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COMPARISON OF HAEMATOLOGICAL AND BIOCHEMICAL PARAMETERS IN LATE-GESTATION SINGLE-BEARING AND TWIN-BEARING ILE DE FRANCE EWES

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ABSTRACT

The aim of this study was to evaluate the effect of fetal number on selected haematological and biochemical blood parameters in late-gestation Ile de France ewes. A total of 40 clinically healthy ewes aged 2–5 years, with an average body weight of 68.0 ± 3.58 kg, were included in the experiment. All ewes were between days 110 and 130 of gestation. Based on ultrasonographic examination, the animals were divided into two groups: ewes carrying a single fetus (n = 20) and ewes carrying twin fetuses (n = 20). The independent samples Student's t-test for comparison of group means was used.

Biochemical analysis revealed significant differences between the experimental groups. Ewes carrying a single fetus exhibited significantly higher concentrations of total protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, creatinine, calcium, and iron ($P \leq 0.001$). In addition, higher white blood cell, lymphocyte, and granulocyte counts were observed in this group. Conversely, ewes carrying twin fetuses showed significantly higher concentrations of globulin, triglycerides, cholesterol, β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA), phosphorus, haemoglobin, and platelet counts ($P \leq 0.001$).

Twin pregnancy markedly affected several metabolic and haematological indicators. Elevated concentrations of BHB and NEFA in twin-bearing ewes indicate increased mobilisation of body fat reserves and the development of a negative energy balance during late gestation.

Keywords: sheep, Ile de France, pregnancy, fetal number, haematological parameters, biochemical parameters

INTRODUCTION

Sheep production represents an important component of agricultural livestock farming, contributing substantially to the production of meat, milk, wool, and hides. In addition to their economic importance, sheep play a significant role in maintaining agricultural landscapes through the utilisation of permanent grasslands and pasture ecosystems. The Ile de France breed is one of the most important meat-type sheep breeds, characterised by rapid growth, excellent carcass quality, and favourable reproductive performance. Owing to its high production potential, optimal nutritional management and health care are particularly important during pregnancy. Pregnancy imposes considerable physiological demands on the maternal organism, affecting not only the reproductive tract but also the overall metabolic status of the ewe. The magnitude of these physiological changes depends on several factors, including the stage of gestation, nutritional status, age, breed, and, most importantly, the number of developing fetuses **(Antunović *et al.*, 2011; Bezerra *et al.*, 2017)**. During the final third of gestation, fetal growth accelerates rapidly, with approximately 70–80% of total fetal development occurring during this period. Consequently, the increasing nutritional and energy requirements of the fetus induce substantial metabolic adaptations in the dam, frequently leading to the mobilisation of body reserves **(Sormunen-Cristian & Jauhiainen, 2001)**. These demands become even greater in multiple pregnancies, as twin-bearing ewes require larger amounts of glucose, energy, minerals, and other essential nutrients to support fetal growth. Pregnancy-associated metabolic alterations are reflected in changes in serum biochemical parameters. Increased concentrations of β-hydroxybutyrate (BHB) and non-esterified fatty acids (NEFA) are recognised indicators of negative energy balance and enhanced lipid mobilisation, both of which are major risk factors for the development of pregnancy toxaemia in ewes **(Herdt, 2000; Moallem *et al.*, 2012; Raoofi *et al.*, 2015)**. Haematological parameters are valuable indicators of the physiological and health status of animals and provide important information regarding adaptive responses during different physiological stages. Pregnancy induces several changes in the haematopoietic system, which may result from endocrine adaptations as well as differences in fetal number **(Pereira *et al.*, 2015; Santarosa *et al.*, 2022)**. Mineral metabolism is also profoundly influenced during pregnancy due to the increasing requirements of the developing fetuses. Calcium and phosphorus are particularly important for fetal skeletal development and maternal preparation for lactation. Blood concentrations of these minerals may vary according to both the stage of pregnancy and the number of fetuses carried **(Yildiz *et al.*, 2005; Stojković *et al.*, 2014)**.

Therefore, the aim of the present study was to compare selected haematological and biochemical blood parameters between late-gestation Ile de France ewes carrying single and twin fetuses and to evaluate the influence of fetal number on the metabolic and physiological status of ewes during late pregnancy.

