J I T A A
Journal of the Indonesian Tropical Animal Agriculture
Accredited by Ditjen Riset, Teknologi dan Pengabdian kepada Masyarakat No. 164/E/KPT/2021

J. Indonesian Trop. Anim. Agric.
pISSN 2087-8273 eISSN 2460-6278
http://ejournal.undip.ac.id/index.php/jitaa
48(2):76-88, June 2023
DOI: 10.14710/jitaa.48.2. 76-88

Effects of rumen-protected fat on changes of metabolites and reproductive genes in testes of Malin rams

A. Ismail[1], A. I. Muhammad[1,2], L. T. Kee[1], L. T. Chwen[1], and A. A. Samsudin[1*]
[1] Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia, Serdang 43400, Malaysia;
[2] Department of Animal Science, Faculty of Agriculture, Federal University Dutse, Dutse 7156, Nigeria
**Corresponding author: anjas@upm.edu.my*

Submitted February 14, 2023, Accepted March 28, 2023

ABSTRACT

The effects of RPF on metabolites and reproductive genes in testes of Malin ram was investigated. Twenty Malin rams (36.6kg ± 5.57 kg of bodyweight), were subjected to four dietary treatments; A: basal diet without rumen-protected fat (RPF), B; basal diet with 2% prilled fat, C; basal diet with 2% calcium salt and D; basal diet with 2% canola oil. At the end of the experiment four out of five animals from each group were slaughtered. The testes were excised for metabolites and gene expression studies. The genes tested were associated to testes development and spermatogenesis (ODF1, SERPINA10, CatSper4, AdipoR2 and DAZL). Feeding RPF with calcium salt (Treatment C) has resulted in the up-regulation more than two folds in all reproductive genes. There were metabolites changes occurred between the groups and identified 44 important putative metabolites present in the testes. In conclusion, feeding of RPF to the animals as a source of energy has up-regulated the genes and identified the metabolites involve in the male reproductive tissues and activities.

Keywords: *Energy, Fat, Gene expression, Metabolites, Sheep.*

INTRODUCTION

Animal feeding is one of the main factors that can alter the performance of male animals. In the reproduction activities, rams that are not allowed to have proper feeding and nutrition will impact the breeding performance such as undeveloped testes, low sperm production, and quality which in turn will lead to reduce the fertility of the animals (Lone *et al.,* 2017; Singh *et al.,* 2018). To overcome these limitations, the farmers need to provide the rams with proper feeds, especially during breeding seasons. Grains that were incorporated in the pellet are a common practice to overcome these issues. However, as the price of the grains is getting higher these days, it would affect the farmers in terms of their finances. Alternatively, the use of fats as feed supplements during breeding seasons will help to resolve these issues (Schoech *et al.,* 2004).

Currently, there is a technology to produce insoluble fats from microbial fermentation and

biohydrogenation which is called rumen-protected fats (RPF) (Behan *et al.,* 2019 and De Silva *et al.,* 2020;). This helps the RPF to escape the rumen microbial fermentation, absorbed through the small intestine and then converted to a source of energy (Owens and Basalan, 2016). There have been a few studies on the effects of RPF on metabolites and gene expression in female animals (Behan *et al.,* 2019; Chavda *et al.,* 2022), however, the current study focused on metabolites changes and reproductive genes in the ram's testes.

The sperm fatty acid composition is characterized by very high proportions of omega- 3 PUFAs (n−3), particularly Docosahexaenoic acid (DHA; 22:6 n −3) (Hosseini *et al.,* 2019; Ngcobo *et al.,* 2021). The ratio of omega-6 (n−6) to n−3 PUFAs may play a significant role in several aspects of animal production and reproduction (Khoshniat *et al.,* 2020; Ngcobo *et al.,* 2021). Furthermore, there is substantial evidence that the lipid composition of the sperm membrane is a major determinant of motility, overall viability, cold sensitivity, fusion capacity of sperm, and lipid metabolism (Wysokińska and Szablicka, 2021). There have been several studies on the effect of RFP on metabolites in ewes, however, our research focused on metabolites in the ram's testes. RPF increased Malin ram reproduction performance by enhancing sperm quality (Ahmad *et al.,* 2018; Manriquez *et al.,* 2019).

The development of testis and spermatogenesis are frequently regulated by core genes (Du *et al.,* 2021; Zhang *et al.,* 2019). There are many genes associated with nutrition and reproduction (Ma *et al*., 2019; Heng Yang *et al.,* 2018), therefore the current study focused on five differential genes (DEGs) which were; Outer density fiber protein 1 (ODF1), SERPINA 10, Cat ion channel of Sperm4 (CatSper 4), AdipoR2 and DAZL. These genes were chosen because of their functions in testis development and spermatogenesis (Bai *et al.,* 2017; Qu *et al.,* 2019). Gene expression is a process that involves transcription, translation, and turnover of messenger Ribonucleic acids (mRNAs) and proteins (Naval-Sanchez *et al.,* 2018). It is regarded as one of life's most fundamental processes, and the genes expressed in an organism define its characteristics or features (Buccitelli and Selbach, 2020). To improve animal reproductive performance, many farmers now use rumen-protected fat. RPF improved Malin ram reproduction performance by improving sperm quality (Ahmad *et al.,* 2018), and the current work was a continuation of that research to further understand RPF effects at the molecular level. Therefore, this study aimed to investigate how rumen-protected fat altered metabolite changes, and mRNA expression of reproductive genes involved in testes development and spermatogenesis in Malin ram testes.

