doi: 10.17221/8595-CJAS

Effect of the ratios of acetate and β-hydroxybutyrate on the expression of milk fat- and protein-related genes in bovine mammary epithelial cells

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ABSTRACT: The objective of this study was to evaluate the effects of the different ratios of acetate and β-hydroxybutyrate (BHBA) on cell viability, triacylglycerol (TAG) content, and mRNA expression of the genes related to lipid and protein synthesis in bovine mammary epithelial cells (BMECs). Primary cells were isolated from the mammary glands of Holstein dairy cows and were passaged twice. Then, the cells were cultured with different ratios of acetate and BHBA (1 : 3, 1 : 2, 1 : 1, 2 : 1, 3 : 1, 4 : 1, and 1 : 1, Group 1 to Group 7, respectively) for 48 h, and the fetal bovine serum in the culture media was replaced with fatty acid-free bovine serum albumin (BSA) (1 g/l). The control culture media contained only fatty acid-free BSA without unsaturated fatty acids (0mM). Cell viability was not affected by adding different ratios of acetate and BHBA, but TAG accumulation was significantly influenced by supplementing the culture media with different ratios of acetate and BHBA. The expression levels of genes related to milk fat (*FASN, ACACA, CD36, SCD, FABP3, LPL, PPARG,* and *SPEBF1)* and milk protein-related genes (*CSN1S1*, *CSN3*, *mTOR*, *4E-BP1, S6KB1*, *STAT5*, *JAK2,* and *LEPTIN*) were significantly affected by the addition of different ratios of acetate and BHBA to the BMECs. Our results suggested that Groups 3 and 4 (1 : 1 and 2 : 1) had a stronger acceleration of milk fat synthesis, and Group 4 (2 : 1) had the strongest effect. The expression of the *CSN1S1* and *LEPTIN* mRNAs was more effectively promoted in Groups 3 and 4 (1 : 1 and 2 : 1), and Group 3 (1 : 1) had the strongest acceleration. Expressions of genes related to milk protein synthesis (*mTOR*, *4E-BP1*, *S6KB1, JAK2,* and *STAT5*) were up-regulated using a ratio of acetate and BHBA of 2 : 1. Taken together, the 2 : 1 ratio of acetate and BHBA had the best effect for both the milk fat synthesis and milk protein synthesis genes. However, further studies are necessary to elucidate the mechanism for regulating milk fat and protein synthesis by different ratios of acetate and BHBA.

Keywords: ratio of short chain fatty acids; milk fat precursor; dairy cow; milk fat; milk protein; gene expression

List of abbreviations: BHBA = β-hydroxybutyrate, TAG = triacylglycerol, BMECs = bovine mammary epithelial cells, FBS = fetal bovine serum, UFA = unsaturated fatty acids, BSA = bovine serum albumin, FAs = milk fatty acids, *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase, *FASN* = fatty acid synthase, *ACACA* = acetyl-CoA carboxylase, *SCD* = stearoyl-CoA desaturase, *CD36* = cluster of differentiation 36, *FABP3* = fatty acid-binding protein 3, *LPL* = lipoprotein lipase, *PPARG* = peroxisome proliferator-activated receptor γ, *SREBF1* = sterol regulatory element binding transcription factor 1, *CSN1S1* = αs1-casein, *CSN3* = κ-casein, *mTOR* = mammalian target of rapamycin, *4EBP1* = eukaryotic translation initiation factor 4E, *RPS6KB1* = ribosomal protein S6 kinase 1, *STAT5* = signal transducer and activator of transcription 5, *JAK2* = Janus kinase 2, *LEPTIN* = leptin, PBS = phosphate buffer saline, DMEM/F12 = Dulbecco's modified Eagle's medium/F12, MTT = methyl thiazolyl tetrazolium, RGR = relative cell growth rate, CLA = conjugated linoleic acid, LCFA = long chain fatty acid, MFP = milk fat precursor, 4E-BP1 = eukaryotic initiation factor 4E-binding protein 1, S6K1 = ribosomal protein S6 kinase-1

Supported by the National Key Basic Research Program of China (Project No. 2011CB100803).

doi: 10.17221/8595-CJAS

INTRODUCTION

Milk protein and fat content as well as the milk fatty acids (FAs) composition are the important indicators for measuring milk quality (Palmquist 2006; Harvatine et al. 2009). Improving milk fat synthesis and composition is an important method for enhancing milk quality (Keenan and Mather 2006). Therefore, it is important to discuss the regulation of milk component precursors on milk fat and protein synthesis to guide future studies aimed at improving milk quality and ensuring milk production. Acetate and β-hydroxybutyric acid (BHBA) are the main short-chain fatty acids produced by microbial fermentation in the rumen and hindgut that are taken up into the blood. Circulating acetate can be taken up by the mammary gland and incorporated into milk FAs (Chilliard et al. 2000; Bernard et al. 2008; Jacobs et al. 2013). Almost 50% of the FAs in ruminant milk are synthesized in the mammary gland from acetate and BHBA (Mensink et al. 2003; Gebauer et al. 2007). It was reported that acetate affects milk fat synthesis and lipogenic gene expression (Purdie et al. 2008; Maxin et al. 2011; Jacobs et al. 2013) as well as milk protein synthesis (Purdie et al. 2008). The limited information available indicated that BHBA stimulated the accumulation of triglycerides (TAG) in the BMECs, but it was not involved in forming lipid droplets (Yonezawa et al. 2004). Data in the same experiment also indicated that BHBA inhibited leptin expression and lipid synthesis in the BMECs (Yonezawa et al. 2004). Jacobs et al. (2013) found that BHBA upregulated the expression of stearoyl-CoA desaturase 1 (*SCD1*, 44%), acetyl-coenzyme A carboxylase α (*ACACA*, 28%), and fatty acid synthase (*FASN*, 29%). BMECs treated with different concentrations of BHBA (0–1.25mM) significantly increased TAG accumulation and upregulated the expression of *FASN* and *ACACA* mRNAs.

