Original Research Article

AMPK-ChREBP axis mediates de novo milk fatty acid synthesis promoted by glucose in the mammary gland of lactating goats

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ABSTRACT

To investigate the role of glucose in regulating milk fatty acid synthesis, 6 lactating Guanzhong dairy goats were infused with 0, 60, or 100 g/d glucose via the external pubic artery in a 3×3 repeated Latin square experiment. A concomitant in vitro experiment was conducted to investigate possible mechanisms whereby glucose regulates milk fatty acid synthesis. RNA sequencing was used for cellular transcriptome analysis. Drugs, MK-2206, rapamycin, and dorsomorphin were used to block cellular mammalian AMP-activated protein kinase (AMPK), AKT serine/threonine kinase 1, and mechanistic target of rapamycin kinase signaling pathways, respectively. Carbohydrate response element binding protein (ChREBP) was knockdown and overexpressed to investigate its role in regulating milk fatty acid synthesis in mammary epithelial cells. Glucose infusion linearly elevated the concentration of C8:0 ($P = 0.039$) and C10:0 ($P = 0.041$) in milk fat while it linearly decreased ($P = 0.049$) that of C16:0. This result was in agreement with the upregulation of genes related to de novo synthesis of fatty acids and lipid droplet formation, including adipose differentiation-related protein, butyrophilin subfamily 1 member A1, fatty acid synthase (FASN) and ChREBP. Their expression increased ($P < 0.05$) linearly in the lactating goat mammary gland. In vitro, glucose linearly stimulated the expression of genes related to de novo synthesis of fatty acids and cellular triacylglycerol in cultured mammary epithelial cells. RNA sequencing and inhibition studies revealed that glucose induced transcriptomic changes increasing lipogenic pathways, with AMPK responding to glucose by controlling ChREBP and FASN. Knockdown and overexpression of ChREBP highlighted its essential role in lipogenesis. The knockdown and overexpression of ChREBP protein also revealed an essential role in regulating the de novo synthesis of fatty acids. Collectively, our data highlight that glucose supplementation promotes de novo fatty acid synthesis via the AMPK-ChREBP axis, hence increasing milk fat yield in the goat mammary gland. Results from the current study provide possible strategies to manipulate the fatty acid composition as well as improve ruminant milk quality.

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1. Introduction

Bioactive components in milk lipids contribute to human health (Lock and Bauman, 2004; Mohan et al., 2021). Among total milk fatty acids, short- and medium-chain (4 to 14 carbons) and a portion of the 16-carbon fatty acids are derived from de novo synthesis, which is controlled by fatty acid synthase (FASN) and acetyl-CoA carboxylase alpha (ACACA). Nutrients such as acetate or glucose are required for lipogenesis in ruminants and non-

ruminants. Although the data in non-ruminants clearly demonstrates that glucose supply is essential for milk fat synthesis, there have been contrasting responses reported in ruminants. For instance, in dairy cows, increased doses of glucose infused via the abomasum had a negative effect on milk fat yield and content (Curtis et al., 2014), while no significant changes in fatty acids content were detected (Hurtaud et al., 2000). In contrast, recent work in dairy goats revealed that an increase in the systemic supply of glucose had a positive effect on milk fat yield (Cai et al., 2018). These inconsistencies highlight that the mechanisms whereby glucose availability at the mammary gland may alter milk fat synthesis are unclear.

De novo synthesis of fatty acids in the mammary gland is regulated by key transcription factors including sterol regulatory element binding transcription factor 1 (SREBP1) (Li et al., 2014; Xu et al., 2016) and carbohydrate response element binding protein (ChREBP) (Chen et al., 2021). Numerous studies have reported how altering the activity of pathways involved in de novo synthesis changes the nutritive quality of milk, including fat and protein (Shi et al., 2013a; Xu et al., 2016). In addition to molecular and enzyme-level regulation of de novo synthesis, diet and post-ruminal substrate availability are also important factors that could alter profiles of milk fatty acids.

The protein ChREBP is a whole-body glucose sensor that, at least in non-ruminants, is an important regulator of lipogenesis in response to high dietary carbohydrate (Herman et al., 2012; Ortega-Prieto and Postic, 2019). For example, increased glucose supply led to O-GlcNAcylation and acetylation of ChREBP, thereby increasing its transcriptional activity by enhancing binding to promoters of target genes including *FASN* and *ACACA* (Abdul-Wahed et al., 2017). Recent data revealed a crosstalk between ChREBP (activated by glucose) and SREBP-1c and peroxisome proliferator activated receptor alpha, suggesting interdependence of ChREBP activity with other signaling pathways (Iroz et al., 2017; Pulkoski-Gross et al., 2018).

Mammalian AMP-activated protein kinase (AMPK) is known to be activated by low concentrations of glucose via increases in AMP/ATP and ADP/ATP ratios (Zong et al., 2019). Milk protein and fatty acid synthesis were associated with the AMPK pathway in mammary gland tissue (Cai et al., 2020b; Zhang et al., 2018b). Increased supply of glucose to the liver of mice led to the stimulation of AMPK, but also the phosphorylation and activation of ChREBP and fatty acid synthesis (Kawaguchi et al., 2002). Our previous data with goat mammary gland tissue suggested a role for glucose in the stimulation of lipogenesis (Cai et al., 2018), but the responsible mechanisms remain unclear. Thus, together, these data prompted us to question whether AMPK-ChREBP through glucose played a role in lipogenesis in the ruminant mammary gland.

The objective of this study was to investigate the crosstalk between glucose and milk fat, and whether supplementation with glucose altered the composition of milk fatty acids. To investigate this, we used in vivo glucose infusions and in vitro culture of primary goat mammary cells.

2. Materials and methods

2.1. Animals and sampling

Details of the animal feeding and experimental design were described previously (Cai et al., 2020a). Briefly, 6 lactating Guanzhong dairy goats (aged 3 years; days-in-milk: 185 ± 6 d; and BW: 43.6 ± 3.0 kg) were infused via the external pudendal artery with 0, 60, and 100 g/d glucose in a 3 × 3 replicated Latin square design. Each period was 12 d in length and consisted of 7 d of treatment where glucose was infused each day for 5 h, followed by 5 washout days without glucose infusion before the next period began.