MATERIALS AND METHODS

Experimental Design, Animals and Diets

Twenty Malin rams (BW of 36.6 kg ± 5.6 kg at 10-14 months of age, with body scores 3.0 - 3.5) were reared at the National Institute Animal Biodiversity Jerantut, Pahang and approved by the Universiti Putra Malaysia animal care and

Table 1: Feed ingredients of experimental diet

Ingredients	Experimental diets			
	Control	Prilled	Casa	Canola
Brachiaria grass	66.00	76.00	75.00	77.00
Commercial Sheep Concentrate	34.00	22.00	23.00	21.00
Prilled Fat	00.00	02.00	00.00	00.00
Calcium Salt of Fatty Acid	00.00	00.00	02.00	00.00
Canola Oil	00.00	00.00	00.00	02.00
Total (%)	100.00	100.00	100.00	100.00
Calculated Analysis				
ME (Kcal/kg)	2217	2218	2210	2212
Crude Protein (%)	12.20	11.30	11.30	11.20

use committee (IACUC) guidelines (Reference # R064/2016). The experimental animals were divided randomly into four treatment groups, each of which received a different feeding treatment: A: basal diet without rumen-protected fat (RPF); B: a basal diet with RPF (as 2% prilled fat from palm oil source); C: basal diet with 2% RPF (as calcium soap, Casa); and D: basal diet with 2% canola oil (Table 1). The 12-week experiment included a two-week adaptation period and ten weeks of feeding trials. Each group received isocaloric and isonitrogenous formulated feed according to the maintenance requirement of sheep with water provided *ad-libitum*. The animals were dewormed with Fenbendazole 10%, 1ml/10kg two weeks before the feeding trials.

Testes Collection

Four Malin rams' testes (four from each treatment group) were taken as described in our earlier work (Ahmad *et al.,* 2018) to determine metabolites and gene expression in testes. The testes samples were stored at -80 °C for further gene expression study.

Sample Preparation and LC-MS Analysis

The testes' tissues were crushed into a fine powder using a pestle and mortar with liquid nitrogen. The testes tissue samples were prepared as described in previous studies (Chen *et al.,* 2015; Fraser *et al.,* 2021), with slight modifications. Briefly, approximately 150 mg of each pulverized sample was homogenized in 450 µl methanol in two steps to make a final volume of 900 µl to precipitate the proteins. The solution was vortexed three times for five minutes each, then centrifuged at 16000 x g for 15 minutes at 4 °C. A centrifugal evaporator was used to concentrate the sample to a final volume of 75 µl. The sample was then centrifuged at 17,500 × g for 5 min at 4 °C and the supernatant was transferred to a new 1.5 ml microcentrifuge tube for LC-MS analysis. Each biological sample was replicated four times.

Fingerprinting sample was performed using Agilent 1200 LC system with an auto-sampler and binary pump coupled to 4000 Q-TRAP (AB Sciex, USA). Chromatographic separation was performed on zorbax eclipse xdb – C18 150 x 4.6 mmx 5 u. Mobile phases consisted of solution A; 0.1% formic acid in ultrapure water and solution B; 100% acetonitrile with the following gradient conditions; 0-1 minute 97% of solution A and 3% of solution B. From minutes 1 to 19 minutes, solution B was from 3% to 97% of concentration. Then it was maintained until 22 minutes. The column was equilibrated before each analysis. The flow rate was 0.5ml/min and the volume of the sample injected was 15µl. Analysis was performed at 45°C. The mass spectrometer was operated in a positive mode in scan type of Enhance MS (EMS). The scan rate was 1000Da/s and the scan range of 50-2800 *m/z*.

Total RNA Extraction and Purification

Total RNA was extracted from sample tissues using the RNeasy® Lipid Tissue Mini kit (Cat. No. 748sw04, Qiagen, Hilden, Germany) according to the manufacturer's protocol. Next, using a spectrophotometer (NANODROP (ND 1000), 1 µl of RNA was used to evaluate the purity of RNA by measuring the absorbance ratio at 260 nm/280 nm, and only samples with a ratio of ~2.0 were generally accepted as "pure". Furthermore, the purification of RNA was determined by the evaluation of cDNA using standard curves.

Quantitative Real-Time Polymerase Chain Reaction (q-PCR)

The synthesis of first-strand cDNA was run by reverse transcription of 3 µg isolated total RNA (20 µl reaction mixture) as per kit protocol. The reaction was placed in the thermal cycler MJ Research PTC-100, USA. The master mix was prepared as per the manufacturer's protocols. The master mix was prepared according to the manufacturer's protocol. The 2x QuantiNova SYBR Green PCR Master Mix, QuantiNova Yellow Template Dilution Buffer, cDNA, primers, and RNase-Free water were thawed. The primers sequences of the selected gene which were forward and reverse are presented in Table 2. Each reaction (20 µL) contained 10 µL 2x SYBR PCR

Master Mix, 1 μL of each forward and re- verse primers, 7 μL of nuclease-free water, and 1 μL of cDNA. The qPCR reactions were carried out following standard cycling mode as per kit protocol. A melting curve was also generated to confirm the sequence-specific PCR products. Two house-keeping genes of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Beta-actin (β-actin) were used in triplicates to determine the stable house-keeping gene in tissues. Real-time PCR was then performed on a Bio-Rad CFX Manager™ 3.1 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Genes of interest were amplified through the 3 steps cycling program; step 1; denaturation for 15 seconds at 95°C; step 2; annealing for 20 seconds at 60°C; and step 3, extension for 20 seconds at 72°C. A standard curve was constructed to determine if the sample can be either a gene-specific plasmid or a cDNA preparationin which the gene of interest is present. The R^2 of the standard curve should be > 0.980 (Qiagen, Germany). For the efficiency, reproducibility, and dynamic range of an SYBR Green assay, the standard curve was constructed using serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. The efficiency of the assay should be 90-105%. Quantification analysis was performed by measuring the average cycle threshold (CT)value ΔΔ (2-ΔΔCT method) described by Livak and Schmittgen, (2001) using the formula below, following the standard curve method after normalization with reference genes.

$$Ratio = \frac{(E\ target)^{\Delta CT,\ target\ (calibrator\ test)}}{(E\ ref)^{\Delta CT,\ ref\ (calibrator\ test)}}$$

In the above equation, Etarget and Eref are the amplification efficiencies of the target and references genes, respectively. ΔCT target (calibrator-test) = CT of the target gene in the calibrator minus the CT of the target gene in the test sample and ΔCT, ref (calibrator – test) is the CT of the reference gene in the calibrator minus the CT of the reference gene in the test sample.

Data Processing

The success of the LC-MS technique can be determined by its ability to give three dimensional (3D) data. First, the compounds were separated in time by LC (retention time). Secondly, the ions generated in the ionization source were then separated according to their mass to charge ratio, and *m/z* ratios in the mass analyser of MS, and finally, the MS detector measured the abundance of each ion (intensify). For data processing, the ion sources data then went to further screening, verify and quantitate using the Analyst 1.5.1 software. During the pre-screening, the outlier ions were removed from the data. The cleaned data were then reformed into an excel matrix for further analysis.