Our previous work indicated that the ratios of acetate and BHBA entering into mammary artery were different when the dairy cows were fed different feedstuffs, which further affected milk fat and milk protein content, and that milk protein and milk fat content were increased when the ratio of acetate and BHBA entering into mammary artery was 2 : 1. A recent study (Jacobs et al. 2013) found that a combination of acetate and BHBA (5mM and 5mM, 1 : 1) also increased the transcript levels of *SCD1*, *ACACA*, and *FASN* compared to the control (0mM acetate and BHBA). Sheng et al. (2015) indicated that appropriate ratios of unsaturated fatty acids (UFAs) in milk fat presursor (MFP) could regulate milk fat and milk protein synthesis, and ratios of UFAs entering into mammary artery kept almost the same ratios. Thus the varying ratios of acetate and BHBA likely had an impact on milk fat synthesis, but little information is available. Consequently, the present study was conducted to determine the influence of the acetate and BHBA ratios on cell proliferation, TAG accumulation, and the mRNA expression of αs1-casein (*CSN1S1*), κ-casein (*CSN3*), and genes involved in lipid and protein synthesis to further illustrate the regulatory mechanism by which the acetate and BHBA ratios regulate milk fat and protein synthesis.

MATERIAL AND METHODS

Cell culture and treatments. The primary cells were isolated from the mammary glands of 8 Holstein dairy cows (primiparous and mid-lactating) at a local abattoir. Several pieces (of approximately 1 cm^3) of the mammary gland tissues were aseptically removed and washed with cold phosphate buffered solution (PBS) (HyClone, NWJ0467, Shanghai, China) containing 100 U/ml penicillin and 100 μg/ml streptomycin. The mammary tissue fragments were minced with sterile scissors and digested by collagenase Ⅱ for 1 h at 37°C and 5% CO_2 and were shaken every 20 min. The digested tissues were filtered through a 200 micron nylon mesh to remove large tissue fragments, the filtered liquid was centrifuged at 179 *g* for 5 min, and the supernatant was removed. The cell pellet was resuspended in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) (GIBCO; Gibco BRL Co. Ltd., USA) media supplemented with 10% fetal bovine serum (FBS) (GIBCO), 0.5% insulin (Sigma-Aldrich, St. Louis, USA), 4 μg/ml prolactin (Sigma-Aldrich), 1 μg/ml hydrocortisone (Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich), and 100 μg/ml streptomycin (GIBCO) at 37°C in a humidified atmosphere of 5% CO_2. The cells were passaged twice and then cryopreserved.

The BMECs were randomly allocated to eight treatments, including one control and seven treatment groups. For all treatments, the FBS in the culture solution was replaced with fatty acid-free bovine serum albumin (BSA) (1 g/l), and the

doi: 10.17221/8595-CJAS

Table 1. Experimental design

Treatment	Acetate:BHBA	Level of acetate (mM)	Level of BHBA (mM)
Control	0	0	0
Group 1	1:3	2.08	6.24
Group 2	1:2	2.77	5.54
Group 3	1:1	4.16	4.16
Group 4	2:1	5.54	2.27
Group 5	3:1	6.24	2.08
Group 6	4:1	6.66	1.66
Group 7	5:1	6.93	1.39

BHBA = β-hydroxybutyrate

cells were treated with various ratios of acetate and BHBA (acetate:BHBA – 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, and 5:1) in the culture media for 48 h at 37°C and 5% CO_2. The control culture solution contained 0mM acetate and BHBA (Table 1). We chose 6mM and 2.32mM as the concentrations of the acetate and BHBA, according to previous *in vitro* data, and the total fatty acid concentration was 8.32mM (Table 1). Seven treatment groups, Group 1–Group 7, were subsequently designed. Each treatment was performed six times.

Cell viability assay. Cell viability was determined by the methyl thiazolyl tetrazolium (MTT) assay (Liu et al. 1997). The cells were plated in each well of 96-well plates at 1×10^4 cells/well, and each treatment was performed six times. Briefly, after a 48-h incubation, 20 ul of 5 mg/ml MTT was added to each well and incubated for 4 h at 37°C. The formazan crystals were then dissolved in 100 µl dimethyl sulphoxide (DMSO). The absorbance of each well was read at 490 nm using a microplate reader (BioTek, Vermont, USA). The relative cell growth rate (RGR) was calculated according to the formula:

$$RGR = (A_{490} \text{ of treated cells}/A_{490} \text{ of control cells}) \times 100\%$$

Oil Red O staining and analysis. Intracellular triglyceride accumulation was measured by Oil Red O staining, according to the method of Ramirez-Zacarias (1992). The Oil Red O method was performed to detect the lipid droplet staining in cells. The cells were seeded (5×10^4 cells/well) and treated with different ratios of acetate and BHBA for 48 h. After washing two times with PBS, the cells were fixed with 4% paraformaldehyde for 1 h. The fixed cells were washed with PBS, and stained with 0.5 ml of Oil Red O for 2 h. The cells were then rinsed three times with PBS. For quantification, the cells were incubated with 0.3 ml of isopropanol for 20 min to extract the Oil Red O staining, and the optical density was measured at 510 nm by a microplate reader (BioTek).

