Animal Nutrition 18 (2024) 272–283

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

Influences of lauric acid addition on performance, nutrient digestibility and proteins related to mammary gland development in dairy cows

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ARTICLE INFO

Article history:
Received 17 August 2023
Received in revised form
6 May 2024
Accepted 6 June 2024
Available online 2 July 2024

Keywords:
Lactation performance
Lauric acid
Mammary gland development
Milk fat synthesis
Nutrient digestibility

ABSTRACT

Lauric acid (LA) has the possibility to improve milk production in dairy cows by improving mammary gland development, however, the mechanism by which it might regulate mammary gland development is unclear. The influence of LA on milk production, nutrient digestibility and the expression of proteins related to mammary gland development in dairy cows were evaluated. Forty primiparous Holstein dairy cows were divided into 4 groups in a randomized block design. Four treatments included the control (0 g/d LA per cow), low-LA (100 g/d LA per cow), medium-LA (200 g/d LA per cow), and high-LA (300 g/d LA per cow). Yields of milk, fat-corrected milk, and energy-corrected milk quadratically increased ($P < 0.05$), and yield and content of milk fat linearly increased ($P < 0.05$) with LA supplementation. Percentages of C12:0, C18:1 and C20:1 fatty acids in milk fat linearly increased ($P < 0.05$), but that of C16:0 fatty acid linearly decreased ($P = 0.046$). Supplementation of LA led to a linear and quadratical increase ($P < 0.05$) in digestibility of dry matter, organic matter, neutral detergent fibre and acid detergent fibre, and ruminal total volatile fatty acid concentration but a linear reduction ($P = 0.018$) in the ratio of acetate to propionate. The enzymatic activities of ruminal pectinase, xylanase, and α-amylase, and populations of total bacteria and anaerobic fungi increased linearly ($P < 0.05$), while populations of total protozoa and methanogens decreased linearly ($P < 0.05$) with increased LA addition. Following LA addition, blood glucose, triglyceride, estradiol, prolactin, and insulin-like growth factor 1 concentrations increased linearly ($P < 0.05$) and albumin and total protein concentrations increased quadratically ($P < 0.05$). Moreover, addition of 200 g/d LA promoted ($P < 0.05$) the expression of protein involved in mammary gland development and fatty acids synthesis. These results suggested that LA addition enhanced milk production and fatty acids synthesis by stimulating nutrient digestion, the expression of proteins associated with milk fat synthesis and mammary gland development.

1. Introduction

The goal of the dairy cow industry is to provide large quantities of high-quality milk. As a medium-chain fatty acid (MCFA), lauric acid (C12:0, LA) can be extracted from coconut, palm kernel, and babassu seed oils (Dayrit, 2015). Although LA is a saturated fatty acid (SFA), it is related to a lower risk of cardiovascular disease than other SFA and has various beneficial impacts on health (Ong et al., 2019). Approximately two-thirds of coconut oil-derived LA is transported via the portal vein, whereas the remainder is carried to the lymph and stored in chylomicrons in rats (Dayrit, 2015; Yang et al., 2020). Previous researches found that dietary addition with 1% of LA increases the dilation of mammary ducts in pubertal mice and that 100 μmol/L LA stimulates the proliferation of HC11 mouse mammary epithelial cells (Meng et al., 2017, 2018). Furthermore, an in vivo study in lactating mice suggested that LA improved lactation function in breast-feeding mice reflected by the increased body

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Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2024.06.002

weight of offspring mice, stimulated mammary gland development reflected by the number of alveoli, and increased the protein expression levels of β-casein and Elf5 (Yang et al., 2020). Therefore, LA is expected to be an additive to promote mammary gland development in dairy cows.

Varying influences of LA on milk yields, nutrient digestion, and ruminal fermentation have been reported, implying that only a suitable dose of LA could exert beneficial impacts on the performance of dairy cows. Several of these researches have demonstrated the potent antiprotozoal impacts of LA addition in dairy cows (Lee et al., 2011; Faciola and Broderick, 2013), which result in the decreased dry matter intake (DMI; Dohme et al., 2004; Faciola and Broderick, 2013; Külling et al., 2002), neutral detergent fibre (NDF) and acid detergent fibre (ADF) digestibility (Faciola and Broderick, 2013, 2014), total volatile fatty acid (VFA) concentration (Faciola and Broderick, 2014), acetate to propionate ratios (Faciola and Broderick, 2014), and milk yields and components (Hristov et al., 2011; Faciola and Broderick, 2014). In contrast, several other studies reported that LA supplementation in dairy cows has no influence on DMI (Faciola and Broderick, 2014), milk yields and components (Faciola et al., 2013; Hristov et al., 2009), and ruminal pH (Faciola and Broderick, 2013, 2014). Nevertheless, Kim et al. (2018) found that the addition of LA in steer diets decreased methanogen and *Fibrobacter succinogenes* populations, but increased ruminal *Ruminococcus flavefaciens* populations, total VFA, and propionate concentrations. The propionate produced by ruminal fermentation is transported to the liver and subsequently converted to glucose (Chan and Freedland, 1972), and will most likely result in an increase in glucose availability. Additionally, it has been demonstrated that peroxisome proliferator-activated receptor (PPAR) activated by several fatty acids, including LA in immortalized cell culture model of bovine liver, both when supplied individually and in combination (Busato and Bionaz, 2021). Moreover, PPAR regulates important metabolic processes in monogastric and ruminant animals, such as the metabolism of fatty acids and the production of milk fat (Busato and Bionaz, 2021; Ma and Corl, 2012). Therefore, we speculate that LA might promote fatty acid synthesis in the mammary gland of dairy cow.

Accordingly, we hypothesized that LA addition could enhance lactation performance and milk fatty acid synthesis by stimulating nutrient digestion of dairy cows and the expression of proteins associated with milk fatty acid synthesis and cell proliferation of mammary gland. Therefore, this study was to determine the influences of LA addition on lactation performance, nutrient digestion, and the expression of proteins related to milk fatty acid synthesis and cell proliferation of mammary gland in dairy cows.

2. Materials and methods

2.1. Animal ethics statement

The research proposal of this investigation was evaluated and authorized by the Animal Care and Use Committee of Shanxi Agriculture University, Taigu, China, prior to study commencement (IACUC Issue No. SXAU EAW-2021C.FU.00301413).

2.2. Holstein cows, design, and diets

The experiment was undertaken from August 2021 to December 2021 at a dairy farm (Datong Sifang Hi-Tech Dairy Farm, Datong, China). A randomized block design experiment was carried out with 40 primiparous Holstein dairy cows (662 ± 13.9 kg BW, 71.3 ± 6.82 DIM, 36.1 ± 1.32 kg/d milk yield) at the beginning of the study. The feeding experiment was conducted for 120 d, comprising a 15-d covariate period followed by a 15-d adaptation period and subsequent 90-d sampling period. The diet (Table 1) was formulated according to NRC (2001) recommendations for a 680 kg cow producing 35 kg/d of milk containing 35 g/kg of milk fat and 35 g/kg crude protein. Cows were blocked by DIM and milk yield together and were randomly divided into 1 of 4 groups in a randomized block design. Four treatments were control (0 g/d of LA per cow), low-LA (LLA; 100 g/d of LA per cow), medium-LA (MLA; 200 g/d of LA per cow) and high-LA (HLA; 300 g/d of LA per cow). The amount of LA added was based on previous study (Faciola and Broderick, 2014) who found that 1.3% of LA addition to the basal diet has no influence on DMI. The LA additive (feed grade, contained 996 g/kg of LA [C12:0], 1.5 g/kg of decanoic acid [C10:0], 0.8 g/kg of decanoic acid [C14:0] and 1.7 g/kg of others; Wuxi Odio Technology Development) was blended into the basal diet (Table 1). The basal diet and LA additive was mixed as total mixed ration (TMR) once daily. Cows were housed in a naturally ventilated, two-row, head-to-head, free-stall barn equipped with Calan gates Feeding System for monitoring individual intake. Cows were milked three times daily (at 05:30, 13:30, and 20:30); fed the corresponding TMR ad libitum; and had free access to water.

2.3. Data and sample collection

The cows were weighed on 2 consecutive days at 16:00 on d 1 of the covariate period, and d 1 and 90 of the sampling period. The provided and refused TMR were determined daily for each dairy cow during the entire experiment to estimate the DMI. Samples of TMR were collected every 5 d of the covariate period and every 10 d during the sampling period, and stored at −20 °C for subsequent analyses. Milk production was measured daily for each cow during the entire experiment. Milk samples were collected every 5 d of the covariate period and every 10 d during the sampling period from each milking on 3 consecutive milkings within a day. Concurrently, samples were collected and stored at 4 °C with antimicrobial agent 2-bromo-2-nitropropane-1, 3-diol for subsequent analyses. At 06:30 and 18:30 during d 1 to 15 of the covariate period and d 70 to 87 of the sampling period, all cows were dosed with 5 g of chromic

Table 1
The ingredients of the basal diet and its nutritional content (%, DM basis).

