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Comparative analysis of reproductive organs, hormones and blood metabolism of MSTN mutated and non-mutated cows during gestation

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Abstract

Background Long breeding cycle, long calving intervals and typical single calves limit the potential for improving their economic benefits. Ensuring the reproductive performance and efficiency of cows are crucial to increasing their economic value. Factors affecting the reproductive performance of cows include breed, pre-pregnancy maternal preparation, nutrition during pregnancy, and perinatal management. The gene editing of MSTN gene can improve the development of skeletal muscles and provide a new way for the promotion of existing beef cattle breeds. However, little has been reported about the reproductive performance and pregnancy state of MSTN gene-edited animals. In order to evaluate the reproductive safety and physiological changes during pregnancy of MSTN gene-edited cows, this study compared the sizes of reproductive organs, reproductive hormones, blood metabolic indicators, and metabolomic profiles at different stages of pregnancy, including period to be insemination, first trimester, second trimester, and late third trimester in MSTN gene-edited Luxi cattle (MT) and non-edited Luxi cattle (WT).

Results The results showed no significant differences in ovary and uterus sizes between MT and WT cows. However, MT cattle exhibited a larger pelvic area and higher calf birth weight. Compared to WT cattle, MT cattle showed enhanced glucose metabolism, reduced lipid synthesis, increased protein synthesis and absorption capacity, and decreased tryptophan synthesis at different stages of pregnancy. The hormone levels showed decreased E2 and increased P4 in MT cattle.

Conclusion The study demonstrates that MSTN gene editing has no significant impact on the reproductive safety of dairy cows and provides a deeper understanding of the feasibility of MSTN mutations for beef cattle breeding.

Keywords Myostatin, Gene editing, Luxi Beef Cattle, Reproductive performance, Hematological and biochemical physiology, Progesterone, Metabolome

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Background

Myostatin (*MSTN*) is an important negative regulator of skeletal muscle development and plays a crucial role in maintaining animal body characteristics and overall muscle structure [1]. The *MSTN* gene has a highly conserved genetic structure in a variety of species, including cattle, sheep, rabbits, dogs, pigs, and primates, with over 90% C-terminal sequence homology [2]. *MSTN* gene mutation leads to double-muscle phenotype by affecting protein structure. To date, naturel mutations in the *MSTN* gene have been observed in several breeds, including Belgian Blue Cattle [3], Piedmontese [3], Charolais [4], Limousin [5], Blonde d'Aquitaine [6], and German Gelbvieh [7], all of which exhibit muscular hypertrophy phenotypes. Compared to ordinary cattle, the growth rate and muscle yield of *MSTN* mutant cattle show significantly increased, the beef fat content is reduced, the lean meat amount is increased, and the slaughter rate and net meat yield have been significantly improved [8]. However, the study found that compared to normal calves, MSTN-mutated Charolais cows have reduced pelvic area, prolonged gestation of calves, and significantly higher birth weights of offspring. This affects the dilation of the pelvis, leading to a significant increase in the incidence of dystocia and higher postnatal calf mortality [9]. Interestingly, animals with heterozygous MSTN gene mutations also exhibit a double-muscle phenotype, but their pelvic muscles develop normally, avoiding the difficulty of delivery experienced by homozygous mutant animals and thus reducing the risk of fetal mortality [10].

Studies have confirmed that mammalian blood physiological and biochemical indexes change at different stages of pregnancy, and these changes are related to reproductive efficiency [11–14]. During early gestation, maternal malnutrition affects oocyte viability and development, thereby reducing pregnant rates. During the last trimester of pregnancy, the weights of fetuses can increase by about 75%, during which the nutritional requirements of the mothers are significantly higher [15, 16]. After the dietary crude protein level was increased, the concentration of the plasma urea nitrogen was increased. High levels of plasma urea nitrogen can have toxic effects on oocytes, sperm and embryos, reducing prostaglandin synthesis and lower progesterone levels, thereby delaying ovulation and decreasing conception rates. Common indicators for assessing mammalian health include aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, creatine kinase (CK), and bicarbonate levels [17, 18]. Progesterone level in the body can also significantly affect maternal pregnancy. High levels of progesterone (P4) in the uterus are more conducive to embryo implantation and reduce the probability of early miscarriage. Elevated P4 concentrations during early pregnancy help maintain pregnancy, but excessively high a level of P4 can inhibit uterine muscle contractions. Therefore, P4 levels gradually decrease with the progression of pregnancy [19]. Estradiol (E2) is synthesized and secreted by the ovarian follicle membrane, corpus luteum, and placenta during pregnancy, and is an important hormone regulating estrus in cows during non-gestation period [[20]]. In addition, E2 promotes cervical tissue softening and lochia discharge, contributing to uterine repair. It acts on the smooth muscle of the uterus, increasing its sensitivity to oxytocin and enhancing its contraction ability, ensuring a smooth delivery [20].

The Luxi Yellow Cattle is one of the major indigenous breeds mainly distributed in Shandong Province, East of China. Known for their large sizes and excellent meat quality, it is considered a significant genetic resource [21]. Due to its primary role in draft work, the Luxi cattle grow relatively slowly and have underdeveloped hindquarters, but the breeding ability and the environmental adaptability are strong. Based on this background, the *MSTN* gene-mutant Luxi cattle through CRISPR/Cas9 technology and the wild-type ordinary Luxi cattle were used in the present study [22]. First, the reproductive organ sizes of the two groups were compared to study whether *MSTN* gene mutations affected the reproductive phenotype of Luxi cattle. Secondly, by measuring blood metabolic indicators and reproductive hormone levels at different stages of pregnancy, we can understand whether *MSTN* gene mutations affect the pregnancy process of Luxi cattle. Finally, the differential metabolites and their physiological effects were identified and analyzed through the analysis of blood metabolomics at different stages of pregnancy. In this study, relevant indicators related to the reproductive and breeding performance of *MSTN* gene-edited Luxi cows were detected. By exploring whether *MSTN* gene editing would affect the reproductive and breeding performance of beef cattle while influencing muscle development, this study aims to provide data support for better promoting the application of gene-editing technology in the field of beef cattle breeding.

Results

Reproductive traits analysis of WT and MT cattle

The statistical analysis of reproductive traits data of MT and WT cattle is shown in Table 1. Compared to WT cattle, the estrus rate, pregnancy rate, birth rate, and calf survival rate of MT cattle were slightly decreased, and the dystocia rate was slightly increased, but the difference was not significant ($P > 0.05$). This indicated that MSTN gene editing had no substantial effect on the reproductive traits of Luxi cattle.

Table 1 Reproductive performance statistics

Group	Synchronous estrus quantity	Number of cow in estrus(%)	Number of pregnant cows(%)	Number of calves born(%)	Number of difficult calving (%)	Calf survival (%)
WT	101	85 (84.2%) [a]	73 (85.9)[b]	67 (91.7)[a]	9 (13.4)[a]	62 (92.5)[a]
MT	111	89 (80.2) [a]	74 (83.1)[b]	66 (89.1)[a]	12 (18.2)[a]	60 (91.0)[a]

Different letters represent significant differences ($P < 0.05$), while the same letter represents no significant differences ($P > 0.05$)

The state of ovaries and uterus are key factors affecting the reproduction of cows. Measurements of ovary and uterus size showed that the ovaries and uterus of MT cows were slightly smaller than those of the WT cows. Notably, the thickness of the right ovaries was significantly lower in the MT group than in the WT group (Fig. 1A, B).

Successful calving in cows may be influenced by pelvic size [23, 24]. Measurement of pelvis size in MT and WT cows revealed that MT cows had significantly greater pelvic height, width, and area than WT cows (Fig. 1D, E). Calves from the MT group had a higher birth weight (Fig. 1C). The pelvis-to-weight ratio in MT cows was higher but the difference was non-significant (Fig. 1F). The higher calf birth weight and pelvis-to-weight ratio might contribute to the slightly higher dystocia incidence in the MT group.