MATERIAL AND METHODS

Animals and experimental design

The experiment was conducted using the university flock of Ile de France sheep maintained at the University Agricultural Enterprise in Oponice, Slovak University of Agriculture in Nitra, Slovak Republic. Throughout the experimental period, all animals were under continuous veterinary supervision, and all procedures were performed in accordance with the provisions of Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. Furthermore, according to the State Veterinary and Food Administration of the Slovak Republic, this study was assessed as an unverified agricultural practice, to which the provisions of Government Regulation of the Slovak Republic No. 377/2012 of 14 November 2012, laying down requirements for the protection of animals used for scientific or educational purposes, do not apply.

A total of 40 clinically healthy late-gestation Ile de France ewes were randomly selected for the study. The animals were between 2 and 5 years of age and had an average body weight of 68.0 ± 3.58 kg. The pregnancy stage ranged from 110 to 130 days of gestation. Fetal number was determined by ultrasonographic examination, after which the animals were assigned to one of two experimental groups: single-bearing ewes (n = 20) and twin-bearing ewes (n = 20). Pregnancy diagnosis and fetal counting were performed using a portable ultrasound scanner (HS-103V, Honda Electronics Co., Ltd., Japan) equipped with a 5-MHz transducer. All animals received routine husbandry and veterinary care throughout the study. Particular attention was paid to minimising animal stress during ultrasonographic examination and blood sampling.

Feeding management

During late gestation, all ewes received the standard diet routinely used on the farm. Feed was offered twice daily, in the morning and afternoon, in quantities and with a composition designed to meet or slightly exceed the sheep's daily nutritional requirements. The daily ration per ewe consisted of maize silage, meadow hay, alfalfa hay. Fresh drinking water and mineral salt licks were available *ad libitum* throughout the experiment. The nutrient composition of the daily diet is shown in Table 1.

Table 1 Nutritional characteristics of the daily diet for ewes during late pregnancy (values expressed as feed)

	kg	NEL	PDIN	CP	CF	Ca	P	Mg	Fe
Alfalfa hay	0.43	2.06	24.51	58.62	125.86	3.61	1.12	1.06	0.09
Meadow hay	0.70	3.18	25.63	41.30	253.77	5.67	2.21	1.26	0.26
Maize silage	0.15	0.95	6.20	10.21	34.73	0.39	0.32	0.08	0.10
Daily diet in total*	1.28	6.19	56.34	110.13	414.36	9.67	3.65	2.41	0.45

* without the mineral salt lick, which the sheep had available *ad libitum*; NEL - net energy for lactation (MJ); PDIN - protein digestible in the intestine when rumen-degradable nitrogen intake is not limiting (g); CP - crude protein (g); CF - crude fibre (g); Ca - calcium (g); P - phosphorus (g); Mg - magnesium (g); Fe - iron (g)

Blood sampling and haematological analysis

Immediately after ultrasonographic examination, blood samples (12 mL) were collected from the jugular vein (vena jugularis externa) of each ewe. For haematological analysis, blood was collected into tubes containing K3-EDTA anticoagulant (MEUS s.r.l., Italy). Complete blood counts were determined using an automated veterinary haematology analyser (Abacus Junior Vet, Diatron Messtechnik GmbH, Austria).

The following haematological parameters were evaluated: red blood cell count (RBC), haemoglobin concentration (HGB), haematocrit (HCT), white blood cell count (WBC), lymphocyte count (LYM), granulocyte count (GRA), and platelet count (PLT).

Biochemical analysis

For biochemical analysis, 10 mL of blood was collected into tubes without anticoagulant. Samples were centrifuged at 3000 rpm for 15 min (Labofuge 300, Heraeus, Thermo Fisher Scientific Inc., USA). The obtained serum was transferred into Eppendorf tubes and stored at −40 °C until biochemical analyses were performed. Serum biochemical parameters were determined using commercial diagnostic kits (Randox Laboratories, Crumlin, United Kingdom) and an automated biochemical analyser (Randox RX Monaco). The following variables were assessed: Protein profile: albumin, globulin, total protein. Enzyme profile: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP). Metabolic profile: glucose, urea, creatinine, uric acid, total bilirubin, cholesterol, triglycerides, β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA). Mineral profile: calcium (Ca), phosphorus (P), magnesium (Mg), iron (Fe).

