COMPARATIVE MAPPING OF CROSS-REACTIVITIES OF MILK PROTEINS FROM DIFFERENT GOAT BREEDS WITH COW'S MILK ALLERGENS BY PROTEOMIC APPROACH

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

November 2022

IPTSM 2022 11

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Cow's milk allergy is prevalent among atopic children and the exclusion of milk proteins from their diet is the most common solution in managing the allergic symptoms. Milk from other ruminants such as goats have previously been thought to be a good alternative to cow's milk for cow's milk protein allergic (CMPA) individual but there are increasing evidence of their proteins to have cross-reactivities to cow's milk allergens. However, no conclusive studies have been done in comparing the allergenic potential between the different goats breed that cross-reacted to cow's milk allergens. This study therefore aimed to profile and compare milk proteins from different goat breeds that have cross-reactivities to cow's milk allergens using proteomic approach. Two-dimensional gel electrophoresis (2DE) coupled with immunoblotting with allergen-specific serum IgE and mass spectrometry was applied to accurately identify and profile multiple allergens in protein samples. Efficacies of three protein extraction methods; a milk dilution method in urea/thiourea based buffer (Method A), a triphasic separation protocol in methanol/chloroform solution (Method B), and a dilution in sulphite-based buffer (Method C), were first evaluated for the 2DE-proteomic on milk from two different goat breeds, Saanen and Jamnapari. Method A was selected as the most suitable method with 72.68% and 71.25% protein recovery, 199±16.1 and 267±10.6 total spots resolved on 2D gels, optimal spot resolution and minimal streaking for Saanen and Jamnapari samples. Protein profiles of skimmed milk extracts from Saanen, Jamnapari, and Toggenburg (n = 6 animals/ breed) were then compared by 1D- and 2D-gel electrophoresis (2DE). Cow's milk was used as a control (n = 6). Proteins that cross-reacted with serum IgE of CMPA patients (n = 10) were compared and identified by IgE-immunoblotting and mass spectrometry. Matrix-assisted laser desorption ionisation time-of-flight tandem mass spectrometry (MALDI-TOF/TOF MS) analysis of IgE-reactive proteins detected in the milk of the three goat breeds revealed that the protein spots identified with high confidence were proteins that are homologous to common cow's milk allergens such as α_{S1}-

casein (α_{S1}-CN) (spots 402, 1239, 1282, 385, 2664, 1263, 2667), β-casein (β-CN) (spot 1334), κ-casein (κ-CN) (spots 1438, 1392, 1388), and beta-lactoglobulin (β-LG) (spot 2661). Among the dairy goat breeds evaluated in this study, Jamnapari's milk proteins were shown to have cross-reactivities with four main 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 main milk allergens, α_{S1}-CN and β-LG, while Toggenburg goat's milk proteins exhibit reactivity to only one of the main milk allergens; κ-CN. Although milk from different goat breeds had similar protein composition, the variation in breed was observed to have affected the IgE- reactivity of the milk protein. The findings of this study may provide more information for future research on the hypoallergenic potential of milk from different breeds or species, as well as the effect of genetic variation on the composition and structure of protein on cellular activities.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PEMETAAN PERBANDINGAN REAKTIVITI SILANG PROTEIN SUSU DARIPADA BAKA KAMBING YANG BERBEZA DENGAN ALAHAN SUSU LEMBU MENGIKUT PENDEKATAN PROTEOMIK.