Comparison of blood physiological and biochemical indicators at different gestational stages between WT and MT cows

To further explore the reasons for the gestation period differences between WT and MT cows, we analyzed the blood physiological and biochemical indicators and their trends during different gestational periods in the two groups. The main indicators included those for glucose metabolism (Glucose (Glu), Lactic acid (LA), Lactate dehydrogenase (LDH)), lipid metabolism (Triglyceride

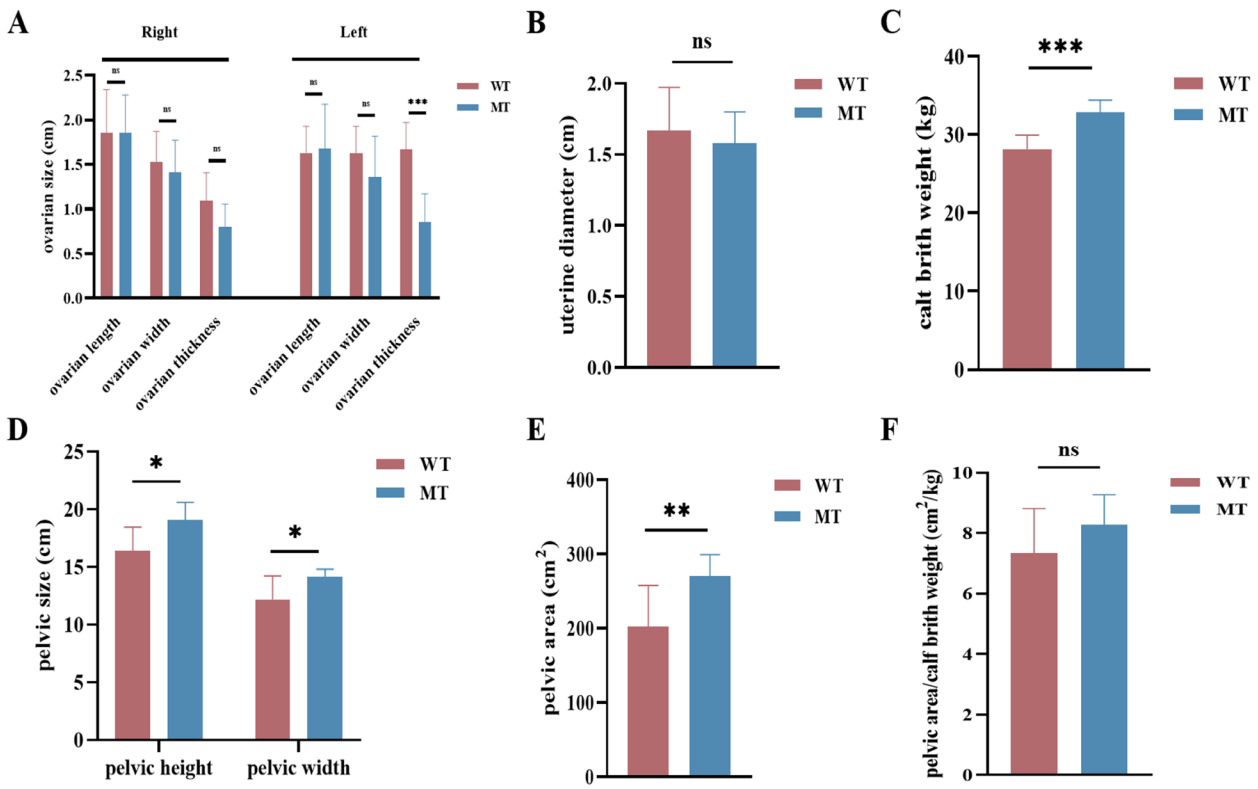

Fig. 1 Analysis of reproductive performance indicators for WT and MT cows. **A** Statistical comparison of left and right ovarian sizes in WT and MT cows. **B** Comparison of uterine diameter sizes between WT and MT cows. **C** Statistical analysis of calf birth weight in WT and MT cows. **D** Comparison of pelvis size between WT and MT cows. **E** Comparison of pelvis area between WT and MT cows. **F** Analysis of pelvis area to calf birth weight ratio (pelvis-to-weight ratio) in WT and MT cows

(TG), Cholesterol (CHO), High-density lipoprotein (HDL), Low-density lipoprotein (LDL)), protein metabolism (Blood urea nitrogen (BUN), Total protein (TP), Albumin (ALB)), and health (Aspartate aminotransferase (AST), Creatinine, Creatine kinase (CK), Bicarbonate (BC)).

The results (Fig. 2) revealed differences in blood glucose, lipid, protein metabolism, and health indicators

