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EFFECTS OF DIFFERENT TYPES OF PROTECTED FAT ON RUMEN METABOLISM, MEAT QUALITY AND METABOLOMICS IN DORPER CROSSBRED SHEEP

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By

ATIQUE AHMED BEHAN

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

February 2018

DEDICATION

This thesis is dedicated to

MY LATE FATHER

AND

MY MOTHER WITH LOVE

who always supported and encouraged me to do the best

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

EFFECTS OF DIFFERENT TYPES OF PROTECTED FAT ON RUMEN METABOLISM, MEAT QUALITY AND METABOLOMICS IN DORPER CROSSBRED SHEEP

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Chairman : Associate Professor Anjas Asmara @ Ab. Hadi Samsudin, PhD
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The prilled fat, lecithinized prilled fat and calcium soap (calcium salts of palm fatty acids) are the extensively used rumen protected fats (RPF). These are used in ruminant diets to protect dietary lipids from rumen biohydrogenation and to prevent detrimental effects of high fats on rumen fermentation. Supplementation of the protected fats could improve *in vitro* and *in vivo* rumen metabolism, nutrient intake and digestibility, meat quality and metabolomics; and modify meat fatty acid profile. There is very limited number of studies in the literature discusses the use of RPF and their impact on performance, meat quality characteristics and fatty acid profile in sheep. However, the influences of RPF supplementation on rumen metabolism have been highly variable and inconsistent and their impacts on meat quality remain obscure. Therefore there is a need for specific studies to permit personalized decisions and informed choices in the utilization of protected fats. Thus, the present study was conducted to examine the effects of different types of protected fats on *in vitro* and *in vivo* rumen metabolism, nutrient intake and digestibility, serum biochemistry; meat quality and fatty acid profile and meat metabolomics in Dorper crossbred sheep.

In vitro experiment was conducted using ruminal fluid from fistulated Dorper sheep. Treatment consisted of basal diet (70:30 concentrate to rice straw) with no added RPF (T1), basal diet plus prilled fat (T2), basal diet plus prilled fat with lecithin (T3) and basal diet plus calcium soap of palm fatty acids (T4). Completely randomized design (CRD) was followed. *In vitro* gas production, fermentation kinetics, *in vitro* dry matter digestibility (IVDMD), *in vitro* organic matter digestibility (IVOMD), rumen fermentation characteristics and apparent biohydrogenation of fatty acids were determined. The cumulative gas production and gas production kinetics were not affected by RPF. Prilled fat with lecithin increased IVDMD and IVOMD significantly.

Metabolizable energy was not affected by addition of RPF. The RPF did not influence significantly pH, ammonia nitrogen, methane, VFA and molar proportion of VFA. However, the diet containing prilled fat with lecithin (T3) reduced acetate to propionate ratio, decreased methane numerically and increased biohydrogenation of C18:2n-6 and C18 UFA without disrupting rumen fermentation.

For the *in vivo* experiment, 36 male Dorper crossbred sheep about 18 months of age were used to evaluate the effects of different protected fats on nutrient intake, nutrient digestibility, serum biochemistry, meat quality and fatty acids. The animals were fed with the four experimental diets for 90 days (including last 10 days for digestibility trial) and were slaughtered. The diets did not affect body weight (BW), feed conversion ratio (FCR) and feed efficiency. There was no significant difference (P>0.05) seen in the intake and digestibility of all nutrients except ether extract (EE) and crude fibre (CF).

The rumen fermentation characteristics including pH, methane (CH_4), VFA and molar proportions of VFA, acetate to propionate ratio differ significantly (P<0.05). The maximum CH_4 reduction was observed in the diet T3 while T2 showed the least reduction. Concentration of ammonia nitrogen was not different significantly (P>0.05) among the treatments. Numerically the lowest total VFA concentration was seen in the diet prilled fat with lecithin (T3). Fatty acid profile of rumen digesta was significantly different for ∑SFA, ∑MUFA, ∑PUFA, ∑n-3 and ∑n-6. Neither the diet nor the sampling time influenced serum cholesterols (total, HDL, LDL and VLDL), triglycerides, glucose and fatty acids. Serum fatty acids including ∑SFA, ∑UFA, ∑MUFA, ∑PUFA did not significantly differ (P>0.05). Sheep fed with diet containing RPF had higher (P<0.05) n-3 PUFA as compared to control. The diets did not influence (P>0.05) the levels of ALT, ALP and AST but the concentrations of ALP and AST were affected by sampling day.