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Alahan susu lembu lebih lazim dalam kalangan kanak-kanak atopik dan pengecualian susu daripada diet mereka adalah penyelesaian yang terbaik dalam mengawal gejala alahan. Susu daripada ruminan lain seperti kambing sebelum ini dianggap sebagai alternatif yang baik kepada susu lembu untuk individu yang mempunyai alahan protein susu lembu (CMPA), tetapi terdapat peningkatan kes protein di dalam susu kambing mempunyai tindak balas silang dengan alergen susu lembu. Walau bagaimanapun, tiada kajian konklusif dilakukan dalam membandingkan potensi alahan antara baka kambing yang berbeza yang bertindak balas silang kepada alergen susu lembu. Oleh itu, kajian ini bertujuan untuk memprofil dan membandingkan protein susu daripada baka kambing yang berbeza yang mempunyai tindak balas silang dengan alergen susu lembu menggunakan pendekatan proteomik. Gabungan elektroforesis gel dua dimensi (2DE) dan immunoblotting, menggunakan IgE serum yang bertindak balas secara khusus kepada susu lembu dan spektrometri jisim mampu untuk mengenal pasti dan memprofilkan pelbagai alergen dengan tepat dalam sampel protein. Keberkesanan tiga kaedah pengekstrakan protein; pencairan susu dalam penimbal berasaskan urea/tiourea (Kaedah A), pemisahan trifasa dalam larutan, metanol/kloroform (Kaedah B), dan pencairan dalam penimbal berasaskan sulfit (Kaedah C), pada dinilai untuk 2DE-proteomik susu daripada dua baka kambing berbeza, Saanen dan Jamnapari. Kaedah A dipilih sebagai kaedah yang paling sesuai dengan 72.68% dan 71.25% kadar perolehan semula protein, 199±16.1 dan 267±10.6 jumlah tompok dipisahkan dalam gel 2D, resolusi tompok optimum dan coretan minimum untuk sampel Saanen dan Jamnapari. Ekstrak protein susu skim dari Saanen, Jamnapari, dan Toggenburg (n=6 haiwan/baka) kemudiannya dibandingkan dengan elektroforesis gel 1D dan 2D. Susu lembu digunakan sebagai kawalan (n=6). Protein yang bertindak balas silang dengan serum IgE pesakit CMPA (n=10) dibandingkan dan dikenal pasti melalui IgE-immunoblotting dan spektrometri jisim. Melalui analisis spektrometri jisim masa penerbangan bantuan matriks

(MALDI-TOF/TOF MS), tompok protein yang dikenal pasti dengan keyakinan tinggi didalam susu ketiga-tiga baka kambing adalah protein yang homolog dengan alergen susu lembu. Alergen susu lembu yg dikenalpasti adalah α-$_{S1}$-casein (α_{S1}-CN) (bintik 402, 1239, 1282, 385, 2664, 1263, 2667), β-kasein (β-CN) (bintik 1334), κ-casein(κ-CN) (bintik 1438, 1392, 1388), dan beta-lactoglobulin (β-LG) (bintik 2661). Antara baka kambing tenusu yang dinilai dalam kajian ini, protein susu Jamnapari menunjukkan tindak balas silang dengan empat alergen susu utama; α_{S1}-CN, β-CN, κ-CN dan β-LG. Protein susu kambing Saanen, sebaliknya, bertindak balas silang dengan dua alergen susu utama, α_{S1}-CN dan β-LG, manakala protein susu kambing Toggenburg mempamerkan tindak balas kepada hanya satu daripada alergen susu utama; κ-CN. Walaupun susu daripada baka kambing yang berbeza mempunyai komposisi protein yang sama, variasi dalam baka diperhatikan telah mempengaruhi kereaktifan IgE protein susu. Penemuan kajian ini mungkin dapat memberikan lebih banyak maklumat untuk penyelidikan masa depan tentang potensi hipoalergenik susu daripada baka atau spesies yang berbeza, serta kesan variasi genetik terhadap komposisi dan struktur protein ke atas aktiviti selular.

ACKNOWLEDGEMENT

In the name of Allah, the Most Gracious and the Most Merciful. Alhamdulillah, all praises to Allah for the strengths and His blessing in completing this thesis. Special appreciation goes to my supervisor, Dr Nuzul Noorahya binti Jambari, for her supervision, patience, tolerance and constant support throughout the completion of this project. I would like to also express my deepest gratitude to Associate Prof. Dr. Intan Hakimah binti Ismail for her support and valuable advices on the allergy and clinical aspects of the project and also, Dr. Jameel R. Al-Obaidi for his continuous guidance on the proteomic aspect throughout the completion of this project. Last but not least, the staffs and officers from Institute of Tropical Agriculture and Food Security, Faculty of Food Science and Technology and Service and Training Unit, Agro-Biotechnology Institute Malaysia, for their endless assistance throughout this Master's project.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

Abbreviation	Description
1DE	One dimensional gel electrophoresis
2DE	Two-dimensional gel electrophoresis
2-ME	2-Mercaptoethanol
AA	Amino Acid
CAN	Acetonitrile
ANOVA	Analysis of variance
AP	Alkaline phosphatase
BAT	Basophil activation test
BSA	Bovine serum albumin
CBB	Coomassie Brilliant Blue
CHAPS	3-[(3-cholamidopropyl) dimethylammoniocholamidopropyl) dimethylammonio]-1-propanesulfonate
CMPA	Cow's Milk Protein Allergy
CN	Casein
DTT	1,4-dithiothreitol
ECL	Enhanced Chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
FTICR	Fourier-transform ion cyclotron resonance
HCCA	α-Cyano-4-hydroxycinnamic acid
HPLC	High performance liquid chromatography
HRP	Horse Radish Peroxidase
IAA	Iodoacetamide
IEF	Isoelectric focussing