Data Analysis

The excel matrix was exported to SIMCA-P software (version 14.1, MKS Umetrics, Sweden) for further multivariate statistical analysis. The data were employed to identify biochemical patterns using principal component analysis (PCA), partial least squares- discriminate analysis (PLS-DA) and orthogonal partial least squares discriminate analysis (OPLS-DA). Some outlier ions also need to be removed along the process to get the most relevant ions. The values of variables' importance in the projection (VIP) in the OPLS-DA model were used to determine important metabolites. The characteristic of metabolites (the exact molecular mass and m/z) were identified using the Human Metabolome Database (http://hmdb.ca/spectra/ms/search), and the MassBank database (www.massbank.jp/search), Metabolites and Tandem MS Database (http://metlin.scripps.edu) and the RIKEN integrated database of mammals (http://scinets.org/db/mammal).

Statistical Analysis

Data were analyzed using Statistical Analysis Software (SAS, version 9.4). General Linear Model (GLM) was used to analyse the data and Duncan's Multiple Range Test (DMRT) was used to compare the mean between the treatments. Data were expressed as (mean ± SE) and statistical analysis that has a value of $P < 0.05$ was considered significantly different (one-way

Table 2. Primer sequence of selected genes (Bai *et al.,* 2017)

Name of Target gene	Primer sequence	Product Size
Cat ion channel of Sperm4 (CatSper4)	**F**: TCGGCTGGTTAAATGGTTTC R: CGACGGCACTGAGTTCATTA	114
SERPINA10	F: TCTTACCCTGGGCTGACCTA R: CTGCCATTGCCTCTGTACCT	117
DAZL	F: TTATCATGTGCAGCCACGTC R: AGGGTTCATCATGGTTGGAG	118
ADIPOR2	F: GAGGAGTGTGAGTGCGATGA R: CGACCTTCCCAGACCTTACA	128
Outer density fibre protein 1 (ODF1)	F: CGCGAGAACAGATACGACTG R: GAGCCCGTAGGAGTACGTCA	117
β-actin	F: GCTCTCTTCCAGCCTTCCTT R: CGTGTTGGCGTAGAGGTCTT	114
GADPH	F: CATGGCCTTCCGTGTTCCTA R: TACTTGGCAGGTTTCTCCAGG	460

ANOVA).

RESULTS AND DISCUSSION

PCA, PLS-DA and OPLS-DA of Metabolomics Profile

PCA, an unsupervised pattern recognition method, was used to determine the presence of inherent similarities in spectral profiles. Each scatters represented the testes sample in every treatment group. The PCA and PLS-DA result showed that there were not any separation between the groups or overlapping each other. To differentiate the testicular metabolites, we applied the OPLS-DA model to characterize the control and each different dietary group. There was a distinct clustering between the control group and each treatment group. However, the clustering between group C and group D was not well separated (Figure 1; OPLS-DA). The completely separated clustering between the treatment groups indicated that control group A could

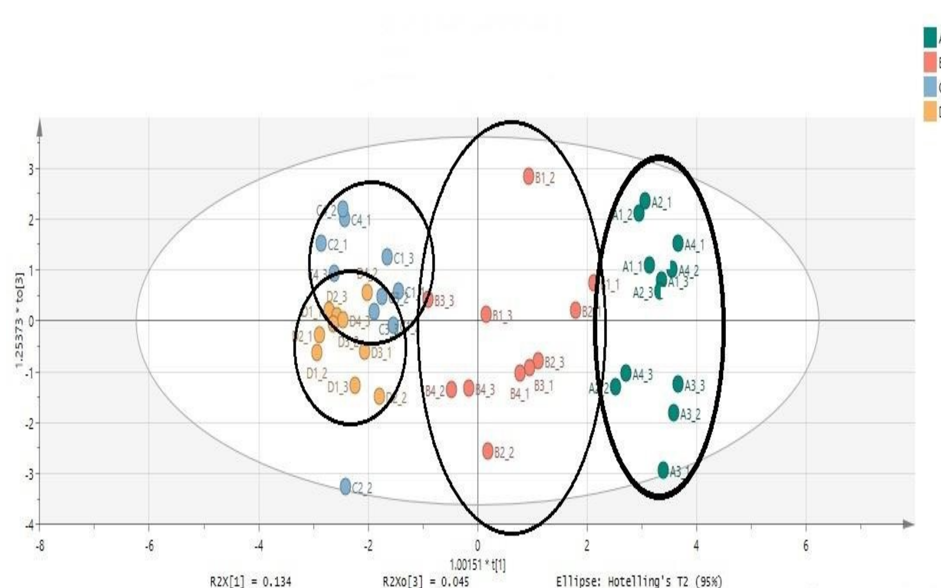


Figure 1. Multivariate analysis, OPLS-DA. A - control, B - prilled fat, C - calcium salt, and D - canola oil.

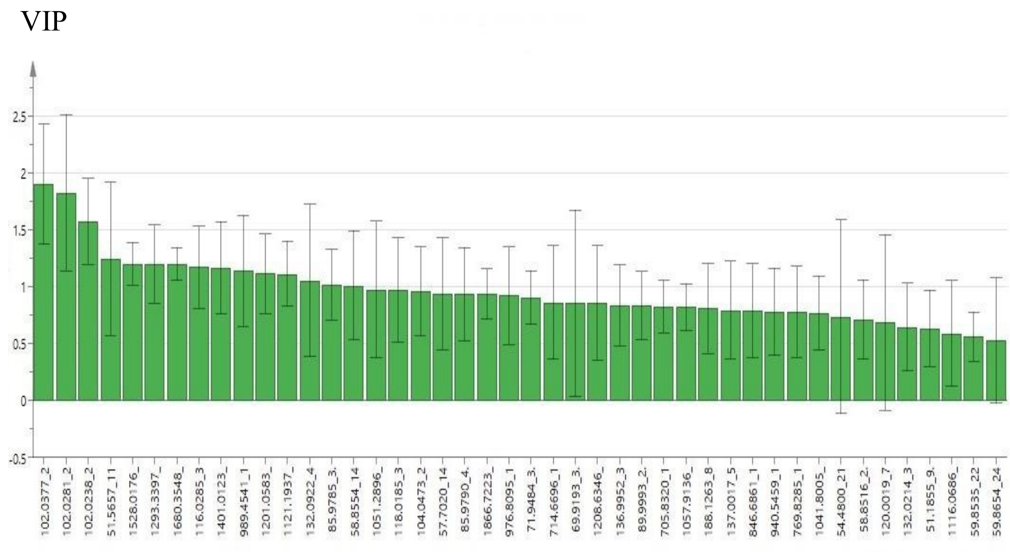
Table 3. Identified putative metabolites in the testes
(Human metabolome database;http://hmdb.ca/spectra/ms/search, Mass Bank database; www.massbank.jp/search, Metabolites and Tandem MS database http://metlin.scripps.edu)