Item	Contents
Ingredients	
Corn fodder silage	24.9
Alfalfa hay	12.1
Oat hay	13.0
Corn grain	25.5
Wheat bran	6.00
Soybean meal	9.20
Cottonseed cake	5.00
Rapeseed meal	2.50
Calcium carbonate	0.50
Salt	0.50
Dicalcium phosphate	0.30
Mineral and vitamin premix [1]	0.50
Nutrient levels	
Organic matter	94.4
Crude protein	16.7
Ether extract	3.23
Neutral detergent fiber	31.1
Acid detergent fiber	19.2
Calcium	0.72
Phosphorus	0.45
NE_L[2], MJ/kg	6.57

[1] Per kilogram premix: 20,100 mg Fe, 1620 mg Cu, 8100 mg Mn, 7122 mg Zn, 1.20 mg I, 62 mg Se, 21 mg Co, 840,000 IU vitamin A, 320, 000 IU vitamin D, and 12, 000 IU vitamin E.
[2] In accordance with NRC (2001), net energy for lactation (NE_L) was calculated.

oxide powder placed in a gelatin capsule to serve as a digestion marker. Approximately 250 g fecal samples were collected from each cow's rectum at 07:00, 13:00, 19:00, and 01:00 during d 8 to 15 of the covariate period and d 78 to 87 of the sampling period, and stored at −20 °C for subsequent analyses. During d 88 to 89, samples of TMR, refusals, and feces of each cow were composited, dried at 55 °C for 72 h, and mashed to pass through a 1 mm screen with a cutter mill.

At 06:30, 09:30, 12:30, and 15:30 on d 5 of the covariate period and d 44 and 89 of the sampling period, the ruminal fluid was collected from each animal via an oral stomach tube. The initial ruminal fluid of approximately 150 mL was discarded to minimize saliva contamination, whereafter the next 200 mL was preserved. The ruminal pH of each cow was determined once using a portable pH meter (5011B; Shanghai Shuo optoelectronic Technology Co., Ltd., China). The ruminal fluid samples were filtered through four layers of medical gauze. Thereafter, 5 mL of the filtrate was blended with 1 mL of 250 g/L meta-phosphoric acid before being stored at −20 °C for VFA determination. Furthermore, 5 mL filtrate was blended with 1 mL of 20 g/L sulfuric acid before being stored at −20 °C for the analysis of ammoniacal nitrogen. For microbial DNA extraction and enzymatic activity analyses, 50 mL filtrate was placed in liquid nitrogen and subsequently stored at −80 °C.

At 10:30 on d 15 of the covariate period and d 90 of the sampling period, blood samples from each dairy cow were collected into 10 mL evacuated tubes (Serum separation gel coagulant tube, Hunan Liuyang Medical Instrument Factory, Liuyang, China) via the coccygeal vessel. Blood samples were transported to the laboratory to separate the serum by centrifugation at 2000 × *g* and 4 °C for 12 min, whereafter the serum samples were stored at −20 °C.

For each cow in groups supplemented with 0 and 200 g/d LA per cow, mammary tissue biopsies were carried out from 16:00 to 20:00 on d 15 of the covariate period and d 90 of the sampling period. About 1 g of secretory tissues in the mammary gland of each cow was collected via surgical biopsy as described by Farr et al. (1996) from the midpoint section of the rear quarter. Tissue biopsy samples for total RNA extraction were rapidly frozen in liquid nitrogen and kept in a low temperature refrigerator at −80 °C for total RNA extraction.

2.4. Chemical analyses

The contents of DM (method 934.01), nitrogen (method 976.05), ether extract (EE; method 973.18), and crude ash (method 942.05) in TMR, refusal, and feces samples were determined according to the method of AOAC (2000). The organic matter (OM) content was calculated as the differences between the DM and crude ash contents. The NDF content was determined as elaborated by Van Soest et al. (1991), using heat-stable alpha-amylase and sodium sulfite, and expressed including residual ash. The ADF content was analyzed based on the method described in AOAC (2000, method 973.18). Calcium (Ca) and phosphorus (P) were determined via the disodium ethylenediaminetetraacetate complexometric titration method and ammonium vanadate molybdate colorimetric method according to the methods of National Standards of the People's Republic of China GB/T 6436-2018 (China National Standard, 2018a) and GB/T 6437-2018 (China National Standard, 2018b), respectively. The fat, true protein, and lactose contents of the milk were analyzed using a Milko Scan FT-120 unit (Foss Electric) based on the method described in AOAC (2000, method 972.16). An aliquot of milk was centrifuged to obtain the milk fat cake. The milk fat was then extracted using the procedure of (Hara and Radin, 1978), and transmethylation of the esterified fatty acids was performed according to the method of Chouinard et al. (1999). Fatty acid methyl esters (FAMEs) were used for gas chromatographic analysis of total fatty acids. According to the method of Liu et al. (2018), fatty acid composition was determined using gas chromatography (GC) on a CP SIL 88, 100 m × 0.25 mm × 0.25 μm capillary column (Agilent J&W Advanced Capillary GC Columns, the Netherlands) in an Agilent 7890 A (Agilent Technologies, Santa Clara, CA, USA) with an auto sampler, flame ionization detector and split injection. The standards were Supelco 37 Component FAME Mix C4–C24 Unsatures (Catalog No. Sigma, 18919-1AMP, Sigma–Aldrich, USA), Supelco PUFA No. 1 (Marine Source, Catalog No. 47033, Supelco Chemical, USA), Methyl trans-11C18:1 (Sigma 46905, Sigma–Aldrich, USA), Methyl cis-9, trans-11 CLA (catalog no. Matreya1255, Cayman Chemical, USA), and Methyl cis-5,8,11,14,17-eicosapentaenoic acid (catalog no. Supelco 44864, Supelco Chemical, USA). The FAME were identified by comparisons with the retention times of the standards. The chromium content of the feces was measured using atomic absorption spectrophotometry (AAA320N; Shanghai Yidian Instrument Co., Ltd., China) based on the method of Williams et al. (1962). Ruminal VFA conctent were determined using gas chromatography (GC-7890; Agilent Technologies, Santa Clara, CA, USA). According to the method of Weatherburn (1967), ammoniacal nitrogen content was analyzed by a colorimetric spectrophotometer (UV759; Qingdao Juchuang Instrument Co., Ltd., China). The samples of ruminal fluid for the measurement of enzymatic activity were immediately taken to the laboratory. Activities of cellobiose, carboxymethyl cellulase [CMCase], α-amylase, xylanase, pectinase, and protease were determined as described by Agarwal et al. (2002). Biochemical kits (Nanjing Jiancheng Bioengineering Institute) of glucose (no. A154-2-1), total protein (A045-3-1), albumin (no. A028-1-1), blood urea nitrogen (BUN; no. C013-2-1), triglyceride (no. A110-2-1) and non-esterified fatty acids (NEFA; no. A042-2-1) were used to analyze serum content of glucose, total protein, albumin, urea nitrogen, triglyceride) and NEFA by using an automatic biochemical analyzer (BS-400, Nanjing Badeng Co., Ltd. China), The ELISA kits (Beijing Biolide Biotechnology Co., Ltd. China) of estradiol (E2; bovine, no. BL-E28820M), prolactin (bovine, no. BL-E28845M), and insulin-like growth factor 1 (IGF-1; bovine, no. BL-E21687M) were used to analyze E2, prolactin, and IGF-1 by using a Konelab auto-analyzer (Thermo Fisher Scientific, USA).

