2	R.DMPIQAFLLYQEPVLGPVR.G R.DMPIQAFLLYQEPVLGPVR.G	8	Determination of the surface properties of the casein micelles	7.57 ± 0.130	7.62 ± 0.042	7.43 ± 0.093
1438#	κ-CN, partial	Cap h 12	425862705 / K7Z0L5	53	5.34	4.88	19,162.03	25	1	R.YPSYGLNCYQQR.P	6	Stabilizer for micelle formation, preventing casein precipitation in milk.	7.25 ± 0.140	6.48 ± 0.880	7.14 ± 0.029
1392#	κ-CN, partial	Cap h 12	425862705 / K7Z0L5	51	5.34	4.75	19,162.03	26	1	R.YPSYGLNCYQQR.P	6	Stabilizer for micelle formation, preventing casein precipitation in milk.	6.79 ± 0.180	6.18 ± 0.590	6.69 ± 0.120
1388#	κ-CN, partial	Cap h 12	425862705 / K7Z0L5	39	5.34	5.05	19,162.03	26	1	R.YPSYGLNCYQQR.P	6	Stabilizer for micelle formation, preventing casein precipitation in milk.	6.82 ± 0.067	6.16 ± 0.610	6.64 ± 0.000

Spots with ANOVA p-value < 0.5 and maxfold change > 2.0
a Protein spots that intersected between Saanen and Jamnapari
b Protein spots that intersected between Jamnapari and Toggenburg
* Spots with ANOVA p-value < 0.05

Table 6
Identified IgE-reactive protein in Saanen, Jamnapari and Toggenburg.

Spot ID	Protein	Function	Average normalised volumes		
			Saanen	Toggenburg	Jamnapari
1239 ^a	α -S ₁ -CN	Important role in the capacity of milk to transport calcium phosphate.	6.73 ± 0.064	6.57 ± 0.150	6.46 ± 0.110
1282 ^a	α -S ₁ -CN	Important role in the capacity of milk to transport calcium phosphate.	7.17 ± 0.200	7.11 ± 0.083	7.03 ± 0.039
2664 ^a	α -S ₁ -CN	Important role in the capacity of milk to transport calcium phosphate.	6.6 ± 0.170	6.52 ± 0.350	6.37 ± 0.210
1263 ^a	α -S ₁ -CN	Important role in the capacity of milk to transport calcium phosphate.	7.02 ± 0.170	6.88 ± 0.110	6.77 ± 0.027
2667 ^a	α -S ₁ -CN	Important role in the capacity of milk to transport calcium phosphate.	7.21 ± 0.099	7.22 ± 0.130	7.06 ± 0.035
2661 ^a	β -LG	Major component of whey, probably involved in the transport of retinol	7.5 ± 0.091	7.65 ± 0.120	7.52 ± 0.049
1334 ^a	β -CN	Determination of the surface properties of the casein micelles	7.57 ± 0.130	7.62 ± 0.042	7.43 ± 0.093
1438 [#]	κ -CN, partial	Stabilizer for micelle formation, preventing casein precipitation in milk.	7.25 ± 0.140	6.48 ± 0.880	7.14 ± 0.029
1392 [#]	κ -CN, partial	Stabilizer for micelle formation, preventing casein precipitation in milk.	6.79 ± 0.180	6.18 ± 0.590	6.69 ± 0.120
1388 ^b	κ -CN, partial	Stabilizer for micelle formation, preventing casein precipitation in milk.	6.82 ± 0.067	6.16 ± 0.610	6.64 ± 0.000

Spots with ANOVA p-value < 0.5 and maxfold change > 2.0

a Protein spots that intersected between Saanen and Jamnapari

b Protein spots that intersected between Jamnapari and Toggenburg

* Spots with ANOVA p-value < 0.05

et al., 2013) was. Further allergenicity testing, such as the in vitro basophil activation test (BAT), is needed to confirm the allergenic properties of the proteins, as it has been done previously for the assessment of milk allergy (Rubio et al., 2011) and the assessment of β -LG allergenicity post milk processing (Yang et al., 2020). While genetic variation accounts for the majority of differences in protein composition, the lactation stage, physical traits, and proteolytic digestion by milk enzymes can also influence the composition of milk proteins (Currò et al., 2019; Vacca et al., 2018). By analysing milk of the does from the same breeds in the same environment, with standardised diets and conditions, we were able to eliminate these extrinsic factors. However, because the goat farming industry in Malaysia is small, it is difficult to find pure goat breeds because most goats in Malaysia are crossbreeds (Adawiyah Zayadi, 2021), our sample size for the milk samples used in this study is small and may not fully represent the genetic variation of the specific breeds. To represent the whole breed-specific variants in future research, larger

sample size is required.

This proteomic study of IgE-reactive proteins in pooled goat milk samples could be used as a starting point in the development of hypoallergenic goat milk. Future research should concentrate on individual goats from Toggenburg that have been shown in this study to have low milk-specific IgE-binding protein variants, as previous studies have clearly shown that genetic variation in caseins and whey proteins may occur at the individual goat level rather than interspecies or intraspecies (Ballabio et al., 2011). Current attempts to reduce the allergenicity of milk by using gene-editing technology to knock out or knock down allergens such as β -LG in cow's milk (Sun et al., 2018) and β -CN in goat's milk (Hazebrouck et al., 2013) have had limited success due to the presence of multiple milk allergens and a limited database on milk proteins and milk protein variants. The results of this study can be used as a database to compare allergens and putative allergens in different goat breeds, as well as used in selective breeding to develop hypoallergenic goat milk.

4. Conclusion

In conclusion, milk protein blots with different intraspecies and interspecies IgE-reactive spots profiles were obtained after 2DE IgE-immunoblotting of cow's and goat's milk protein with CMPA patient serum. Cross-reacted protein spots on goat's milk protein blot have been identified as α S₁-CN, κ -CN and β -LG; major milk allergens, and five other proteins not associated with milk allergens. In this study, goat's milk protein was proven to be less IgE-reactive compared to cow's milk in CMPA patient sera. Plus, the genetic variance and polymorphism within goat species were observed to have affected the IgE-binding capabilities of milk protein produced. Although goat's milk may not be the 'perfect' alternative to cow's milk, proteomic information on milk protein from different goat breeds in this study should provide a glimpse of its hypoallergenic potential or the potential for future study on the effect of genetic variance on the milk produced.

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CRediT authorship contribution statement

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Declaration of interest

The authors declare no conflict of interest.

Data availability

Data available within the submitted manuscript.

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