No.	VIP	*m/z*	Putative Metabolites	Formula
1	1.89877	102.0377	10-hydroxy-(2E,8E)-decadien-4-ynoic Acid	$C_{10}H_{12}O_3$
2	1.82653	102.0281	albendazole S-oxide	$C_{12}H_{15}N_3O_3$
3	1.57053	102.0238	Nicotinuric acid	$C_8H_8N_2O_3$
4	1.24246	51.5657_11	Methylcyclopentane	C_6H_{12}
5	1.2031	1528.0176	CL(20:1(11Z)/18:2(9Z,12Z)/18:1(11Z)/18:1(11Z)	$C_{83}H_{152}O_{17}$P
6	1.19827	1293.3397	18(R)-Hydroxy-20-oxo-20-CoA-LTE4	$C_{44}H_{69}N_8O_2$
7	1.19754	1680.3548	CL(i-13:0/a-21:0/i-24:0/a-25:0)[rac]	$C_{92}H_{180}O_{17}$P
8	1.17195	116.0285_3	Methylpentanoic acid 1 Hexanoic acid	$C_6H_{12}O_2$ $C_6H_{12}O_2$
9	1.16402	1401.0123	DG(20:5(5Z,8Z,11Z,14Z,17Z)/ 22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)	$C_{45}H_{66}O_5$
10	1.13656	989.4541	Fluspirilene	$C_{29}H_{31}F_2N_3$
11	1.11573	1201.0583	CE(12:0)	$C_{39}H_{68}O_2$
12	1.11306	1121.1937	Anhydrosafflor Yellow B	$C_{48}H_{52}O_{26}$
13	1.05437	132.0922	5,10-Pentadecadien-1-ol	$C_{15}H_{28}O$
14	1.01708	85.9785	Crotonoic acid	$C_4H_{10}N_2$
15	1.01065	58.8554_14	1,1-Dimethylbiguanide	$C_4H_{11}N_5$
16	0.974863	1051.2896	Cyanidin	$C_{47}H_{55}O_{27}$
17	0.971319	118.0185	Succinic acid DL-2-Hydroxyvaleric acid	$C_5H_{12}NO_2$ $C_5H_{10}O_3$
18	0.97953	104.0473	DL-2,3-Diaminopropionic acid Choline 3-Cyanopyridine	$C_3H_8N_2O_2$ $C_5H_{14}N_O$ $C_6H_4N_2$
19	0.939066	57.7020	2-Amino-5-phenyl pyridine	$C_{11}H_{10}N_2$
20	0.936192	85.9790	Piperazine	$C_4H_{10}N_2$
21	0.934178	1866.7223	Glycerol trinonadecanoate	$C_{60}H_{116}O_6$
22	0.921368	976.8095	TG(20:3(5Z,8Z,11Z)/15:0/20:3(5Z,8Z,11Z))	$C_{58}H_{100}O_6$
23	0.9064	71.9484	Superoxide	O_2
24	0.861032	714.6696	Cer(d18:1/23:0)	$C_{41}H_{81}NO_3$
25	0.854799	69.9193	Propionic acid	$C_3H_2N_2$

Table 3. Identified putative metabolites in the testes (continued)

No.	VIP	*m/z*	Putative Metabolites	Formula
26	0.854243	1208.6346	M(IP)2C(d18:0/16:0 Preprosomatostatin	$C_{52}H_{101}NO_{24}P_2$ $C_{52}H_{83}N_{17}O_{15}$
27	0.836234	136.9952	Antharilic acid	$C_7H_7NO_2$
28	0.834576	89.9993	DL Lactic acid Lactic acid	$C_3H_6O_3$ $C_3H_6O_3$
29	0.826173	705.8320	Guanosine 3'-diphosphate 5'-triphosphate	$C_{10}H_{18}N_5O_{20}P_5$
30	0.820011	1057.9136	1-Heneicosanoyl-2-docosanoyl-3-sn glycerol	$C_{68}H_{122}O_6$
31	0.808577	188.1263	Antipyrine Gly-Leu	$C_{11}H_{12}N_{2O}$ $C_8H_{16}N_2O_3$
32	0.796197	137.0017	Anthranilic acid Trigonelline	$C_7H_7NO_2$ $C_7H_7NO_2$
33	0.786638	846.6861	PC(18:0/22:0) PC(20:0/20:0) PC(16:0/24:0) PE(17:0/26:0)[U] PE(24:0/19:0)[U] PE(22:0/21:0)[U]	$C_{48}H_{96}NO_8P$ $C_{48}H_{96}NO_8P$ $C_{48}H_{96}NO_8P$ $C_{48}H_{96}NO_8P$ $C_{48}H_{96}NO_8P$ $C_{48}H_{96}NO_8P$
34	0.780037	940.5459	Dehydrosoyasaponin I	$C_{48}H_{76}O_{18}$
35	0.776376	769.8285	PE 38:3 PC 35:3 Phosphatidylcholine 17:1-18:2 Phosphatidylethanolamine 18:0-20:3	$C_{43}H_{80}NO_8P$ $C_{43}H_{80}NO_8P$ $C_{43}H_{80}NO_8P$ $C_{43}H_{80}NO_8P$
36	0.768842	1041.8005	PS(24:0/24:1(15Z))	$C_{54}H_{104}NO_{10}P$
37	0.735888	54.4800	Peroxynitrite	HNO_3
38	0.712305	58.8516	N4-Acetylaminobutanal	$C_6H_{11}NO_2$
39	0.684643	120.0019	(-)-1-(Methylthio)propyl 1-propenyl disulphide	$C_6H_{12}S_2$
40	0.647569	132.0214	L-Ornithine	$C_5H_{12}N_2O_2$
41	0.63501	51.1855	Ethylbenzene	C_8H_{10}
42	0.591421	1116.0686	TG(20:0/24:1(15Z)/22:1(13Z))	$C_{69}H_{130}O_6$
43	0.562731	59.8535	Acetate	$C_2H_2O_2$
44	0.527496	59.8654	Acetate	$C_2H_2O_2$

VIP



EXACT MASS

Figure 2. Viable importance for the projection (VIP) plot showed the summarized of the important metabolites (sorted from most important to lower) in the samples.

be attributed to have different metabolites from groups B, C, and D.