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA); the experimental unit was the individual ewe (n = 20 per group). The normality of the data distribution was tested within each group by the Shapiro–Wilk test and verified by normal Q–Q plots; the homogeneity of variances was tested by Levene's test based on the mean. Depending on the fulfilment of these assumptions, the groups were compared by the independent-samples Student's t-test (both assumptions met), by the Welch-corrected t-test (Levene's $p < 0.05$) or by the non-parametric Mann–Whitney U test (Shapiro–Wilk $p < 0.05$ in at least one group). The results are presented as mean ± standard deviation and differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

The biochemical profiles of late-gestation Ile de France ewes carrying either a single fetus or twin fetuses are presented in Table 2. Significant differences were observed between the experimental groups for several biochemical variables.

Ewes carrying a single fetus exhibited significantly higher serum total protein concentrations than twin-bearing ewes ($P \leq 0.001$). The lower total protein concentration observed in twin pregnancies may be attributed to the increased demand for amino acids required for fetal growth and development, as well as for colostrum synthesis during the prepartum period. **Balikci *et al.* (2007)** reported that serum total protein concentrations decline during the final weeks of pregnancy due to the increased utilisation of proteins for fetal development and mammary gland preparation. Similar findings were described by **Safsaf *et al.* (2012)** and **Haffaf *et al.* (2024)**, who associated the reduction in serum protein concentration with enhanced protein synthesis required for fetal muscle development.

In contrast, serum globulin concentration was significantly higher in twin-bearing ewes ($P \leq 0.001$). Elevated globulin levels may reflect increased maternal immune activity during advanced pregnancy and physiological preparation for parturition and the onset of lactation.

A significant difference was also observed in serum creatinine concentration, which was higher in ewes carrying a single fetus ($P \leq 0.001$). Despite this statistical difference, the mean values in both groups remained within the physiological reference range for sheep. The lower creatinine concentration in twin-bearing ewes may be related to enhanced maternal renal adaptation during late gestation, including increased renal blood flow and glomerular filtration, which may promote greater creatinine clearance. Other factors, such as differences in energy metabolism, muscle protein turnover, hydration status, and nutrient utilisation, may also contribute to this variation. Since these factors and glomerular filtration were not directly assessed in the present study, the observed difference cannot be attributed solely to fetal number. **Santos *et al.* (2011), Silva *et al.* (2013)**, and **Santarosa *et al.* (2022)** reported that serum creatinine concentrations generally remain stable during pregnancy and are not markedly influenced by fetal number.

Within the enzyme profile, significant differences were found in the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) ($P \leq 0.001$). Both enzymes showed higher activities in ewes carrying a single fetus; however, the measured values remained within the normal physiological range in both experimental groups. **Wang *et al.* (2023)** and **Chalmeh *et al.* (2019)** suggested that increased hepatic enzyme activity during late pregnancy may result from elevated metabolic demands on the liver combined with reduced dry matter intake. Conversely, **Berlinguer *et al.* (2020)** reported higher ALT and AST activities in ewes carrying multiple fetuses, whereas **Yenilmez *et al.* (2021)** observed higher values in single-bearing ewes, which is consistent with the present findings.

Serum glucose concentration also differed significantly between the groups ($P \leq 0.001$). Ewes carrying a single fetus had higher mean glucose concentrations than twin-bearing ewes, although glucose values remained within the physiological range in both groups. The lower glucose concentration observed in multiple pregnancies is most likely associated with increased fetal glucose utilisation and may contribute to the development of negative energy balance during late gestation **(Lacetera *et al.*, 2001)**. Similar decreases in blood glucose concentrations in twin-bearing ewes have been reported by **Moallem *et al.* (2012)** and **Raoofi *et al.* (2013)**.