RNA extraction and real-time PCR. The cells were plated in 6-well plates at 1×10^5 cells/well in medium. After 48 h, the culture medium was changed for DMEM/F12 induction medium containing different concentrations of acetate and BHBA, and FBS was replaced with fatty acid-free BSA (1 g/l). The cultures were maintained at 37°C and 5% CO_2 for 48 h. Total RNA was harvested from the cells using the RNAprep pure Cell Kit (DP430) (Tiangen, Beijing, China), according to the manufacturer's instructions. RNA quality and purity were assessed by 2% agarose gel electrophoresis for 20 min at 100 V and compared to DNA Marker DL 1000 standards. The cDNAs for real-time PCR were generated in a 10 µl volume using the PrimeScript RT Master Mix (No. DRR036A; TaKaRa, Tokyo, Japan). Real-time PCR was performed in 20 µl reactions containing 10 µl of 2× SYBR® Premix Ex Taq™, 2 µl of cDNA, 0.4 µl each of the 10µM forward and reverse primers (Table 2), and 0.8 µl of RNase free water. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the internal control. The reactions were performed in a MxPro-Mx3000P Real-time PCR machine (Agilent Technologies, Santa Clara, USA) with an initial denaturing step at 95°C for 30 s followed by 40 cycles at 95°C for 30 s (denaturation), at 60°C for 30 s (annealing), and at 72°C for 20 s (extension). The quality and specificity of the PCR products were assessed by melting curve analysis and subsequent agarose gel electrophoresis. The quantitative real-time PCR data were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Statistical analysis. The data were analyzed by one-way ANOVA and Duncan's Multiple Range Test using the SAS software (Statistical Analysis System, Version 8, 2000) to test the statistically significant differences between the treatments. A level of $P < 0.05$ was significant, and a level of $P < 0.10$ was a statistical trend.

RESULTS

Cell viability. The RGR was not affected after adding different ratios of acetate and BHBA ($P =$

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Table 2. Primer sequences

Genes	Primer sequences (5'-3')	GenBank Acc. No.	Length (bp)	Tm (°C)
GAPDH	F: GGGTCATCATCTCTGCACCT R: GGTCATAAGTCCCTCCACGA	XM_001252479	177	60
FASN	F: AGGACCTCGTGAAGGCTGTGA R: CCAAGGTCTGAAAGCGAGCTG	NM_001012669	85	62
ACACA	F: CATCTTGTCCGAAACGTCGAT R: CCCTTCGAACATACACCTCCA	AJ132890	101	58
SCD	F: TCCTGTTGTTGTGCTTCATCC R: GGCATAACGGAATAAGGTGGC	AY241933	101	58
CD36	F: CCTCTTGGCAACCACTTTCA R: GCTTTGACACCCGAGTAACG	BC103112	113	62
FABP3	F: GAACTCGACTCCCAGCTTGAA R: AAGCCTACCACAATCATCGAAG	DN518905	102	62
LPL	F: ACACAGCTGAGGACACTTGCC R: GCCATGGATCACCACAAAGG	BC118091	101	60
PPARG	F: CCAAATATCGGTGGGAGTCG R: ACAGCGAAGGGCTCACTCTC	NM_181024	101	62
SREBF1	F: CTGACGACCGTGAAAACAGA R: AGACGGCAGATTTATTCAACTT	NM_001113302	334	60
CSN1S1	F: ACATCCTATCAAGCACCAAGGACTC R: GACGAAATGCTTTCAGCTTCCA	NM_181029	192	60
CSN3	F: CCAGGAGCAAAACCAAGAAC R: TGCAACTGGTTTCTGTTGGT	NM_174294	148	56
mTOR	F: TGAACTGGAGGCTGATGGACAC R: TGACTGGCCAGCAGAGTAGGAA	XM_001788228	83	58
4EBP1	F: GGCAGGCGGTGAAGAGTC R: CCTGGGCTGCGGGAT	BC120290	302	58
RPS6KB1	F: CAAGCTTGCATGCTAATTTGTCC R: TTGAGTCCTGATCATGTCGAAGA	DN544771	101	62
STAT5	F: AAGACCCAGACCAAGTTCGC R: AGCACCGTGGCAGTAGCAT	NM_001012673	422	60
JAK2	F: TGAAGAAAACAGGTAATCAGACTGG R: AACATTTTCTCGCTCAACAGCA	DT897449	101	60
LEPTIN	F: GGAGAAGGTCCCGGAGGTT R: GGACCAGACATTGGCGATCT	NM_173928	102	58

GAPDH = glyceraldehyde 3-phosphate dehydrogenase, *FASN* = fatty acid synthase, *ACACA* = acetyl-CoA carboxylase, *SCD* = stearoyl-CoA desaturase, *CD36* = cluster of differentiation 36, *FABP3* = fatty acid-binding protein 3, *LPL* = lipoprotein lipase, *PPARG* = peroxisome proliferator-activated receptor γ, *SREBF1* = sterol regulatory element binding transcription factor 1, *CSN1S1* = αs1-casein, *CSN3* = κ-casein, *mTOR* = mammalian target of rapamycin, *4EBP1* = eukaryotic translation initiation factor 4E, *RPS6KB1* = ribosomal protein S6 kinase 1, *STAT5* = signal transducer and activator of transcription 5, *JAK2* = Janus kinase 2, *LEPTIN* = leptin

0.243) (Table 3), but the RGR of Group 4 was slightly higher than the control. Moreover, the cell viability in the other treatment groups was downregulated.

