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PROTEOMICS OF BROILER'S LIVER

(The near Consumer insight on Liver Proteomics of Malaysian Broiler in Comparison with Indigenous Chicken)

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Summary Statement
The study aims to investigate the effect of the advancements in selective breeding to the liver function in broilers which have much faster growth compared to indigenous chicken in near consumer experience.

Abstract
In this study, the differences in protein expression from chicken liver between commercial broilers (CB) and indigenous chicken (IC) have been analysed by using two-dimensional gel electrophoresis (2D-PAGE). The study was conducted adjacent to consumer experience in order to find out the differences of the protein profile in liver tissues between the two popular types of chickens that have been offered to the Malaysians consumer for over 50 years. In 2D-PAGE, six spots were successfully identified differentially expressed in term of intensity. Those proteins are phosphoenolpyruvate carboxykinase (PEPCK) (2 spots), delta-1-pyrolline-5-carboxylate (P5CDH), soluble epoxide hydrolase (sEH), heat shock protein 90 (HSP90) and γ-actin. PEPCK and P5CDH enzymes were expressed higher in CB compared to IC, suggesting the utilization of alternative metabolic pathways for broilers' energy metabolism. The sEH expression was found higher in CB compared to IC probably due to the process of xenobiotic metabolic activity to eliminate harmful epoxide inside the CB's liver. HSP90 was found less in CB compared to IC, which may indicate the lack of regulation of heat stress response in CB. The higher expression of actin in IC liver suggested a good form of structure integrity in IC liver compared to CB, which may happen due to the age differences.Most of the differentially expressed proteins are involved in energy metabolism, stress regulation, and cellular structure.These differences could arise from many variables involved which may include breeds, age, feed regimen, drug treatment and rearing environment.

Keywords - Chicken, Broilers, Indigenous, Proteomics, 2D-Electrophoresis, Liver

I. INTRODUCTION

Poultry, notably chicken is one of the major meats or protein source in Malaysia and the demands for chicken keep increasing drastically every year. The consumption of chicken meat by Malaysians has shown an increment from 36 to 39 kg per capita from the year2000 to 2011(Jayaraman et al., 2013). The increased demand in chicken consumption is due to the fact that the chicken is universally accepted as common food by people from various religious and culture, as well as the fact that it is relatively cheap compared with other types of meats.

Two types of chickens that are mainly reared for meat are broilers and indigenous chickens. In Malaysia, the broilers are mostly originated from Cobb & Ross strain while the indigenous chickens are likely to be originated from Rhode Island Red strain. Broilers are farmed by modern integrated poultry raising facilities and are chosen by the farmers due to their ability to produce high feed-meat conversion ratio(Torok et al., 2011). The high feed-meat conversion ratio depends mostly on the rearing environment, diet and also the breed of chickens(Willems et al., 2013). This conventional type of chicken is grown to be marketed within 6-8 weeks and has a high yield of breast meat because the farmers focus on maximizing the production in the shortest time(Silva et al., 2016). In many cases, due to rapid juvenile growth in broilers, they are susceptible to diseases. Health issues such as ascites, sudden death syndrome, excessive fatness and also leg weakness have become known threats tobroilers. Metabolic disorderscouldalso pose the threats to broilers and causing more economic loss compared toinfections(Kumari et al., 2016). On the other hand, the growth rate of indigenous chickens is slower and may take about 12-14 weeks before they can be sold. Although this type of chicken is regarded as less efficient meat producer, they have better liveability, more active and reported to givea different meat quality compared to broilers(Sokołowicz et al., 2016).

Despite the incredibly fast growth of commercial broilers, theyhave been sold to the consumer for a long time without us knowing the quality of their meat, especially in molecular perspective. As liver is one of the main organs that involves in several important metabolic processes, we postulate that variety of factors that involved in broilers growth could affect the condition of the liver in comparison with the normal growth rate of chicken. Previous finding suggested that adiverse multifactorial causes

such as environmental, nutritional, metabolic and hormonal factors, hadimposes a negative effect on the liver quality(Rozenboim et al., 2016). Thus, proteomics approaches involving two-dimensional gel electrophoresis (2D-PAGE) wereconducted to analyse and compare the expression of proteins in theliver tissues in broilerand indigenous chicken.

II. MATERIALS AND METHODS

2.1 Animals and Husbandry

Chicken, *Gallus gallus sp.*is the main animals used in this study. The two lines of chickens were used which is the broiler and indigenous chicken. The broiler is originated from Cobb Ross strain while the indigenous chicken isoriginated from Rhode Island Red strain. The experiment was conducted as near as consumer experience as possible. Both lines of chicken were left treated as the same as the real-lifetime took place. The primary aim was to see the differences protein expression that could rise from different lines of chicken in the liver that might be expressed unusual to the chicken as well as to the consumer. Thus, there are many variables that could arise from the experiment. All those variables are described in **Table 1**.