There was no difference (P>0.05) in slaughter weight, hot and cold carcass weights, dressing percentage, chilling loss, rib eye area, non-carcass components, non-carcass fats, and primal cuts. However, back fat thickness was significantly affected among the treatments. Chemical composition of *longissimus dorsi* (LD) and *semitendinosus* (ST) muscles, meat cholesterol, meat pH, drip loss, cooking loss and shear force were not significantly affected.

The muscle and liver metabolomics was conducted using ^{1}H NMR spectroscopy. Six metabolites were identified from the muscle tissues including choline, creatine, glycerophosphocholine, inosine, isoleucine and lactate. The concentration of choline, creatine, glycerophosphocholine, inosine and lactate were significantly different but there was no significant difference observed in the concentration of isoleucine.

The supplementation of prilled fat with lecithin (T3) decreased SFA and increased MUFA and PUFA in the meat. Also, it increased the concentrations of C18:1n-9, CLA *Cis*-9 *Trans*-11, CLA *Trans*-10 *Cis*-12, C18-2n-6, C18-3n-3 and reduced n-6:n-3 which is beneficial to human health making the meat from Dorper crossbred sheep, free from negative effect.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN JENIS LEMAK BERBEZA TERHADAP METABOLISME RUMEN, KUALITI DAGING DAN METABOLOMIK PADA KAMBING BIRI-BIRI DORPER

Oleh

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Februari 2018

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Lemak terlindung, lemak terurai dan tepu kalsium (garam kalsium daripada asid lemak sawit) adalah lemak rumen terlindung yang digunakan secara meluas (RPF). Ia digunakan dalam diet ruminan untuk melindungi lipid pemakanan daripada biohidrogenasi rumen dan untuk mencegah kesan-kesan sampingan lemak tinggi pada penapaian rumen. Tambahan lemak terlindung dapat meningkatkan rumen metabolisme *in vitro* dan dalam *in vivo*, pengambilan nutrien dan kecernaan, kualiti daging dan metabolomik; dan mengubah suai profil asid lemak daging. Terdapat sangat banyak kajian dalam kajian lepas dalam membincangkan penggunaan RPF dan kesannya terhadap prestasi, ciri-ciri kualiti daging dan profil asid lemak pada kambing. Walau bagaimanapun, pengaruh suplemen RPF terhadap metabolisme rumen sangat jauh berubah dan tidak konsisten dan impak mereka terhadap kualiti daging tetap tidak jelas. Oleh itu, terdapat keperluan untuk penyelidikan khusus untuk membenarkan keputusan dan pilihan peribadi yang dimaklumkan dalam penggunaan lemak terlindung. Oleh itu, kajian ini dijalankan untuk mengkaji kesan pelbagai jenis lemak terlindung pada *in vitro* dan dalam metabolisme *iv vivo*, pengambilan nutrien dan pencernaan, biokimia serum; kualiti daging dan profil asid lemak dan metabolisma daging pada kambing biri-biri Dorper.