The variable importance for the projection (VIP) summarizes the importance of the variables to explain and correlate to Y. The plot was sorted from high to low, to show the confidence intervals for the VIP value. VIP values were arger than 0.5 indicated "important" metabolites and lower indicated "unimportant" (Figure 2). Using VIP > 0.5 as the cut-off, the important metabolites were identified in the testes and listed in Table 3.

The corresponding Loading's scattered (LS) plot showed the distribution of different variables between control and treatment groups. Each point in the LS plot represents an ion. Ions far away from the origin are significantly important to the differences between groups. Ions in Figure 3, were illustrated and given the identification according to their groups as shown in Table 4.

The OPLS-DA plots of the metabolites in the testes differed between the control group (A) and the treatment group fed with RPF. This finding indicates the presence of different metabolites. There were 44 differentiated metabolites identified in testes using the VIP analysis (VIP>0.5). The important putative metabolites in this study are; 10 – hydroxy - (2E,8E) – decadiene – 4 - ynoic acid (organic compounds known as medium- chain fatty acids), albendazole S-oxide, Nicotinuric acid, Methylcyclopentane (saturated monocyclic hydrocarbons), CL (20: 1 (11Z) / 18: 2(9Z,12Z)/18: 1(11Z)/18:1 (11Z) glycerophospholipids, 18(R) hydroxy – 20 – oxo – 20 – CoA - LTE4 (metabolitethrough lipid oxidation of Leukotriene E4 (LTE4)), CL(i-13:0/a-21:0/i-24:0/a- 25:0)[rac]('double' phospholipids), Methylpentanoic acid (saturated fatty acids with anacyl chain that has a methyl branch).

In the control group (A), 10 – hydroxy - (2E,8E) – decadiene – 4 - ynoic acid was mainly identified. We found the metabolite in the group fed with Prilled fat (B) was different from the group (A), (C) and (D). The metabolite in group B was, 1 - Heneicosanoyl – 2– docosanoyl – 3 - (7Z, 10Z, 13Z, 16Z, 19Z, docosapentanoyl) – sn – glyceryl is categorized as Glycerolipids [GL], main class: Triradylglycerols [GL03] and subclass: Triacylglycerols [GL0301] (TAGs). The metabolites went through further changes however, we could not identify the specific metabolites in group calcium salt and canola oil which

Table 4. Identified putative metabolites in treatment groups.

Groups*	*m/z*	Putative metabolites
A	102.0377	10-hydroxy-(2E,8E)-decadien-4-ynoic acid
B	1057.9136	1-Heneicosanoyl-2-docosanoyl-3-sn glycerol
C and D	705.8320	Adenosine 5' pentaphosphate
		PE (24:0/24:1(15Z)

*A- Control; B-Prilled fat; C- Calcium salt; D-Canola oil

are Adenosine 5' pentaphosphate and PE (24:0/24:1(15Z)). Adenosine is a product of complete dephosphorylation of adenine nucleotides which are presence in various compartments of the cell. PE (24:0/24:1(15Z)) is a phosphatidylethanolamine (PE or GPEtn). Fatty acids containing 16, 18 and 20 carbons are the most common, however PE (24:0/24:1(15Z)), in particular, consists of one chain of lignoceric acid at the C-1 position and one chain of nervonic acid at the C-2 position. While most phospholipids have a saturated fatty acid on C-1 and an unsaturated fatty acid on C-2 of the glycerol backbone, the fatty acid distribution at the C-1 and C-2 positions of glycerol within phospholipids are continually in flux, owing to phospholipid degradation and the continuous phospholipid remodeling that occurs while these molecules are in membranes. PEs are neutral zwitterions at physiological pH. They mostly have palmitic or stearic acid on carbon 1 and a long chain of unsaturated fatty acid (e. g. 18:2, 20:4 and 22:6) on carbon 2 (PubChem, U.S. National Library of Medicine). The synthesis of Adenosine and PE could be due to the increment of the metabolism rate in the cells and the various process involved in the spermatogenesis in the testes in the animals' group fed with calcium salt and its efficiency to produce energy (Pavkovych *et al.,* 2015).

Basch *et al*. (1992) reported that a Ca2+- and Mg2+ stimulated adenosine 5' diphosphatase has been found in lactating bovine mammary glands. The enzyme is associated with membranes of mitochondrial, microsomal, and Golgi