2.2 Sample Collection

The male broilers and indigenous chickens were purchased from the Balok Poultry Farm and from the local farmers at Cherok Paloh Village, Kuantan, Malaysia respectively. The chickens were purchased alive to avoid from postmortem protein degradation and the effect from different slaughtering approaches. The chickenswere randomly selected from the population in triplicates (n = 3 per line). The chickens were selected to the highest maturity to imitate the condition of chicken that are currently being offered to theconsumer. The weights of the chickens were ranged between 2.2-2.8 kg for broilers at the age of 7 weeks, and 1.3-1.7 kg for indigenous chicken at the age of 14weeks. The animals have been carefully handled following the guidelines from Animal Ethics Policy by International Islamic University Malaysia.The birds were slaughtered followingIslamic slaughtering method which basically being practice by Malaysians today. The Islamicslaughtering methodhas been considered humane by some authors and religious scholars (Farouk et al., 2014). Asharp knife was used to cutoffchicken'sthroat, windpipe and blood vesselson theneck. The bodies were dissected and the livers were taken out and stored at -80°C until further used.

2.3 Protein Extraction

The protein was extractedaccording to the optimized method from previous research (Wu et al., 2009). The liver (100 mg) was homogenized under liquid nitrogen with pre-chilled mortar and pestle. The grounded liver was dissolved in 1.0 ml of extraction buffer (7 M Urea, 2 M Thiourea, 2% CHAPS, 65 mM DTT, 0.2% Bio-Lyte 3/10, 1 mM protease inhibitor, 20 U/ml DNase I, 0.25 mg/ml RNase A) and shaken on ice for 1 hour. Then, the mixture was mixed vigorously using vortex for 2 min and left on ice for another 2 min. The process was repeated five times before the sample was shaken on ice for another 1 hour and centrifuged at 14,000 × g at 15°C for 1 hour. The supernatant was taken out and the pellet was discarded.

2.4 Protein Quantification

The total protein concentration of the samples was determined by using Bradford assay (Bradford, 1976; Cheng et al., 2016). A series of serial dilution of bovine serum albumin (BSA) was mixed with 10% extraction buffer solution. Then, 1000 µl of Bradford reagent (Sigma-Aldrich, USA) was added to the BSA mixture and left for 2 min. The absorbance value was measured at 595 nm by protein quantification mode using WinLab software (Perkin Elmer, USA) to generate the standard curve. For each sample, the quantification was performed in triplicates.

2.5 SDS-PAGE

The electrophoresis was carried out by using mini-gel of 12% running gel (1.5 M Tris-HCl pH 8.8, 10% SDS, 30% acrylamide/bis, TEMED, 10% APS) and 4% stacking gel (0.5 M Tris-HCl pH 6.8, 10% SDS, 30% acrylamide/bis,TEMED, 10% APS). A protein marker, Novex Sharp Pre-stained protein standard (Thermo Fisher Scientific, USA), was used as a molecular weight marker. A constant amount of samples (50 µg)premixed with Laemlli sample buffer were pipetted into the specified well. Running Electrode Buffer Tris/Glycine/SDS (Bio-Rad) was prepared and filled upto the tank. The gel was run at 200 V for 50 min until the bromophenol blue reached the bottom of the plate. The gel was then stained with BioSafe Coomassie Blue G-250 for 1 hour on the table shaker. Then, it was destained with distilled water overnight.

2.6 Two-Dimensional Gel Electrophoresis

The sample (400 µg) was added to the rehydration buffer (7 M Urea, 2 M thiourea, 2% CHAPS, 65 mM DTT, 0.2% Bio-Lyte 3/10, few grains of Bromophenol blue) resulting in a total volume of 125 µl. The non-linear (NL) immobilized pH gradient (IPG) strips (Bio-Rad) (pH 3-10, 7cm) were rehydrated overnight with the premixed protein sample mentioned above. The isoelectric focusing (IEF) was performed in PROTEAN IEF Cell (Bio-Rad) at 20 °C, using modes 100 V, 20 min; 350 V, 1 hour; 3500 V, 2 hours; 10000 Volt•Hour and hold at 100 V. After IEF, the strips were equilibrated with a buffer containing 6 M urea, 20% glycerol, 2% SDS, 1.5 M Tris pH 8.8 and 1% (w/v) DTT for 15 min followed by an additional 15 min with the same equilibration buffer in which DTT was replaced with

2.5% (w/v) iodoacetamide. The equilibrated strips were moved onto 12% polyacrylamide gel. The strips were covered with 0.5% agarose, and a second-dimensional separation was performed using mode 150 V for 1 hour until the bromophenol blue reached the bottom of the gel. The gel was stained withCoomassie Brilliant Blue G-250 solution for 1 hour and destained with distilled water overnight.