Eksperimen *in vitro* dijalankan menggunakan cecair ruminal dari kambing biri-biri Dorper yang difistula. Rawatan terdiri daripada diet asas (70:30 menumpukan konsentrat kepada jerami padi) tanpa tambahan RPF (T1), diet basal ditambah lemak terlindung (T2), diet basal ditambah lemak terlindung dengan lesitin (T3) dan diet asas serta kalsium asid lemak sawit (T4). Reka bentuk rawak sepenuhnya (CRD) diikuti. Pengeluaran gas *in vitro*, kinetik penapaian, penghadaman bahan kering dalam *in vitro* (IVDMD), *in vitro* bahan organik pencernaan (IVOMD), ciri penapaian rumen dan

biohidrogenasi asid lemak ditentukan. Pengeluaran gas kumulatif dan kinetik pengeluaran gas tidak terjejas oleh RPF. Lemak yang terlindung bersama dengan lesitin meningkat IVDMD dan IVOMD dengan ketara. Tenaga metabolizable tidak terjejas oleh penambahan RPF. RPF tidak mempengaruhi pH, amonia nitrogen, metana, VFA dan bahagian molar VFA yang ketara. Walau bagaimanapun, diet yang mengandungi lemak terlindung dengan lesitin (T3) mengurangkan asetat kepada nisbah propionat, menurunkan kadar metana dan peningkatan biohydrogenasi C18: 2n-6 dan C18 UFA tanpa mengganggu penapaian rumen.

Untuk eksperimen *in vivo*, 36 ekor kambing biri-biri Dorper jantan berusia kira-kira 18 bulan digunakan untuk menilai kesan-kesan lemak yang berlainan pada pengambilan nutrien, kecernaan nutrien, biokimia serum, kualiti daging dan asid lemak. Haiwan ini diberi makan dengan empat diet percubaan selama 90 hari (termasuk 10 hari terakhir untuk percubaan pencernaan) dan disembelih. Diet tidak mempengaruhi berat badan (BW), nisbah penukaran makanan (FCR) dan kecekapan suapan. Tidak terdapat perbezaan yang signifikan (P> 0.05) yang dilihat dalam pengambilan dan penghadaman semua nutrien kecuali keputusan ekstrak ether (EE) dan serat mentah (CF).

Ciri-ciri penapaian rumen termasuk pH, metana (CH4), VFA dan bahagian molar VFA, asetat kepada nisbah propionat berbeza dengan ketara (P <0.05). Pengurangan CH4 adalah maksimum diperhatikan dalam diet T3 manakala T2 menunjukkan pengurangan yang paling rendah. Kepekatan nitrogen ammonia tidak banyak berbeza (P> 0.05) di antara semua rawatan. Secara numerik jumlah kepekatan VFA yang paling rendah dilihat dalam diet lemak terlindung dengan lesitin (T3). Profil asid lemak rumen yang dicernakan adalah berbeza dengan ketara untuk ΣSFA, ΣMUFA, ΣPUFA, Σn-3 dan Σn-6. Diet atau masa pensampelan tidak mempengaruhi kolesterol serum (jumlah, HDL, LDL dan VLDL), trigliserida, glukosa dan asid lemak. Asid lemak serum termasuk ΣSFA, ΣUFA, ΣMUFA, ΣPUFA adalah tidak berbeza jauh (P> 0.05). Kambing yang diberi makanan yang mengandungi RPF lebih tinggi n-3 PUFA secara ketara (P <0.05) berbanding dengan kawalan. Diet tidak mempengaruhi (P> 0.05) tahap ALT, ALP dan AST tetapi kepekatan ALP dan AST dipengaruhi oleh hari sampling.

Tidak ada perbezaan yang ketara (P> 0.05) dalam berat penyembelihan, berat badan panas dan sejuk, peratusan bersama kulit, kehilangan kerengsaan, kawasan mata rusuk, komponen bukan karkas, lemak bukan karkas, dan potongan awal. Walau bagaimanapun, ketebalan lemak belakang telah terjejas dengan ketara di kalangan rawatan. Komposisi kimia longissimus dorsi (LD) dan semitendinosus (ST) otot, kolesterol daging, pH daging, kehilangan titisan, daya masakan dan daya ricih tidak terjejas dengan ketara.