2.5. Extraction of microbial DNA and real-time PCR

Exactly 1.5 mL of homogeneous ruminal fluid was used to extract microbial DNA using the repeated bead-beating plus column (RBB + C) as elaborated by (Yu and Morrison, 2004). The integrity and purity of the extracted DNA were evaluated via agarose gel electrophoresis and a NanoDrop 2000 Spectrophotometer (Thermo Scientific, NanoDrop Technologies), respectively. The target microbial population comprised total bacteria, protozoa, anaerobic fungi, methanogens, *R. flavefaciens*, *Ruminococcus albus*, *Butyrivibrio fibrisolvens*, *F. succinogenes*, *Ruminobacter amylophilus*, and *Prevotella ruminicola*. The target microbial primer set sequences are listed in Table 2. For absolute quantification of the gene copy numbers, 10 sample-derived standards were prepared from the microbial DNA treatment pool using conventional PCR. The PCR products were purified, using the PureLink Quick Gel Extraction and PCR Purification Combo Kit (Thermo Fisher Scientific, USA), and quantified using a spectrophotometer. The copy number of each standard was calculated using the mass concentration and length of the PCR products (Yu et al., 2004). The target DNA was quantified using 10-fold serial dilutions from 10^1 to 10^8 DNA copies. The real-time PCR amplification and detection was conducted in triplicate using a Chromo 4 system (Bio-Rad). The 20 μL reaction mixture contained 10 μL SYBR Premix Taq II (TaKaRa Bio), 2 μL DNA template, 0.8 μL forward primer (10 μmol/L), 0.8 μL reverse primer

Table 2
Real-time PCR primers used for microbial DNA.

Target species	Primer sequence (5′–3′)	GenBank accession no.	Annealing temperature, °C	Size, bp
Total bacteria	F: CGGCAACGAGCGCAACCC R: CCATTGTAGCACGTGTGTAGCC	CP058023.1	60.0	147
Total fungi	F: GAGGAAGTAAAAGTCGTAACAAGGTTTC R: CAAATTCACAAAGGGTAGGATGATT	GQ355327.1	57.5	120
Total protozoa	F: GCTTTCGWTGGTAGTGTATT R: CTTGCCCTCYAATCGTWCT	HM212038.1	59.0	234
Total methanogens	F: TTCGGTGGATCDCARAGRGC R: GBARGTCGWAWCCGTAGAATCC	GQ339873.1	60.0	160
Ruminococcus albus	F: CCCTAAAAGCAGTCTTAGTTCG R: CCTCCTTGCGGTTAGAACA	CP002403.1	60.0	176
Ruminococcus flavefaciens	F: ATTGTCCCAGTTCAGATTGC R: GGCGTCCTCATTGCTGTTAG	AB849343.1	60.0	173
Butyrivibrio fibrisolvens	F: ACCGCATAAGCGCACGGA R: CGGGTCCATCTTGTACCGATAAAT	HQ404372.1	61.0	65
Fibrobacter succinogenes	F: GTTCGGAATTACTGGGCGTAAA R: CGCCTGCCCCTGAACTATC	AB275512.1	61.0	121
Ruminobacter amylophilus	F: CTGGGGAGCTGCCTGAATG R: GCATCTGAATGCGACTGGTTG	MH708240.1	60.0	102
Prevotella ruminicola	F: GAAAGTCGGATTAATGCTCTATGTTG R: CATCCTATAGCGGTAAACCTTTGG	LT975683.1	58.5	74

(10 μmol/L), 0.4 μL of ROX Reference Dye II (TaKaRa), and 6.0 μL dH_2O. The PCR cycling conditions were detailed as follows: 1 cycle at 50 °C for 2 min and 95 °C for 2 min for the original denaturation step, followed by 40 cycles at 95 °C for 15 s, anneal at annealing temperature of each target microbial DNA for 30 s, and extension at 60 °C for 1 min.

2.6. Western blot analysis

Six mammary tissue samples were selected randomly from the same block of two groups, from which total protein was extracted from 100 mg of ground bovine mammary tissue using radioimmunoprecipitation assay buffer (RIPA buffer) containing protease/phosphatase inhibitors (Roche Diagnostics, Shanghai, China). Protein content was analyzed using the Thermo Scientific Pierce BCA protein assay kit (23225; Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Beta-actin was used as the loading control. Equal quantities of protein (20 μg) were separated on a 12% SDS-PAGE, whereafter the separated proteins were transferred onto nitrocellulose membranes (1704271; Bio-Rad). The membranes were blocked with 6% (wt/vol) BSA in tris-buffered saline plus Tween (TBST) for 2 h at 25 °C. Thereafter, the membranes were incubated overnight at 4 °C with the following primary antibodies diluted in TBST: mouse anti-cyclin A1 (1:2000; cat. no. NB100–2660; Novusbio Biologicals, USA), mouse anti-proliferating cell nuclear antigen (PCNA; 1:2000; cat. no. 2586; Cell Signaling Technology, USA), rabbit anti-Akt (1:2000; cat. no. 9272; Cell Signaling Technology, USA), rabbit anti-phosphorylated-Akt_{Ser473} (1:2000; cat. no. bs-0876R; Bios Antibodies, USA), rabbit anti-mTOR (1:2000; cat. no. bs-1992R; Bios Antibodies, USA), rabbit anti-phosphorylated-$mTOR_{Ser2448}$ (1:2000; cat. no. bs-3495R; Bios Antibodies, USA), rabbit anti- G-protein-coupled receptor 84 (GPR84; 1:2000; cat. no. bs-13507R; Bios Antibodies, USA), rabbit anti-peroxisome proliferator-activated receptor gamma (PPARγ, 1:2000; cat. no. bs-4590R; Bios Antibodies, USA), rabbit anti-sterol regulatory element-binding protein 1 (SREBP1; 1:2000; cat. no. NB100–2215; Novus Biologicals, USA), rabbit anti-phosphorylated-acetyl-coenzyme A carboxylase-α (ACACA; 1:2000; cat. no. bs-12954R; Bios Antibodies, USA), rabbit anti-ACACA (1:2000; cat. no. bs-11912R; Bios Antibodies, USA), rabbit anti-fatty acid synthase (FASN, 1:2000; cat. no. bs-60347R; Bios Antibodies, USA), rabbit anti-stearoyl-CoA desaturase 1 (SCD1; 1:2000; cat. no. bs-3787R; Bios Antibodies, USA), and rabbit anti-β-actin (1:10,000; cat. no. 4970; Cell Signaling Technology, USA). In order to remove excess antibodies, membranes were washed five times with 1 × TBST for 5 min. Thereafter, the membranes were incubated at 25 °C for 2 h with either of the following secondary antibodies diluted in TBST: goat anti-rabbit (1:5000; cat. no. E-AB-1003; Elabscience, USA) or goat anti-mouse (1:5000; cat. no. RS0001; Immunoway, USA). SuperSignal West Pico Chemiluminescence Substrate (Thermo Fisher Scientific, USA) was used to visualize the western blots prior to being semi-quantified using the ImageJ software (version 1.4.3.67).

2.7. Calculation and statistical analysis

Dietary net energy for lactation was estimated by multiplying the $NE_{L\text{-}3X}$ density of the feed ingredient by its dietary content (NASEM, 2021). Energy-corrected milk (ECM) and fat-corrected milk (FCM) were estimated according to NRC (2001), where ECM = 0.327 × milk (kg/d) + 12.95 × fat (kg/d) + 7.65 × protein (kg/d), 4% FCM = 0.4 × milk (kg/d) + 15 × fat (kg/d). Feed efficiency was estimated for each animal as milk yield (actual milk and ECM yield) divided by dietary DM intake. Nutrient digestibility (%) was calculated by the following formula: $(1 - bc/ad) \times 100$, where a was nutrient concentration in feed; b was nutrient concentration in feces; c was chromium concentration in feed; d was chromium concentration in feces (Salehi et al., 2023).

Data were analyzed as a randomized complete-block design via the mixed procedure of SAS (PROC MIXED; SAS, 2002). Data for DMI, milk production, feed efficiency and milk fatty acid composition were analyzed using the following statistic model:

$$Y_{iklm} = \mu + \beta V_k + B_i + T_j + C_{k(i)} + L_l + TL_{jl} + E_{ijkl},$$

where Y_{iklm} was the dependent variable; μ was the overall mean; β was regression coefficient; V_k was covariate measurement; B_i was the random effect of block i; T_j was the fixed effect of time j; $C_{k(i)}$ was the random effect of cow k within block i; L_l was the fixed effect of LA treatment l; TL_{jl} was interaction between time and LA treatment; E_{ijkl} was residual error.