2.7 Gel Image Analysis

All the gel images were obtained using GS-800 Densitometer (Bio-Rad). The gel images from SDS-PAGE were analysed using Quantity One (Bio-Rad) application by comparing the number of resolved protein bands and also its intensity between all the samples while the images from 2D-PAGE were analysed by using PDQuest application (Bio-Rad) by comparing protein spots between the two types of samples. Image spots were detected using an automatic method and manual corrections. The analysis set was created according to the quantitative method between replicates gel. The Student's *t*-test analysis set with 95% confidence level was used to determine if two sets of data are significantly different from each other. In addition, the method of above upper limit was chosen and set to be in 2.0 fold in order find the spots in broilers which have at least twice the intensity compared to the corresponding spots in the indigenous liver samples, or vice versa. The intersection of analysis between Student's *t*-test and Quantity 2.0 fold were performed to narrow down the spots that have higher intensity.The spots that have observable differences in images and gels were carefully selected for MALDI-TOF/ MS analysis.

2.8 Peptide Sequencing and Homology Examination

The selected spots were manually excised from the gel with a clean razor blade. The in-gel digestion was done following protocols from PROMEGA by using Trypsin Gold. The 500 µl microcentrifuge tube was prewashed with 50% acetonitrile (ACN) / 0.1% trifluoroacetic acid (TFA). Then, the excised gel was inserted into the tube. The gel was destained twice with a total of 200µl NH_4HCO_3 / 50% ACN (1:1, vol/vol) for 45 min at 37°C. The gel was then dehydrated in SpeedVac for 15 min to remove excess ACN. The amount of 1µg/µl trypsin goldwas resuspended in 50mM acetic acid.Next, the mixture was diluted in 40mMNH_4HCO_3 / 10% ACN (9:1, vol/vol) to the final concentration of 20 µg/ml. The gel was incubated with 15µl volume of trypsin solution for 1 hour at room temperature.Then, the digestion buffer (40mMNH_4HCO_3/10% ACN) was added to completely cover the gel. The mixture was incubated overnight at 37°C. The gel buffer was removed to a new microcentrifuge tube and dried in SpeedVac for 3 hours at room temperature before it went for mass spectrometric analysis. The extracted protein was analyzed using MALDI-TOF/MS Proteomics Analyzer (5800 ABSCIEX, USA) from Walter and Eliza Institute of Medical Research, Melbourne, Australia.The data from MALDI-TOF/MS were searched against Ludwig non-redundant (http://jpsl.ludwig.edu.au), SwissProt (http://www.uniprot.org/uniprot/) and non-redundant NCBI databases(http://www.ncbi.nlm.nih.gov/) by using Mascot Peptide Mass Fingerprint application(http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF). The fixed modification of Carbamidomethyl (C) and Oxidation (M) were selected with peptide tolerance of ±100 ppm and known contaminant keratins were excluded.The taxonomy of *Gallus gallus* organism was selected for primary reference. MASCOT protein score greater than 52 was considered statistically significance ($p \leq 0.05$). The best ion score with aconfidence interval of ≥ 95% and lowest E-value was selected as the identified protein.

III. RESULTS

3.1 Extracted Protein Yield

Protein extraction was aimed to yield as much protein as possible for all the samples. The protein concentrations extracted from 100 mg of commercial broiler's (CB) and indigenous chicken's (IC) tissue liver were found to be 12.57 ± 3.523 mg/ml and 8.69 ± 3.632 mg/ml respectively.The differences in protein concentration might be due to the myoglobin content on CB liver which was observed as more 'reddish' in colour compared to IC's liver.

3.2 Protein separation using SDS-PAGE

Protein samples were run on apolyacrylamide gel and the separated proteins are presented in **Figure 1**. In general, each sample showsaround 28-33 bands.Based on the results, the highest protein intensities can be found at60 kDa, 40 kDa, 35 kDa, 25 kDa, 17 kDa, 15 kDa and 13 kDa, which depicted theoverall electrophoretic pattern of chicken's liver. Eleven bands were found to be different in intensity when the two types of chickenwere compared, as marked by arrows. No differences could be seen in terms of absence or presence of protein bands.The 6 bands that have the high differences were selected and searched through Expasy Swiss-2DPAGE website against the database from mouse liver samples. Based on the putative protein listed in **Table 2**, it is shown that 11 out of 18 of the identified proteins are involved in metabolic related function.The actual proteins composition that expressed higher or lower in the listed bands may vary due to the vastness of proteins list that located on the same molecular weight. However, these predictive findings have shown a general idea of the differences that may occur in the composition of protein in the liver samples in the comparison between two samples. The samples were further

resolved in 2D-PAGE to analyse the protein aggregates in more details.