TAG content. Different ratios of acetate and BHBA in the culture medium significantly affected the intracellular TAG accumulation ($P < 0.0001$) (Table 3). The TAG accumulation was maximal

doi: 10.17221/8595-CJAS

Table 3. Effect of ratios of acetate and BHBA on cell viability and TAG content in BMECs

Item	Treatment								SEM	*P*-value
	Control	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7		
RGR	100.000	95.013	95.602	98.671	106.704	92.472	84.342	83.730	0.078	0.243
TAG	0.102^{c}	0.097^{c}	0.099^{c}	0.115^{b}	0.127^{a}	0.114^{b}	0.100^{c}	0.100^{c}	0.002	< 0.0001

BHBA = β-hydroxybutyrate, TAG = triacylglycerol, BMECs = bovine mammary epithelial cells, RGR= relative growth rate, Group 1 = acetate/BHBA ratio was 1 : 3, Group 2 = acetate/BHBA ratio was 1 : 2, Group 3 = acetate/BHBA ratio was 1 : 1, Group 4 = acetate/BHBA ratio was 2 : 1, Group 5 = acetate/BHBA ratio was 3 : 1, Group 6 = acetate/BHBA ratio was 4 : 1, Group 7 = acetate/BHBA ratio was 5 : 1

$^{a–c}$means within a row without the same superscripts differ significantly ($P < 0.05$)

in Group 4 and was significantly higher than in the control and the other treatments. The TAG content in Groups 3 and 5 was significantly up-regulated compared to the control and the other treated groups, but it was lower than in Group 4. The values in Groups 1, 2, 6, and 7 were not different from the control.

mRNA abundance of genes associated with lipid synthesis in BMECs. There were significant influences of the different ratios of acetate and BHBA on *FASN, ACACA, SCD, CD36, FABP3, LPL, PPARG,* and *SREBF1* mRNA expression levels (Table 4). The *FASN*, *SCD,* and *CD36* mRNA expression levels in the BMECs were up-regulated after adding different ratios of acetate and BHBA ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$), and the expression values for all of the treated groups were significantly higher than for the control. The expression of *FASN* in Groups 4 and 5 was higher than in the control; Group 4 attained the highest value, and Group 1 had the lowest value, Group 2 followed. The expressions of *SCD* and *CD36* were up-regulated in all treatment groups compared to the control. Groups 3–5 had a greater effect on the *SCD* expression, while Groups 2 and 3 promoted the expression of *CD36* more than the other groups.

The abundance of the *ACACA* mRNA in Groups 4–7 was dramatically higher than in the control and the other treatments ($P < 0.0001$). Group 4 had the highest value, while groups 1 and 2 demonstrated the opposite tendency. There was no difference between Group 3 and the control.

The abundance of *FABP3* mRNA in Groups 4 and 5 was dramatically higher than that observed in

Table 4. Effect of ratios of acetate and BHBA on the mRNA expression of genes involved in milk fat synthesis in BMECs

Gene	Treatment								SEM	*P*-value
	Control	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7		
FASN	1.00^{h}	1.64^{g}	1.89^{f}	2.28^{e}	3.83^{a}	3.06^{b}	2.68^{c}	2.47^{d}	0.05	< 0.0001
ACACA	1.00^{e}	0.56^{g}	0.74^{f}	1.11^{e}	3.18^{a}	2.17^{b}	1.59^{c}	1.32^{d}	0.06	< 0.0001
SCD	1.00^{f}	1.22^{de}	1.34^{bc}	1.73^{a}	1.41^{b}	1.41^{b}	1.18^{e}	1.15^{e}	0.04	< 0.0001
CD36	1.00^{e}	1.48^{c}	1.64^{b}	1.73^{a}	1.47^{c}	1.40^{d}	1.37^{d}	1.36^{d}	0.04	< 0.0001
FABP3	1.00^{c}	0.41^{f}	0.51^{e}	0.63^{d}	1.61^{a}	1.20^{b}	1.03^{c}	0.62^{d}	0.06	< 0.0001
LPL	1.00^{c}	0.93^{c}	1.01^{c}	1.28^{b}	1.97^{a}	0.84^{d}	0.49^{e}	0.47^{e}	0.06	< 0.0001
PPARG	1.00^{e}	0.52^{f}	0.64^{f}	2.04^{b}	2.59^{a}	1.76^{c}	1.45^{d}	1.05^{e}	0.05	< 0.0001
SREBF1	1.00^{d}	0.46^{f}	0.75^{e}	2.02^{b}	2.33^{a}	1.60^{c}	1.03^{d}	0.56^{f}	0.04	< 0.0001