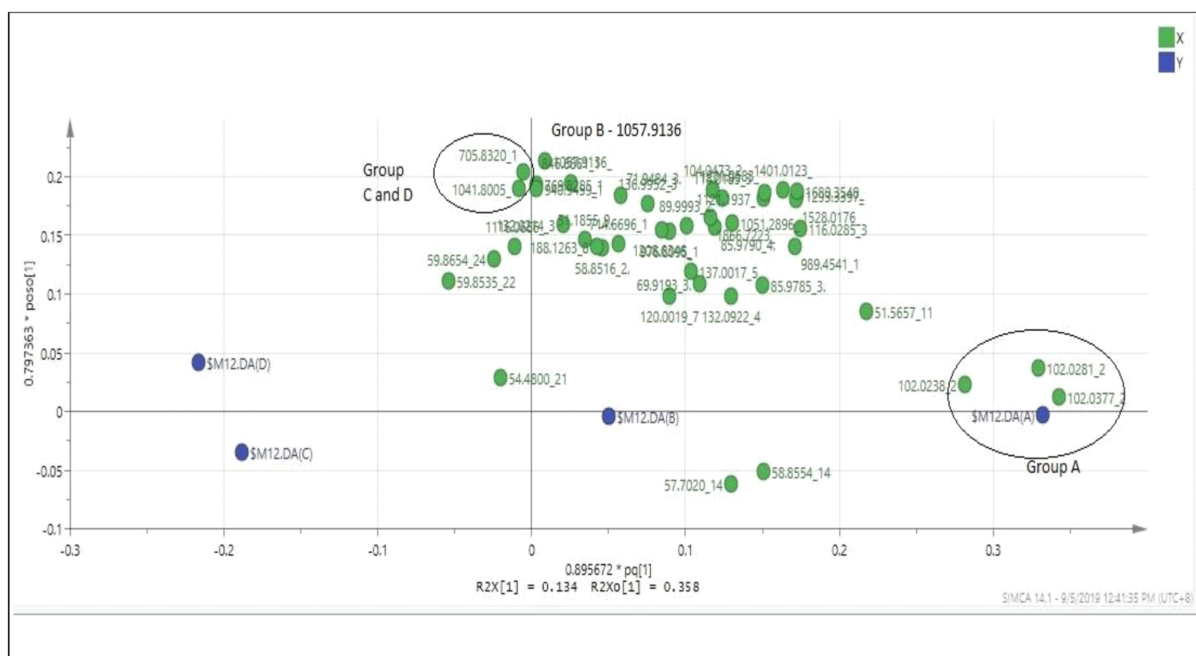


Figure 3. Loading's scatter (LS) plot illustrated the metabolites that consist in different treatment groups

apparatus fractions in the mammary gland which indicates a possible role for this enzyme in the milk secretory process, particularly in ATP cycling in vesicles. Therefore, Kurebayashi *et al*., (1980) mentioned that Adenosine 5' pentaphosphate represents an extremely useful tool in experiments with fragmented sarcoplasmic reticulum, such as studies of H^+ movement accompanying Ca^+ movement, ATP-ADP exchange reaction, and calorimetry of the Ca^+ uptake process in bullfrog skeletal muscle. Cusostomo *et al.,* (2020) reported that the increase in testicular content in ATP and ATP/ ADP ratio was correlated with sperm counts. He also identified polar lipid metabolite in testicular of mice which are Glycerol, Ethonolamine, Phosphoethamolamine and Myoinositol using the H-NMR.

In general, the LIPID MAPS comprehensive classification system for lipids is comprised of eight lipid categories: Fatty acyls (FA), Glycerolipids (GL), Sphingolipids (SP), Glycerophospholipids (GP), Saccharolipids (SL), Prenol lipids (PR), Polyketides (PK), and Sterol lipids (ST). Each lipid category has its subclassification hierarchy. Glycerolipids (GL) are mono-, di-, and tri-substituted glycerol's, the most well-known being the TAGs, fatty acid esters of glycerol, formerly termed as triglycerides. TAGs represent the most abundant lipid class in oils and fats of animal origin and comprise the bulk of storage fat in mammalian tissue. These molecules exist as enantiomers since a centre of asymmetry is created upon enzymatic biosynthesis at carbon 2 of the glycerol backbone (Donato *et al*., 2015). From this finding, we could understand that the important putative metabolites in the testes were fatty acids and their derivatives. This is consistent with Jafaroghli *et al*. (2014) who mention that lipids are abundant in testicles, playing a crucial role in membrane structure and function, energy storage and cell signaling.

Reproductive Genes

The relative expression of the outer dense fiber protein 1 (ODF 1), SERPINA10, CatSper4, AdipoR2 and DAZL are presented in Figure 4. In the group fed with calcium salt and canola oil, the expression levels of ODF 1 were five-fold and seven-fold compared to the control. SERPINA10 has expressed up-regulation compared to control which is four-fold, eight-fold, and 15-fold in the group fed with prilled fat, calcium salt, and canola oil. CatSper4 was expressed up to four- fold in calcium salt meanwhile, in canola oil two-fold compared to control. The AdipoR2 gene was only expressed in the group fed with calcium salt which is ten-fold compared to the control. DAZL gene was expressed five-fold in both groups; the group was fed with calcium salt and canola oil, respectively.

Ram fertility is important and it is influenced by testis development and spermatogenesis. Both testis development and spermatogenesis are often regulated by core genes (Bai *et al.,* 2017). Feeding RPF with calcium salt resulted in the up-regulation of all genes studied in this study (ODF1, SERPINA10, CatSper4, AdipoR2 and DAZL), with more than two fold increment. This is because calcium salts have the highest digestibility among the other unsaturated fatty acid sources, providing the highest digestible energetic value which leads to the up-regulation of these core genes (Block *et al.,* 2006).

ODF1 and Catsper4 are the genes related to sperm total number, concentration, and progressive motility (Ahmad *et al.,* 2018). SERPINA10 gene can improve fertilization and sperm development (Bai *et al*., 2017). Lipid metabolism with PUFAs improves gene expression of AdipoR (Mazaherioun *et al.,* 2017), and DAZL gene which will bring up the meiosis and spermatogenic process in the testes (Ma *et al.,* 2013). The present findings are congruent with the report made by Pavkovych *et al.,* (2015), where supplementation of protected fats and polyenoic fatty acids of vegetable origin in a diet of cattle stimulates metabolism in the animals, increases their productivity, and improves the quality of milk and meat. Supplements of calcium salts of fatty acids, made of palm oils, soybean, sunflower, rapeseed, and flaxseed are best given in a diet to young animals. Present finding suggested that supplemen-