Data for rumen fermentation, microbial enzyme activity and microbiota were analyzed using the following statistic model which there were repeated measurements over time (pH, VFA, enzymatic activity and microbiota):

$$Y_{iklm} = \mu + \beta V_k + B_i + D_j + C_{k(i)} + L_l + DL_{jl} + E_{ijkl} + T_m + LT_{ml} + S_{ijklm},$$

where, Y_{iklm} was the dependant variable; μ was the overall mean; β was regression coefficient; V_k was covariate measurement; B_i was the random effect of block i; D_j was the fixed effect of day j; $C_{k(i)}$ was the random effect of cow k within block i; L_l was the fixed effect of LA treatment l; DL_{jl} was interaction between day and LA treatment; E_{ijkl} was whole plot error; T_m was effect of time m; LT_{ml} was interaction between LA treatment and time; S_{ijklm} was subplot error. Original data was tested in SAS for homogeneity of variance and normality; moreover, rumen microbiota residuals were tested for normality. Log-transformed microbiota data only marginally improved normality; therefore, analysis was done using original data. The covariance structure for variables was first-order autoregressive. Mean separations using probability of difference tests (PDIFF in SAS) were conducted only for influences that were statistically significant ($P < 0.05$). Data for nutrient digestibility and blood parameters were analysed with the same model as suggested above, but time and the interaction between time and treatment were omitted. The CONTRAST statement of SAS was used to compute linear and quadratic orthogonal contrasts based on the LA application rates.

SigmaPlot version 12.5 statistical analysis package (Systat Software, Inc., San Jose, CA, USA) was used to analyze the western blotting results. Student's *t*-test (*t*-test) was used to analyze statistical differences between treatments. Effects of the factors were considered significant at $P < 0.05$ unless other trends were declared at $P < 0.10$.

3. Results

3.1. Lactation performance

The DMI decreased linearly ($P = 0.042$) with increased LA addition (Table 3). Conversely, the yields of actual milk ($P = 0.036$), 4% FCM ($P = 0.037$), and ECM ($P = 0.042$) increased quadratically with an increase in LA addition (Table 3). Milk fat content ($P = 0.014$) and production ($P = 0.038$) increased linearly with increased LA supplementation, whereas milk true protein content ($P = 0.014$) and production ($P = 0.013$) increased quadratically following LA supplementation. However, the production and content of milk lactose did not change with increased LA addition. Feed efficiency, described as milk yield to dietary DM intake ratio ($P = 0.032$) or ECM yield to dietary DM intake ratio ($P = 0.019$), also increased linearly with increased LA dose.

3.2. Milk fatty acid composition

Supplementation of LA had an effect on de novo fatty acids (DN FA; Σ FA < 16C; $P = 0.038$) (Table 4). Most of the increase in DN FA was depend on an elevation in the proportion of C12:0 in milk ($P = 0.018$). However, supplementation of LA decreased ($P = 0.045$) the proportions of mixed sourced fatty acids (MSFA; Σ FA = 16C) in milk due to the linearly decreased proportion of C16:0 in milk ($P = 0.046$). Although supplementation of LA increased the proportions of C18:1 ($P = 0.038$) and C20:1 ($P = 0.046$) in milk, there were no difference in the proportions preformed fatty acids (PFA; Σ FA > 18C). Proportions of odd- and branched-chained fatty acids (OBCFA) was also not impacted by LA addition.

3.3. Digestibility and rumen fermentation

The digestibility of dietary DM, OM, NDF and ADF, increased linearly and quadratically ($P < 0.05$), but that of CP ($P = 0.018$) and EE ($P = 0.024$) increased linearly with increasing LA dose (Table 5). Although ruminal pH did not decrease, ruminal total VFA content increased quadratically ($P = 0.034$) with increased LA dose. The molar proportion of acetate was unaffected by LA addition, whereas that of propionate increased linearly ($P = 0.021$) with increased LA addition. Moreover, the acetate to propionate ratio decreased linearly ($P = 0.018$) with an increase in LA addition. Conversely, the molar percentages of butyrate, valerate, isobutyrate and isovalerate were unaffected by LA addition. Rumen ammonia N content decreased linearly ($P = 0.024$) with increased LA addition.

3.4. Microbial enzymatic activities and microbiota

Although the enzyme activities of CMCase and cellobiase were unaffected by LA addition, the activity of xylanase increased linearly ($P = 0.012$) with increased LA dose (Table 6). The activity of pectinase ($P = 0.022$) and α-amylase ($P = 0.008$) increased linearly with an increase in LA dosage. Populations of total anaerobic fungi

Table 3
Impacts of lauric acid addition on lactation performance and feed efficiency.

Item	Treatments[1]				SEM	*P*-value		
	CON	LLA	MLA	HLA		Treatment	Linear	Quadratic
DMI, kg/d	22.0^{a}	21.5^{ab}	21.2^{ab}	20.7^{b}	0.14	0.023	0.042	0.976
Milk production, kg/d								
Actual	34.6^{b}	35.4^{ab}	36.4^{a}	35.4^{ab}	0.24	0.042	0.615	0.036
4.0% FCM[2]	31.2^{b}	32.5^{ab}	34.0^{a}	33.1^{ab}	0.26	0.049	0.474	0.037
ECM[3]	34.3^{b}	35.1^{ab}	36.7^{a}	36.1^{ab}	0.24	0.038	0.523	0.042
Milk fat	1.16^{b}	1.22^{ab}	1.29^{a}	1.26^{a}	0.016	0.026	0.038	0.427
Milk true protein	1.09^{b}	1.13^{ab}	1.17^{a}	1.12^{ab}	0.011	0.023	0.755	0.013
Milk lactose	1.85	1.87	1.95	1.89	0.017	0.654	0.766	0.331
Milk composition, g/kg								
Fat	33.5^{b}	34.5^{ab}	35.4^{a}	35.6^{a}	0.04	0.043	0.014	0.928
True protein	31.6^{b}	31.8^{ab}	32.2^{a}	31.5^{ab}	0.03	0.037	0.962	0.014
Lactose	53.4	52.7	53.4	53.4	0.04	0.893	0.857	0.633
Feed efficiency[4], kg/kg								
Milk yield to DMI ratio	1.58^{b}	1.65^{ab}	1.72^{a}	1.71^{a}	0.005	0.016	0.032	0.204
ECM to DM intake ratio	1.56^{b}	1.64^{ab}	1.73^{a}	1.74^{a}	0.006	0.023	0.019	0.507

^{a,b} Means with different superscripts in each row differ significantly ($P < 0.05$).
[1] Control (CON), 0 g/d per cow lauric acid (LA); low-LA (LLA), 100 g/d per cow LA; medium-LA (MLA), 200 g/d per cow LA; high-LA (HLA), 300 g/d per cow LA.
[2] Fat-corrected milk (FCM) was estimated as 4.0% FCM = 0.4 × milk (kg/d) + 15 × fat (kg/d).
[3] Energy-corrected milk (ECM) was estimated as ECM = [0.327 × milk (kg/d)] + [12.95 × fat (kg/d)] + [7.65 × protein (kg/d)].
[4] Estimated as milk yields (milk or ECM yields) divided by DMI for each cow.

Table 4
Impacts of lauric acid addition on milk fatty acid composition (g/100 g of total milk fatty acids).