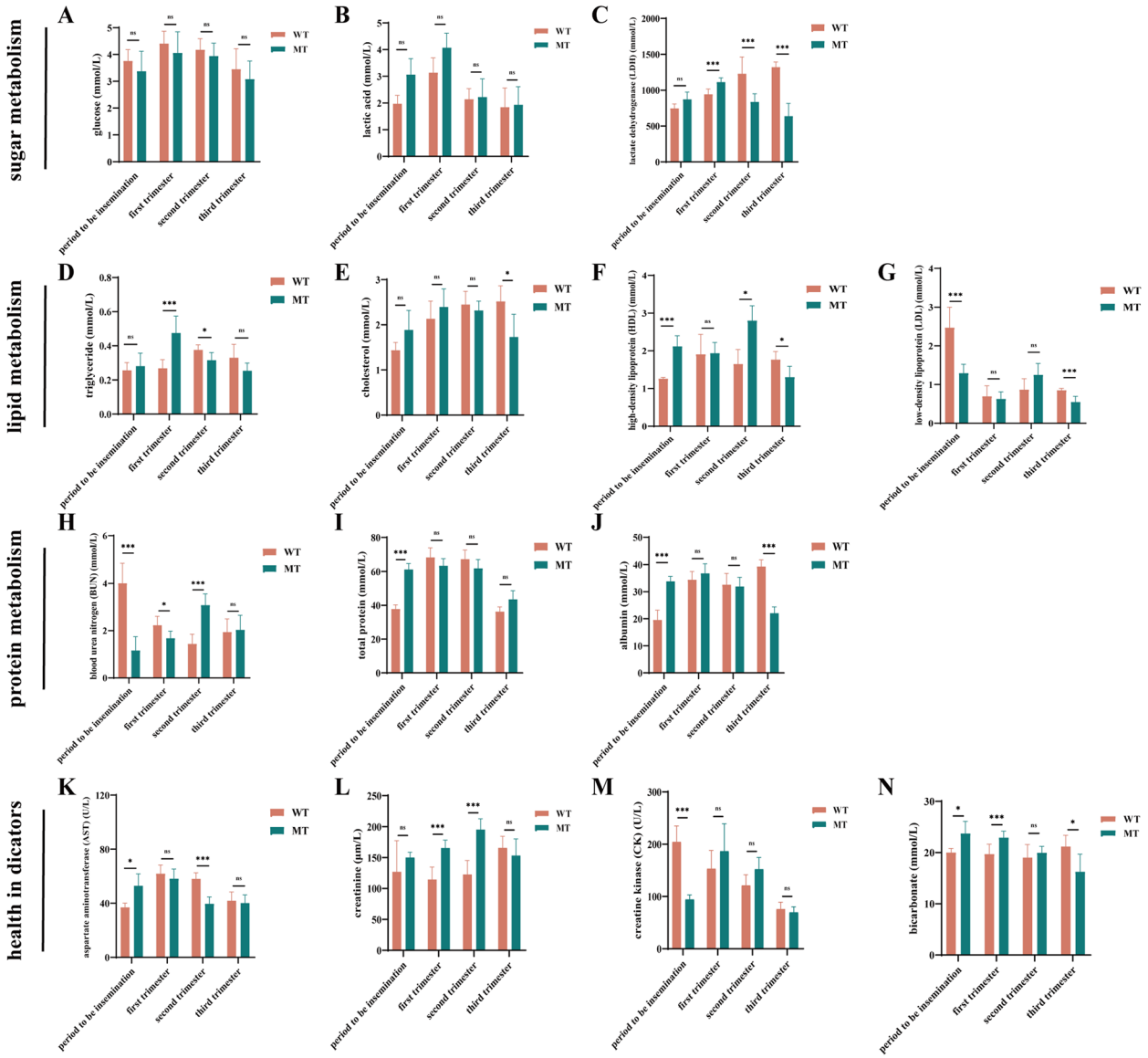

Fig. 2 Analysis of blood physiological and biochemical indicators at different gestational stages in WT and MT cows. **A-C** Blood glucose metabolism indicators in wt and mt cows at different gestational stages. **A** Blood glucose levels in WT and MT cows at different gestational stages. **B** Blood lactate levels in WT and MT cows at different gestational stages. **C** Blood lactate dehydrogenase (LDH) levels in WT and MT cows at different gestational stages. **D-G** Blood lipid metabolism indicators in WT and MT cows at different gestational stages. **D** Blood triglyceride levels in WT and MT cows at different gestational stages. **E** Blood cholesterol levels in WT and MT cows at different gestational stages. **F** Blood high-density lipoprotein (HDL) levels in WT and MT cows at different gestational stages. **G** Blood low-density lipoprotein (LDL) levels in WT and MT cows at different gestational stages). **H-J** Blood protein metabolism indicators in WT and MT cows at different gestational stages. **H** Blood urea nitrogen (BUN) levels in WT and MT cows at different gestational stages. **I** Total protein levels in WT and MT cows at different gestational stages. **J** Blood albumin levels in WT and MT cows at different gestational stages. **K-N** Blood health indicators in WT and MT cows at different gestational stages. **K** Blood aspartate aminotransferase (AST) levels in WT and MT cows at different gestational stages. **L** Blood creatinine levels in WT and MT cows at different gestational stages. **M** Blood creatine kinase levels in WT and MT cows at different gestational stages. **N** Blood bicarbonate levels in WT and MT cows at different gestational stages

between MT and WT cows. Compared to period to be insemination cows, Glu, LA, and LDH levels in MT and WT cows' blood initially rose and then declined with pregnancy progression. Glu and LA peaked in first trimester. MT cows had lower Glu and higher LA levels than WT cows at different pregnancy stages, but the differences were non-significant. LDH in MT cows was higher than in WT cows during period to be insemination and the first trimester, significantly so in first trimester, but became lower as pregnancy advanced. Fluctuations in LDH may affect LA levels.

For lipid metabolism indicators TG and CHO, both cow types showed a rise after conception followed by a decline. MT cows had higher TG and CHO levels than WT cows during period to be insemination and first trimester but lower levels from the second to third trimester. HDL and LDL levels had no significant change; MT cows had higher levels than WT cows in the second trimester.

BUN level had no significant change during pregnancy; MT cows had lower BUN in non-gestation and early gestation but higher BUN in the second and third trimesters. TP levels increased in both types with pregnancy, with MT cows having higher levels overall but lower levels in the first and second trimesters. ALB levels in MT cows were higher than in WT cows during period to be insemination and the first trimester but lower in the second and third trimesters.

AST, a health indicator, was higher in period to be insemination MT cows but lower from gestation to delivery. Creatinine in MT cows was higher than in WT cows from period to be insemination to the second trimester but lower in the third trimester. Creatine kinase in MT cows gradually decreased, while in WT cows it peaked in early gestation and then declined. Bicarbonate levels in both types decreased with pregnancy except in the last stage, where WT cows had higher levels. In MT cows, glucose metabolism increased, and lipid synthesis capacity decreased.

Serum reproductive hormone levels of MT and WT cows

P4 and E2 are key reproductive hormones regulating estrus and pregnancy progression, and their levels are crucial for maintaining pregnancy. To investigate differences between WT and MT cows during pregnancy, ELISA was used to measure serum P4 and E2 levels. Results showed that serum E2 levels in both MT and WT cows gradually increased with pregnancy progression, but those in WT cows were higher (Fig. 3A). As gestation prolonged, serum P4 levels in both groups increased, with significantly higher levels in MT cows (Fig. 3B).

Blood metabolomic analysis of WT and MT cows

Based on previous results, differences in reproductive traits, blood physiological and biochemical indicators, and reproductive hormones were found between MT and WT cows. To further explore the effect of MSTN gene-editing on reproductive indicators, a metabolomic analysis of blood from WT and MT cows at different gestation stages was conducted. The OPLS-DA model was used to identify group differences. OPLS-DA results showed clear separation of metabolites between MT and WT cows in non-gestation and gestation, with significant differences. The OPLS-DA permutation test confirmed the model's validity and non-overfitting. There were significant differences in blood metabolites between MT and WT cows at different gestation stages (Fig. 4A, B; Fig. 5A, B; Fig. 6A, B;

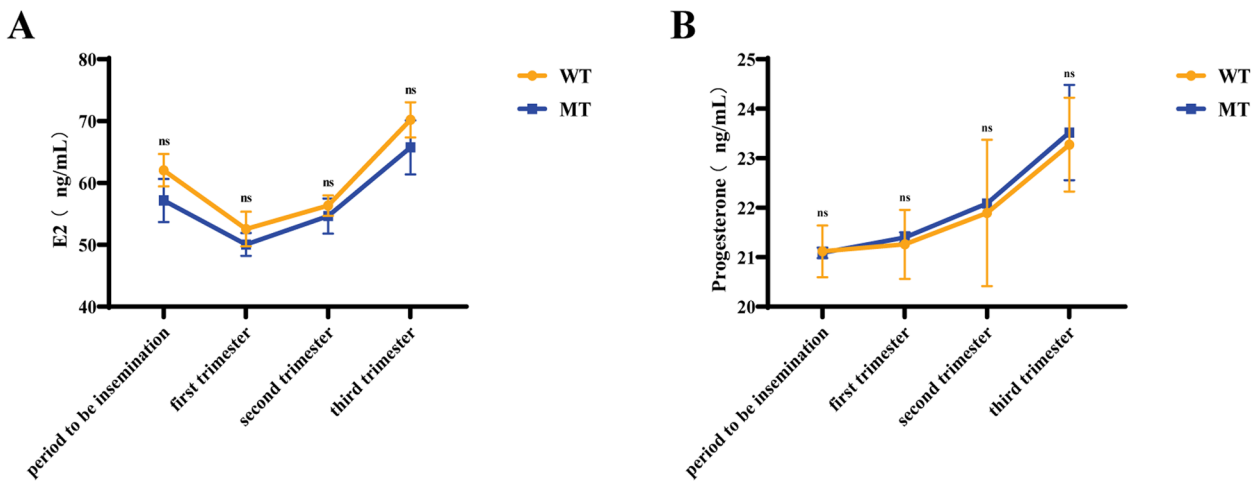

Fig. 3 Detection of serum reproductive hormone levels in WT and MT groups at different gestational stages. **A** Estradiol levels in the serum of WT and MT groups at different gestational stages. **B** Progesterone levels in the serum of WT and MT groups at different gestational stages