BHBA = β-hydroxybutyrate, BMECs = bovine mammary epithelial cells, *FASN* = fatty acid synthase, *ACACA* = acetyl-CoA carboxylase, *SCD* = stearoyl-CoA desaturase, *CD36* = cluster of differentiation 36, *FABP3* = fatty acid-binding protein 3, *LPL* = lipoprotein lipase, *PPARG* = peroxisome proliferator-activated receptor γ, *SREBF1* = sterol regulatory element binding transcription factor 1, Group 1 = acetate/BHBA ratio was 1 : 3, Group 2 = acetate/BHBA ratio was 1 : 2, Group 3 = acetate/BHBA ratio was 1 : 1, Group 4 = acetate/BHBA ratio was 2 : 1, Group 5 = acetate/BHBA ratio was 3 : 1, Group 6 = acetate/BHBA ratio was 4 : 1, Group 7 = acetate/BHBA ratio was 5 : 1

$^{a–h}$means within a row without the same superscripts differ significantly ($P < 0.05$)

doi: 10.17221/8595-CJAS

Table 5. Effect of ratios of acetate and BHBA on the mRNA expression of genes involved in milk protein synthesis in BMECs

Gene	Treatment								SEM	*P*-value
	Control	Group 1	Group2	Group 3	Group 4	Group 5	Group 6	Group 7		
CSN1S1	1.00^{d}	1.17^{c}	1.51^{b}	2.44^{a}	1.55^{b}	1.16^{c}	0.73^{e}	0.67^{e}	0.08	< 0.0001
CSN3	1.00^{d}	0.54^{e}	0.61^{e}	0.56^{e}	0.98^{d}	1.72^{a}	1.56^{b}	1.13^{c}	0.10	< 0.0001
mTOR	1.00^{d}	0.31^{f}	0.76^{e}	1.27^{c}	1.62^{a}	1.48^{b}	1.47^{b}	1.33^{c}	0.04	< 0.0001
4E-BP1	1.00^{d}	0.83^{e}	0.85^{e}	1.30^{c}	1.75^{a}	1.44^{b}	0.59^{f}	0.29^{g}	0.05	< 0.0001
S6KB1	1.00^{d}	0.47^{f}	0.76^{e}	1.45^{b}	1.85^{a}	1.47^{b}	1.36^{c}	1.01^{d}	0.04	< 0.0001
STAT5	1.00^{c}	0.42^{f}	0.74^{d}	1.23^{b}	1.61^{a}	1.22^{b}	0.61^{e}	0.42^{f}	0.05	< 0.0001
JAK2	1.00^{e}	0.53^{g}	0.84^{f}	1.28^{c}	1.96^{a}	1.55^{b}	0.94^{d}	0.85^{f}	0.07	< 0.0001
LEPTIN	1.00^{d}	0.53^{f}	0.74^{e}	2.50^{a}	2.17^{b}	1.62^{c}	1.50^{c}	0.92^{d}	0.05	< 0.0001

BHBA = β-hydroxybutyrate; BMECs = bovine mammary epithelial cells, *CSN1S1* = αs1-casein, *CSN3* = casein κ, *mTOR* = mammalian target of rapamycin, *4EBP1* = eukaryotic translation initiation factor 4E, *RPS6KB1* = ribosomal protein S6 kinase 1, *STAT5* = signal transducer and activator of transcription 5, *JAK2* = Janus kinase 2, *LEPTIN* = leptin, Group 1 = acetate/BHBA ratio was 1 : 3, Group 2 = acetate/BHBA ratio was 1 : 2, Group 3 = acetate/BHBA ratio was 1 : 1, Group 4 = acetate/BHBA ratio was 2 : 1, Group 5 = acetate/BHBA ratio was 3 : 1, Group 6 = acetate/BHBA ratio was 4 : 1, Group 7 = acetate/BHBA ratio was 5 : 1

$^{a–g}$means within a row without the same superscripts differ significantly ($P < 0.05$)

the control and the other treatments ($P < 0.0001$), and Group 4 had the highest value. Compared to the control and the other treatments, the *FABP3* mRNA expression in the BMECs was dramatically down-regulated in Groups 1, 2, 3 and 7 ($P < 0.0001$), while there was no effect in Group 6. The abundance of the *LPL* mRNA in Groups 3 and 4 was dramatically increased, whereas in Groups 5–7 the expression of *LPL* was significantly decreased compared to the control and the other treated groups ($P < 0.0001$). However, there was no difference between Groups 1 and 2 and the control.

The expression of the *PPARG* and *SPEBF1* mRNAs in BMECs was significantly affected by supplementing the cultures with different ratios of acetate and BHBA ($P < 0.0001$). Groups 3–6 expressed more *PPARG* mRNA than the control and the other treated groups; Group 4 attained the highest value. Groups 1 and 2 exhibited notably lower expression of the *PPARG* mRNA than the control and the other treatments, while Group 7 was not significantly different from the control. Groups 3–5 expressed more *SPEBF1* mRNA than the control and the other treated groups; Group 4 attained the highest value. However, Groups 1, 2, and 7 dramatically inhibited the mRNA expression of *SPEBF1*, and no difference was observed between Group 6 and the control.

mRNA abundance of genes associated with protein synthesis in BMECs. Table 5 shows that the *CSN1S1* mRNA expression in the BMECs was significantly modulated by adding different ratios of acetate and BHBA ($P < 0.001$). Group 3 attained the highest value, which was significantly higher than in the control and the other treated groups. The values in Groups 1, 2, 4, and 5 were also higher than in the control and the other treated groups, but Groups 6 and 7 exhibited the opposite tendency and the values were significantly lower than in the control and other treated groups. The *CSN3* mRNA expression in the BMECs was also significantly affected after adding different ratios of acetate and BHBA ($P < 0.0001$). Groups 5–7 had significantly higher values than the control and the other treatments. Group 5 achieved the highest value, while Groups 1–3 had the opposite tendency and did not exhibit differences.