3.3 2D profiling from Chicken's Liver

The protein spots detected in both groupis shown in**Figure 2**. Based on the Student's *t*-test analysis and quantitative 2.0-fold increase, 6 spots were found to be significantly different between each other. The spots were selected and successfully identified with MALDI-TOF/MS.

The four spots (Spot ID: 1, 2, 3 and 4) were detected to be quantitatively expressed with a much higher intensity in broiler compared to indigenous chicken'sliver. On the other hand, another two spots (Spot ID: 5 and 6) were detected and quantitatively expressed with a much higher intensity in indigenous chicken compared to broiler'sliver (**Table 3**).The proteins that were successfully identified with MASCOT Peptide Mass Fingerprint are summarized in**Table4**.

IV. DISCUSSION

Following differential analysis of liver proteins from each type of chicken, six spots were successfully identified by MALDI-TOF/MS. The results showed the involvement of various proteins in several metabolic activities in mitochondria and cytosol of the liver tissues.

The proteins identified were involved in gluconeogenesis, proline metabolism, secondary metabolites hydrolases, stress responses andcellular structure. Four spots, identified as phosphoenolpyruvate carboxykinase (PEPCK) (2 spots), delta-1-pyrroline-5-carboxylate dehydrogenase (P5CDH), and soluble epoxide hydrolase 2 (sEH) were highly expressed in commercial broilers (CB) compared to indigenous chicken (IC) liver. On the contrary, two spots, identified as HSP90 and actin were detected to be more highly expressed in IC liver compared to CB.

4.1 Proteins Related to Energy Metabolism

PEPCK enzyme was detected and highly expressed in CB compared to IC liver. PEPCK enzyme is involved in the*de novo* synthesis of glucose from non-hexose precursors in gluconeogenesis(Croniger et al., 2002). Apart from that, PEPCK enzyme also takes part in glyceroneogenesis that plays a big role in the overall triglyceride/fatty acid cycle between liver and adipose tissue(Hanson and Reshef, 2003).

In normal healthy mammals, gluconeogenesis is a metabolic pathway that occurs in thetime of fasting or exercise where glucose was depleted. This pathway will utilize non-carbohydrate precursors such as malate, lactate, and amino acids to produce glucose for body consumption. Apparently, the most important step in the gluconeogenesis involves PEPCK enzyme which is positioned at thestarting point of the long chain reaction.Due to this, PEPCK enzyme has been considered as the rate-limiting stepin gluconeogenesis reaction(Chakravarty et al., 2005; Chichelnitskiy et al., 2009) (**Figure 3**).

The higher expression of PEPCK enzyme couldbe due tothe large muscle mass of broilers, which urges broilers to utilize more energy compared to indigenous chicken which resulted in faster glucose depletion. A study on cellular energy utilization explained that different body mass and phylogeny of animals would produce different standards of metabolic rate that change the whole energy metabolism(Brzek et al., 2016). The depletion of glucose will allow broilers to use another metabolic pathway in order to provide energy for their cellular activity. These findings may explain the need for broilers to undergo gluconeogenesis pathways in their mitochondria as an alternativesupply for energy generation.

The next protein is adelta-1-pyrroline-5-carboxylate dehydrogenase (P5CDH) enzyme that originates from mitochondria. In this study, it was detected to be highly expressed in CB compared to IC. This enzyme is an NAD+ dependent aldehyde dehydrogenase that catalyzes the last step of proline catabolism in mitochondria(Pemberton and Tanner, 2013). Currently, the study of P5CDH on mammals is quite limited. However, the enzyme has been extensively studied on plants that are related to osmotic stress, which prevents P5C/Proline from intensive cycling and avoids reactive oxygen species (ROS) production from electron run-off(Ben Rejeb et al., 2014; Deuschle et al., 2001; Pérez-Arellano et al., 2010; Servet et al., 2012) (**Figure 4**). A study of P5CDHon Drosophila showed that the deficiency of this enzyme can lead to hyperprolinemia(He and DiMario, 2011). These observations suggested that P5CDH plays a protective role against ROS generation, mitochondria and cell damage, as well as apoptosis(Hu and Hou, 2014).

The broilers have a higher metabolic and growth rates compared to indigenous chicken.Therefore, they need more energy to supply the demands of their own bodies.It was reported that glycolytic activity increased further and faster in fast growth poultry strains compared to slow strains poultry(Petracci et al., 2017). We suggest that the broilers might undergo proline catabolism to form glutamate inside mitochondria of the hepatocyte cells. This reaction would give NAD+ electrons that will contribute to the respiratory electron transport chain, which indirectly producing energy for cellular activityvia proline catabolism pathways.