Ig	Immunoglobulin
IL-	Interleukin
IPG	Immobilized pH gradient
LB	lysis buffer
LC-MS	Liquid chromatography mass spectrometry
LF	lactoferrin
LIT/LTQ	Linear ion trap
MALDI-TOF	Matrix assisted laser desorption ionisation time-of-flight
MREC	Medical Research and Ethics Committee
MRM	Multi reaction monitoring
MS	Mass Spectrometry
MW	Molecular weight
NC	Nitrocellulose
NCBI	National Centre for Biotechnology Information
OVA	ovalbumin
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-tween 20
pI	Isoelectric point
PVDF	Polyvinylidene difluoride
Q	Quadrupole
QIT	Quadrupole ion trap
SB	Solubilising buffer
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SPT	Skin prick test
TEMED	Tetramethylethylenediamine

TFA	Trifluoracetic acid
TOF	Time of flight
UHPLC	Ultra-high performance high-performance liquid chromatography
UPLC	Ultra-performance liquid chromatography
WB	Western blot
WHO/IUIS	World Health Organization and International Union of Immunological Societies
α_{S1}-CN	α_{S1}-casein
α-LA	α-Lactalbumin
α_{S2}-CN	α_{S2}-casein
β-CN	β-casein
β-LG	β-lactoglobulin
κ-CN	κ-casein
Ɣ-CN	Ɣ-casein

CHAPTER 1

INTRODUCTION

1.1 Background of study

Children are more likely than adults to suffer from a milk allergy as the milk is a vital food source for most infant in their early year of life (Yadav & Naidu, 2015). Primarily, colostrum is secreted by female mammal's mammary gland, which contains antibodies that help infants to strengthen their immune system to fight infection and bacteria followed by breast milk which contain nutrients such as carbohydrate, protein, fat, minerals and vitamins to supply new-borns throughout their infancy (Donovan, 2019). As cow's milk has been traditionally consumed, it is usually chosen as an alternative to feed infant who may have limited access to breast milk. When cow's milk is introduced into the digestive system, the immune system may perceive it as foreign and cause an allergic reaction. Allergy is an immune mediated reaction caused by substance, particularly food, pollen or fur (Center for Disease Control and Prevention, 2013). According to World Health Organization and International Union of Immunological Societies (WHO/IUIS) allergen nomenclature, common milk allergens that are found in the casein fraction are α-$_{S1}$-casein (α_{S1}-CN), α-$_{S2}$-casein (α_{S2}-CN), kappa casein (κ-CN), and beta casein (β-CN) whereas β-lactoglobulin (β-LG), α-lactalbumin (α-LA), serum albumin and immunoglobulin (Ig) in whey fraction. According to cases that have been reported and research on cow's milk protein allergies, neither a specific reactive protein nor all milk proteins have ever caused an allergic reaction in CMPA patients (Ah-Leung et al., 2006; Voskamp et al., 2014). Differences in the immunity of each patient showed differences in their allergic reactions towards specific milk proteins. Sensitization of food protein or antigen occurred not only via gastrointestinal tract after ingestion of food but also through food antigen contact on oral cavity and skin. These immune responses can be divided into IgE-mediated and non-IgE-mediated categories. the presence of food antigens that the immune system perceives as foreign, triggering the plasma cell to produce specific IgE which would then bind to the food antigen, then to the mast cells, which trigger the chemical mediator such as histamine to be secreted and initiate either non-lethal reaction such as rashes or lethal reaction such as anaphylaxis (Sampson et al., 2018; Yu et al., 2016). The immunological mechanism underlying immunoglobulin E (IgE)-mediated cow's milk allergy has been the subject of extensive research for many years. The identification of the key immune cells (mast cells, B cells) and molecules (IgE) in the allergic process has led to the realisation that avoiding IgE-crosslinking epitopes is effective in reducing allergic symptoms but cannot be considered a treatment (Knol et al., 2019).