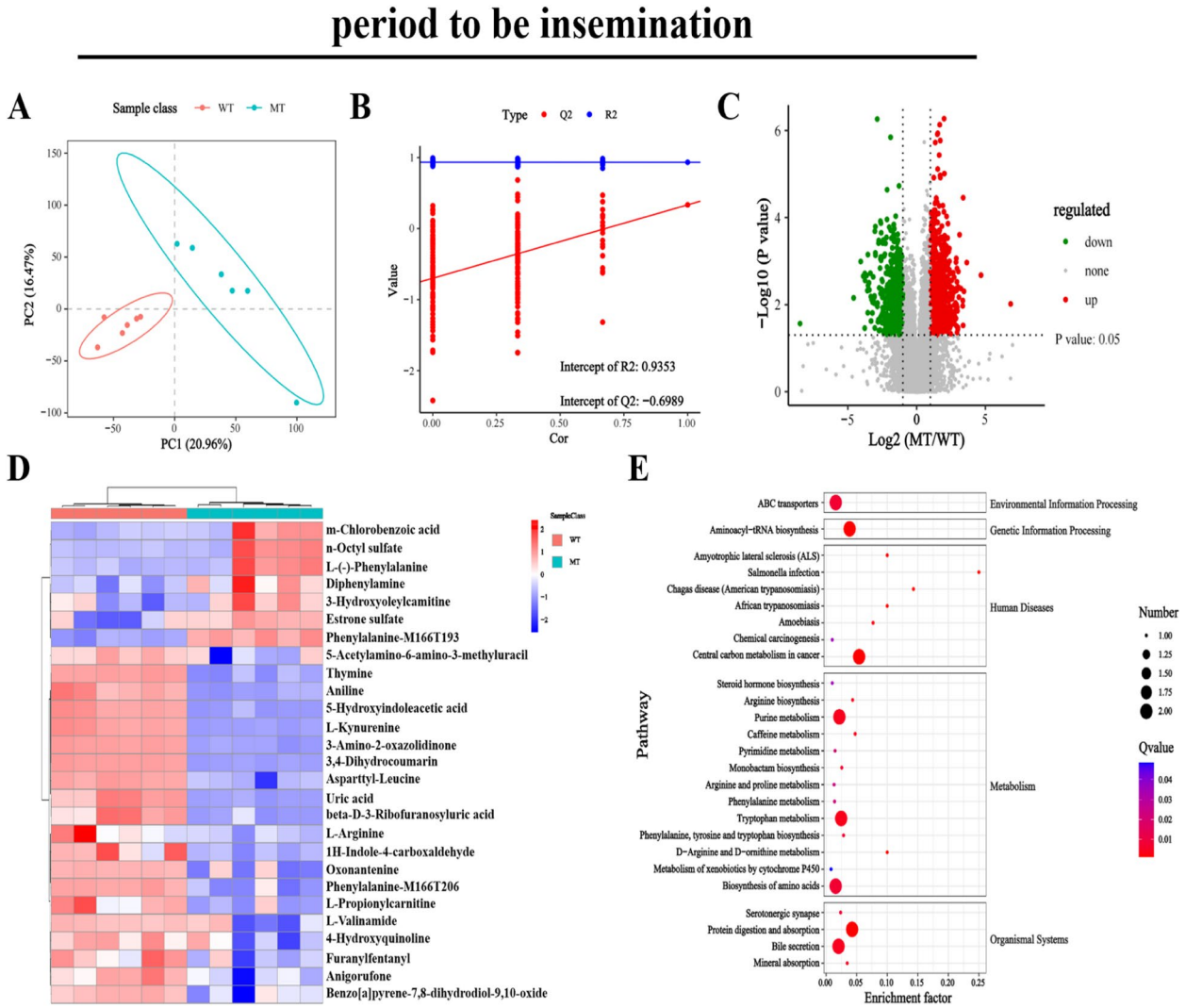

Fig. 4 Metabolomic analysis of blood in WT and MT cows at period to be insemination. **A** OPLS-DA analysis of blood metabolomics in WT and MT cows at period to be insemination. **B** PLS-DA permutation test of blood metabolomics in WT and MT cows at period to be insemination. **C** Volcano plot showing differentially expressed metabolites in the blood metabolomics of WT and MT cows at period to be insemination. **D** Clustering analysis of differential metabolites in the blood of WT and MT cows at period to be insemination. **E** KEGG pathway enrichment analysis of differentially expressed metabolites in the blood of WT and MT cows at period to be insemination

Fig. 7A, B). Differential metabolites were selected and clustered according to radio ≥ 2 or radio ≤ 2, VIP > 1.0 and $P < 0.05$ (Fig. 4C; Fig. 5C; Fig. 6C; Fig. 7C). Cluster analysis showed that in period to be insemination, MT cows had upregulated metabolites like phenylalanine, uracil and estrone sulfate, and downregulated ones such as 5-hydroxyindoleacetic acid, L-kynurenine, uric acid and L-arginine compared to WT cows (Fig. 4D). KEGG pathway enrichment analysis indicated that differential metabolites in non-gestation MT and WT cows' blood were mainly enriched in pathways including ABC transporters, Aminoacyl-tRNA biosynthesis, etc. (Fig. 4E).

Screening and clustering differential metabolites in WT and MT cows during first trimester showed that, compared to WT cows, MT cows had upregulated deoxycholic acid, cholic acid and ursocholic acid, and downregulated L-propionylcarnitine, phenyl glucuronide and phenylacetyl-L-glutamine (Fig. 5D). KEGG pathway enrichment analysis of these differential metabolites indicated they were mainly enriched in fatty acid biosynthesis, glycerophospholipid metabolism, arachidonic acid metabolism, and biosynthesis of unsaturated fatty acids (Fig. 5E).

Screening and cluster analysis of differential metabolites in WT and MT cows during the second trimester

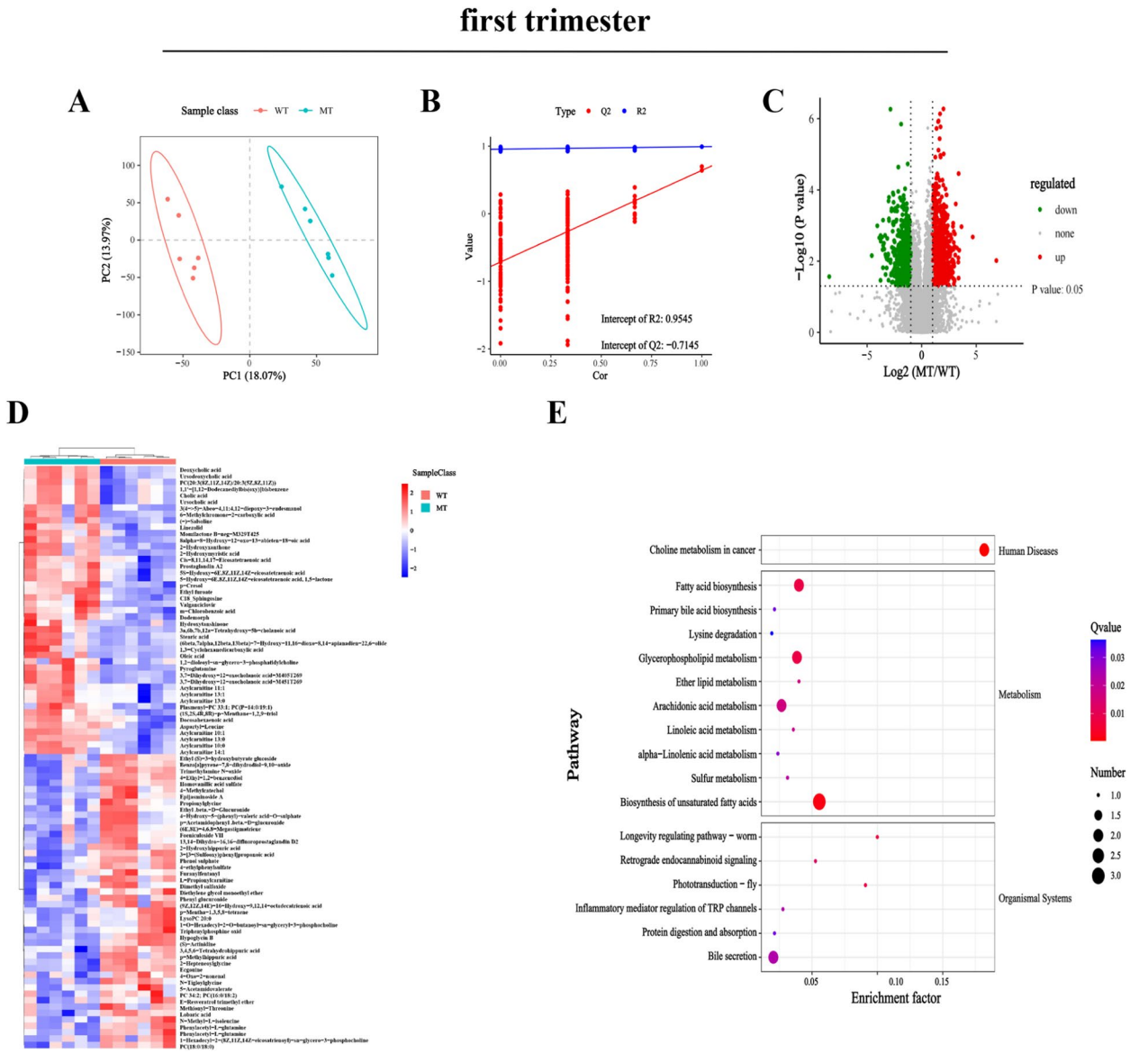

Fig. 5 Metabolomic analysis of blood in WT and MT cows during the first trimester. **A** OPLS-DA analysis of blood metabolomics in WT and MT cows during the first trimester. **B** PLS-DA permutation test of blood metabolomics in WT and MT cows during the first trimester. **C** Volcano plot showing differentially expressed metabolites in the blood metabolomics of WT and MT cows during the first trimester. **D** Clustering analysis of differential metabolites in the blood of WT and MT cows during the first trimester. **E** KEGG pathway enrichment analysis of differentially expressed metabolites in the blood of WT and MT cows during the first trimester