A pronounced difference was observed for β-hydroxybutyrate (BHB), with twin-bearing ewes exhibiting significantly higher serum concentrations (0.91 mmol/L) than single-bearing ewes (0.53 mmol/L). Elevated BHB concentration reflects increased ketone body production resulting from insufficient energy availability. According to **Herdt (2000)**, BHB concentrations ranging from 0.80 to 1.60 mmol/L indicate the presence of negative energy balance. The present findings are in agreement with those of **Moallem *et al.* (2012)** and **Raoofi *et al.* (2015)**, who likewise reported increased BHB concentrations in ewes carrying multiple fetuses.

A similar trend was observed for non-esterified fatty acids (NEFA). Twin-bearing ewes exhibited significantly higher NEFA concentrations (0.57 mmol/L) than ewes carrying a single fetus (0.39 mmol/L). Increased circulating NEFA concentrations indicate enhanced mobilisation of adipose tissue reserves during negative energy balance **(Mohammadi *et al.*, 2016)**. In the present study, the mean NEFA concentration in twin-bearing ewes slightly exceeded the recommended threshold of 0.50 mmol/L, suggesting intensified lipolysis during the final stage of pregnancy.

Serum cholesterol and triglyceride concentrations were also significantly higher in ewes carrying twin fetuses ($P \leq 0.001$). Elevated concentrations of these lipid metabolites are likely associated with the increased energy requirements imposed by the rapid growth of multiple fetuses.

The mineral profile revealed significant differences in calcium, phosphorus, and iron concentrations. Serum calcium concentration was significantly higher in single-bearing ewes, whereas phosphorus concentration was significantly greater in twin-bearing ewes ($P \leq 0.001$). **Yildiz *et al.* (2005)** reported that mineral concentrations in pregnant ewes are influenced by both fetal number and the stage of gestation. The resulting serum calcium-to-phosphorus ratio (0.73:1 in single-bearing and 0.50:1 in twin-bearing ewes) was markedly lower than the physiological optimum of 1.5:1 to 2:1 reported by **Stojković *et al.* (2014)**, indicating a mineral imbalance that was more pronounced in twin-bearing ewes. Serum phosphorus concentrations also exceeded the physiological reference range

for sheep (1.62–2.36 mmol/L; Merck & Co., Inc., 2024) in both groups. This elevation may reflect physiological changes in mineral metabolism during late gestation, combined with dietary phosphorus intake: the daily ration provided approximately 3.65 g of phosphorus and 9.67 g of calcium per ewe, corresponding to a dietary Ca:P ratio of 2.65:1 — notably higher than the recommended range, despite the low serum ratio observed. Since individual intake of the ad libitum mineral lick was not monitored, its contribution to total phosphorus intake could not be quantified. Iron concentration was significantly higher in ewes carrying a single fetus, whereas the mean iron concentration in twin-bearing ewes was slightly below the physiological reference range. A similar decline in serum iron concentration during the progression of pregnancy has been reported by **Gurdogan *et al.* (2006)**.

Table 2 Biochemical parameters of late-gestation Ile de France ewes carrying single fetus or twin fetuses