The abundance of the *mTOR*, *4E-BP1, S6KB1, STAT5,* and *JAK2* mRNAs in Group 4 was dramatically affected after adding different ratios of acetate and BHBA ($P < 0.0001$). The abundance of the *mTOR* mRNA in Groups 3–7 was dramatically higher than in the control and the other treatments, and Group 4 had the highest value. In contrast, Groups 1 and 2 demonstrated the opposite tendency ($P < 0.0001$). Groups 3–5 had

doi: 10.17221/8595-CJAS

higher *4E-BP1* mRNA expression, and Group 4 attained the highest value. Groups 1, 2, 6, and 7 exhibited notably lower expression of the *4E-BP1* mRNA than the control and the other treatments ($P < 0.0001$). The *S6KB1* mRNA expression in Groups 3–6 was significantly higher than in the control and Groups 1, 2 and 7, and the expression levels of Groups 1 and 2 were dramatically lower than of the control ($P < 0.0001$). The *STAT5* and *JAK2* mRNA expression data demonstrated that Groups 3–5 had significantly increased values compared to the control, and Group 4 had the best effect. In contrast, the values in Groups 1, 2, 6, and 7 were dramatically lower than in the control ($P < 0.0001$).

In addition, Groups 3–6 had dramatically higher *LEPTIN* mRNA expression ($P < 0.0001$); Group 3 attained the highest value. However, Groups 1, 2, and 7 exhibited the opposite trend, and Group 1 attained the lowest value.

DISCUSSION

Kadegowda et al. (2008) examined the effect of an abomasal infusion of butterfat, conjugated linoleic acid (CLA) or long chain fatty acids (LCFAs) on milk fat and found that abomasally infusing CLA decreased the MFP and yield, while abomasally infusing butterfat increased the MFP and yield. However, infusing LCFAs had no effect on milk fat synthesis, suggesting that MFP containing the same FAs composition as milk fat could provide a perfect precursor for milk fat synthesis. Sheng et al. (2015) indicated that appropriate ratios of UFAs in MFP could regulate milk fat and milk protein synthesis, and ratios of UFAs entered into mammary artery kept almost the same ratios as in the MFP. Acetate and BHBA are the main MFP for milk FAs *de novo* synthesis, and had effect on milk fat and milk protein synthesis in the BMECs. Jacobs et al. (2013) found that the 1 : 1 ratio of acetate and BHBA increased the transcript levels of *SCD1*, *ACACA*, and *FASN*. Our previous work indicated that the ratios of acetate and BHBA entering into mammary artery were 3 : 1, 2 : 1, and 1 : 1 when the dairy cows were fed different feedstuffs, which further affected milk fat and milk protein content. When the ratio was 2 : 1, milk protein and milk fat content were increased. Thus there was a hypothesis that ideal model of MFP could regulate milk fat and milk protein synthesis, but little information is available. Therefore, the present research examined the effects of different ratios of acetate and BHBA on the expression of milk fat- and protein-related genes in BMECs. We chose 1 : 3, 1 : 2, 1 : 1, 2 : 1, 3: 1, 4 : 1, and 5 : 1 as treatment ratios of acetate and BHBA.

Milk fat is primarily composed of TAG secreted from the BMECs in variably sized droplets; therefore, the TAG content directly reflected milk fat synthesis. The current research indicated that different ratios of acetate and BHBA significantly influenced TAG accumulation. Additionally, when the ratios of acetate and BHBA were 1 : 1, 2 : 1, and 3 : 1 (Groups 3–5), TAG accumulation was up-regulated, particularly in Group 4, which reached significantly higher levels than the control or the other treatments. When the ratios were 1 : 3, 1 : 2, 4 : 1, and 5 : 1, there was no significant difference from the control. Moreover, the ratios of 1 : 1, 2 : 1, and 3 : 1 promoted milk fat synthesis, and the 2 : 1 ratio had the best effect. However, few data are available regarding the regulation of ratios of acetate and BHBA on TAG content.

ACACA and *FASN* are two important genes involved during the *de novo* synthesis of FAs in the milk from bovine mammary tissues (Bionaz and Loor 2008). In the current study, *ACACA* and *FASN* mRNA expressions were promoted by 2 : 1, 3 : 1, 4 : 1, and 5 : 1 ratios of acetate and BHBA, and the 2 : 1 ratio attained the highest value. However, the ratios of 1 : 3 and 1 : 2 inhibited expression of the *ACACA* mRNA, depressing the *de novo* synthesis of milk FAs. These results indicated that the proper ratios of acetate and BHBA promoted the *de novo* synthesis of milk FAs, and the 2 : 1 ratio, in particular, had the best effect. Few data are available; therefore, more studies regarding the effects of different ratios of acetate and BHBA on the *de novo* synthesis of FAs in the BMECs are needed.