revealed that, compared to WT cows, MT cows had down-regulated 5-hexyltetrahydro- 2-furanocrylic acid and methionine, and up-regulated glycodeoxycholic acid, creatine and phenylalanine (Fig. 6D). KEGG pathway enrichment analysis showed these metabolites were mainly enriched in purine, tryptophan, glycerophospholipid metabolism and metabolic pathways (Fig. 6E).

In the third trimester, MT cows had down-regulated citric acid, phenylalanine and thymine, and up-regulated isoleucine, PH-tryptophan and valine (Fig. 7D). KEGG analysis indicated enrichment in metabolism-related pathways like phenylalanine, tryptophan, metabolic and 2-oxy-carboxylic acid metabolism (Fig. 7E).

Blood metabolomic analysis of WT and MT cows at different gestational stages showed that differential metabolites in MT cows were mainly related to fatty acid biosynthesis, tryptophan and phenylalanine metabolism, and protein digestion and absorption. This implies higher protein metabolism capacity in MT cows during gestation, which might explain the reduced blood levels of

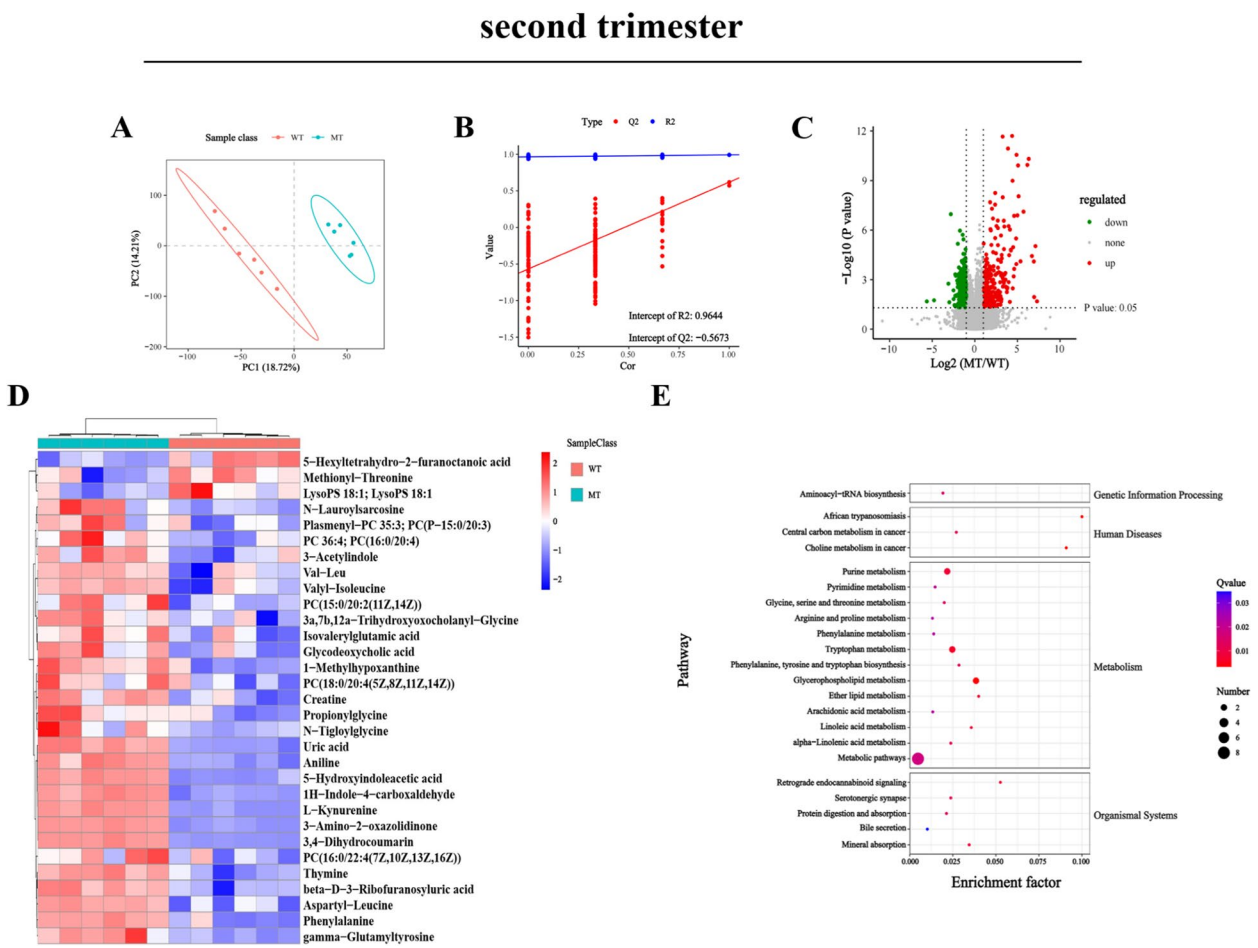

Fig. 6 Metabolomic analysis of blood in WT and MT cows the second trimester. **A** OPLS-DA Analysis of Blood Metabolomics in WT and MT cows during the second trimester. **B** PLS-DA permutation test of blood metabolomics in WT and MT Cows During the second trimester. **C** Volcano plot showing differentially expressed metabolites in the blood metabolomics of WT and MT Cows during the second trimester. **D** Clustering analysis of differential metabolites in the blood of WT and MT cows during the second trimester. **E** KEGG pathway enrichment analysis of differentially expressed metabolites in the blood of WT and MT cows during the second trimester

protein-metabolism-related indicators. Down-regulated metabolites such as uric acid, indoles and pyrimidines in MT cows suggested reduced tryptophan metabolism and nucleotide degradation.

Discussion

The results of this study show that, compared with WT cows, there are no obvious structural differences in the reproductive organs of MT cows. Meanwhile, there are no significant changes in the estrus rate during pregnancy, pregnancy rate, calf birth rate, and survival rate. Through the dynamic monitoring of reproductive hormone levels at different pregnancy stages and the detection and analysis of the blood metabolome, it was found that MT cows exhibit unique metabolic characteristics. Specifically, the glucose metabolism level of MT cows is significantly increased, while the lipid synthesis ability is slightly reduced. In addition, MT cows have a stronger protein synthesis ability, but the tryptophan metabolism level decreases. Based on the above results, we can further confirm that *MSTN* gene editing does not have an adverse effect on the reproductive potential of cattle while increasing the meat yield. This finding provides important scientific evidence for the wide-scale application of *MSTN* gene-editing technology in the beef cattle farming industry.