Metabolisma otot dan hati telah dijalankan menggunakan spektroskopi 1H NMR. Enam metabolit dikenal pasti dari tisu otot termasuk choline, creatine, glycerophosphocholine, inosine, isoleucine dan lactate. Kepekatan choline, creatine, glycerophosphocholine, inosine dan lactate sangat berbeza tetapi tiada perbezaan yang signifikan dalam kepekatan isoleucine.

Suplemen lemak terlindung dengan lesitin (T3) menurunkan SFA dan meningkatkan MUFA dan PUFA dalam daging. Selain itu, ia meningkatkan kepekatan C18: 1n-9, CLA Cis-9 Trans-11, CLA Trans-10 Cis-12, C18-2n-6, C18-3n-3 dan mengurangkan n-6: n-3 dimana ia bermanfaat untuk kesihatan manusia yang menjadikan daging dari kambing biri-biri Dorper bebas dari kesan negatif.

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

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ADF	Acid detergent fibre
ADG	Average daily gain
ADL	Acid detergent lignin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
BH	Biohydrogenation
BW	Body weight
CCW	Cold carcass weight
CF	Crude fibre
CH_4	Methane
CLA	Conjugated linoleic acid
cm	Centimetre
cm^2	Centimetre square
CO_2	Carbon dioxide
CP	Crude protein
d	Day
DM	Dry matter
DMD	Dry matter digestibility
DMI	Dry matter intake
EBW	Empty body weight
EE	Ether extract
FA	Fatty acid

FAME	Fatty acid methyl esters
FCR	Feed conversion ratio
g	Gram
GLM	Generalized linear model
h	Hour
HCW	Hot carcass weight
HDL	High density lipoprotein
IU/L	International units per litre
IVDMD	*In vitro* dry matter digestibility
IVOMD	*In vitro* organic matter digestibility
kg	Kilogram
KPH	Kidney, pelvic and heart fat
L	Litre
LD	*longissimus dorsi*
LDL	Low density lipoprotein
ME	Metabolizable energy
mg	Milligram
MJ	Mega joule
mL	Millilitre
mm	Millimetre
mM	Millimole
mmol/L	Millimole per litre
MUFA	Mono unsaturated fatty acid
N	Nitrogen
NDF	Neutral detergent fibre
NGP	Net gas production

NH_3-N	Ammonia-nitrogen
NRC	National Research Council
OM	Organic matter
OMD	Organic matter digestibility
PUFA	Poly unsaturated fatty acid
RPF	Rumen protected fat
SEM	Standard error of means
SFA	Saturated fatty acid
ST	*Semitendinosus*
TVFA	Total volatile fatty acid
UFA	Unsaturated fatty acid
VFA	Volatile fatty acid
VLDL	Very low density lipoprotein
WG	Weight gain
WHC	Water holding capacity
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Feed is considered a major proportion of the cost of raising ruminants. The raw ingredients for animal feed such as cereal grains, vegetable and animal proteins, mineral sources, micro-ingredients and other additives are not produced in Malaysia but are imported from other countries (Loh, 2004). So as to reduce the cost incurred on the import of these raw ingredients there is a need of utilizing available low-cost indigenous resources for animal feeds to fulfil their energy needs by producing more with spending less. In this connection, there has been a recent emphasis to utilize by-products of oil palm industry as animal feed (Alimon and Wan Zahari, 2012).

In order to fulfil the energy needs, dietary lipids are being used in ruminant nutrition. However, feeding lipids in high concentrations could adversely influence rumen microbial metabolism, affecting nutrient digestibility and animal performance (Hartati *et al.*, 2012; Szumacher-Strabel *et al.*, 2009; Naik *et al.*, 2007a). The adverse effects of lipid supplementation are because of extensive biohydrogenation (BH) of fatty acids (FA), especially BH of unsaturated fatty acids (UFA) to saturated fatty acids (SFA) by rumen microbes. Thus, the negative impact of lipid supplementation can be easily overwhelmed by feeding rumen protected fats (RPF) or rumen inert or rumen bypass fats to ruminants.