4.2 Proteins Related to Stress Response

Soluble epoxide hydrolase (sEH) is the xenobiotic metabolic enzyme that appears in almost every cell in

the body especially in hepatocyte and adipose tissues. It has a cytotoxic activity by mediating the formation of cytotoxic dihydrodiol fatty acids at the expense of cytoprotective epoxides of fatty acids(El-Sherbeni and El-Kadi, 2014).

A study on mice revealed that sEH expression was significantly increased in the liver of mice fed withahigh-fat diet, indicating a chronic endoplasmic reticulum stress(Bettaieb et al., 2013). Moreover, homocysteine, which can lead to ischemic injury, was also reported to increase the expression of sEH in endothelial cells(Zhang et al., 2012). Apart from that, other studies on the effects of antibiotic administration to broilers have shown the increment of oxidative stress in the blood sample of the broilers(Settle et al., 2014). During biotransformation of xenobiotic, epoxides, a reactive intermediate can be formed through the oxidative metabolism that contributes to cytotoxic damage mediated by oxidative stress(Farin et al., 2001). Epoxide could pose potential hazards to the biological systems, but mammals' bodies have been equipped with several ways to detoxify the epoxides. One of the ways is through the sEH enzyme. In our study, the factor differences between broilers and indigenous chicken that related with sEH expression may come from the antibiotic treatment. Broilers have been known to receive vaccine and antibiotics in order to improve their health, feed efficacy and growth. Nonetheless, this is in contrast with the indigenous chicken, where the indigenous chickens from this study were not treated with any vaccine and antibiotics. Thus, we postulate that the expression of thesEH enzyme might help broilers to overcome the cytotoxic effects of secondary metabolitesfrom drugs treatment.

Another stress protein called Heat Shock Protein 90 (HSP90) expressed lower in CB as compared to IC liver. The HSP90 plays many roles in cellular activity including protein folding, stress response and signal transduction(Imai et al., 2003). A previous study showed that HSP90 played a big role in the elevation of temperature in organisms in response to heat stress(Al-Zghoul et al., 2014). The protein was rapidly synthesized in tissues that were subjected to heat stressors and HSP90 helped protein to maintain the integrity of its structure by preventing protein aggregation, and aided in folding-refolding of damaged proteins(Morimoto, 2008). We hypothesize that HSP90 would be expressed higher in broilers compared to indigenous chickensdue to their rapid metabolism and high body temperature(Belhadj Slimen et al., 2016).

However, in our study, HSP90 protein was expressed more in IC compared to CB liver. It remains unclear whether the IC liver may be sensitive to heat changes, or the regulation of heat stress is more efficient in hepatocytes to prevent cell damages. Further studies need to be conducted to address these questions. HSP90 also functions as a housekeeping protein in normal conditions and this aids in protein signaling and folding/refolding, as discussed earlier. Thus, the general housekeeping functions of HSP90 may possibly cause the increase of HSP90 expression in IC liver.

On the other hand, the low expression of HSP90 in the broiler's liver might signalpoor overall metabolic system of the broilers. A decrease in intracellular HSP90 has been reported to increase the mortality of mammalian cells at an elevated temperature(Al-Zghoul et al., 2014). These findings may suggest that the broilers are moresusceptible to heat stress compared to IC.

4.3 Proteins Related to Cellular Structure

Actin is another protein that was found to be expressed lower inCB compared to IC. Actin is known to maintain the shape and structure integrity of plasma membrane and plays a dynamic role in several membrane-associated events including cell migration, cell adhesion, phagocytosis and mobility(Nowak et al., 2013). Based on the *actg1* gene, the actin expressed in this study belongs to γ-actin, a globular type of actin which is abundance in the cytosol of muscle and non-muscle cells. The cellular actin isoforms have distinct roles in regulating thestructure and maintaining thepermeability of tight junctions and adherence junction between cells(Baranwal et al., 2012). Moreover, a different study concluded that γ-actin is required for maintaining long-term stability of filamentous actin (F-actin) based structures(Belyantseva et al., 2009).

The cytosolic actin isoform is found to be essential in hepatocyte cells viability. Higher expression of actin by indigenous chickensmay indicate their good structure integrity and efficient membrane-associated events, compared to broilers. The high expression of actin found in indigenous chickenmay also be due to the differencesin their age, where indigenous chicken are much older compared to the broilersat the time of slaughtering.

4.4 Summary

To recap, the study was conducted to denote the current nature of meat being served to the consumer. Thus, this kind of proteomics approach should be considered as exploratory. The findings in this study from both samples of hepatocytes have shown several differencesof protein expressions, which are related to energy metabolic pathways, stress response and cellular structure. These differences could arise from many variables involved during their lifetime including breeds, age, feed regimen, antibiotic treatment, and rearing environment.This current discovery will be useful tools to uncover the molecular basis of physiological differences in

theliver that undergo rapid growth and stress response in the future.