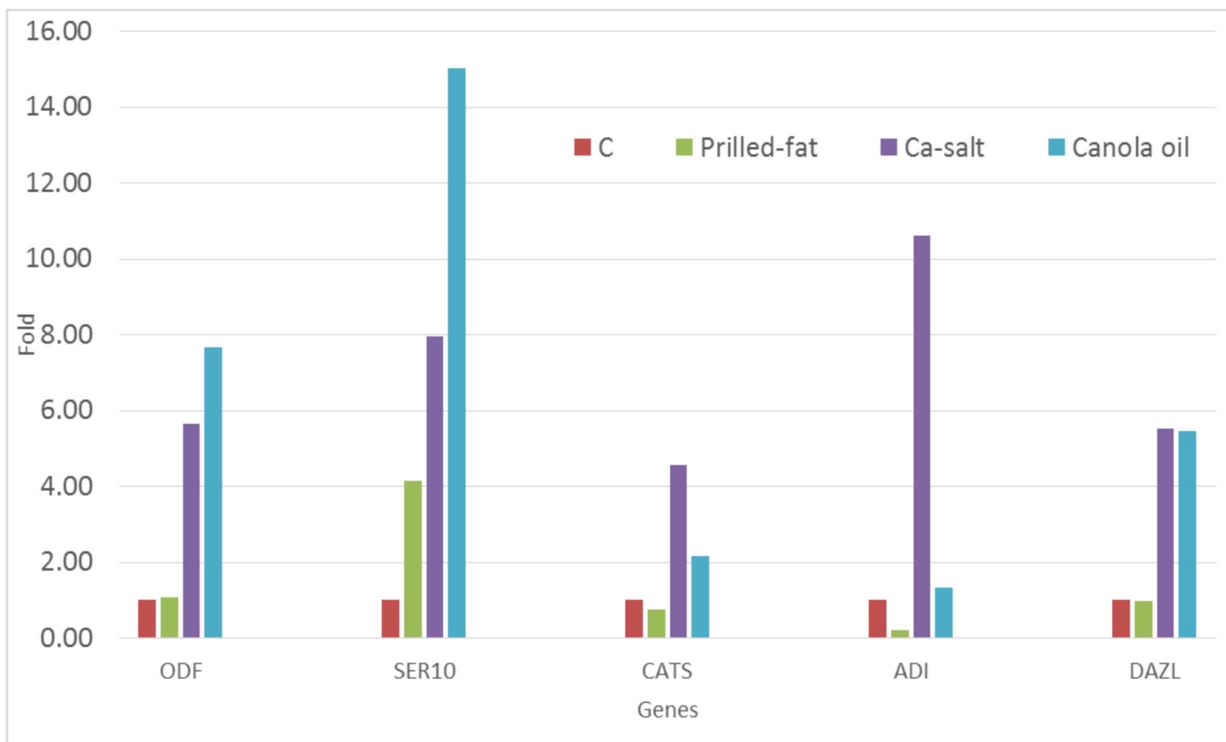


Figure 4. The fold change of reproductive genes: outer dense fiber protein 1 (ODF 1), SERPINA10 (SER10), CatSper4 (CATS), AdipoR2 (ADI) and DAZL

tation of RPF in the form of calcium salt may improve the fertility of Malin ram through sperm total number, concentration, and progressive motility.

CONCLUSION

It could be concluded that feeding lipid as RPF with calcium salt had converted PUFAs to Adenosine, or energy for the spermatogenesis process in the testes. The OPLS-DA model showed that there was a metabolites difference or changes between the control and RPF dietary group. However, no changes were identified between the calcium salt and canola oil. The changes indicated the efficiency of the RPFmetabolism to produce PUFAs, TAG, PE, and Adenosine during the spermatogenesis in the testes. The present study also revealed 44 important putative metabolites via preliminary screening of LC/MS. However, this study only gives an overview of the metabolites in the testes and the changes when the animals are fed with prilled fat, calcium salt, and canola oil. The NMR or LC-MS/MS and Proteomic integration with pathway analysis should be performed to confirm the putative metabolites and pathways in this study. Similarly, feeding RPF with calcium salt gave up-regulation effects on reproductive gene expression such as ODF1, SERPINA10, CatSper4, AdipoR2, and DAZL in Malin ram. This finding gave the impression that supplementation of RPF with calcium salt will improve the reproductive efficiency in male ruminants by improving the motility of thesperm as well as the meiosis and spermatogenic process in testes.

ACKNOWLEDGEMENT

A.I. was a recipient of a scholarship from Federal Training Award (HLP), The Government of Malaysia.

REFERENCES

Ahmad, M. H., L. T. Chwen, and M. S. Maidin. 2018. Effect of different rumen protected fat from palm oil on testosterone level and tes-

ticular traits in Malin rams. Mal. J. Anim. Sci. 21: 27–38.

Bai, M., L. Sun, J. Zhao, L. Xiang, X. Cheng, J. Li, C. Jia, and H. Jiang. 2017. Histological analysis and identification of spermatogenesis-related genes in 2-, 6-, and 12-month-old sheep testes. Naturwissenschaften. 104: 84.

Basch, J., C. Leung, E. Wickham, and H. Farrel. 1992. Distribution of Adenosine 5 diphosphatese activity in the lactating bovine mammary gland. J. Dairy Sci. 75: 732-738.

Behan, A. A., T. C. Loh, S. Fakurazi, U. Kaka, A. Kaka, and A. A. Samsudin. 2019. Effects of supplementation of rumen protected fats on rumen ecology and digestibility of nutrients in sheep. Anim. 9: 400.

Block, B. E., W. Chalupa, D. Palmquist, and C. Sniffen. 2006. Calcium salts are highly digestible. Meyler's Side Effects of Drugs: The International Encyclopedia of Adverse Drug Reactions and Interactions. 77, 610–611.

Buccitelli, C. and M. Selbach. 2020. mRNAs, proteins and the emerging principles of gene expression control. Nat. Rev. Genet. 21: 630–644.

Chavda, M. R., H. H. Savsani, M. R. Gadariya, K. B. Vala, A. J. Dhami and V. K. Karangiya. 2022. Effect of peripartum supplementation of rumen protected choline and rumen protected fat on energy status, insulin, Igf-1 and postpartum fertility in gir cows. The Indian J. Vet. Sci. and Biotechnol. 18: 34–38.

Chen, X., C. Hu, J. Dai C.u, and L. Chen. 2015. Metabolomics analysis of seminal plasma in infertile males with Kidney-Yang deficiency: A preliminary study. Evid. Based Complementary Altern. Med. 2015:892930.

Cusostomo, L., R. Videira and I. Jaral. 2020. Diet during early life define testicular lipid content and sperm quality in adulthood. Am. J. Physiol. Endocrinol. Metab. 319:E1061-E1073

De Silva, S. M. H. H., E. D. N. S. Abeyrathne, W. M. P. B. Weerasinghe, K. K. T. N. Ranaweera and M. B. P. K. Mahipala. 2020. Rumen protected fat preparation using by products generated in coconut processing industry. Proceedings of the International Research Conference of Uva Wellassa University, 15.