Mammary tissue was sampled via biopsy after the afternoon milking on the last day of infusion. Milk samples were collected before the biopsy. Tissues and milk were stored in liquid nitrogen before analysis.

2.2. Cell culture and treatment

Goat mammary epithelial cells (GMEC) were isolated from peak lactation Saanen goats individually, as described previously (Shi et al., 2014). The 293A cells, gifted from Professor Shu Zhu's lab in Zhejiang University, were used for adenovirus generation. Details of cell culture were described in a recent paper (Shi et al., 2013b). Briefly, culture medium for GMEC was composed of DMEM/F12 (Hyclone, Beijing, China) containing insulin (5 mg/L, Sigma, USA), hydrocortisone (5 mg/L, Sigma, USA), penicillin-streptomycin (10,000 units/L, Harbin Pharmaceutical Group, China), epidermal growth factor (1 mg/L, Sigma, USA) and fetal bovine serum (10%, Gibco, USA). The 293A cells were cultured in basal DMEM medium (Gibco, USA), containing 10% fetal bovine serum and penicillin-streptomycin (10,000 units/L, Harbin Pharmaceutical Group, China).

For glucose stimulation, when cells reached 90% confluence, the culture medium was changed to basic medium for 12 h. The basic medium contained DMEM (DMEM without glucose, 11966025, Gibco, Invitrogen, USA) supplemented with insulin (5 mg/L, I6634, Sigma, USA), hydrocortisone (1 mg/L, H0888, Sigma, USA), and penicillin-streptomycin (10,000 U/L). After 12 h of starvation, cells were incubated with basic medium containing glucose (D9434, sigma, USA) at 0, 1, 2.5, and 4.5 g/L for 24 h. Cells were harvested for RNA extraction, triacylglycerol (TAG) concentration and lipid droplet staining using BODIPY (D3922, Invitrogen, USA).

2.3. RNA sequencing data generation and processing

Total RNA was extracted from cells stimulated with glucose at 0 and 4.5 g/L using the RNA Prep Pure Cell Kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer's instructions. After assessing the quantity and quality of RNA using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA), mRNA libraries were constructed using the TruSeq RNA Sample Preparation Kit (Illumina Inc., San Diego, CA, USA). The Illumina HiSeq 2500 platform was used for sequencing. RNA expression and microRNA (miRNA) expression matrices for mammary tissue were obtained after RNA-sequencing data processing, genome alignment, functional annotation, and reported as counts per million (CPM). Raw gene counts were used to identify differential expression (DE) of genes (4.5 g/L group vs. 0 g/L group) using the Bioconductor package edgeR (v3.4.1).

The cutoff for differential expression was set at 2-fold change and false discovery rate < 0.01. The $\log_2$ (Fold Change) and $-\log10$ (*P*-value) were applied to generate volcano plots and display the DE gene results. The identifications of DE of genes were submitted to KABOS (http://kobas.cbi.pku.edu.cn/) for KEGG analysis. The KEGG enrichments for metabolism categories were displayed using ggplot2.

2.4. Luciferase analysis

GMEC at 80% confluence in 48-well plates were transfected with 0.2 μg of Luciferase reporter vector containing the promoter of FASN along with a Renilla vector (pRL-TK) as a control at a ratio of 29:1 using the Lipofectamine 2000 (Thermo Fisher Scientific Inc., Waltham, MA) (Shi et al., 2017b). After a 12-h starvation in basic medium, cells were treated with glucose at final concentrations of 0, 1, and 4.5 g/L and harvested 24 h later. Lysates were made using reporter lysis buffer (Dual-Luciferase Reporter Assay System,

Promega, USA) according to the manufacturer's instructions. Luciferase activity in the cell extract was determined using luciferase assay buffer and luciferase assay substrate according to the manufacturer's protocol.

2.5. Pathway blocking assay

The drugs MK-2206 (S1078, Selleck Chemicals, Houston, TX, USA), rapamycin (S1039, Selleck Chemicals) and dorsomorphin (S7306, Selleck Chemicals) were used for blocking the activity of AMPK, AKT serine/threonine kinase 1 (Akt) and mechanistic target of rapamycin kinase (mTOR), respectively. GMEC were treated with basic medium containing glucose at high (4.5 g/L) or low (1 g/L) levels. The dimethyl sulfoxide (DMSO), MK-2206 (500 nM), rapamycin (10 nM) or dorsomorphin (10 μM) were incubated with cells for 24 h before harvesting for RNA extraction.

2.6. RNA interference

Sequences of the siRNA oligonucleotides for *ChREBP* (Accession: XM_018040995) (siRNA) were designed and synthesized by Jima Biotechnology Co., Ltd (Shanghai, China). To assess interfering efficiency, 2 designed siRNA were transfected into GMEC at 5 or 30 nM using transfection reagent (Lipofectamine RNAiMAX, Thermo Fisher Scientific Inc., Waltham, MA). Transfected GMEC were harvested at 48 h for RNA extraction. Sequences for the siRNA targeting *ChREBP* are described in the supplementary file (Table S1). Scrambled siRNA, a functional non-targeting siRNA, was used as a negative control (NC).

The siRNA1 was selected for the interference of *ChREBP* expression. Transfected GMEC were cultured with glucose (4.5 g/L) or control (0 g/L) after 24 h of initial culture, and then harvested at 48 h (24 h later) for RNA extraction. In total, there were 4 treatments (NC, NC + glucose, siRNA, siRNA + glucose) in the RNAi experiment.

2.7. RNA extraction and qPCR

Total RNA from mammary tissue and GMEC was extracted using the RNA Prep Pure Cell Kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer's protocol. Genomic DNA contamination was removed using DNase provided with the kit. Synthesis of cDNA from 1 μg RNA was conducted using the PrimeScript RT kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed according to the manufacturer's instructions using a kit (SYBR Premix Ex Taq II, Perfect Real Time, Takara Bio Inc., Otsu, Japan).