Due to these circumstances, Families with kids who are hypersensitive to cow's milk may need to choose different milk replacements. Goat, sheep, horse, camel, and donkey milk from ruminants have all had their hypoallergenic potentials investigated (Bernard et al., 1999; Hinz et al., 2012). As an alternate solution,

plant-based milks like soy and nut milk have been taken into consideration. However, mammals' milk is a better option when it comes to supplements for postnatal development of infants because it innately contains the vital nutrients for the growth of the newborn (Vojdani et al., 2018). Availability of these alternatives are also limited to the climate of the area, as the climate differences between the continent or area affect the type of crop or livestock that can be farmed or reared. In Malaysia, according to the statistics of national milk product output, production of milk increased rapidly between the years 2013 and 2019 (FAO, 2020), with dairy cattle and goats making up the majority of the agricultural production. There aren't a lot of large-scale dairy goat farms in Malaysia because the large number of dairy goat farms are owned by local farmers (Shahudin et al., 2018). Among dairy goat breeds reared around Malaysia are Saanen, Jamnapari, Toggenburg, Alpine and Shami (Liang & Paengkoum, 2019). These breeds have been largely known for their high dairy yield, thus in order to enhance this quality, cross-breeding with other breed with other quality such as high meat yield, quality or better resistance toward disease has been practised among farmers(Gipson, 2019).

However, goat's milk as a subtitute to milk allergy patients may possess some issues due high similarities between the two species milk proteins (Bernard et al., 2012). The casein and whey protein fractions of milk are approximately equivalent among phylogeny species. It has been documented that the milk proteins from ruminants of multiple species exhibit cross-reactivities (Bellioni-businco et al., 1999; El-Agamy et al., 2009; Goodman et al., 2007). However, there have been instances of patients with goat's milk allergies who had never experienced an allergic reaction to cow's milk, and the opposite is also the case (Ah-Leung et al., 2006; Bellioni-businco et al., 1999; Goh et al., 2019). IgE responses to sheep and goat milk were relatively weak than most of those to cow and buffalo milk, as demonstrated by a study on the cross-reactivity of casein fraction among numerous ruminant species, indicating that some CMPA patients may have tolerance to ewe's and goat's milkv(Clark & García, 2017). Besides, past studies on the genetic variations between goat breeds has demonstrated that these variations have an impact on the yield and chemical compositions of goat milk. (Selvaggi et al., 2014;Idowu & Adewumi, 2017). Hypoallergenic potential of goat's milk could be further assessed through proteomic means rather than dismissing it entirely.

Allergenomic remains a powerful tool that can be used for rapid and comprehensive identification and characterisation of multiple allergens in specific sources and allows further direct assessment of cross-reactivity of allergens with other closely related sources (Di Girolamo et al., 2015). Protein analysis methods such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoassay techniques such as enzyme-linked immunosorbent assay (ELISA) and Western blotting (WB) have been utilized for allergen detection in allergenomics studies (Raikos et al., 2006; Sharma et al., 2017). In bottom-up proteomic studiesBefore peptide products are analysed using mass spectrometry (MS), a mixture of proteins would be subjected to several processes, such as isolation via SDS-PAGE and proteolytic cleavage. For specific detection of allergenic protein, 2-dimensional SDS-PAGE

(2-DE) allows further separation of proteins based on their molecular weight (MW) and isoelectric point (pI) to generate protein dispersion patterns. From the protein spots produced from 2DE, further analysis on the allergen-specific IgE reactivity can be conducted using Western blotting. Western blotting is a technique where protein from SDS-PAGE gel was electroblotted, then treated with antibody of the target proteins. Antibody of the target protein will bind to the protein on the blot then treated with labelled secondary antibody which will bind to the primary antibody forming an antibody complex that can indicate the location of the target protein on the SDS-PAGE gel. Target proteins can be identified with the help of MS analysis techniques including matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF/TOF) and liquid chromatography mass spectrometry (LC-MS/MS). The combination of 2DE-immunoblotting and MS analysis has been widely used in allergen detection and identification because of its accuracy when dealing with food proteins, which may contain a wide variety of isoforms and are susceptible to post-translational modifications and structural changes during storage and processing (Le et al., 2017; Villa et al., 2018). Previous studies shown differences between species and polymorphism within a species has produced milk protein with different composition and properties, and the variation has also been reported to affect the allergenicity of the protein (Razmkabir et al., 2021; Selvaggi et al., 2014). Several research have looked into how genetic variation influences allergenicity in relation to a specific protein source, such as casien or whey (Cong et al., 2020; Kordesedehi et al., 2018). However, the actual hypoallergenic capability of goat's milk has not been completely investigated due to a lack of data on the allergenicity of total protein in milk of different species and breeds.