Reproductive performance and pregnancy outcomes of cows are important indicators of reproductive production. Factors affecting reproductive performance in cattle include uterine, ovary morphology and size, pelvic area, calf birth weight, nutritional levels and reproductive hormones during gestation [16, 25]. Previous studies on *MSTN*-edited cows [26] and mice [25–27] have indicated that *MSTN* gene-edited animals exhibit a slight

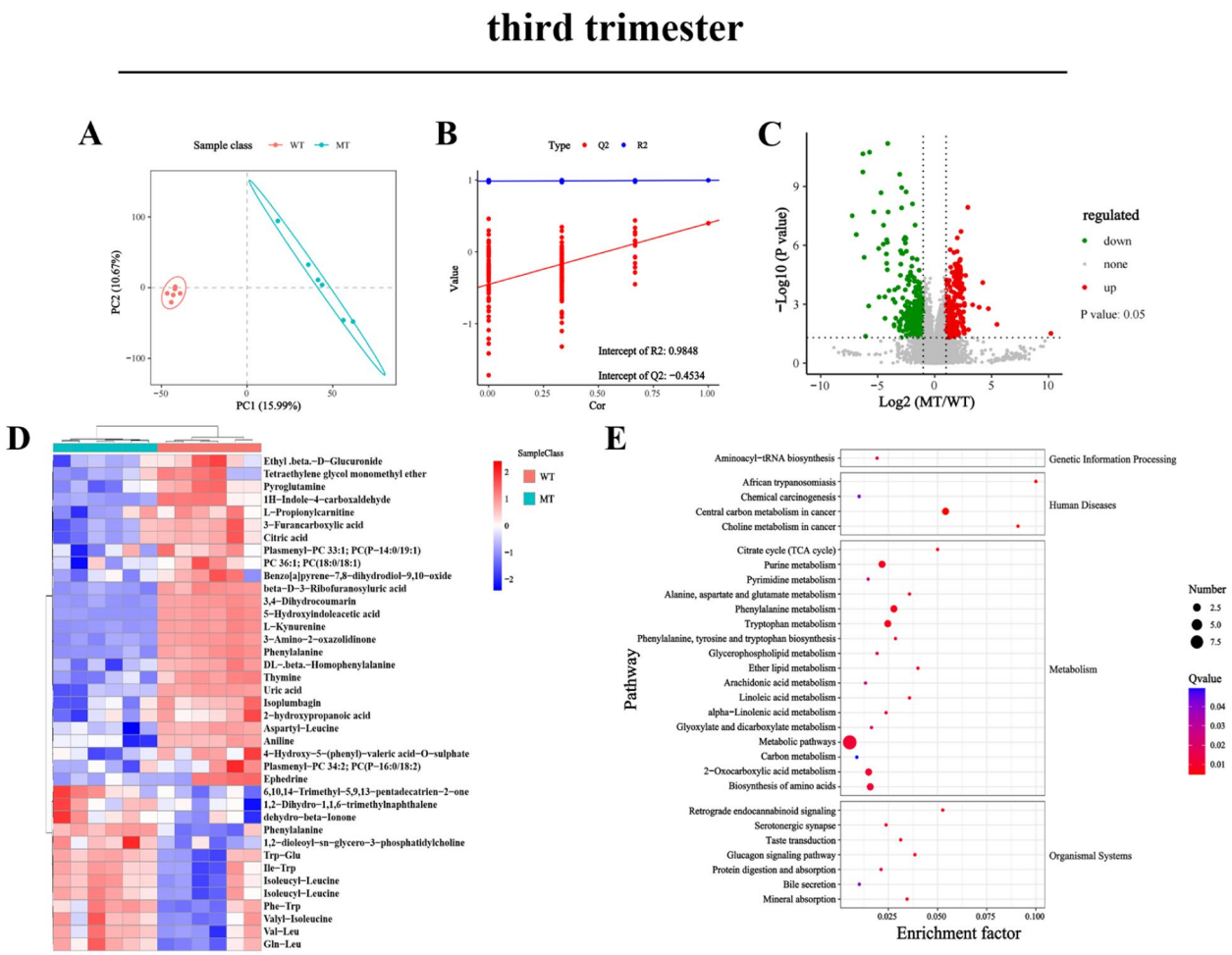

Fig. 7 Blood metabolomic analysis of WT and MT cows during the third trimester. **A** OPLS-DA analysis of blood metabolomics in WT and MT cows during the third trimester. **B** PLS-DA permutation test of blood metabolomics in WT and MT cows during the third trimester. **C** Volcano plot showing differentially expressed metabolomics of WT and MT cows during the third trimester. **D** Clustering analysis of differential metabolites in the blood of WT and MT cows during the trimester. **E** KEGG pathway enrichment analysis of differentially expressed metabolites in the blood of WT and MT cows during the third trimester

decrease in follicle number, but the oocyte quality and embryo development remain unaffected. For female animals, larger ovaries can support larger dominant follicles, which can produce larger pre-ovulatory follicles, giving them a reproductive advantage. In larger follicles, the levels of glucose, glutathione, and superoxide dismutase activity in the follicular fluid are slightly increased, which is beneficial for successful pregnancies [25]. It was found that the external diameter of the uterine horns of pregnant cows decreased sharply from 14.0 cm to 3.9 cm on the 7 th to 28 th day after delivery [28]. An increase in the diameter of uterus has an adverse effect on the fertility of cows. The smaller the diameter of the uterus, the more complete of the uterine remodeling. This process is influenced by a variety of factors, including maternal nutrition, hormone levels, and metabolic diseases [28, 29]. In this study, the ovaries and uteruses of WT and MT cows were ultrasoundly measured 35 days before artificial insemination. The results showed that there was no significant difference in ovarian size and uterine diameter between WT and MT cows, indicating that *MSTN* gene editing does not affect ovarian and uterine development. Compared to homozygous mutations, *MSTN* heterozygous mutations are associated with normal pelvic muscle development in pregnant cows, increased pelvic area, and reduced incidence of calving difficulties [9, 30]. Calf birth weight is the key indicator in beef cattle breeding. *MSTN* editing significantly increases calf birth weight. Calves with Homozygous *MSTN* mutant calves from Belgian Blue cattle are 3.5 kg heavier at birth than heterozygous mutant calves and 5.5 kg heavier than wild type calves. Similarly, homozygous *MSTN* mutant calves of Aquitanian cattle are 2.6 kg heavier at birth than heterozygous mutant calves and 5.7 kg heavier than wild

types. Moreover, homozygous *MSTN* mutant calves of Aquitanian cattle are 2.6 kg heavier at birth than heterozygous mutant and 5.7 kg heavier than wild types [31, 32]. Basarab et al. found that when the ratio of pelvic area to calf birth weight of cows is 4.5, dystocia generally do not occue, and the higher the ratio, the easier calving is [33]. This study found that the dystocia rate of MT cows was higher than that of WT cows. Moreover, the pelvic height, width, and area of MT cows were significantly larger than those of WT cows, and the birth weight of their calves was also significantly higher than that of calves from WT cows. Although the ratio of pelvic area to calf birth weight in both MT and WT cows exceeded 4.5, the birth weight of MT calves was significantly higher, and there was no significant difference in this ratio between the two groups. The increased incidence of dystocia in MT cows may be due to the increase in pelvic muscle mass caused by *MSTN* gene editing, which in turn affects the expansion of the pelvis during parturition.

Our previous report has shown that *MSTN* gene editing enhance glucose metabolism in cattle by promoting glycolysis and the tricarboxylic acid cycle [34]. In this study, we observed that the concentrations of glucose and lactate in MT and WT cows during pregnancy showed a trend of increasing first and then decreasing. Meanwhile, as pregnancy progressed, the level of lactate dehydrogenase gradually increased. This result is consistent with our expectations, as during pregnancy, the metabolic requirements of cows' bodies change dynamically with the development of the fetus. Notably, compared with WT cows, the concentrations of glucose and lactate in MT cows decreased more significantly during the mid- and late-pregnancy periods. This phenomenon may be related to the fact that the fetuses of *MSTN* gene-edited cows are larger and require more energy consumption. Larger fetuses need more nutrients and energy to support their growth and development. Therefore, more glucose and lactate in the cows' bodies are used to meet the needs of the fetuses, resulting in a decrease in the concentrations of these two substances in the maternal blood. This finding further confirms the impact of *MSTN* gene editing on the metabolism of cows. It also reminds us that when raising *MSTN* gene-edited cattle, we need to pay more attention to the energy supply during their pregnancy to ensure the normal development of the fetuses. In addition, MT hybrids showed changes in muscle content and lower fat content compared to WT cows [35]. This result may be closely related to the function of the *MSTN* gene. As a factor that negatively regulates muscle growth, gene editing of *MSTN* may lead to enhanced muscle growth, thereby relatively reducing the fat content. In terms of lipid metabolism indicators, both MT and WT cows showed a trend of first increasing and then decreasing. This trend indicates that in the first trimester, cattle experience fat accumulation, which may be a strategy for the body to store energy for subsequent pregnancy and parturition. As pregnancy progresses, the development of the fetus and the subsequent processes of parturition and lactation require more energy. Therefore, the fat metabolism of cattle gradually increases to avoid a negative energy balance. This dynamic change in fat metabolism is crucial for the normal growth and development of the fetus, smooth parturition, and postpartum recovery of cows. Total protein and albumin, as indicators reflecting liver synthesis ability, did not show significant changes between the MT group and the WT group. This result indicates that *MSTN* gene editing did not have an obvious impact on the liver synthesis ability of cows. As an important metabolic and synthetic organ in the body, the stable function of the liver is crucial for maintaining the overall health and normal physiological functions of cattle. The results of this study suggest that although *MSTN* gene editing has had a certain impact on the metabolism, growth, and development of cows, the synthetic function of the liver has not been significantly disturbed.