Rumen protected fats are generally, a by-product of palm oil industry, considered as insoluble fats due to their protection from microbial fermentation and biohydrogenation. They remain insoluble at normal rumen pH range of 6 to 7 and escape rumen fermentation. They are then utilized as a source of energy when absorbed through the small intestine (Warner *et al.*, 2015). The use of RPF enhance fibre digestibility in high fat supplemented diets by forming insoluble soaps (Palmquist and Jenkins 1980). Moreover, supplementation of RPF improves energy efficiency as a result of reduced production of methane from the rumen and direct use of long-chain fatty acids (Park *et al.*, 2010). Owing to their inert nature, RPF can be successfully used in relatively large amounts without compromising rumen function (Ayasan and Karakozak, 2011) and reducing feed intake (Gooden, 1977).

The common available RPF include calcium soaps (calcium salts of palm fatty acids), hydrogenated fats from palm oil, fractionated palm oil fatty acids (C16), straight fats (tallow) and dry fat premixes (blends of vegetable and/or animal fats). The common methods of RPF preparation include microencapsulation with a water-insoluble lipid coating, formaldehyde treatment of a lipid-protein matrix, the formation of calcium salts of fatty acids and preparation of fatty acyl amides (Bauman *et al.*, 2003; Putnam *et al.*, 2003).

The calcium soap of palm fatty acids and the prilled fat are the most extensively used protected fats, both of which are highly digestible. Calcium soap of palm fatty acids, prilled fat and lecithinized prilled fat are the highly concentrated source of energy supplement fats, specially produced from 100 percent fully refined palm oil fraction which are non-hydrogenated and free from trans fatty acids (TFA). The high palmitic acid contents in these fats can bypass rumen and become a direct energy source for the ruminants. Moreover, lecithinized prilled fats improve the emulsifying properties and thus increase the digestibility of animals.

The calcium salts of palm fatty acids are produced by reacting palm fatty acids distillate with calcium hydroxide to form calcium soaps. The calcium soaps would not be influenced in the rumen (pH 6.5 to 6.8), and finally are fully opened making the fatty acids accessible for absorption in abomasum and duodenum (pH 3.5) (Mierlita, 2018). It was reported that the RPF in the form of calcium soap allows normal rumen fermentation and digestibility of nutrients (Schauff and Clark, 1989; Jenkins and Palmquist, 1984). Prilled fats are made by liquefying a mixture of fatty acids high in saturated fatty acid content and spraying the mixture under pressure into a cooled atmosphere in order to form a dried prilled fatty acid supplement that is inert in the rumen and does not alter rumen fermentation (Grummer, 1988; Chalupa, 1986). Furthermore, the prilled fat with lecithin act as an emulsifier by dispersing fatty acids and enhancing fatty acid absorption (Wettstein *et al.*, 2011).

Bhatt *et al.* (2015); (2013b) reported that supplementation of protected fat of industrial grade of improved nutrient digestibility, body condition and carcass characteristics in sheep. There was no adverse effect of supplementation of RPF on the rumen fermentation (Naik *et al.*, 2013). Alexander *et al.* (2002) reported that calcium soap prepared from sunflower acid oil at 10 % of DM can be fed to sheep without affecting fibre digestibility in sheep. However, there was a significant reduction in *in vitro* DM degradability (IVDMD) with an increase in the level of bypass fat (Tangendjaja *et al.*, 1993). Manso et al. (2006) reported that supplementation of calcium soaps of palm fatty acids improved EE digestibility and FCR in growing lambs. Therefore protected fat supplementation could improve digestibility and FCR in adult sheep as well so there is a need to evaluate the effects of protected fats in adult sheep.

Red meat is one of the major dietary sources of protein and essential nutrients such as vitamins and minerals that play vital role in human health. However, consumption of red meat may increase the risk of cardiovascular disease (CVD) and cancer in the colon (McAfee *et al.,* 2010) because of high SFA contents in it. Thus, reducing SFA content and the n-6/n-3 ratio is of major importance in meat research (Mierlita, 2018). Therefore, modifying the FA composition of ruminant meat is of prime significance (Mapiye *et al*., 2015; Scollan *et al*., 2014).