Genes studied include those involved in de novo fatty acid synthesis (*FASN, ACACA*), elongation (long-chain fatty-acyl elongase 5 [*ELOVL5*], *ELOVL6*, *ELOVL 7*), lipid droplet formation (adipose differentiation-related protein [*ADRP*], butyrophilin subfamily 1 member A1 [*BTN1A1*]), and transcription factors (*SREBP1* and *ChREBP*). All qPCR reactions were performed in an ABI7500 (Thermo fisher) sequence detector. Ubiquitously expressed transcript (*UXT*), mitochondrial ribosomal protein L39 (*MRPL39*), and ribosomal protein S9 (*RPS9*) were used as internal controls. Primer sequences are reported in the supplementary file (Table S2).

2.8. Adenovirus generation and ChREBP overexpression

The coding sequence of *ChREBP* (GenBank No.: XM_018040995) was linked to an adaptor containing flag sequence at the C terminal and then subcloned into the pAdTrack-CMV, which was a gift from Bert Vogelstein (Addgene plasmid # 16405; http://n2t.net/addgene:16405; RRID: Addgene_16405), plasmid vector to generate pAdTrack-ChREBP-flag. The vector was inserted into an adenoviral vector (pAdEasy-1) to generate adenoviral plasmids in BJ5183 cells. The adenoviral plasmids linearized by P*ac* I (New England Biolabs, USA) were transfected into 293A cells to generate the adenovirus Ad-ChREBP. The recombinant adenovirus expressing green fluorescent protein (Ad-GFP) was used as a control. The GMEC at about 80% confluence were infected with adenovirus supernatant (Ad-ChREBP or Ad-GFP). The infected GMEC were collected after 48 h of culture for lipid and protein extraction.

2.9. Lipid extraction and analysis

Milk lipids were extracted according the protocol from Liu et al. (2018). For fatty acid profiling, collected GMEC were scraped off the culture dish using a 2-mL aliquot of 2.5% (vol/vol) vitriol: methanol. Then, total lipid extraction and methylation were performed according to Shi et al. (2013a). Methylated lipid samples were analyzed using a Gas Chromatography-Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) installed with an HP-88 column (100 m × 0.25 mm i.d. × 0.25 μm film thickness, Agilent Technologies) following a published procedure (Shi et al., 2019). The relative proportion of each fatty acid was determined as the ratio of the fatty acid peak to the total peaks in each run. Data for each fatty acid was analyzed as a proportion of the total fatty acids.

2.10. Western blotting

Total proteins were extracted from GMEC with different treatments using radioimmunoprecipitation assay buffer (RIPA buffer) containing protease/phosphatase inhibitors (Roche Diagnostics, Shanghai, China). Antibodies against flag (20543-1-AP, diluted at 1:1,000, Proteintech, Wuhan, China) were used. The secondary antibody was IgG with horseradish peroxidase (SA00002-2, diluted at 1:1,500, Proteintech, Wuhan, China).

2.11. Statistical analysis

Homogeneity of variance was examined by Levene statistics, and deviations from normality were examined by the Kolmogorov−Smirnoff test with Lilliefors correction. Data from goats infused with glucose were analyzed using a MIXED model procedure (SAS Inst. Inc., Cary, NC) as a 3 × 3 replicated Latin square design. Treatment and period were the fixed effects. The individual goat was considered as the random effect. The residual effect was used to test the significance of treatment, goat, and period. Data from qPCR were analyzed using the $2^{-\Delta\Delta Ct}$ method. Each in vitro experiment with glucose included 3 biological replicates, and results are expressed as means ± standard error of means. Differences between treatments were analyzed by orthogonal polynomial linear and quadratic contrasts using the MIXED protocol in SAS 8.2 (SAS Inst. Inc., Cary, NC). Differences in fatty acid profiles in GMEC were tested via Student's *t*-test. Statistical significance was declared when $P < 0.05$.

3. Results

3.1. Glucose infusion regulates de novo synthesis of fatty acids in the mammary gland

Glucose infusion at 60 g/d enhanced the milk fat yield as previously described (Cai et al., 2018), suggesting a direct effect on de novo synthesis of fatty acids. To assess alterations in lipid metabolism induced by glucose infusion in the mammary gland, the profiles of milk fatty acids were measured by GC−MS. As shown in Table 1, the ratio of C4:0 changed quadratically ($P = 0.049$), decreasing from 0 to 60 g/d and then increasing from 60 to 100 g/d of glucose infusion. Linear increases in C8:0 ($P = 0.039$) and C10:0

($P = 0.041$) as well as a linear decrease in C16:0 ($P = 0.049$) were observed with increasing amounts of infused glucose.

Linear increases in *ChREBP* ($P = 0.03$), *FASN* ($P < 0.01$), *ACACA* ($P = 0.09$), *BTN1A1* ($P = 0.02$), *ADRP* ($P = 0.01$), *ELOVL5* ($P = 0.02$) were detected with increasing glucose infusion (Fig. 1A–D). Glucose infusion had no effect on the relative mRNA level of *SREBP1* ($P_{linear} = 0.95$, $P_{quadratic} = 0.95$), *ACACA* ($P_{linear} = 0.09$, $P_{quadratic} = 0.53$), *ELOVL6* ($P_{linear} = 0.27$, $P_{quadratic} = 0.45$) and *ELOVL7* ($P_{linear} = 0.89$, $P_{quadratic} = 0.07$).

3.2. Glucose promotes de novo fatty acid synthesis and cellular TAG accumulation in mammary epithelial cells

The effect of glucose on fatty acid metabolism was assessed through stimulation of GMEC at doses of 0, 1, 2.5 and 4.5 g/L, respectively. Linear increases in *ChREBP* ($P = 0.02$), SREBP ($P = 0.03$), *FASN* ($P < 0.01$), *ACACA* ($P < 0.01$), *BTN1A1* ($P = 0.02$), *ADRP* ($P = 0.04$), and *ELOVL6* ($P = 0.03$) were detected with increasing glucose stimulation (Fig. 2A–D). No significant changes were observed for the level of *ELOVL7* (P-linear = 0.28, P-quadratic = 0.09) after incubation with glucose (Fig. 2D). A quadratic change of *ELOVL5* was observed, decreasing at doses of 1 and 2.5 g/L, and increasing at dose of 4.5 g/L.