Previous studies have shown that the expression of the *MSTN* gene can promote the synthesis of E2 in the ovaries while inhibiting the secretion of P4, thereby reducing the estrus rate of cows [36]. The research results show that, compared with WT cows, the level of E2 in the blood of MT cows decreased significantly. Regarding the P4 concentration, although there was a certain increase in MT cows, there was no significant difference statistically compared with WT cows. Meanwhile, there was also no significant difference in the estrus rate between MT and WT cows. Upon further analysis of the dynamic changes of hormone levels across different pregnancy stages, we found that in the period to be insemination, the P4 content showed a significant upward trend, which may provide necessary support for embryo implantation and early development. E2 remained at a relatively high level in the third trimester and when the cows were period to be insemination. It is speculated that this high-level estradiol may play an important role in maintaining the normal function of the cows' reproductive system, promoting the parturition process, and facilitating postpartum recovery.

Tryptophan is an essential amino acid for ruminants. In cattle, tryptophan is metabolized through four pathways: rumen microbial degradation, protein synthesis, serotonin pathway, and kynurenine pathway [36]. Through the analysis of the blood metabolomics of cows at different pregnancy stages, we found that there were significant differences in tryptophan-related

metabolites such as kynurenine and indole compounds between MT cows and WT cows. Although the total protein level of MT cows was lower, their protein synthesis ability was stronger and protein metabolism was more active. This phenomenon may be related to the lower tryptophan level in MT cows. Previous studies have shown that a high concentration of tryptophan can significantly enhance the immune function of the fetus and ensure the smooth progress of pregnancy. Based on this, we believe that the lower tryptophan level in MT cows may have a certain impact on the immune function of the fetus and the pregnancy process. Subsequent studies can further explore the effect of tryptophan supplementation on improving the reproductive performance of MT cows. In late pregnancy, the developing fetus and the cow require energy from fat and nucleotide metabolism to meet their needs [37]. In this study, we found that the nucleotide degradation metabolites such as purines and pyrimidines in MT cows with the decrease of tryptophan metabolites during late pregnancy. Deletion of *MSTN* gene can increase the AMP/ATP ratio by activating the AMPK signaling pathway, thereby enhancing mitochondrial energy metabolism and promoting lipid metabolism [38]. Our results indicate that the decrease in nucleotide metabolism in MT cows may be precisely because the knockout of the *MSTN* gene promotes lipid metabolism, enabling lipids to provide substrates for glycolysis or be converted into sugars through gluconeogenesis. This means that although *MSTN* gene editing promotes glycolysis, leading to a decrease in blood glucose levels, due to the enhancement of lipid metabolism, cows do not need to promote nucleotide metabolism for glycogen compensation. In addition, the decrease in citric acid content in MT cows further suggests that *MSTN* gene editing promotes bile acid synthesis and enhances the capacity of the TCA cycle. The increase in bile acid synthesis may contribute to improving the efficiency of fat digestion and absorption, providing more energy sources for cows in the late stage of pregnancy. The enhanced capacity of the TCA cycle helps to utilize nutrients more efficiently to meet the energy requirements of the fetus and the cows themselves.

Based on the comprehensive analysis of reproductive phenotypes, blood physiological and biochemical parameters, reproductive hormones and metabolic data, the present study indicates the feasibility of *MSTN* gene editing in beef cattle breeding. Although certain research results have been achieved in this study, there are still some limitations. Although the sample sizes of WT and MT cows used in this study are somewhat representative, the sources of the samples are relatively concentrated in specific regions. As a result, it may not fully cover the characteristics of genetically mutated animals under all geographical and environmental conditions.

Conclusions

In conclusion, this study shows that *MSTN* gene editing has no significant impact on the reproductive safety of Luxi cows. Specifically, *MSTN* gene editing has no effect on the estrus rate, conception rate, calving rate of Luxi cattle, and even the birth weight and survival rate of calves. Blood physiological and biochemical tests, reproductive hormone level and metabolomics analyses reveal that during the pregnancy of *MSTN* gene-edited cows, glucose metabolism increases, lipid synthesis ability slightly decreases, protein synthesis improves, tryptophan metabolism decreases, the level of E2 decreases, but the concentration of P4 increases. Based on the above results, in subsequent studies, the sample size can be increased, and the reproductive traits of *MSTN* gene-edited cows in different regions can be detected. This provides an important theoretical basis for promoting the application of *MSTN* gene editing in the field of beef cattle breeding.

Methods

Animals

The MT and the WT animals selected for this study were all sourced from the Beef Cattle Breeding Base of Inner Mongolia University, located in the Helingeer New Area of Hohhot City. The construction process of MT cattle in this study is described in the research by Zhao et al. [39]. Briefly, using the CRISPR-Cas9 gene-editing technology with the *MSTN* gene as the target gene, site-specific knockout was carried out at different loci of the *MSTN* gene. Subsequently, Luxi cattle with the MSTN gene knocked out were successfully bred through somatic cell cloning technology. All cattle are raised under standard feeding conditions on the ranch. The feed composition and nutritional content are shown in Additional file 1: Table S1. A total of 100 MT and 100 WT Luxi cows were selected to statistically analyze their reproductive data, The selected cows were all aged between 2 and 2.5 years. They had never given birth, were in good health, could come into estrus normally, and were in a good physiological state. The typical gestation period for cows is 285 days, with artificial insemination marked as day 0. The first trimester lasts from 0 to 95 days, the second trimester from 95 to 190 days, and the third trimester from 190 days to delivery. Twenty MT and twenty WT Luxi cows were selected for reproductive organ examination and blood sampling for physiological and biochemical analysis. Before insemination, the blood samples were collected from the vena caudalis of the cows. The sizes of their ovaries, pelvis, and uterus were measured.

Thirty-three days later, PGF was intramuscular administered, and cows began to estrus 24 h later. The estrus status was observed and recorded, and artificial insemination was performed 12 h later, with two consecutive inseminations at an interval of 8–10 h. Sixty days after insemination, cows were tested for pregnancy and blood samples were collected. Then, the pregnant cows were selected for blood collection at 155 and 250 days, respectively. After delivery, the cow serum samples were selected for physiological, biochemical, and metabolomic analysis (Fig. 8). Finally, six WT and six MT Luxi cows were selected to analyze blood metabolomics during the insemination period, first trimester, second trimester, and third trimester.

Measurement of pelvic size

Wash the vulva and anal region of the cows thoroughly before measurement to avoid contamination of the samples. The height and width of the pelvic cavity were measured using a special custom-made pelvimetric caliper. Specifically, the width is defined as the maximum distance between the bones on both sides of the pelvic cavity, that is, the pelvic inlet width; while the height refers to the distance of the symphysis pubis to the nearest point of the sacrum, namely, the height of the pelvic entrance (Fig. 9). The pelvic area was calculated by multiplying the width of pelvic inlet by the height of the pelvic entrance. During the measurement process, the ends of the caliper should not slide out of the measuring point, and should be gently pressed to avoid injuring the uterus.