CD36, *FABP*, and *LPL* participate in the uptake and intracellular transport of LCFAs in many tissues (Lehner and Kuksis 1996). *FABP* and *CD36* had a synergistic action (Spitsberg and Matitashvili 1995). *SCD* catalyzes the synthesis of monounsaturated fatty acids from saturated fatty acids. The present results showed that the expression of *CD36* and *SCD* was higher in all groups compared to the control, and Group 3 (1 : 1 ratio) had the highest value. The abundance of the *FABP3* and *LPL* mRNAs in Group 4 (2 : 1 ratio) was dramatically higher than that observed in the control and the

doi: 10.17221/8595-CJAS

other treatments, and attained the highest value. The *FABP3* mRNA expression in the BMECs was dramatically down-regulated in Groups 1, 2, 3, and 7, and the expression of the *LPL* mRNA was down-regulated in Groups 1, 5, 6, and 7 compared to the control and the other treated groups, suggesting that a proper ratio of acetate and BHBA was beneficial to LCFA uptake in the BMECs.

PPARG and *SREBF1* are two important factors that regulate milk fat synthesis in the mammary glands (Salter and Tarling 2007). Bionaz and Loor (2008) reported that lipogenic genes, such as *ACACA, FASN, SREBF1* and *SREBF2*, were upregulated when MAC-T cells were incubated with rosiglitazone, a *PPARG* agonist, compared with the control, suggesting that *PPARG* helped regulate bovine milk fat synthesis and most likely upregulated the *SREBF1* expression. Ma and Corl (2012) showed that the mRNA levels of *FASN, ACACA, SCD,* and *FABP3* decreased after adding 100nM *SREBF1* small-interfering RNA to the BMECs, indicating that the expression levels of the *FASN, ACACA, SCD,* and *FABP3* mRNAs were regulated by *SREBF1*. The present study indicated that the mRNA expression of *PPARG* and *SPEBF1* in the BMECs was significantly affected by supplementing the media with different ratios of acetate and BHBA. The 1 : 1, 2 : 1, and 3 : 1 ratios expressed more *PPARG* and *SPEBF1* mRNAs than the control and the other treated groups; the ratio of 2 : 1 attained the highest value. However, the 1 : 2 and 1 : 3 ratios exhibited notably lower expression of the *PPARG* and *SPEBF1* mRNAs than the control, and the 1 : 2 and 1 : 3 ratios exhibited reduced *ACACA, LPL,* and *FABP3* mRNA expression. All of these results suggested that the different ratios of acetate and BHBA could modulate the expression of the genes related to milk fat synthesis, thereby altering milk fat synthesis. Meanwhile, the 1 : 1, 2 : 1, and 3 : 1 ratios, particularly the 2 : 1 ratio, most likely upregulated the expression of the *PPARG* and *SREBF1* mRNAs. Consequently, the *FASN* and *ACACA* mRNA expressions were increased, improving the *de novo* synthesis of milk FAs. Moreover, *FABP3* mRNA expression was upregulated, which was related to LCFA uptake and transport. However, the 1 : 2 and 1 : 3 ratios exhibited the opposite trend. Jacobs et al. (2013) suggested that a combination of acetate and BHBA (5mM and 5mM, 1 : 1) increased the transcript levels of *SCD1*, *ACACA,* and *FASN*. However, there were no differences in the expression of *PPARA* and *SREBF1* between the treated groups and the controls (Jacobs et al. 2013). Adding different ratios of UFAs had different results and suppressed the expression of *ACACA* and *FASN,* but had the opposite effect on the abundances of the *FABP3* and *CD36* mRNAs (Sheng et al. 2015). The expression levels of the *PPARG, SPEBF1, CSN1S1,* and *CSN3* mRNAs in the BMECs were significantly affected after adding different ratios of UFAs (Sheng et al. 2015). Little related research has been published; therefore, further studies are needed to reveal the exact mechanism.

In the present research, Groups 1, 2, 6, and 7 had no significant effect on TAG content, and the expression of lipogenic genes was lower than or similar to the control. These results suggested that the 1 : 2, 1 : 3, 4 : 1, and 5 : 1 ratios of acetate and BHBA did not significantly activate milk fat synthesis. The reason is still unclear. In the present study, it is possible that high concentrations of both acetate (in Groups 6 and 7) and BHBA (in Groups 1 and 2) suppressed milk fat synthesis. Kong (2012) indicated that high concentrations of acetate (8mM) or BHBA (1.25mM) had reduced TAG accumulation and *ACACA* and *FASN* expression compared to the lower concentrations of acetate or BHBA. Thus, the reason that the 1 : 2, 1 : 3, 4 : 1, and 5 : 1 ratios of acetate and BHBA had no evident activation on milk fat synthesis is most likely related to inhibitory effects of higher levels of acetate or BHBA in the medium on milk fat synthesis. The reason is unknown, and more research is needed to explore the probable mechanism. Currently, there are no reports on the effect of different ratios of acetate and BHBA on milk protein synthesis. Both *CSN1S1* and *CSN3* play crucial roles in milk protein synthesis. The current research showed that the *CSN1S1* mRNA expression in the BMECs was significantly up-regulated by adding different ratios of acetate and BHBA, and Group 3 (the ratio was 1 : 1) attained the highest value. High levels of acetate down-regulated the expression of the *CSN1S1* mRNA in the BMECs. However, the result of *CSN3* mRNA expression was different; Groups 5–7 significantly promoted the *CSN3* mRNA expression compared to the control and the other groups, while Groups 1–3 had the opposite tendency. These results implied that the BMECs supplemented with a proper ratio of acetate and BHBA expressed dramatically higher

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levels of the *CSN1S1* and *CSN3* mRNAs, which may result in better milk protein synthesis.