As observed with BODIPY staining (Fig. 3A), incubation with glucose gradually increased the amount of lipid droplets containing TAG in cells. The quantification of TAG revealed a strong positive effect of glucose on cellular TAG content (P-linear < 0.01, P-quadratic = 0.03) and led to 1.5- and 2.5-fold increases at 2.5 g/L ($P = 0.12$) and 4.5 g/L ($P = 0.001$), respectively (Fig. 3B). As shown in Fig. 3C, incubation with glucose significantly increased the activity of the *FASN* promoter (P-linear < 0.01, P-quadratic < 0.01).

3.3. Glucose treatment elicits transcriptomic changes toward increased lipid synthesis

Compared with the control group (0 g/L), 971 mRNAs were upregulated in the cells incubated with 4.5 g/L glucose, while 961 mRNAs were downregulated (Fig. 4A and the supplementary file 2). Selected significant pathways related to lipid metabolism are shown in Fig. 4B. The predominant pathways were involved in metabolism, PI3K-Akt signaling, insulin signaling, insulin resistance, AMPK signaling, cell cycle, and mTOR signaling.

3.4. Glucose changes lipid synthesis and is associated with the AMPK pathway

To scan for pathways induced by glucose, MK-2206, dorsomorphin, and rapamycin were incubated with the GMEC. The data

showed that inhibition of AMPK and mTOR signaling significantly upregulated ($P < 0.05$) the expression of *FASN* when incubated with low levels of glucose (Fig. 5A). High concentrations of glucose enhanced ($P < 0.05$) the expression of *FASN* when the AMPK pathway was blocked. Inhibition of the AMPK pathway by dorsomorphin at low concentrations of glucose had no effect on *ChREBP* expression (Fig. 5B). However, an increased expression of *ChREBP* was observed at high concentrations of glucose ($P < 0.05$). There was no significant effect on the expression of *ChREBP* after the inhibition of Akt and mTOR signaling pathways.

3.5. ChREBP is required for the expression of genes involved in de novo fatty acid synthesis

Two siRNA targeting goat ChREBP were selected for incubations with GMEC, respectively. Compared with the control group (NC), the siRNA1 had a higher efficiency (78%) than siRNA2 (62%) and was selected to knockdown *ChREBP* (Fig. 6A). Compared with the control NC group (NC), knockdown of *ChREBP* did not significantly alter the mRNA abundance of *SREBP1*, *ACACA* and *FASN* in the cells without glucose (siRNA) (Fig. 6B). Under the stimulation of glucose, the knockdown of *ChREBP* had no effect on the level of *SREBP1*, but it decreased the mRNA abundance of *ACACA* and *FASN* (Fig. 6C).

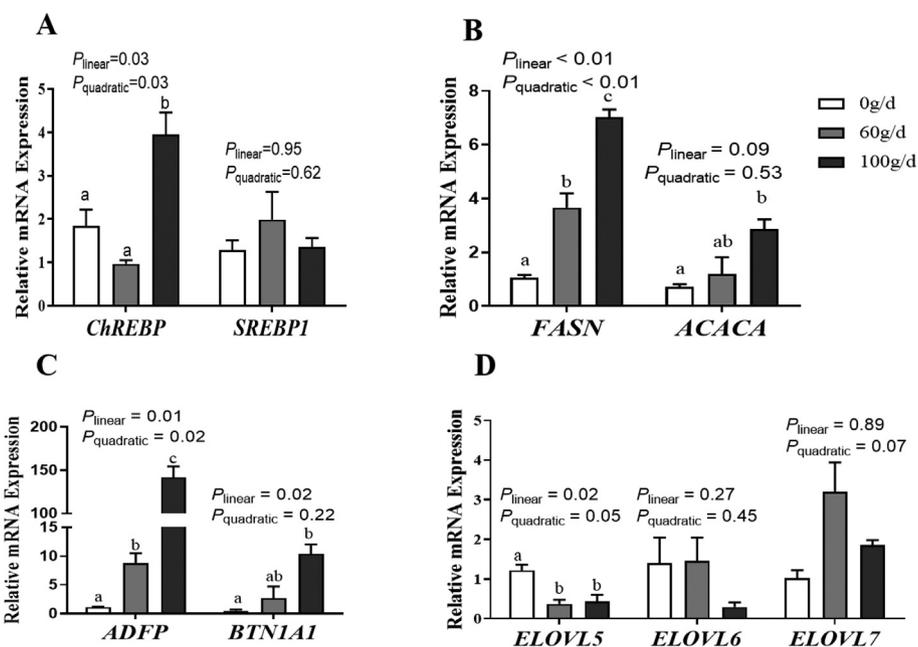### 3.6. ChREBP altered fatty acid profiles

ChREBP was overexpressed in the GMEC using adenovirus (Ad-ChREBP), with Ad-GFP as the control. Western blot data showed that incubation with Ad-ChREBP markedly upregulated ($P < 0.05$) the protein level of ChREBP compared with the control (Fig. 7A). Results of fatty acid profiling demonstrated that activation of ChREBP did not affect the ratio of C16:0, C16:1, C18:0, C18:1trans and C18:1cis in GMEC (Fig. 7B). However, a higher concentration of C14:0 ($P = 0.02$) and lower concentration of C18:2 ($P = 0.01$) were observed after overexpression of ChREBP (Fig. 7B).
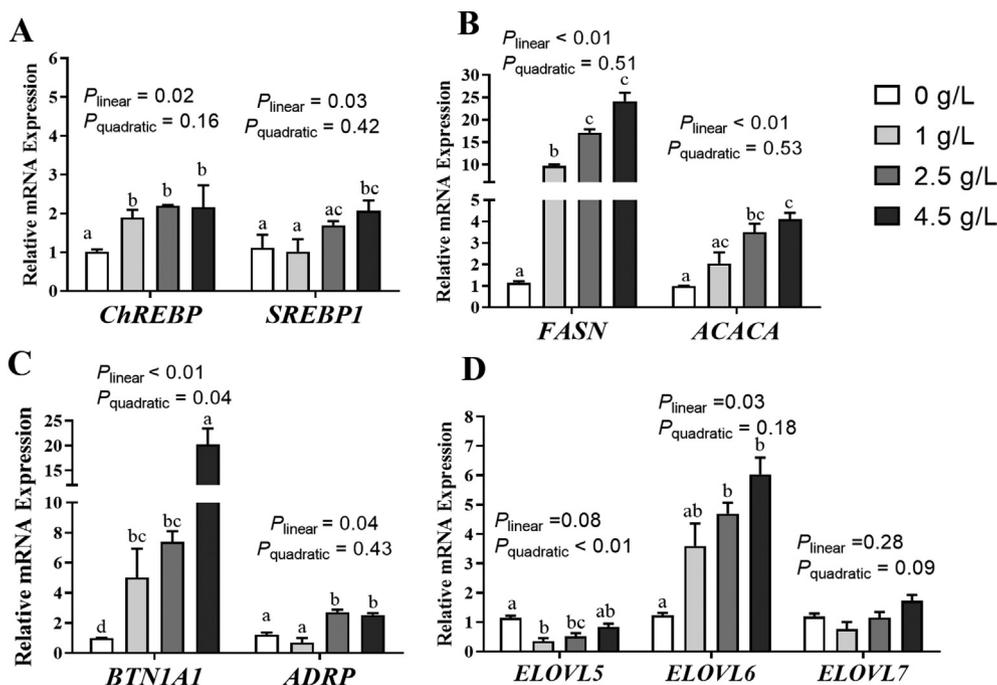
4. Discussion