Measurement of ovarian size and uterine diameter

Cow ovaries were measured using B-mode ultrasound, and uterine diameters were measured using the method described by Baze et al. [40]. In brief, a portable ultrasound machine (Ibex pro; E.I. Medical Imaging, Loveland, CO, USA) equipped with a 7.5 MHz linear array transducer was used to measure uterine diameter. The uterine diameter is defined as twice the distance from the center point of the collapsed uterine horn to the endometrial echo boundary, measured at two distinct vertical cross-sections of each horn.

Detection of physiological and biochemical blood parameters

Blood samples were collected from cows at different stages of pregnancy in a fasting state. The samples were centrifuged at 3500 rpm for 15 min, and the upper serum layer was collected. Then, a MSCAN-II dry chemistry analyzer (ROCHE, C311, Switzerland) was used to analyze physiological and biochemical

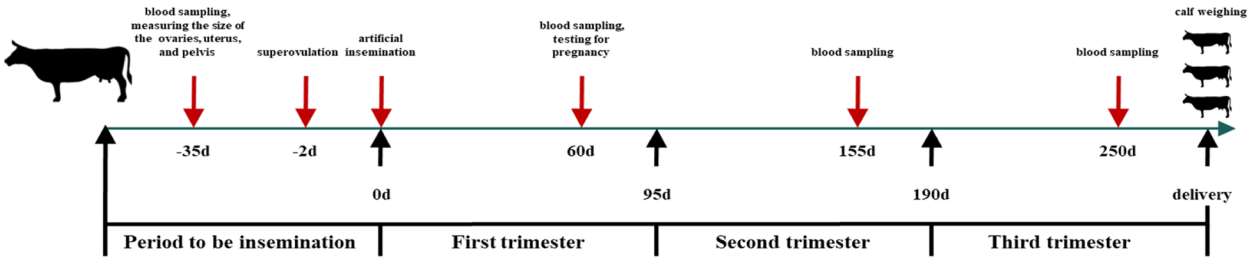

Fig. 8 Division of pregnancy stages and testing flowchart

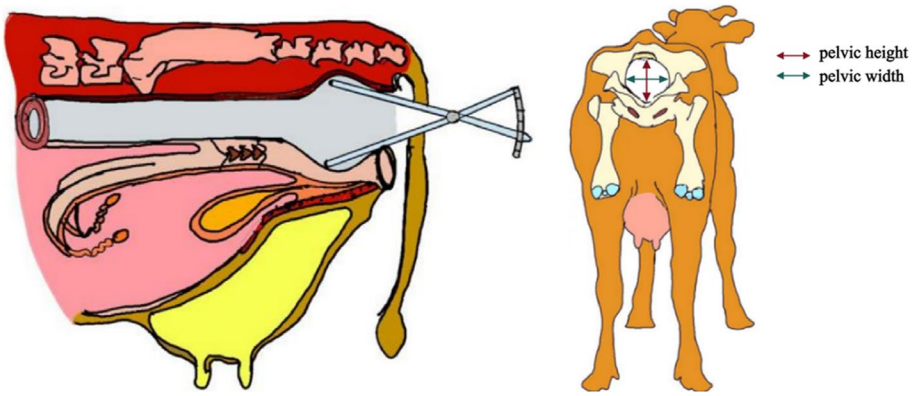

Fig. 9 Schematic diagram of pelvic measurement

parameters such as Glu, LA, LDH, TG, CHO, HDL, LDL, BUN, TP, ALB, AST, CK, and BC.

Measurement of serum P4 and E2 levels

According to the manufacturer's instructions, P4 and E2 levels in serum of MT and WT cattle at different stages of pregnancy were determined using the Bovine Progesterone ELISA Kit (MM- 5091802, MEIMIAN, Jiangsu, China) and the Bovine Estradiol ELISA Kit (MM- 002302, MEIMIAN, Jiangsu, China). Briefly, equilibrate the column at room temperature for 20 min. Set up standard wells and sample wells respectively. Add 50 μL of standard substances at different concentrations to each standard well. For the sample wells, first add 10 μL of the sample to be tested, and then add 40 μL of sample diluent. Additionally, set up a blank well. Next, except for the blank well, add 100 μL of the detection antibody labeled with horseradish peroxidase (HRP) to each well of the standard wells and sample wells, and incubate at 37 °C for 60 min. Discard the liquid, fill each well with washing solution, and wash the plate 5 times, 1 min each time. Add 50 μL of each of Substrate A and Substrate B to each well, and incubate at 37 °C in the dark for 15 min. Then, add the stop solution to terminate the reaction. Use a microplate reader to measure the OD value of each well at a wavelength of 450 nm.

Blood metabolomic analysis

Took 20 μL of serum sample, added 120 μL 50% methanol (A- 456–4, Fisher, USA), shook evenly, and left at room temperature for 10 min to extract metabolites from the samples. The extract solution was stored at − 20 °C overnight to precipitate the proteins in the samples. Spun at 4000 g for 20 min, and transferred the supernatant to a 96-well plate. Took 10 μL of each sample and mixed the same amount to form quality control (QC) sample. The extracted samples were analyzed and sequenced. Proteowizard and MSConvert software were used to convert the raw data into readable mzXML data, and XCMS software was used for peak extraction and quality control. The CAMERA annotated the extracted substances with summed ion annotations, and the metaX software performed primary identification. Matched the identification information to the in-house standard database. Metabolite annotation was performed using HMDB, KEGG, etc. Differential metabolites were screened based on univariate statistical analysis (t-test) combined with multivariate statistical analysis (OPLS-DA/PLS-DA) and fold change values (FC). The default screening criteria are $P < 0.05$ and VIP > 1 and (FC < 1 or FC > 1).

Data analysis

The original data of estrus rate, pregnancy rate, calf birth rate and survival rate, as well as ovarian and uterine size, pelvic area, etc., of WT and MT Luxi cows are all expressed in the form of mean ± standard deviation. The SPSS 22.0 software is used for significance analysis (one-way ANOVA was used for multiple group comparisons and Student's t-test was used for two group comparisons) and $P < 0.05$ was a significant difference, and $P < 0.01$ was extremely significant differences between the different group. An asterisk (*) in the figures indicates a significant difference between two group ($P < 0.05$), and different letters in the tables denote significant differences between two groups ($P < 0.05$). Use GraphPad Prism 8 software to draw graphs.

Abbreviations

MT	MSTN gene-edited Luxi cattle
WT	Non-edited Luxi cattle
MSTN	Myostatin
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
CK	Creatine kinase
P4	Progesterone
E2	Estradiol
QC	Quality control
t-test	Univariate statistical analysis
FC	Fold change values
OPLS-DA	Orthogonal partial least squares discriminant analysis
Glu	Glucose
LA	Lactic acid
LDH	Lactate dehydrogenase
TG	Triglyceride
CHO	Cholesterol
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
BUN	Blood urea nitrogen
TP	Total protein
ALB	Albumin
BC	Bicarbonate

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04742-8.

Supplementary Material 1

Acknowledgements

Not applicable.

Clinical trial number

No applicable.

Authors' contributions

Ghuanghua Su and Guangpeng Li initiated and supervised the study. Xiaoyu Zhao and Jiechuan Xiao performed the majority of experiments and analysis. Yuan Yun and Chunjie Bo conducted hormone content determination. Yuxin Gao, Lishuang Song and Chunling Bai conducted measurements of ovarian and uterine sizes. Zhuying Wei, Li Zhang and Lei Yang have conducted preparations for the pregnancy of cows. Xiaoyu Zhao and Guanghua Su wrote the manuscript. All authors have read and approved the final manuscript.

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Data availability
The datasets produced and/or analyzed the during the current study are available from the corresponding author on reasonable request. The raw metabolomic data analyzed in this study can be obtained from the China National Center for Bioinformation OMIX database (OMIX ID: OMIX008432).

Declarations

Animal ethics and consent to participate declarations
All animals used in the study were treated following the Council of China Animal Welfare guidelines. All protocols were approved by the Institutional Animal Care and Use Committee at Inner Mongolia University (Approval: IMU-CATTLE- 2022–065).

Consent for publication
No applicable.

Competing interests
The authors declare no competing interests.

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