The mTOR protein, which is a conserved serine/threonine protein kinase, is composed of two distinct multi-protein complexes: *mTORC1* and *mTORC2* (Laplante and Sabatini 2009). The mTOR signalling cascade regulates protein synthesis through phosphorylating eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and p70 ribosomal protein S6 kinase-1 (S6K1) (Burgos et al. 2010). The expression of *SREBF1* target genes (*ACACA, FASN,* and *SCD*) was suppressed by rapamycin, suggesting that *mTORC1* participated in the *de novo* synthesis of FAs (Soliman et al. 2011). The mTOR rapamycin-induced inhibition reduced the protein levels and activity of *PPARG in vitro* (Kim and Chen 2004). The current study demonstrated that the abundance of the *mTOR*, *4E-BP1,* and *S6KB1* mRNAs was increased in Groups 3–5, while Groups 1 and 2, with high levels of BHBA, were dramatically lower than the control and the other treatments. Groups 6 and 7, with high levels of acetate, had lower *4E-BP1* mRNA expression. Therefore, the regulation of the different ratios of acetate and BHBA on the expression of *CS1N1* and *CSN3* was most likely through the mTOR pathway. Groups 1 and 2, with high levels of BHBA, had a lower abundance of the *mTOR*, *4E-BP1,* and *S6KB1* mRNAs, which likely resulted in the decreased *CSN3* mRNA expression.

The JAK2/STAT5 pathway is another important mechanism that regulates milk protein synthesis in the BMECs. Prolactin plays a central and crucial role in the regulation of milk protein gene expression. *CS1N1* and *CSN3* expression is activated by the tyrosine kinase Janus kinase 2 (*JAK2*) in mammary epithelial cells, which then activates signal transducers and activators of transcription 5 (*STAT5*) (Aoki and Matsuda 2000). The current study revealed significant differences in the abundance of the *STAT5* and *JAK2* mRNAs with different ratios of acetate and BHBA; Groups 3–5 had higher values, and Group 4 had the best effect. Thus, the 1 : 1, 2 : 1, and 3 : 1 ratios (Groups 3–5) promoted *CS1N1* expression, which was in accord with the higher *STAT5* and *JAK2* mRNA expression in the same group. Moreover, Groups 6 and 7, with high levels of acetate, down-regulated *CS1N1* expression because of the lower *STAT5* and *JAK2* expression. Lower *STAT5* and *JAK2* mRNA expression in Groups 1 and 2, with high levels of BHBA, may result in lower *CSN3* mRNA expression. Little data are available describing the effect of incorporating different ratios of acetate and BHBA on milk protein synthesis; therefore, further studies are needed to reveal the exact mechanism.

Leptin is a hormonal protein that is produced and secreted predominantly by white adipose tissue; it has a critical role during the regulation and coordination of energy metabolism. Leptin enhanced the fatty acid synthesis and the expression of α-casein and α-lactoglobulin in mammary gland explants from lactating cows in the presence of prolactin, but exerted no effect without it. A similar pattern was found for the expression of α-casein and α-lactoglobulin in mammary gland explants from lactating cows (Feuermann et al. 2004). The present study showed that Groups 3–6 had dramatically higher *LEPTIN* mRNA expression; Group 3 attained the highest value. Therefore, supplementing with different ratios of acetate and BHBA could influence the synthesis of milk fat and proteins, likely through the action of *LEPTIN*. Little related research has been published; therefore, further studies are needed to reveal the exact mechanism.

In the present study, *in vitro* experiments validated the significant impact of the varying acetate and BHBA ratios on milk fat synthesis and the expression of genes related to milk fat and protein synthesis. For TAG synthesis and the expression of genes related to milk fat synthesis, the 1 : 1, 2 : 1, and 3 : 1 ratios induced better effects, and the 2 : 1 ratio was the best. For *CS1N1* and *LEPTIN* expression, the 1 : 1 ratio exerted the greatest impact, followed by the 2 : 1 ratio. The 3 : 1 ratio significantly increased *CSN3* mRNA expression, and the 2 : 1 ratio did not inhibit *CSN3* mRNA expression. The expressions of genes related to milk protein synthesis (*mTOR*, *4E-BP1*, *S6KB1, JAK2,* and *STAT5*) were up-regulated when the media were supplemented with a 2 : 1 ratio of acetate and BHBA. Taken together, for both the genes related to milk fat synthesis and milk protein synthesis, the 2 : 1 ratio of acetate and BHBA had the best effect, and high levels of acetate or BHBA were the worst.

CONCLUSION

The varying ratios of acetate and BHBA significantly influenced milk fat synthesis and the expression of milk fat- and milk protein synthesis-related genes. The 1 : 1, 2 : 1, and 3 : 1 ratios were

doi: 10.17221/8595-CJAS

more effective, and the 2 : 1 ratio, in particular, had the best effect. However, the present study only partially examined the regulation of the different ratios of acetate and BHBA on milk fat and protein synthesis at the transcript level; therefore, more studies on the translational mechanisms are needed.

Acknowledgement. The authors are grateful to Qi Lizhi, Zhao Yanli, Jin Lu, and Guo Xiaoyu for their assistance with the experiments.

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Received: 2015–02–07
Accepted after corrections: 2015–07–02

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