Parameter	Group	Mean	SD	SEM	Min.	Max.	p-value
Albumin (g/L)	One fetus	31.29	3.27	1.03	27.77	36.99	0.253
	Two fetuses	29.62	3.06	0.97	23.92	35.72	
Globulin (g/L)	One fetus	39.03[a]	2.52	0.80	35.38	43.65	≤0.001
	Two fetuses	50.11[b]	5.79	1.83	38.46	56.33	
Total protein (g/L)	One fetus	67.67[a]	0.47	0.15	66.99	68.43	≤0.001
	Two fetuses	65.89[b]	0.80	0.25	64.67	66.92	
Urea (mmol/L)	One fetus	5.73	1.34	0.42	4.14	8.51	0.396
	Two fetuses	5.30	0.75	0.24	3.70	6.24	
Creatinine (µmol/L)	One fetus	100.12[a]	2.93	0.93	95.60	105.60	≤0.001
	Two fetuses	61.63[b]	5.76	1.82	53.30	69.80	
Uric acid (µmol/L)	One fetus	4.71	0.93	0.29	3.36	6.37	0.736
	Two fetuses	4.97	2.28	0.72	0.91	7.58	
ALT (U/L)	One fetus	31.49[a]	6.72	2.13	22.30	41.80	≤0.001
	Two fetuses	18.94[b]	3.73	1.18	13.60	24.70	
AST (U/L)	One fetus	136.60[a]	12.58	3.98	119.80	159.60	≤0.001
	Two fetuses	104.96[b]	12.33	3.90	85.30	126.20	
ALP (U/L)	One fetus	78.50	30.95	9.79	51.00	160.00	0.078
	Two fetuses	118.40	60.06	18.99	58.00	226.00	
Total bilirubin (µmol/L)	One fetus	0.25	0.10	0.03	0.10	0.40	0.109
	Two fetuses	0.31	0.06	0.02	0.20	0.40	
Glucose (mmol/L)	One fetus	3.80[a]	0.38	0.12	3.15	4.37	≤0.001
	Two fetuses	2.91[b]	0.30	0.09	2.43	3.31	
β-hydroxybutyrate (mmol/L)	One fetus	0.53[a]	0.09	0.03	0.40	0.68	≤0.001
	Two fetuses	0.91[b]	0.12	0.04	0.79	1.13	
NEFA (mmol/L)	One fetus	0.39[a]	0.07	0.02	0.23	0.49	≤0.001
	Two fetuses	0.57[b]	0.07	0.02	0.43	0.64	
Cholesterol (mmol/L)	One fetus	1.77[a]	0.07	0.02	1.64	1.85	≤0.001
	Two fetuses	2.02[b]	0.13	0.04	1.88	2.28	
Triglycerides (mmol/L)	One fetus	0.25[a]	0.02	0.01	0.21	0.28	≤0.001
	Two fetuses	0.32[b]	0.01	0.00	0.29	0.34	
Calcium (mmol/L)	One fetus	2.48[a]	0.05	0.02	2.39	2.56	≤0.001
	Two fetuses	2.37[b]	0.04	0.01	2.28	2.42	
Phosphorus (mmol/L)	One fetus	3.39[a]	0.58	0.18	2.42	4.28	≤0.001
	Two fetuses	4.76[b]	0.36	0.11	4.22	5.34	
Magnesium (mmol/L)	One fetus	1.05	0.06	0.02	0.95	1.11	0.196
	Two fetuses	1.08	0.04	0.01	1.03	1.15	
Iron (µmol/L)	One fetus	27.23[a]	0.63	0.20	26.30	28.30	≤0.001
	Two fetuses	19.67[b]	1.68	0.53	17.50	23.20	

Legend: SD – standard deviation; SEM – standard error of the mean; ALT – alanine aminotransferase; AST – aspartate aminotransferase; ALP – alkaline phosphatase; NEFA – non-esterified fatty acids; [a,b] - within a row/parameter, means with different superscripts differ significantly at the level of significance given in the p-value column.

The haematological profiles of late-gestation Ile de France ewes carrying either a single fetus or twin fetuses are presented in Table 3. Statistically significant differences were observed in haemoglobin concentration (HGB), white blood cell count (WBC), lymphocyte count (LYM), granulocyte count (GRA), and platelet count (PLT). No significant differences were found in red blood cell count (RBC) or haematocrit (HCT) between single- and twin-bearing ewes ($P > 0.05$). The mean values of both parameters remained within the physiological reference ranges reported for sheep. Similar findings were reported by **Yenilmez *et al.* (2021)**, who observed no significant effect of fetal number on erythrocyte indices in late-pregnant ewes. However, **Metodiev *et al.* (2021)** hypothesized that higher haematocrit (HCT) values may be associated with better body condition in sheep, a higher frequency of twin births, and greater sexual activity. In light of these findings, haematocrit may represent one of the indicators associated with the overall condition and reproductive performance of sheep. Nevertheless, the absence of significant differences in HCT between single- and twin-bearing ewes in the present study suggests that fetal number alone may not be sufficient to influence haematocrit values, and that other factors, such as breed, nutritional status, body condition, physiological stage, and environmental conditions, may also contribute to the observed variation.