Item	Treatments[1]				SEM	*P*-value		
	CON	LLA	MLA	HLA		Treatment	Linear	Quadratic
C4:0	2.89	2.98	3.11	3.18	0.028	0.437	0.312	0.538
C6:0	2.34	2.26	2.25	2.12	0.039	0.168	0.224	0.436
C8:0	1.32	1.29	1.29	1.25	0.018	0.419	0.332	0.544
C10:0	2.97	2.83	2.78	2.70	0.051	0.342	0.289	0.452
C12:0	3.31^{c}	4.47^{bc}	5.56^{ab}	6.69^{a}	0.108	0.018	0.021	0.543
C14:0	10.9	11.5	11.8	11.4	0.11	0.195	0.083	0.621
C15:0	2.46	2.40	2.33	2.30	0.178	0.266	0.092	0.502
C16:0	34.4^{a}	32.8^{ab}	30.9^{b}	29.8^{b}	0.19	0.027	0.046	0.468
C17:0	2.01	1.97	1.99	2.04	0.041	0.428	0.342	0.493
C18:0	7.08	6.94	6.97	6.89	0.227	0.603	0.163	0.776
C20:0	0.53	0.51	0.51	0.48	0.013	0.594	0.371	0.613
C21:0	0.08	0.07	0.08	0.09	0.005	0.782	0.453	0.912
C22:0	0.14	0.13	0.14	0.14	0.006	0.541	0.542	0.673
C23:0	0.06	0.06	0.06	0.07	0.005	0.687	0.571	0.764
C24:0	0.12	0.14	0.13	0.14	0.006	0.543	0.493	0.381
C14:1	0.41^{b}	0.47^{ab}	0.55^{a}	0.62^{a}	0.087	0.036	0.048	0.118
C15:1	0.55	0.55	0.56	0.55	0.004	0.499	0.582	0.442
C16:1	2.44	2.35	2.25	2.30	0.158	0.364	0.314	0.133
C18:1	18.4^{b}	18.7^{ab}	19.4^{a}	19.9^{a}	0.73	0.038	0.027	0.932
C20:1	0.14^{b}	0.16^{ab}	0.17^{a}	0.18^{a}	0.008	0.046	0.032	0.451
C22:1	0.08	0.08	0.08	0.09	0.005	0.259	0.113	0.254
C24:1	0.09	0.09	0.09	0.10	0.005	0.378	0.272	0.426
C18:2, *cis* 9, *cis*12	1.61	1.57	1.50	1.53	0.226	0.206	0.108	0.243
C18:2, *trans* 9, *trans*12	1.03	1.07	1.06	1.02	0.165	0.184	0.532	0.124
C18:2, *cis* 9, *cis* 11	0.55	0.56	0.52	0.52	0.026	0.475	0.324	0.632
C18:2, *trans* 10, *cis* 12	0.93	0.92	0.91	0.92	0.042	0.501	0.431	0.243
C18:3, *cis* 6, *cis* 9, *cis* 12	0.16	0.14	0.17	0.15	0.007	0.372	0.742	0.921
C18:3, *cis* 9, *cis* 12, *cis* 15	1.12	1.11	1.10	1.00	0.026	0.476	0.117	0.264
C20:2	0.14	0.13	0.13	0.12	0.006	0.594	0.204	0.452
C20:3, *cis* 8, *cis* 11, *cis* 14	0.66	0.64	0.60	0.57	0.016	0.713	0.262	0.528
C20:3, *cis* 11, *cis* 14, *cis* 17	0.04	0.04	0.03	0.03	0.004	0.618	0.393	0.514
C20:4	0.31	0.26	0.25	0.27	0.021	0.307	0.542	0.123
C20:5	0.11	0.11	0.13	0.13	0.006	0.349	0.391	0.822
C22:2	0.03	0.03	0.03	0.05	0.004	0.206	0.109	0.154
C22:3	0.03	0.04	0.03	0.05	0.004	0.487	0.223	0.353
C22:4	0.22	0.25	0.26	0.26	0.008	0.245	0.154	0.912
C22:5	0.29	0.30	0.32	0.30	0.009	0.253	0.402	0.217
C22:6	0.05	0.07	0.06	0.05	0.005	0.498	0.273	0.101
Σ de novo FA[2]	24.2^{b}	25.8^{ab}	27.3^{a}	28.0^{a}	0.35	0.038	0.043	0.703
Σ MSFA[3]	36.8^{a}	35.1^{ab}	33.1^{b}	32.1^{b}	0.51	0.045	0.034	0.682
Σ PFA[4]	33.8	34.0	34.6	34.9	0.51	0.349	0.215	0.516
Σ OBCFA[5]	5.15	5.06	5.02	5.05	0.134	0.563	0.393	0.204
Σ SFA[6]	70.6	70.4	69.8	69.3	0.94	0.478	0.192	0.331
Σ MUFA[7]	22.1	22.4	23.1	23.7	0.23	0.625	0.094	0.502
Σ PUFA[8]	7.28	7.22	7.09	6.96	0.186	0.724	0.083	0.547

[a-c] Means with different superscripts in each row differ significantly ($P < 0.05$).
[1] Control (CON), 0 g/d per cow lauric acid (LA); low-LA (LLA), 100 g/d per cow LA; medium-LA (MLA), 200 g/d per cow LA; high-LA (HLA), 300 g/d per cow LA.
[2] Σ de novo FA = sum of C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, C14:1.
[3] Σ mixed sourced fatty acids (MSFA) = sum of C16:0, C16:1.
[4] Σ preformed fatty acids (PFA) = sum of C18:0, C20:0, C22:0, C24:0, C8:1, C20:1, C22:1, C24:1, C18:2, C18:3, C20:2, C20:3, C20:4, C20:5, C22:2, C22:3, C22:4, C22:5, C22:6.
[5] Σ odd- and branched-chained fatty acids (OBCFA) = sum of C15:0, C15:1, C17:0, C21:0, C23:0.
[6] SFA = saturated fatty acid.
[7] MUFA = mono-unsaturated fatty acid.
[8] PUFA = poly-unsaturated fatty acids.

($P = 0.043$) and bacteria ($P = 0.044$) increased linearly with an increase in LA dosage. Nevertheless, populations of total protozoa ($P = 0.019$) and methanogens ($P = 0.017$) decreased linearly with an increase in LA dosage. Populations of *R. flavefaciens* ($P = 0.024$), *R. albus* ($P = 0.015$), and *R. amylophilus* ($P = 0.047$) increased quadratically with an increase in LA dosage. However, populations of *F. succinogenes*, *B. fibrisolvens*, and *P. ruminicola* were unaffected by LA addition.

3.5. Blood metabolites

The blood levels of glucose ($P = 0.030$), triglyceride ($P = 0.014$), E2 ($P = 0.032$), prolactin ($P = 0.024$), and IGF-1 ($P = 0.018$) increased linearly with an increase in LA addition (Table 7). Furthermore, blood albumin ($P = 0.016$) and total protein ($P = 0.013$) concentrations increased quadratically with increased LA dose. Conversely, blood UN content decreased quadratically ($P = 0.013$) with increased LA dose. Furthermore, blood NEFA concentration decreased linearly ($P = 0.013$) with increased LA addition.

3.6. Expression of proteins implicated in fatty acid synthesis

In regard to fatty acid synthesis, the protein levels of PPARγ ($P < 0.01$), SREBF1 ($P < 0.01$), FASN ($P < 0.01$), SCD1 ($P < 0.05$) and the phosphorylation ratio of ACACA were uniformly increased upon the addition of 200 g/d LA compared with the control (Fig. 1A and B).

Table 5
Impacts of lauric acid addition on nutrient digestibility and ruminal fermentation.

Item	Treatments[1]				SEM	*P*-value		
	CON	LLA	MLA	HLA		Treatment	Linear	Quadratic
Digestibility, %								
Dry matter	67.9^{b}	71.2^{ab}	72.7^{a}	72.2^{ab}	0.52	0.034	0.013	0.021
Organic matter	68.9^{b}	72.1^{ab}	73.6^{a}	73.2^{ab}	0.51	0.041	0.031	0.033
Crude protein	71.8^{b}	75.3^{ab}	76.6^{a}	77.8^{a}	0.78	0.028	0.018	0.346
Ether extract	75.7^{b}	78.2^{ab}	79.5^{a}	79.0^{a}	0.53	0.034	0.024	0.287
Neutral detergent fiber	54.1^{b}	59.3^{a}	60.7^{a}	59.2^{a}	0.82	0.042	0.032	0.038
Acid detergent fiber	42.2^{b}	47.8^{ab}	50.5^{a}	48.9^{ab}	1.11	0.046	0.034	0.023
Ruminal fermentation								
pH	6.71	6.68	6.64	6.69	0.019	0.589	0.521	0.267
Total volatile fatty acids, mmol/L	85.6^{b}	92.6^{ab}	93.8^{a}	92.7^{ab}	4.02	0.043	0.038	0.034
Percentage of total VFA								
Acetate	61.2	59.6	59.0	58.0	0.56	0.216	0.104	0.482
Propionate	20.7^{b}	21.4^{ab}	22.5^{a}	22.2^{a}	0.25	0.037	0.021	0.703
Butyrate	14.5	15.1	14.6	15.8	0.36	0.085	0.668	0.124
Valerate	1.72	1.72	1.85	2.02	0.093	0.716	0.262	0.672
Isobutyrate	0.28	0.34	0.33	0.33	0.012	0.512	0.207	0.331
Isovalerate	1.61	1.81	1.76	1.69	0.074	0.875	0.813	0.423
Acetate-to-propionate ratio	2.97^{a}	2.79^{ab}	2.63^{b}	2.61^{b}	0.087	0.026	0.018	0.706
Ammonia N, mg/dL	15.8^{a}	14.7^{ab}	13.0^{b}	12.7^{b}	0.10	0.041	0.024	0.231

^{a,b} Means with different superscripts in each row differ significantly ($P < 0.05$).
[1] Control (CON), 0 g/d per cow lauric acid (LA); low-LA (LLA), 100 g/d per cow LA; medium-LA (MLA), 200 g/d per cow LA; high-LA (HLA), 300 g/d per cow LA.

Table 6
Impacts of lauric acid addition on ruminal microbial enzymatic activities and microbiota in dairy cows.