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COMPETING INTEREST

Authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Bunnori NM is a Chief Investigator who granted with a research grant from the Ministry of Higher Education, the main supervisor for Hassan MSA, Hamdan NA and Saidin MS. Hassan MSA, Hamdan NA and Saidin MS performed experiments; Hamid SA revised the manuscript and suggest the corrections. All authors read and approved the manuscript.

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FIGURE LEGENDS

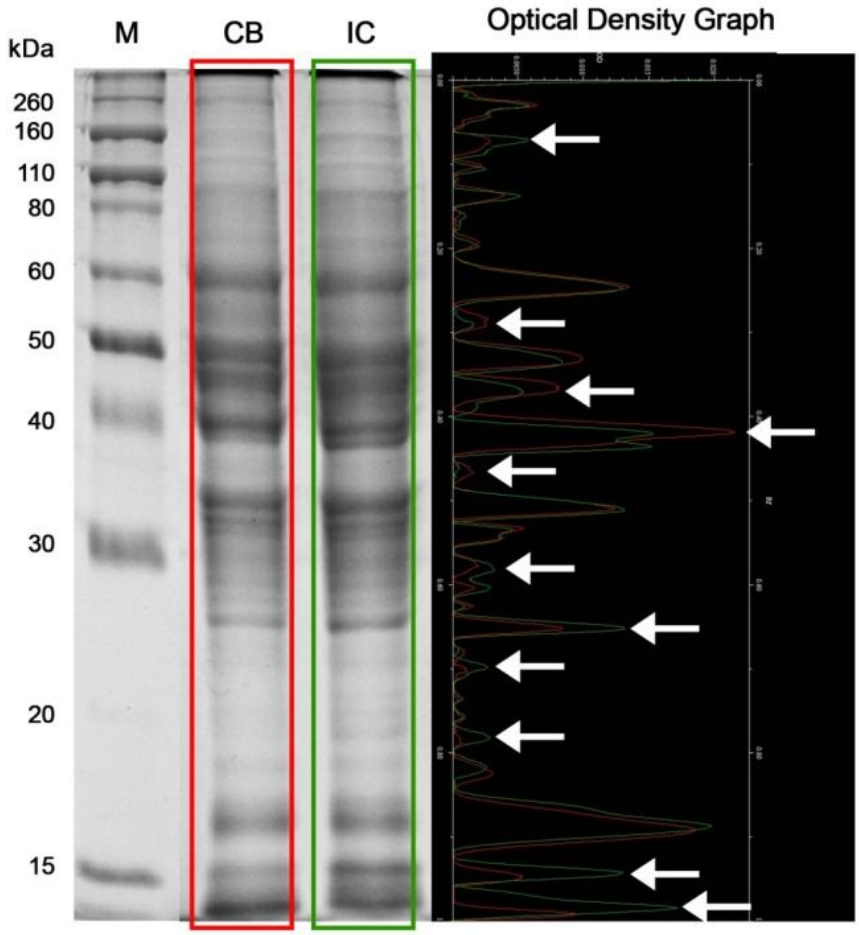


Figure 1

SDS-PAGE electrophoretic patterns of liver protein expression on 12% polyacrylamide gel from two lines of chicken. The arrows show the differences of band expression between both samples.The red and green lines from the optical density graph represent the expression of protein in CB and IC respectively.The annotated bands with roman numbers (i - vi) are searched against mouse liver database on Swiss-2DPAGE Expasy for protein predictions. The SDS gel pattern showed the first evidence of the hypothetical protein differences between CB and IC liver. CB, commercial broiler's liver; IC, indigenous chicken's liver; M, protein marker

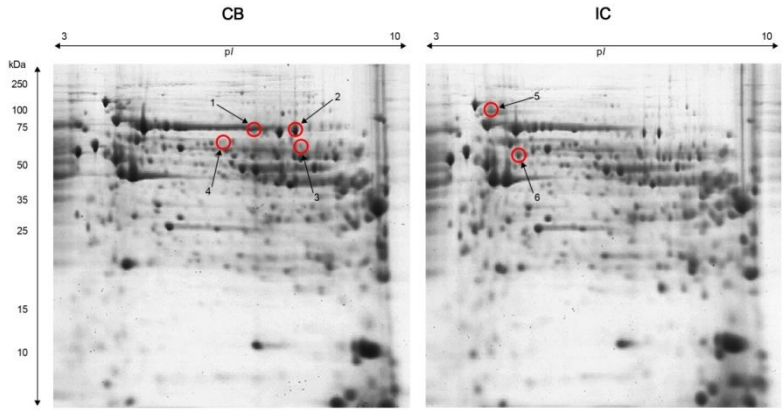


Figure 2

Differential protein expression in chicken's liver sample by using 2D-PAGE. The circles and arrows indicate the excised protein spots that have been successfully identified by using MALDI-TOF/MS. The red circle and green circle showed the higher expression of protein in CB and IC respectively compared to one another.M, protein marker (kDa)*;* p*I*, isoelectric point.