It is demonstrated that infusion with an optimal supply of glucose increases milk fat yield in the lactating dairy goat (Cai et al., 2018). The present study is novel in that in vitro and vivo data were combined to investigate the mechanisms whereby glucose increases milk fat yield. The findings from the current study highlight that glucose promotes de novo fatty acid synthesis via the AMPK-ChREBP pathway; hence, it increases milk fat yield in the lactating mammary gland.

There is evidence suggesting a positive effect of glucose in promoting the synthesis of milk lactose and protein (Cai et al., 2018; Hurtaud et al., 2000). However, inconsistent results have been reported for the effect on milk fat when glucose was infused (Curtis et al., 2014; Hurtaud et al., 2000; Vanhatalo et al., 2003), suggesting an unclear mechanism. Recently, local mammary infusions were shown to be a suitable approach to investigate how glucose supply alters milk synthesis (Cai et al., 2018, 2020a).

Glucose is the major energy source for mammals, but when in excess it can be converted to fatty acids through de novo biosynthesis followed by esterification into lipid droplets. The lipid droplet-associated proteins ADRP and BTN1A1 are associated with milk fat secretion (Wilfling et al., 2014; Zhao et al., 2020). In the current study, the marked increase of both *ADRP* and *BTN1A1* in the mammary tissue infused with glucose and in the GMEC, agrees with the increase in milk fat yield in dairy goats (Cai et al., 2018). In addition, the increase in milk fat yield induced by glucose was confirmed by the increase in TAG content in the mammary cells.

Table 1
Effects of increasing mammary glucose supply on the profiling of fatty acids in the milk.

| Fatty acid (ratio)	Infused glucose, g/d			SEM	P-value	
	0	60	100		Linear	Quadratic
C4:0	0.006	0.002	0.008	0.001	0.380	0.049
C6:0	0.019	0.017	0.021	0.001	0.341	0.254
C8:0	0.029	0.031	0.039	0.002	0.039	0.297
C10:0	0.117	0.125	0.144	0.010	0.041	0.208
C12:0	0.191	0.061	0.083	0.067	0.364	0.450
C14:0	0.152	0.159	0.158	0.008	0.542	0.675
C16:0	0.407	0.390	0.354	0.013	0.049	0.180
C16:1	0.004	0.004	0.004	0.001	0.997	0.959
C18:0	0.102	0.085	0.081	0.100	0.274	0.718
C18:1trans	0.002	0.003	0.002	0.001	0.964	0.303
C18:1cis	0.064	0.058	0.058	0.007	0.606	0.798

Fig. 1. Glucose enhanced the expression of genes related to fatty acid de novo synthesis in the goat mammary gland. Lactating dairy goats were infused with glucose at doses of 0, 60 and 100 g/d in a 3 × 3 replicated Latin square design. Each repeated measure was 12 d periods consisting of 7 treatment days where glucose was infused each day for 5 h, followed by 5 transition days without glucose infusion. Mammary tissue was collected for mRNA expression analysis of genes related to transcription control (A), de novo fatty acid synthesis (B), milk lipid droplet formation (C) and fatty acid elongation (D). Value are means ± standard error of means. Different superscripts denote significant ($P < 0.05$) differences in abundance. ACACA = acetyl-CoA carboxylase alpha; ADRP = adipose differentiation-related protein; BTN1A1 = butyrophilin subfamily 1 member A1; ChREBP = carbohydrate response element binding protein; ELOVL5 = long-chain fatty-acyl elongase 5; ELOVL6 = long-chain fatty-acyl elongase 6; ELOVL7 = long-chain fatty-acyl elongase 7; FASN = fatty acid synthase; SREBP1 = sterol regulatory element binding transcription factor 1.


Fig. 2. Glucose enhanced the expression of genes related to fatty acid de novo synthesis in goat mammary epithelial cells. After a 12-h starvation, cells were incubated with basic medium containing glucose (D9434, Sigma, USA) at 0, 1, 2.5, and 4.5 g/L for 24 h. Cells were collected for mRNA expression of genes related to transcription control (A), de novo fatty acid synthesis (B), milk lipid droplet formation (C) and fatty acid elongation (D). Value are means ± standard error of means. Different superscripts denote significant ($P < 0.05$) differences in abundance. ACACA = acetyl-CoA carboxylase alpha; ADRP = adipose differentiation-related protein; BTN1A1 = butyrophilin subfamily 1 member A1; ChREBP = carbohydrate response element binding protein; ELOVL5 = long-chain fatty-acyl elongase 5; ELOVL6 = long-chain fatty-acyl elongase 6; ELOVL7 = long-chain fatty-acyl elongase 7; FASN = fatty acid synthase; SREBP1 = sterol regulatory element binding transcription factor 1.