Item	Treatments[1]				SEM	*P*-value		
	CON	LLA	MLA	HLA		Treatment	Linear	Quadratic
Enzyme activity								
Carboxymethyl-cellulase, μmol glucose/min per mL	0.167	0.219	0.223	0.228	0.0231	0.368	0.134	0.391
Cellobiase, μmol glucose/min per mL	0.173	0.198	0.205	0.194	0.0142	0.187	0.411	0.364
Xylanase, μmol xylose/min per mL	0.384^{b}	0.598^{a}	0.592^{a}	0.616^{a}	0.0301	0.023	0.012	0.035
Pectinase, μmol D-galacturonic acid/min per mL	0.445^{b}	0.493^{b}	0.556^{a}	0.557^{a}	0.0183	0.037	0.022	0.945
α-Amylase, μmol maltose/min per mL	0.517^{b}	0.542^{ab}	0.552^{a}	0.587^{a}	0.0132	0.045	0.008	0.370
Protease, μg hydrolyzed protein/min per mL	0.605	0.632	0.724	0.689	0.0415	0.264	0.473	0.030
Microbiota, copies/mL								
Total bacteria, $\times 10^{11}$	3.65^{c}	4.73^{bc}	5.71^{a}	5.24^{ab}	0.315	0.047	0.044	0.521
Total anaerobic fungi, $\times 10^{7}$	1.70^{b}	2.23^{ab}	2.58^{a}	2.51^{a}	0.132	0.036	0.043	0.301
Total protozoa, $\times 10^{5}$	7.12^{a}	6.54^{ab}	5.26^{b}	4.95^{b}	0.223	0.032	0.019	0.429
Total methanogens, $\times 10^{9}$	8.64^{a}	8.05^{ab}	7.03^{bc}	6.70^{c}	0.294	0.041	0.017	0.823
Ruminococcus albus, $\times 10^{8}$	3.42^{c}	5.77^{b}	7.75^{a}	5.01^{b}	0.253	0.029	0.508	0.015
Ruminococcus flavefaciens, $\times 10^{9}$	2.17^{b}	3.27^{ab}	3.76^{a}	3.38^{ab}	0.193	0.032	0.268	0.024
Fibrobacter succinogenes, $\times 10^{10}$	1.77	2.40	3.99	2.74	0.527	0.579	0.372	0.402
Butyrivibrio fibrisolvens, $\times 10^{9}$	4.22	4.80	5.46	4.57	0.321	0.313	0.380	0.106
Prevotella ruminicola, $\times 10^{10}$	2.30	3.88	4.41	4.05	0.427	0.396	0.148	0.268
Ruminobacter amylophilus, $\times 10^{10}$	2.08^{c}	3.15b	3.62^{a}	3.06^{b}	0.081	0.046	0.180	0.047

$^{a-c}$ Means with different superscripts in each row differ significantly ($P < 0.05$).
[1] Control (CON), 0 g/d per cow lauric acid (LA); low-LA (LLA), 100 g/d per cow LA; medium-LA (MLA), 200 g/d per cow LA; high-LA (HLA), 300 g/d per cow LA.

Table 7
Effects of lauric acid addition on blood parameters in lactating dairy cows.

Item	Treatments[1]				SEM	*P*-value		
	CON	LLA	MLA	HLA		Treatment	Linear	Quadratic
Glucose, mmol/L	3.81^{b}	4.24^{ab}	4.51^{a}	4.44^{a}	0.113	0.045	0.030	0.125
Total protein, g/L	70.2^{b}	82.2^{ab}	84.7^{a}	81.8^{ab}	1.95	0.016	0.496	0.023
Albumin, g/L	28.5^{c}	29.9^{bc}	36.3^{a}	34.5^{ab}	0.86	0.027	0.342	0.016
Urea nitrogen, mmol/L	7.47^{a}	7.27^{ab}	6.62^{b}	6.90^{ab}	0.213	0.049	0.225	0.013
Triglyceride, mmol/L	1.97^{c}	2.10^{bc}	2.35^{ab}	2.50^{a}	0.081	0.028	0.014	0.394
Non-esterified fatty acids, μmol/L	287^{a}	281^{ab}	271^{bc}	257^{c}	3.5	0.018	0.013	0.264
Estradiol, pg/mL	48.4^{b}	52.6^{ab}	65.1^{a}	66.4^{a}	1.72	0.047	0.032	0.411
Prolactin, mIU/L	569^{b}	588^{ab}	647^{a}	675^{a}	16.1	0.029	0.024	0.503
Insulin-like growth factor 1, ng/mL	202^{b}	216^{ab}	238^{a}	235^{a}	3.9	0.032	0.018	0.682

$^{a-c}$ Means with different superscripts in each row differ significantly ($P < 0.05$).
[1] Control (CON), 0 g/d per cow lauric acid (LA); low-LA (LLA), 100 g/d per cow LA; medium-LA (MLA), 200 g/d per cow LA; high-LA (HLA), 300 g/d per cow LA.

3.7. Expression of proteins implicated in cell proliferation

The protein levels of cyclin A1 ($P < 0.01$) and PCNA ($P < 0.05$) were uniformly increased upon the addition of 200 g/d LA compared with those in the control (Fig. 2A and B). As a receptor for LA, the GPR84 regulates cell proliferation and apoptosis via the Akt/mTOR signalling pathway. The protein levels of GPR84 was increased ($P < 0.01$) following 200 g/d LA addition. The phosphorylation ratios of Akt and mTOR were also increased ($P < 0.01$) following 200 g/d LA addition (Fig. 3A and B).

4. Discussion

4.1. Lactation performance

The linear reduction in the DMI of dairy cows is in consonance with the results obtained by Dohme et al. (2004), who found that LA (C12:0) addition reduces DMI to a greater extent than the C14:0 and C18:0 addition. In keeping with the current findings, Külling et al. (2002) also found a decrease in DMI with LA addition. Moreover, 1.3% of LA addition to the basal diet has no influence on DMI (Faciola and Broderick, 2014); however, larger doses of LA in the TMR (480 and 720 g/d) drastically reduce DMI (Faciola and Broderick, 2013). The decreased DMI upon LA supplementation may be ascribed either to the poor palatability of LA (Külling et al., 2002) or impaired rumen degradation of fibre (Dohme et al., 2004) and resultant increased ruminal retention time of feed (Klop et al., 2017a). Previous studies reported that yields of milk and milk components is either unimpacted (Faciola et al., 2013; Hristov et al., 2009) or decreased (Hristov et al., 2011; Faciola and Broderick, 2014; Klop et al., 2017b) following LA supplementation. Conversely, we found that LA addition quadratically increased milk production of actual, 4% FCM, and ECM, quadratically increased production and content of milk protein, and linearly increased production and content of milk fat. This is mainly ascribed to the chosen LA dose, since Faciola and Broderick (2014) used an LA dose of 1.3% of dietary DM, whereas that in our study was 0.46% (LLA), 0.94% (MLA), and 1.45% (HLA) of dietary DM. Furthermore, the quadratic respond to LA addition with no further elevation of milk production indicate that increasing LA dose from 0.94% to 1.45% of dietary DM was not conducive to improving lactation performance. The quadratic response in milk production following LA supplementation might be due to the linear and quadratic changes in the digestibility of DM, OM, NDF and ADF, further research is needed to verify this explanation.

4.2. Milk fat composition

As expected, elevating the supply of C12:0 in the cow diet elevates the proportion of C12:0 which is inflating the proportions of DN FA. Similarly, Dohme et al. (2004) found a greater proportion of C12:0 in milk fat of cows following LA addition compared to C14:0 and C18:0. The decreased proportions of MSFA was mainly resulted

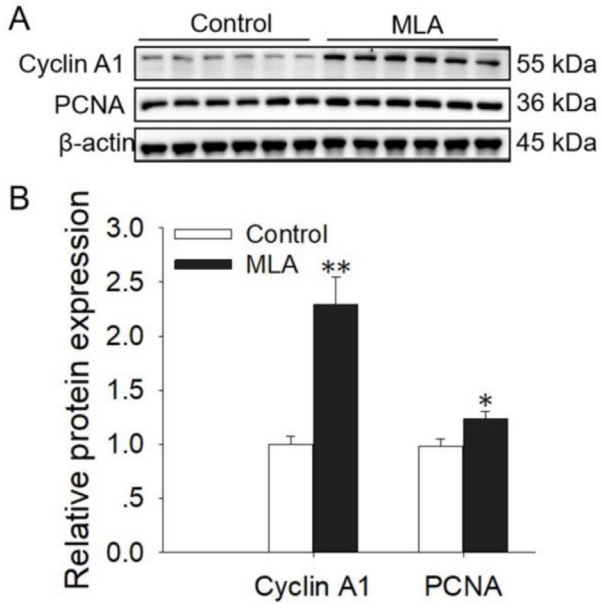

Fig. 2. Impacts of medium lauric acid (MLA) addition on proliferation-related mRNA and protein expression in the bovine mammary gland. (A) Western blot analysis of cyclin A1 and proliferating cell nuclear antigen (PCNA) in bovine mammary gland tissues in response to 0 g/d LA (Control) and 200 g/d LA (MLA) addition. Beta-actin was used as an internal control. $n = 6$ per group, selected randomly from the same block of two groups. (B) Mean ± SEM of immunoblotting bands for cyclin A1 and PCNA. $^{*}P < 0.05$ and $^{**}P < 0.01$ against the control group were used.