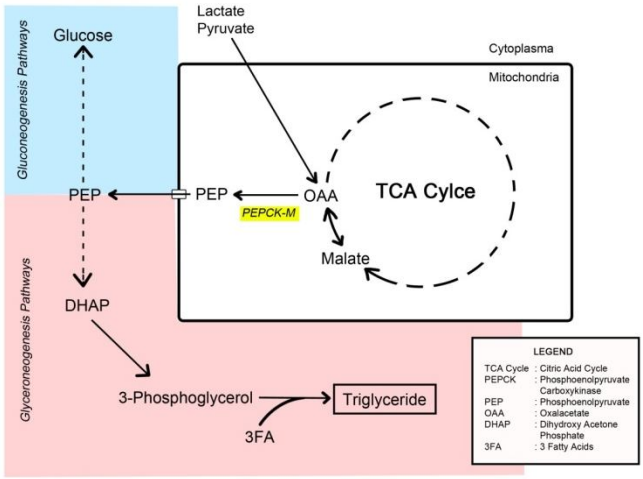


Figure 3

The action of PEPCK-M inside mitochondria catalyses the reaction of OAA to PEP. It is the first step reaction in the gluconeogenesis and glyceroneogenesis pathways. Image adapted from Chakravarty et al. (2005).

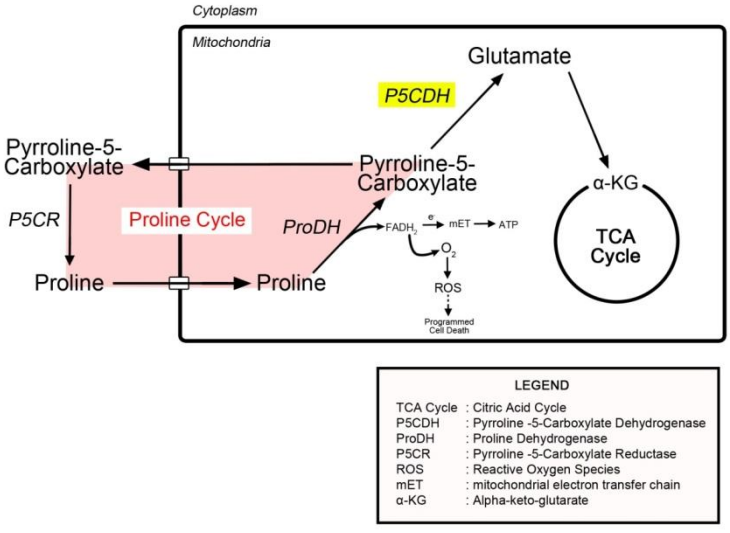


Figure 4

The biochemical pathways of proline metabolism. It is suggested that the P5CDH enzyme helps to avoid from excessive cycling of Proline cycle that will generate ROS which leads to cell death. Image adapted from Servet et al. (2012).

TABLES

Factor	Commercial broilers	Indigenous chicken
Breeds	Cobb & Ross	Rhode Island Red
Age	7 weeks	14 weeks
Weight	2.2 - 2.8 kg	1.3 - 1.7 kg
Environment	Closed Systemwith controlled environment (27-30°C, 18h illumination per day, ventilation, 60-70% humidity)	Open spaceenvironment with a shaded poultry house
Feed Regimen	0 - 21 days: Crumbles Broilers Grower Starter 21- 42 days: Pellets Broilers Grower Finisher	Soybean meal, Corns, Rice (2 times a day)
Treatment	• 14 days: Infectious Bursal Disease (IBD) vaccine • 18 days: Ranikhet vaccine • Antibiotics (if the bacterial infection detected) - Added into drinking water	No Treatment

Table 1:The condition of selected chicken that had been offered to the Malaysian's customer and been used in this study

SDS-PAGE Band No.	Experimental mW (kDa)	Predicted Protein List	Predicted mW from SWISS-2DPAGE Database (kDa)	Metabolic related proteins
i	160.00	Carbamoyl-phosphate synthase	158.169, 160.000	√
ii	45.00	Keratin	46.684, 46.554	
		Glutamine synthetase	44.527	√
		Alpha-Enolase	46.684	√
		Ornithine aminotransferase	44.157, 43.185	√
		Fumarylacetoacetase	43.547	√
iii	40.00	Aspartate aminotransferase	40.35	√
		40S Ribosomal protein SA	40.89	
		Fumarylacetoacetase	40.89	√
		Cytokeratin	40.53	
iv	26.00	Peroxiredoxin-6	26.25, 26.19	√
		Tropomyosin alpha-3 chain	26.69	
		Triosephosphate isomerase	26.69	√
		Cytochrome c oxidase subunit 2	26.69	√
v	15.00	Peptidyl-prolyl cis-trans isomerase A	15.28, 15.06, 14.96	
		Cytochrome b5	14.99, 14.56	√
		Nucleoside diphosphate kinase B	15.44	
vi	7.00	ATP synthase-coupling factor	9.788	

Table 2: Predicted putative protein list based on selected mW from SWISS-2DPAGE database of mouse liver in comparison with SDS-PAGE result from chicken liver.