1.2 Problem Statement

Goat's milk has always been preferred over cow's milk for CMPA patients due to its nutritional and fat digestion. Some CMPA patients have shown an allergy to goat's milk, and vice versa; there have also been reports of patients showing an allergy to goat's milk but not cow's milk. Researchers have found that the structure of goat's milk protein and its allergenicity to those with milk allergy varies depending on the breed of goat employed. Taking into account the wide range in milk protein quality depending on the breed of goat used and the lack of specificity in the reactions of CMPA patients to milk protein. In order to ascertain the allergenicity of goat's milk protein for CMPA patients, the cross-reactivity of goat's milk protein from the common dairy goat breed farmed in Malaysia, Saanen, Jamnapari, and Toggenburg, should be investigated.

This study aims to compare and discover allergenic proteins in milk from various dairy goat breeds that cross-react with cow's milk allergen by employing a proteomic approach. Due to the qualitative nature of 2DE proteomic analysis, the quality of the protein samples is crucial. The determination of allergenic protein in the latter stages of the investigation will depend on the quality of the image formed on the 2D profile following electrophoresis. Optimizing the protein extraction method for 2DE proteomic analysis of goat's milk enabled us to identify one that yielded the highest protein recovery and the greatest number of

protein spots with the best quality possible and the least streaking issues. Several goat breeds' milk proteins (Saanen, Jamnapari, and Toggenburg) were tested for IgE cross-reactivity with cow's milk allergen utilising 2DE followed by immunoblotting in a serum sample from a patient with CMPA. Any cross-reactivity was visible as specific IgE produced by CMPA patient serum is bound to bind to the specific goat's milk proteins during IgE-immunoblotting. MALDI-TOF/TOF MS analysis for each cross-reactive spot will provide the information on the identity of the cross-reactive spots. The overall research flow of this thesis was summarised in Figure 1.1.

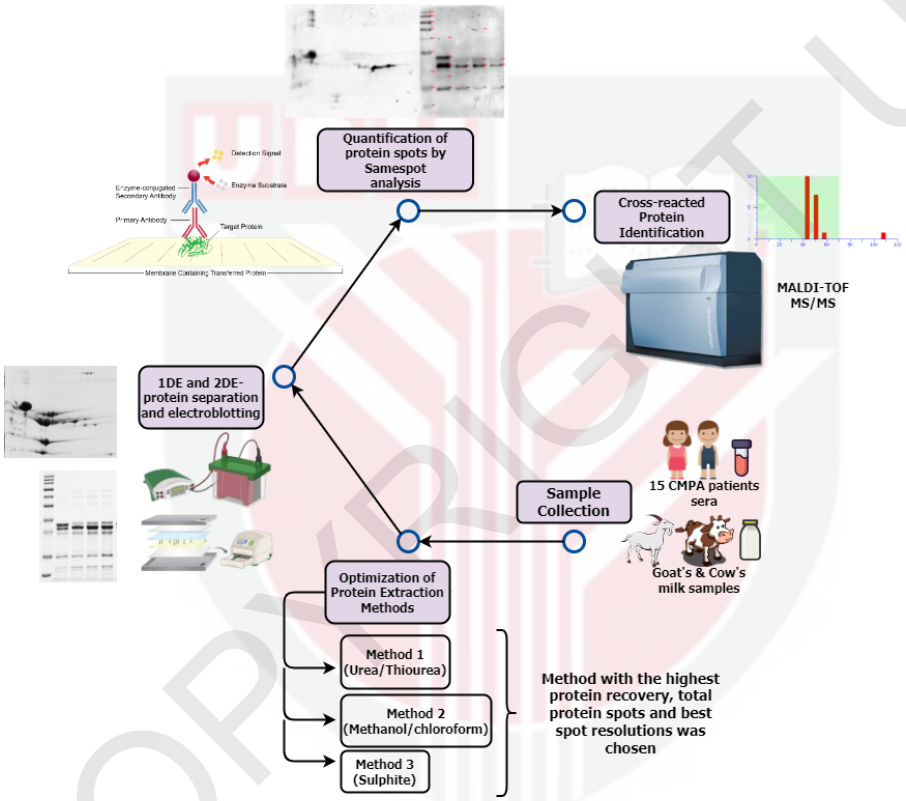

Figure 1.1: Overall research flow chart

1.3 Hypothesis

The differences in goat's breed will affect the milk protein profile and it's cross-reactivity with cow's milk allergen

1.4 Research Objectives

1. To optimize the protein extraction method for goat's milk samples for gel-based proteomic analysis.
2. To compare the profile the milk proteins from Saanen, Jamnapari, and Toggenburg with cross-reactivity to cow's milk allergens by 1D- and 2D-IgE–immunoblotting and MALDI-TOF/TOF MS analysis.

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