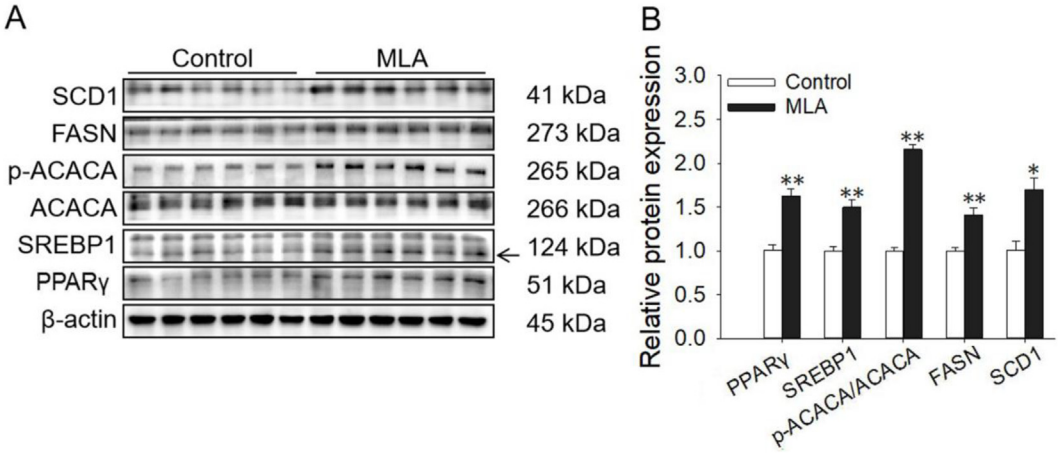

Fig. 1. Effects of medium lauric acid (MLA) addition on the expressions of proteins associated with fatty acid synthesis in the bovine mammary gland. (A) Western blot analysis of peroxisome proliferator activated receptor gamma (PPARγ), sterol-regulatory element binding proteins (SREBP1, the specific bands with an arrow), acetyl coenzyme A carboxylase-α (ACACA), phosphorylated ACACA (p-ACACA), fatty acid synthase (FASN), and stearoyl CoA desaturase 1 (SCD1) in the tissues of bovine mammary gland in response to 0 g/d LA (Control) and 200 g/d LA (MLA) addition. Beta-actin was used as an internal control. $n = 6$ per group, selected randomly from the same block of two groups. (B) Mean ± SEM of immunoblotting bands of PPARγ, SREBP1, ACACA, FASN and SCD1. $^{*}P < 0.05$ and $^{**}P < 0.01$ against the control group were used.

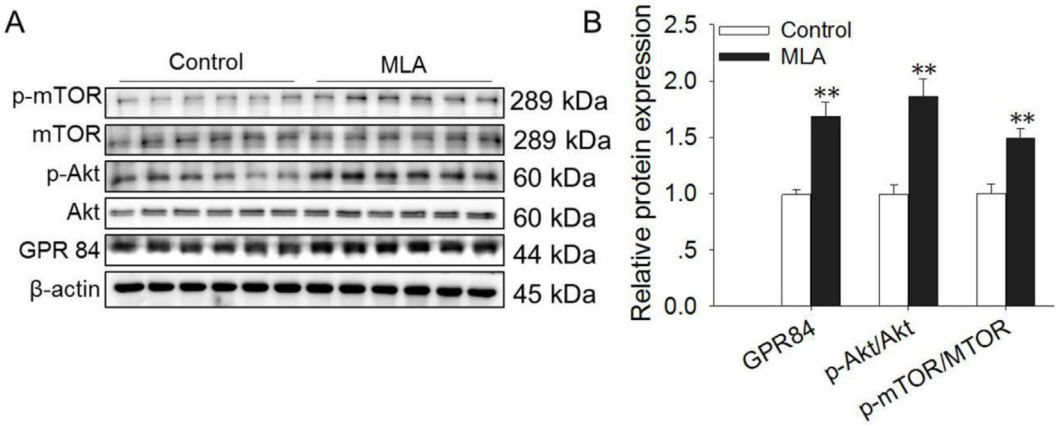

Fig. 3. Effects of medium lauric acid (MLA) addition on the Akt-mTOR signaling pathway in the bovine mammary gland. (A) Western blot analysis of G-protein-coupled receptor 84 (GPR84), Akt, p-Akt, mTORl and p-mTOR in bovine mammary glands treated with 0 g/d LA (Control) and 200 g/d LA (MLA). Beta-actin was used as an internal control. $n = 6$ per group, selected randomly from the same block of two groups. (B) Mean ± SEM of the immunoblotting bands for p-Akt/Akt and p-mTOR/mTOR. $^{**}P < 0.01$ against the control group were used.

from the decreased proportion of C16:0 in milk fat and was due to the decreased DMI. Other previous studies also observed the increased proportion of C12:0 in milk fat and the decreased proportion of C16:0 upon LA addition (Hristov et al., 2011; Klop et al., 2017a,b). The PFA are mostly coming from the diet and from the biohydrogenation of dietary FA. In the current study, the increased proportions of C18:1 and C20:1 in milk fat of cows following LA addition was due to an increase in the apparent digestibility of ether extract and an increase in stearoyl-CoA desaturase. Similarly, Klop et al. (2017a) found that proportions of several C18:1 fatty acids in milk fat of cows were increased with 65 g/kg DM of LA. The unchanged proportions of PFA, OBCFA, SFA, monounsaturated fatty acid (MUFA) or polyunsaturated fatty acids (PUFA) upon LA addition was in keeping with Klop et al. (2017a) and might be due to the comprehensive effect of the decreased DMI and increased nutrient digestibility following LA addition.

4.3. Nutrient digestibility and ruminal fermentation

Previous researches demonstrated digestibility of dietary DM, OM, and CP was not impacted, and both the total-tract and ruminal apparent digestibility of dietary NDF and ADF was reduced upon LA supplementation (Faciola et al., 2013; Faciola and Broderick, 2013, 2014) and after coconut oil feeding (Lee et al., 2011) in dairy cows. However, in the current study the increased nutrient digestibility following LA addition was possibly depend on the increased ruminal bacterial populations and enzymatic activity with an increasing LA dosage (Liu et al., 2018), and resulted in the increased ruminal VFA concentrations, supporting the quadratically improved milk production. Furthermore, the quadratic effect of LA on dietary DM, OM, NDF and ADF digestibility might be depend on the quadratic response of populations of *R. albus*, *R. flavefaciens* and *Rb. amylophilus* to LA addition.

We demonstrated that ruminal pH was unaffected by an increase in LA addition, which concurs with previous observations (Faciola and Broderick, 2013, 2014). Similarly, Kim et al. (2018) demonstrated that LA addition did not impact ruminal pH in Hanwoo steers. Possible explanation for an increase in total VFA and a decrease in ammonia with no change in pH in the current study is an increased absorption of VFA (Suárez et al., 2006) or a net increase of VFA flus (Rémond et al., 2003). Since the VFA flus and production were not measured in this study, it is not possible to tell whether an increase in VFA flow is equivalent to absorption, which was also a limitation of this study. The increased total VFA content was in line with the increased dietary DM digestibility and suggested that ruminal bacterial populations and enzymatic activity were increased following LA addition. Moreover, the linearly decreased ratio of acetate to propionate was depend on the unaltered acetate and linearly increased propionate molar percentages. This implied that the ruminal fermentation pattern tended towards propionate formation under increased LA addition conditions. Correspondingly, Kim et al. (2018) demonstrated that total VFA and propionate content in LA-supplemented Hanwoo steers were greater than those of the control group 6 h after feeding. For glucose and lactose to be synthesized, propionate is an essential substrate (Wang et al., 2009a; Li et al., 2016); therefore, it is possible that LA supplementation enhanced milk production via this mechanism. Nevertheless, Faciola and Broderick (2014) demonstrated that 1.3% LA, based on the dietary DM of dairy cows, caused a small reduction in total VFA concentrations, although the ratio of acetate to propionate was decreased. The linearly decreased rumen ammonia nitrogen content is possibly ascribed to the increased bacterial protein synthesis with LA addition (Cummins and Papas, 1985).