In ruminant milk, the major substrates for de novo synthesis of lipid are the short-chain fatty acids, while glucose alters the biosynthesis of milk through unclear mechanisms. The upregulation of FASN and ACACA in mammary tissue and cells stimulated with glucose in the current study agrees with data in MCF7 cells (Koobotse et al., 2020). Consistent with the increased activity of
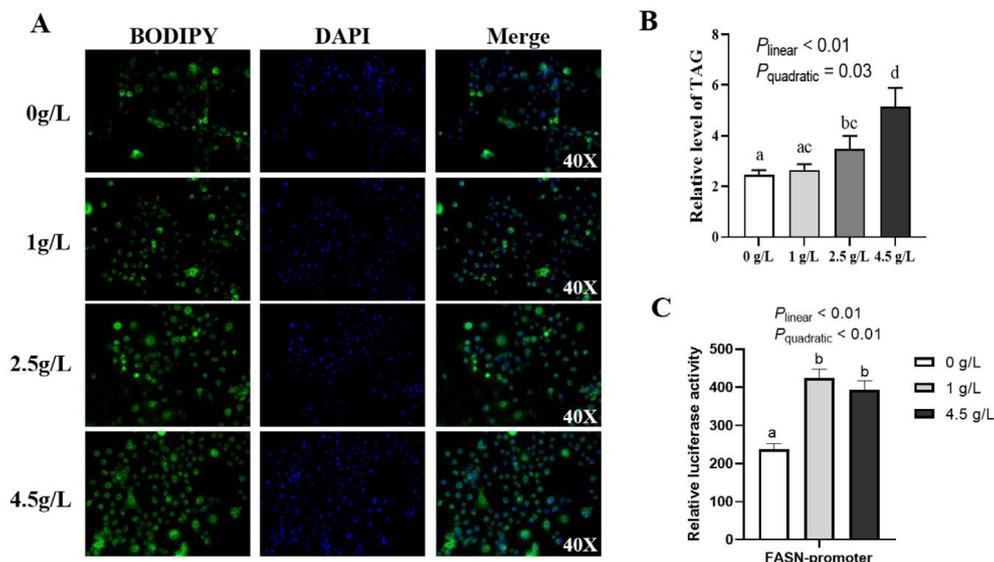
Fig. 3. Glucose promoted cellular triacylglycerol accumulation. After a 12-h starvation, cells were incubated with basic medium containing glucose at 0, 1, 2.5, and 4.5 g/L for 24 h. (A) BODIPY staining of cellular lipid. Cells were collected for BODIPY straining (green). The nucleus was stained using DAPI (blue). (B) Quantification of total triacylglycerol (TAG) mass in glucose-treated goat mammary epithelial cells using image J software. (C) Cultured goat mammary epithelial cells were co-transfected with fatty acid synthase (FASN) promoter and Renilla vectors. After transfection for 24 h, cells were treated with glucose at 0, 1 and 4.5 g/L for 24 h. Relative luciferase activity is expressed relative to the control (0 μM) and normalized with Renilla luciferase activity, respectively. Value are means ± standard error of means. Different superscripts denote significant ($P < 0.05$) differences in abundance.
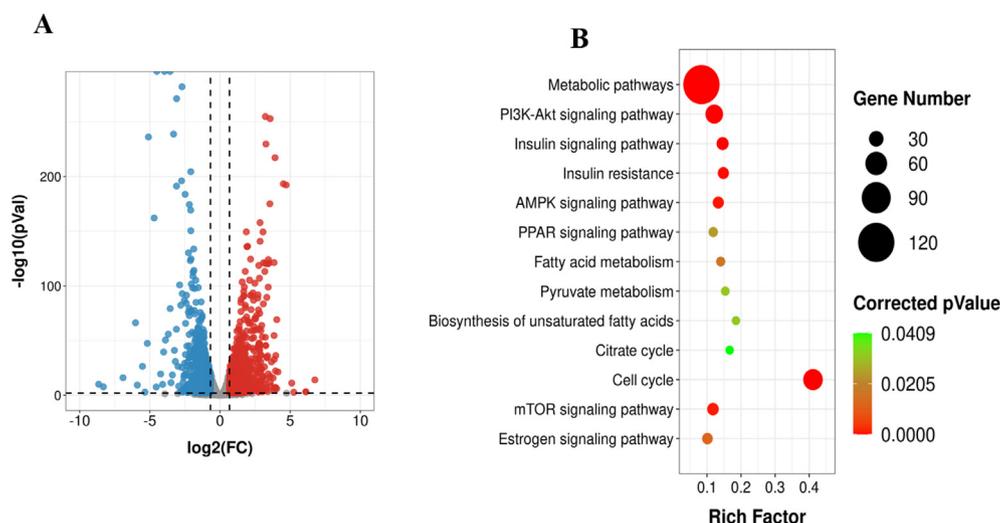

Fig. 4. Differential expression of mRNA and functional enrichment analysis via Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG). After a 12-h starvation, cells were incubated with basic medium containing glucose at 0 and 4.5 g/L for 24 h. (A) Differential expression of mRNA between 0 and 4.5 g/L. The cutoff for differential expression of genes was defined as 2-fold change and P-value < 0.05. (B) Significant categories from the KEGG analysis with the corrected P-value < 0.05.

FASN and ACACA, the level of medium-chain fatty acids (C8:0 and C10:0) increased in milk. These data are consistent with the finding in cows that glucose treatment significantly enhanced the profiles of medium-chain fatty acids (Hurtaud et al., 2000). The quadratic change of C4:0 is due to higher milk production at 60 g glucose/d (Cai et al., 2018), and suggests glucose has a modest effect on the production of C4:0. Although no significant change for total lipids in milk were observed at 100 g glucose/d (Cai et al., 2018), a lower level of C16:0 was also observed in the current study. The decrease in concentration of C16:0 in the milk from goats treated with 100 g glucose/d may have occurred due to a lower mobilization of adipose tissue when glucose was infused; hence, reducing availability of this fatty acid for uptake by the mammary gland (Hurtaud et al., 2000).

These findings highlight a role for glucose in promoting de novo synthesis of fatty acids.