Donato, P., F. Cacciola, M. Beccaria, P. Dugo and L. Mondello. 2015. Lipidomics. Compr. Anal. Chem. 68: 395–439.

Du, L., W. Chen, Z. Cheng, S. Wu, J. He, L. Han, W. Qin and Z. He. 2021. Novel gene regulation in normal and abnormal spermatogenesis. Cells. 10: 1–16.

Fraser, L., K. Wasilewska-Sakowska, Ł. Zasiadczyk, E. Piątkowska and K. Karpiesiuk. 2021. Fractionated seminal plasma of boar ejaculates analyzed by LC–MS/MS: Its effects on post-thaw semen quality. Genes. 12: 1574.

Hosseini, B., M. Nourmohamadi, S. Hajipour, M. Taghizadeh, Z. Asemi, S. A. Keshavarz and S. Jafarnejad. 2019. The effect of omega -3 fatty acids, epa, and/or dha on male infertility: a systematic review and meta-analysis. J. Diet. Suppl. 16: 245–256.

Jafaroghli, M., H. Abdi-Benemar, M. J. Zamiri, B. Khalili, A. Farshad and A. A. Shadparvar. 2014. Effects of dietary n-3 fatty acids and vitamin C on semen characteristics, lipid composition of sperm and blood metabolites in fat-tailed Moghani rams. Anim. Reprod. Sci. 147: 17–24.

Khoshniat, M. T., A. Towhidi, K. Rezayazdi, M. Zhandi, F. Rostami, N. Dadashpour Davachi, F. Khalooee and J. Kastelic. 2020. Dietary omega-3 fatty acids from linseed oil improve quality of post-thaw but not fresh sperm in Holstein bulls. Cryobiology. 93: 102–108.

Kurebayashi, N., T. Kodame and Y. Ogawa. 1980. Adenosine 5 pentaphosphate as an inhibor of adenylate kinase in studies of fragmented Sarcoplasmic reticulum from Bullfrog skeletal muscle. J. Biochem. 88: 871-876.

Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT

method. Methods. 25: 402–408.

Lone, S. A., N. Shah, H. P. Yadav, M. A. Wagay, A. Singh and R. Sinha. 2017. Sperm DNA damage causes, assessment and relationship with fertility: A review. Theriogenology Insight - An Intl. J. Reprod. Anim. 7: 13-20.

Ma, C., J. Li, H. Tao, B. Lei, Y. Li, K. Tong, X. Zhang, K. Guan, Y. Shi and F. Li. 2013. Discovery of two potential DAZL gene markers for sperm quality in boars by population association studies. Anim. Reprod. Sci. 143: 97–101.

Ma, L., J. B. Cole, Y. Da and P. M. VanRaden. 2019. Symposium review: Genetics, genome-wide association study, and genetic improvement of dairy fertility traits. J. Dairy Sci. 102: 3735–3743.

Manriquez, D., L. Chen, P. Melendez and P. Pinedo. 2019. The effect of an organic rumen-protected fat supplement on performance, metabolic status, and health of dairy cows. BMC Vet. Res. 15: 1–14.

Mazaherioun, M., A. Saedisomeolia, M. H. Javanbakht, F. Koohdani, M. R. Eshraghian and M. Djalali. 2017. Beneficial effects of n -3 polyunsaturated fatty acids on adiponectin levels and AdipoR gene expression in patients with type 2 diabetes mellitus: A randomized, placebo-controlled, double-blind clinical trial. Arch. Med. Sci. 13: 716–724.

Naval-Sanchez, M., Q. Nguyen, S. McWilliam, L. R. Porto-Neto, R. Tellam, T. Vuocolo, A. Reverter, M. Perez-Enciso, R. Brauning, S. Clarke, A. McCulloch, W. Zamani, S. Naderi, H. R. Rezaei, F. Pompanon, P. Taberlet, K. C. Worley, R. A. Gibbs, D. M. Muzny, … J. Kijas. 2018. Sheep genome functional annotation reveals proximal regulatory elements contributed to the evolution of modern breeds. Nat. Comm. 9: 1–13.

Ngcobo, J. N., F. V. Ramukhithi, K. A. Nephawe, T. J. Mpofu, T. C. Chokoe and T. L. Nedambale. 2021. Flaxseed oil as a source of omega n-3 fatty acids to improve semen quality from livestock animals: A review. Anim. 11: 1–12.

Owens, F. N. and M. Basalan. 2016. Ruminal Fermentation. In Rumenology. Springer, Cham.

Pavkovych, S., S. Vovk and B. Kruzhel. 2015. Protected lipids and fatty acids in cattle feed rations. Acta Sci. Pol. Zootech. 6145: 3–14.

PubChem, U.S. National Library of Medicine Website, https://pubchem.ncbi.nlm.nih.gov

Qu, Y. H., L. Y. Jian, L. Ce, Y. Ma, C. C. Xu, Y. F. Gao, Z. Machaty and H. L. Luo. 2019. Identification of candidate genes in regulation of spermatogenesis in sheep testis following dietary vitamin E supplementation. Anim. Reprod. Sci. 205: 52–61.

Schoech, S. J., R. Bowman and S. J. Reynolds. 2004. Food supplementation and possible mechanisms underlying early breeding in the Florida Scrub-Jay (A*phelocoma coerulescens*). Horm. Behav. 46: 565–573.

Wysokińska, A. and D. Szablicka. 2021. Integrity of sperm cell membrane in the semen of crossbred and purebred boars during storage at 17◦C: Heterosis effects. Anim. 11: 3373.

Yang, H., X. Liu, G. Hu, Y. Xie, S. Lin, Z. Zhao and J. Chen. 2018. Identification and analysis of microRNAs-mRNAs pairs associated with nutritional status in seasonal sheep. Biochem. Biophy. Res. Comm. 499: 321–327.

Zhang, Q., S. Y. Ji, K. Busayavalasa, J. Shao and C. Yu. 2019. Meiosis I progression in spermatogenesis requires a type of testis-specific 20S core proteasome. Nat. Comm. 10: 1–11.