The supplementation of RPF has been one of the methods to reduce undesirable SFA and increase beneficial UFA in the meat (Warner *et al.*, 2015). In this regard, several studies have been conducted on effects of dietary lipids and their FA composition on various aspects of meat quality (Wood *et al.*, 2008; Schmid *et al.*, 2006; Wood *et al.*, 2004; Palmquist and Jenkins, 1980). However, most of the published studies have been inconclusive in proving whether the use of protected fat increases the content of essential fatty acids in animal meat (Lima *et al.*, 2017). Therefore, there is a need to conduct such experiment in order to evaluate the effects of protected fats.

In previous studies effects of protected fats on live weight, carcass and meat characteristics and fatty acid composition of muscle have been evaluated. However, no characterization of the muscle and liver metabolomes of sheep has ever been evaluated. Therefore the present study was planned to characterize the metabolome of the muscle and liver of sheep, and study the effect of protected fat in these tissues, which are important from the productive and metabolic perspectives. The NMR-metabolomics based approach, which, was for the first time applied to Dorper sheep for dietary fat supplementation.

Although RPF has been extensively evaluated in dairy animals (Gowda *et al.*, 2013; Naik, 2013; Shelke *et al.*, 2012; Wadhwa *et al.*, 2012) and to some extent beef cattle (Mangrum *et al.*, 2016; Long *et al.*, 2014; Hightshoe *et al.*, 1991) yet there is a need to evaluate RPF especially in sheep for meat purpose where there is a very limited number of studies done. The studies conducted on RPF in sheep are in young lambs (Bhatt *et al.*, 2015; Bhatt *et al.*, 2013b) none of those has been conducted in adult sheep, therefore adult animals (about one and half a year of age) were selected to be used in the present study especially with intention to focus on fat deposition. It is well established that fat deposition in the body is age-dependent and subcutaneous fat is accumulated at the later stage of growth (Bhatt *et al.*, 2013a). Leon et al. (1999) reported higher body fat reserves at 12 and 16 months of age as compared to 8 month old lambs.

It is hypothesized that supplementation of protected fat in the form of calcium soap, prilled fat and lecithinized prilled fat will improve *in vitro* and *in vivo* rumen metabolism; nutrient intake and digestibility; meat quality, meat metabolomics and will increase the circulating unsaturated fatty acids in blood and consequently depositing them into the intramuscular meat. However, producing the meat that is low in saturated fatty acids, high in monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), concurrently prevents the development of cardiovascular disease would be an effective approach. Therefore, different types of protected fats were selected to evaluate with the following general objective:

- To determine the effects and efficacy of RPF supplementation in improving the quality of meat production in small ruminants.

The specific objectives of the study include:

1. To evaluate the effects of RPF on *in vitro* gas production, rumen fermentation, and apparent biohydrogenation of fatty acids.
2. To determine the effects of RPF on nutrient intake, digestibility, rumen fermentation, serum biochemistry and serum fatty acid profile in Dorper sheep.
3. To determine the influences of RPF on carcass characteristics, meat quality and meat fatty acid profile.
4. To determine the effects of RPF on muscle and liver metabolites using NMR-based metabolomics.

Presentation of the thesis

The thesis is divided into eight chapters. The first two chapters discuss the framework of the experimental research. Chapter 1 provides the rationale for the focus of the research. Chapter 2 presents the review of literature covering the livestock industry, sheep meat production and consumption in Malaysia, rumen microbial ecosystem, fat supplementation to ruminants and its metabolism in rumen, rumen protected fats (RFP) and significance of RPF supplementation, effects of RPF on body weight changes, nutrient intake and digestibility, rumen fermentation, carcass traits, meat quality and livestock metabolomics. From Chapter 3 to 6 present the experimental works for this study. Chapter 7 describes the major findings and highlights the practical importance. Chapter 8 presents the summary, conclusions and recommendations for future research.

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