After activation by high levels of glucose, ChREBP together with its heterodimerization partner binds to the carbohydrate response element (ChoRE) in the promoter of lipogenic genes, including *FASN* and *ACACA* (Iizuka et al., 2020). The increased levels of *ChREBP* in mammary tissue and epithelial cells after treatment with high concentrations of glucose agree with previous data (Ortega-Prieto and Postic, 2019). Combined with the upregulation of *FASN* and *ACACA* in goat mammary tissue, the finding that knockdown of ChREBP decreased the level of *FASN* and *ACACA* in GMEC treated with glucose, suggesting they are target genes of ChREBP in the goat. This idea is also supported by the response observed in the
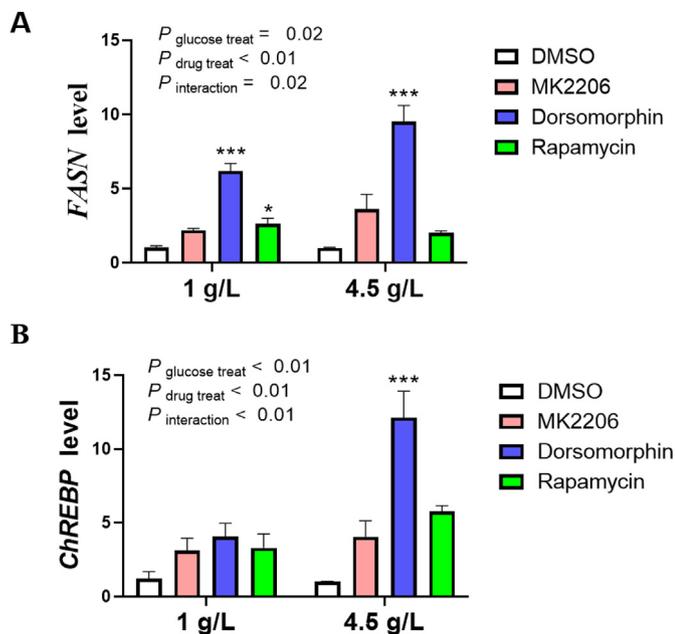
Fig. 5. Signaling pathway blocking assays. The drugs MK-2206 (S1078, Selleck Chemicals, Houston, TX, USA), rapamycin (S1039, Selleck Chemicals) and dorsomorphin (S7306, Selleck Chemicals) were used for the specific blocking of Akt, mTOR and AMPK activity, respectively. Goat mammary epithelial cells were treated with basic medium containing glucose at high (4.5 g/L) or low (1 g/L) levels and were incubated with dimethyl sulfoxide (DMSO; control group), MK-2206 (500 nM), rapamycin (10 nM) or dorsomorphin (10 μM) for 24 h. (A) The expression of *FASN*. (B) Expression of *ChREBP*. Values are means ± standard error of means. *$P < 0.05$. ***$P < 0.001$. AMPK = mammalian AMP-activated protein kinase; Akt = AKT serine/threonine kinase 1; *ChREBP* = carbohydrate response element binding protein; *FASN* = fatty acid synthase; mTOR = mechanistic target of rapamycin kinase.
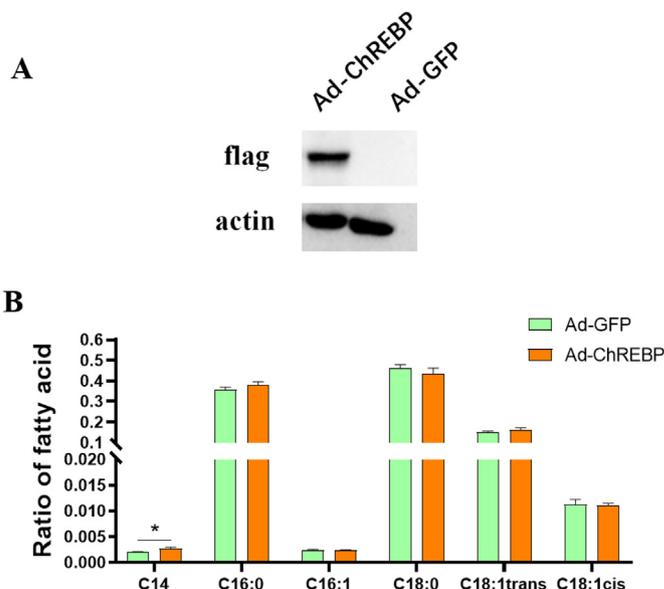
Fig. 7. Effect of ChREBP protein overexpression on fatty acid profiles in goat mammary epithelial cells. Cells were incubated with Ad-ChREBP or negative control (Ad-GFP) for 48 h. Then, cells were collected for fatty acid profiling. Values are means ± standard error of means. *$P < 0.05$. ChREBP = carbohydrate response element binding protein; Ad-GFP = recombinant adenovirus of green fluorescent protein as control; Ad-ChREBP = recombinant adenovirus expression ChREBP-flag.

upregulation of medium-chain (C14:0) concentrations in GMEC after the ChREBP protein level increased.