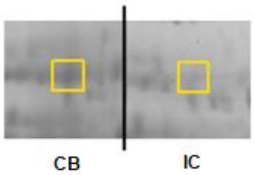
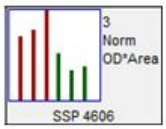
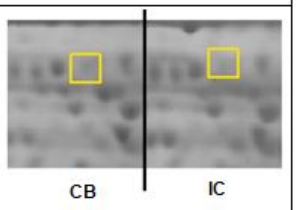
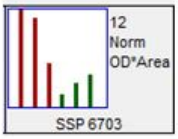
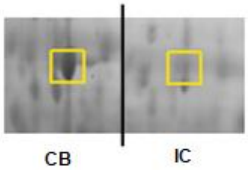
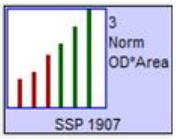
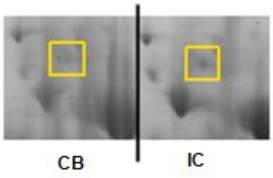
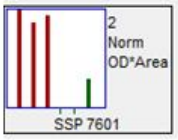
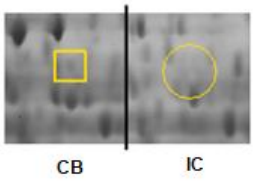
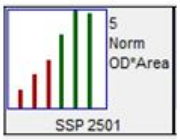
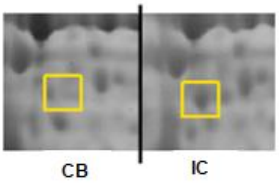
Spot ID	Histogram	Gel Image View	Spot ID	Histogram	Gel Image View
1			4		
2			5		
3			6		

Table 3: Histogram view of the quantitative expression of protein spot corresponding to spot ID.

The table shows the selected spots that have shown prominent variances on intensity differences between CB and IC's liver. These spots were further analyzed with MALDI-TOF/MS for peptide sequence and protein identification. The 3 bars from the left on the histogram are belonged to an optical density of CB spot in triplicates, while the 3 bars from the right on the histogram are belonged to an optical density of IC spot also in triplicates.**CB**, commercial broilers liver; **IC**, indigenous chicken liver.

Spot ID[a]	Protein Name	Higher Expression in CB / IC	Accession Number (NCBI)	Matched Identity / Matched Peptides[b]	Protein Score / Best ion Score[c]	E-Value[d]	Peptides Identified	Mw predicted / experimental (kDa)	pI predicted / experimental
1	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	CB	gi| 229620380	5 / 8	482 / 100	4.6e-07	EYYGENFGADLPR	71.06 / 75.00	7.56 / 6.0
2	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial precursor	CB	gi| 45382653	11 / 15	1076 / 121	2.8e-09	EYYGENFGADLPR	70.92 / 70.00	8.16 / 6.4
3	ALDH4A1 delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	CB	gi| 355390334	6 / 7	560 / 124	3.6e-09	STGAVVAQQPFGGSR	60.59 / 67.00	8.22 / 6.5
4	Soluble epoxide hydrolase 2	CB	gi| 75832164	3 / 5	310 / 101	6.6e-07	YQIPALADAGFR	63.21 / 67.00	5.89 / 5.9
5	HSP90AA1 Heat shock protein HSP 90-alpha	IC	gi| 157954047	3 / 11	611 / 110	8.2e-08	HFSVEGQLEFR	84.01 / 100.00	5.01 / 4.9
6	ACTG1 Actin, Cytoplasmic type 5	IC	gi| 56119084	4 / 5	363 / 126	6.6e-10	SYELPDGQVITIGNER	41.81 / 65.00	5.30 / 5.2

Table 4: Identification of proteins with differential abundance in CB and IC's hepatocytes.

[a] Spot ID is a number corresponding to the spots label as in Figure 2 and Table 1.
[b] The number of peaks which match/unmatched to the trypsin peptides.
[c] Protein score and best ion score were from MALDI-TOF/MS identification. The proteins which have protein score greater than 52 ($p \leq 0.05$) wereregard as successfully identified.
[d] E-value (expectation value) isthe number of matches with equal or better scores that are expected to occur by chance alone in a Blast search result. The lower the expectation value, the more significant the score.

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