4.4. Microbial enzyme activities and microbiota

In cows, dietary fiber is degraded to acetate by the ruminal bacteria, protozoa, and fungi that secrete cellulolytic enzymes (Orpin, 1984). Feed lignocellulosic tissues can be degraded by ruminal fungi, and approximately 30% of fiber digestion and 10% of VFA production could be due to ruminal protozoa (Wang and McAllister, 2002). Therefore, the linear increase in ruminal xylanase activity with an increasing LA dosage was a consequence of the increased populations of total anaerobic fungi, total bacteria, *R. flavefaciens*, and *R. albus*. Moreover, this result supported the increased rumen total VFA and increased digestibility of dietary NDF and ADF.

The increased populations of total anaerobic fungi, total bacteria, *R. flavefaciens*, and *R. albus* might be mainly due to the supply of energy from the degradable LA, since the effective degradability of rumen by-pass fat was about 86% (Soren et al., 2022). The linear reduction in populations of total methanogens and protozoa suggested that LA addition suppressed ruminal CH_4 production (Soliva et al., 2003). Correspondingly, Kim et al. (2018) observed that LA supplementation decreased methanogen and *F. succinogenes* populations and significantly increased *R. flavefaciens* populations. The LA elicits potent antiprotozoal effects (Lee et al., 2011; Faciola and

Broderick, 2013) and 160 g/d of LA provided via ruminal cannula reduces ruminal protozoa proportion by 90% within 2 d of treatment (Faciola et al., 2013). The reduction of protozoa population resulted in the decreased protozoa's ability to phagocytose bacteria, thus changing the structure of microflora (Hristov et al., 2012), increasing the populations of total bacteria, total anaerobic fungi, and promoting ruminal fermentation (Wang et al., 2009b). The linear increase in the activity of α-amylase and pectinase was in agreement with the observed elevation in the *R. amylophilus* population, indicating that LA addition promoted ruminal starch degradation. These results further confirm the increased molar percentage of propionate with an increasing LA dosage. The unaltered ruminal protease activity was mainly associated with the unchanged *P. ruminicola* population.

4.5. Blood metabolites

The propionate produced by ruminal fermentation is transported to the liver and subsequently converted to glucose (Chan and Freedland, 1972). Therefore, the linear increase in blood glucose following LA addition, which is in keeping with the results of previous researches (Li et al., 2016; Wang et al., 2009a), may be ascribed to an increase in ruminal propionate output (Foley et al., 2009). The linear reduce in NEFA concentration and the linear increase in triglyceride concentration following LA supplementation indicated that body fat mobilization was suppressed and fatty acid synthesis was promoted (Coleman et al., 2021). The increase in glucose and the decrease in NEFA following LA supplementation might suggest an increase in insulin resistance, although some literature in monogastric animals appears to support a reverse effect (Tham et al., 2020), while others indicate an increase in insulin resistance in the adipose tissue (Saraswathi et al., 2020) and liver (Kamoshita et al., 2022). Total protein, albumin, and BUN concentrations are indicators of the protein utilization efficiency (Nousiainen et al., 2004). Although we observed the increased albumin and total protein levels and decreased BUN concentrations following LA supplementation, the ability of LA to improve protein utilization needed to be further studied. It is well-known that IGF-1, prolactin, and E2 are vital for mammary duct development (Rosen, 2012). Moreover, ovary-derived IGF-1, prolactin, and E2 stimulate epithelial cell proliferation (Mallepell et al., 2006). Additionally, the PI3K/Akt signaling pathway was activated by IGF-1 through its receptor and promotes the proliferation of epithelial cells (Zhou et al., 2017). Therefore, our results showing LA addition-induced increased serum IGF-1, prolactin, and E2 concentration can provide some support for a possible mechanism of the effect of LA on mammary epithelium. However, further research on the expression of gene and protein related to the above hormone and its receptor are needed to confirm the effect of LA on mammary epithelium.

Based on the quadratic increase of LA in promoting milk yield and ruminal total volatile fatty acid concentration of dairy cows, and the better effect of the MLA group was selected to compare the difference of proteins implicated in fatty acid synthesis and cell proliferation with the control group.

4.6. Expressions of proteins implicated in fatty acid synthesis

Both PPARγ and SREBP1 regulate the expression of ACACA, FASN, SCD, and fatty acid-binding protein 3 (FABP3) in the mammary gland (Ma and Corl, 2012). Moreover, both FASN and ACACA are involved in fatty acids synthesis from acetate and β-hydroxybutyric acid in the mammary gland (Bionaz and Loor, 2008) and are positively correlated with the secretion of $C_{4\text{-}16}$ fatty acids (Bernard et al., 2008). The SCD protein catalyzes MUFA synthesis from SFA (Lehner and Kuksis, 1996; Zhang et al., 2023). The increased protein levels of FASN, ACACA, PPARγ and SREBP1 following LA supplementation indicated that the proteins implicated in the synthesis of de novo FA and MCFA were upregulated, supporting the elevation in milk fat yield. Similarly, Busato and Bionaz (2021) demonstrated that PPARγ activated by LA in immortalized cell culture model of bovine liver.

4.7. Expressions of proteins implicated in cell proliferation

Stimulating cell proliferation in the mammary gland should be the core control point that promotes lactation persistence in dairy cows. The Cyclin A is regarded as indispensable for initiating and completing DNA replication in the S phase (Lim and Kaldis, 2013). Additionally, PCNA is an essential component of both DNA replication and repair (Park et al., 2016). Moreover, previous researches have certificated the involvement of Cyclin D3 and PCNA in modulating mammary epithelial cell proliferation (Zhang et al., 2018). We demonstrated that LA addition increased the protein expressions of cyclin A1 and PCNA, implying that LA stimulates the proliferation of mammary epithelial cells. Traditionally, most of mammary development and cell growth and differentiation occurs before parturition and then net growth during lactation is expected to be minimal. In view of the results of the current experiment, it could provide a way to promote the research of mammary gland development of perinatal cows in the future.

Previous researches have implicated the Akt signaling pathway in modulation of the proliferation and apoptosis of various cells (Huang et al., 2021; Meng et al., 2017). Moreover, the mTOR signaling pathway is concerned with modulating the proliferation of various cells (Li et al., 2017). Existing research has shown that GPR84, which is strongly activated by LA (Miyamoto et al., 2016; Wang et al., 2006), regulates immune responses (Alvarez-Curto and Milligan, 2016) and MCFA taste transduction (Liu, 2016). Furthermore, LA addition increases GPR84 and Cyclin D1 expression and activates the PI3K/Akt signaling pathway of the mammary glands of pubertal (Meng et al., 2017) and lactating (Yang et al., 2020) mice. The addition of LA increased GPR84 expression and subsequently stimulated Akt and mTOR phosphorylation via GPR84 activation. The results suggest that activating the Akt-mTOR signaling pathway might stimulate proliferative markers to be expressed. Considering that the net growth of mammary gland during lactation is expected to be minimal, the activation of AKT and mTOR with LA addition would be likely more in metabolic regulation, especially the regulation of milk synthesis.

5. Conclusions

Supplementation with LA in cow diets improved the milk yields and feed efficiency in the dose-dependent effect by promoting nutrient digestion, ruminal fermentation, and mammary gland fatty acid synthesis. Moreover, addition of LA activated the GPR84-Akt/mTOR pathway and promoted the protein expressions of cyclin A1 and PCNA.

6. Limitations of the study

Although supplementation of LA improved the milk yields and feed efficiency by promoting nutrient digestion, ruminal fermentation, and expressions of protein implicated in fatty acid synthesis and cell proliferation of mammary gland, the results found in the current study could partly also be due to the increase in energy of the diet because of the lack of isocaloric diet between groups. Further study is needed to verify the effect of LA by balancing of the ration with another fatty acid which would not affect the mammary gland development.

Author contributions

Jing Zhang and **Qiang Liu** designed the research; **Lijun Bu**, **Yapeng Liu** and **Wenjie Huo** conducted the research; **Chengqiang Xia** and **Caixia Pei** analyzed the data; **Jing Zhang**, **Lijun Bu** and **Yapeng Liu** wrote the original draft; **Jing Zhang** and **Qiang Liu** had primary responsibility for the final content. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was funded by the Education Department of Shanxi Province, Excellent Doctor Work Award Fund Research Project [SXYBKY2018036] and Modern Agro-industry Technology Research System of Shanxi Province (2023CYJSTX13).

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