SREBP1 is another major transcription factor responsible for the coordinated induction of lipogenic genes involved in de novo
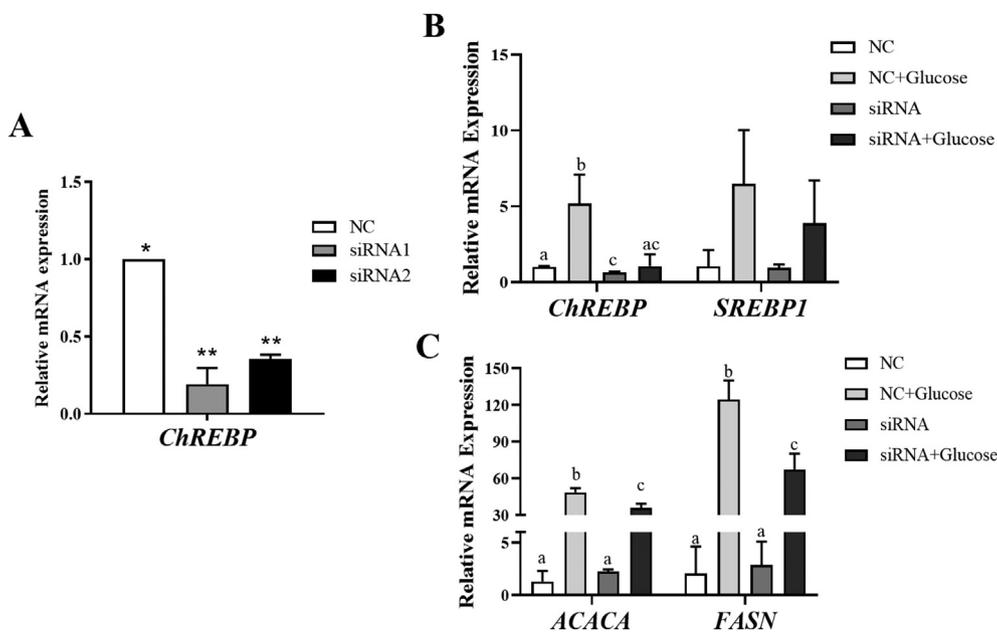
Fig. 6. Knockdown of *ChREBP* altered gene expression. Goat mammary epithelial cells (GMEC) were incubated with siRNA target ChREBP (siRNA) or negative control (siNC) for 48 h and then collected for mRNA extraction for siRNA screening. Transfected GMEC were cultured with glucose (4.5 g/L) or control (0 g/L) after 24 h of initial culture, and then harvested at 48 h (24 h later) for RNA extraction. In total, there were 4 treatments (negative control [NC], NC + Glucose, siRNA, siRNA + glucose) in the RNAi experiment. (A) The efficiency screening of siRNA to knockdown of *ChREBP*. *$P < 0.05$. **$P < 0.01$. (B) The expression of *ChREBP* and *SREBP1*. (C) The expression of *ACACA* and *FASN*. Values are means ± standard error of means. Different superscripts denote significant ($P < 0.05$) differences in abundance (panel B and C). *ACACA* = acetyl-CoA carboxylase alpha; *ChREBP* = carbohydrate response element binding protein; *FASN* = fatty acid synthase; *SREBP1* = sterol regulatory element binding transcription factor 1.

synthesis (Linden et al., 2018). The modest change of *SREBP1* mRNA level in both the mammary tissue and epithelial cells stimulated with glucose suggested that SREBP1 did not respond. ELOVL5 and ELOVL7 are the key enzymes involved in the elongation of very long chain fatty acids in goat mammary gland (Shi et al., 2017a, 2018, 2019), but modest changes in their expression were observed in mammary tissue and cells in the current study, suggesting they are not major targets of glucose. This idea is supported by the lack of change in concentrations of C16:1, C18:0, C18:1 and C18:2 in the milk.

The enzyme ELOVL6 is a target of ChREBP in mouse liver (Iizuka et al., 2020), which agrees with the significant upregulation of ELOVL6 we observed in the GMEC treated with glucose in the current study. The inconsistent result of ELOVL6 in mammary tissue and GMEC in response to glucose might have been caused by differences in cellular substrate levels in vivo and in vitro. Compared with SREBP1, the present data are highly suggestive that ChREBP is the main transcription factor responsive to high levels of glucose and thereby promotes de novo biosynthesis of fatty acids in the goat mammary gland.

AMPK is a sensor of glucose which is activated in response to increases in cellular AMP:ATP ratio. Activity of AMPK inactivates ACACA by phosphorylation, resulting in suppression of fatty acid biosynthesis in lipogenic tissues (Park et al., 2002) and GMEC (Zhang et al., 2018b). The significant alteration of AMPK activity with glucose treatment in the present study is consistent with the fact that AMPK is a sensor of glucose. The data in the present study demonstrate that blocking the AMPK pathway using dorsomorphin significantly upregulated the level of *FASN*, agreeing with the fact that activity of AMPK inhibits de novo synthesis of fatty acids (Zhang et al., 2018b). However, the mechanism whereby the AMPK pathway alters transcription of *FASN* is still unclear.

ChREBP is highly enriched in rodent liver and is known as a master regulator of lipid metabolism (Lin and Hardie, 2018). The binding of ChREBP to promoters of its target genes is decreased by AMPK-induced phosphorylation of ChREBP on residue Ser568 (Kawaguchi et al., 2002; Sato et al., 2016). In the present study, activation of the AMPK pathway by glucose uncovered from the RNA seq data agrees with data in mice and humans (Brun et al., 2020). The increase in transcription of *ChREBP* and *FASN* when AMPK was inhibited by dorsomorphin is suggestive that AMPK mediated by glucose elicits a negative regulation of de novo fatty acid synthesis. In addition, the increased abundance of ChREBP only at 4.5 g glucose/L revealed a potential dose—response for the activation of AMPK in GMEC, which is consistent with the fact that this protein responds to an increase in cellular AMP:ATP ratio (Lin and Hardie, 2018). The Akt and mTOR signaling pathways are involved in de novo fatty acid synthesis via controlling the activity of SREBP1, FASN and ACACA in the mammary gland (Osorio et al., 2016; Zhang et al., 2018a). The finding that inhibition of Akt and mTOR using drugs has a modest effect on the expression of *ChREBP* and *FASN* suggests that the 2 pathways might not play a role in de novo fatty acid synthesis mediated by glucose in vitro. This idea is partially supported by the lack significant change in the expression of *SREBP1* in vitro and in vivo after stimulation with glucose.

5. Conclusion

The goat mammary gland responds to high levels of glucose by increasing the de novo synthesis of fatty acids and milk fat yield. The combination of in vitro and vivo data in the current study reveal that de novo fatty acid synthesis mediated by glucose is dependent on the AMPK-ChREBP axis in the goat mammary gland. The present data clarify a mechanism whereby glucose promotes de novo fatty acid synthesis in the mammary gland. Together, results from the current study provide possible strategies to alter the fatty acid composition as well as improve ruminant milk quality.

Author contributions

Hengbo Shi: Methodology, Funding acquisition, Writing — original draft; **Nannan Jiang**: Methodology, Investigation, Data curation; **Ling Wei**: Methodology, Investigation, Data curation; **Jie Cai**: Methodology; **Wenying Zhang**: Data curation; **Qianming Jiang**: Writing — review & editing; **Juan J. Loor**: Writing — review & editing; **Jianxin Liu**: Project administration.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2022.05.003.

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