Haemoglobin concentration was significantly higher in ewes carrying twin fetuses (118.50 g/L) than in those carrying a single fetus (112.20 g/L) ($P \leq 0.001$). The increased haemoglobin concentration observed in twin-bearing ewes may reflect the greater oxygen demand associated with the rapid growth and development of multiple fetuses. Comparable findings were reported by Khazaal *et al.* (2023), who also demonstrated higher haemoglobin concentrations and several other haematological variables in ewes carrying twin pregnancies. The lower WBC, lymphocyte, and granulocyte counts observed in twin-bearing ewes compared with single-bearing ewes in this study contradict several previous studies reporting leukocytosis in twin-bearing ewes **(Santarosa *et al.*, 2022; Khazaal *et al.*, 2023)**.

Table 3 Haematological parameters of late-gestation Ile de France ewes carrying single or twin fetuses

Parameter	Group	Mean	SD	SEM	Min.	Max.	p-value
RBC (×10¹²/L)	One fetus	10.63	0.32	0.10	10.18	11.12	0.086
	Two fetuses	10.32	0.43	0.14	9.47	10.82	
HGB (g/L)	One fetus	112.20[a]	2.70	0.85	107.00	115.00	≤0.001
	Two fetuses	118.50[b]	1.58	0.50	116.00	121.00	
HCT (%)	One fetus	30.87	0.66	0.21	29.85	31.75	0.637
	Two fetuses	30.72	0.75	0.24	29.65	31.60	
WBC (×10⁹/L)	One fetus	11.36[a]	0.76	0.24	10.12	12.38	≤0.001
	Two fetuses	7.76[b]	0.48	0.15	7.21	8.69	
LYM (×10⁹/L)	One fetus	3.74[a]	0.21	0.07	3.42	4.12	≤0.001
	Two fetuses	2.31[b]	0.33	0.10	1.93	2.97	
GRA (×10⁹/L)	One fetus	8.33[a]	0.37	0.12	7.59	8.84	≤0.001
	Two fetuses	5.07[b]	0.31	0.10	4.35	5.41	
PLT (×10⁹/L)	One fetus	308.40[a]	14.36	4.54	289.00	337.00	≤0.001
	Two fetuses	409.30[b]	22.13	7.00	388.00	457.00	

Legend: SD – standard deviation; SEM – standard error of the mean; RBC – red blood cell count; HGB – haemoglobin; HCT – haematocrit; WBC – white blood cell count; LYM – lymphocytes; GRA – granulocytes; PLT – platelets; [a,b] - within a row/parameter, means with different superscripts differ significantly at the level of significance given in the p-value column.

This discrepancy may be related to several physiological mechanisms. In twin-bearing ewes, there is more intense competition for nutrients between the fetuses and a preferential allocation of maternal resources in their favour, which may limit the availability of substrates necessary for leukopoiesis **(Goldansaz *et al.*, 2022; Turín *et al.*, 2023)**. At the same time, the higher antigenic load associated with two semi-allogeneic fetuses may require greater maternal immunotolerance mediated by regulatory T cells, which may reduce the number of circulating leukocytes **(Caspe *et al.*, 2023)**. Haemodilution resulting from a more pronounced expansion of plasma volume and differences in the stage of pregnancy at the time of sampling may also have contributed to these differences, in addition to the previously mentioned differences in breed, nutrition, physical condition, and environment. The most pronounced haematological difference was observed in platelet count (PLT). Twin-bearing ewes exhibited significantly higher platelet counts (409.30 × 10⁹/L) than single-bearing ewes (308.40 × 10⁹/L) ($P \leq 0.001$). Increased platelet number may represent a physiological adaptation of the haemostatic system during advanced pregnancy, reflecting the increased demand for maintaining vascular integrity and preparing for parturition. Although several haematological parameters differed significantly between the experimental groups, all measured values remained within the established physiological reference ranges for sheep. Therefore, the observed differences are considered physiological adaptations associated with fetal number rather than indicators of pathological conditions.

CONCLUSION

The present findings indicate that fetal number is an important factor influencing both the metabolic and haematological status of late-gestation Ile de France ewes. The greater metabolic demands associated with twin pregnancies highlight the need for careful nutritional management, particularly regarding dietary energy and mineral supplementation during the final trimester of gestation. Appropriate nutritional strategies may contribute to reducing the risk of metabolic disorders, including pregnancy toxaemia, while improving maternal health and lamb viability.

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