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Effect of Supplementation of Short-Fatty Acid on PER2 and Genes Related to Volatile Fatty Acid in Isolated Goat Ruminal Epithelial Tissue

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Key words
Circadian clock, Short chain fatty acid uptake, Ruminal epithelial tissue

ABSTRACT

Period circadian regulator 2 (*PER2*) is a core circadian clock gene associated with metabolism, transport and synthesis of lipids. Here, 3-month-old Boer goats were used to explore the relationship between *PER2* and volatile fatty acid-related genes in isolated goat ruminal epithelial tissue explants. Internal and external culture medium was collected at 0, 3, 6, and 24 h to measure pH and VFA concentration to evaluate conditions *in vitro*, research showed pH at 3 h was significant higher than that at other different time, and pH at 24 h was lower than that in the other three groups; The concentration of acetic acid, propionic acid and butyric acid in culture fluid indicated that the concentrations of acetic acid, propionic acid, butyric acid group and control group were not significantly different ($P > 0.05$) on the time axis. Furthermore, Tissue collected at 0 and 24 h after culture with acetic acid, propionic acid, and butyric acid to measure the expression of biological clock genes such as *PER2* and VFA uptake-related genes. Results showed that different VFA treatments inhibited the relative expression of *CLOCK* and *MCT1* genes, a correlation analysis between *PER2* and VFA uptake-related genes was also performed and showed that *PER2* was positively correlated with *AE2,* and *PER2* was negatively correlated with *MCT1* in rumen tissue.

INTRODUCTION

Circadian clock genes are widely-expressed in mammalian cells, and their expression in the gastrointestinal tract could affect the absorption and transport of macronutrients (Pácha and Sumova, 2013). The core circadian clock genes include brain-muscle-arnt-like 1 (*Bmal1*), circadian locomotor output cycles kaput (*Clock*), period1 (*Per 1*), period2 (*Per 2*), period3 (*Per3*), cryptochrome1 (*Cry1*) and cryptochrome2 (*Cry2*) (Cagampang and Bruce, 2012). The cellular oscillator of circadian clock is composed of a positive limb (*CLOCK* and *BMAL1*) and a negative limb (*CRYs* and *PERs*), and several papers have reviewed the core mechanism of the mammalian circadian clock (Froy, 2007). Previous research revealed that the mRNA expression of transporters

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Abbreviations
AE1, Anion exchanger 1; AE2, Anion exchanger 2; BMAL1, Brain and muscle ARNT-like-1; CCNB1, Cyclin B1; CCND1, Cyclin D1; CCNE1, Cyclin E1; CDK, Cyclin-dependent kinases; CLOCK, Circadian locomotor output cycles kaput; CON, Control; CRY, Cryptochrome circadian clock; DMEM/F12, Dulbecco's Modified Eagle Media/ Nutrient Mixture F-12; Dra, Downregulated-in-adenoma; EGF, Epidermal growth factor; ITS, Insulin-transferrin-selenium solution; MCT, Monocarboxylic acid transporter; NHE1, Na+/H+ exchanger 1; Nhe3, Na+/H+ exchanger 3; PAT1, Putative anion transporter 1; PER2, Period circadian clock 2; PER3, Period circadian clock 3; PPARα, Peroxisome proliferator-activated receptor alpha; PPARδ, Peroxisome proliferator-activated receptor delta; PPARγ, Peroxisome proliferator-activated receptor gamma; RECs, Ruminal epithelial cells; RIPA, Radio immunoprecipitation assay; SB, Sodium butyrate; SCFA, short-chain fatty acid; VH+ ATPase, Vacuolar H+ ATPase subunit B.

involved in intestinal absorption of nutrients such as glucose, peptides, and fatty acids have obvious circadian rhythmicity (Tavakkolizadeh *et al*., 2005; Houghton *et al*., 2008). In the intestine of nocturnal rodents, the circadian clock regulates the mRNA expression of ion transporter proteins including Dra (downregulated-in-adenoma), Ae1 (anion exchanger 1), and Nhe3 (Na+/H+ exchanger 3) with the peak expression at the onset of nighttime (Soták *et al*., 2011). Recently, Sussman *et al*. (2019) reported that *BMAL1* (a core circadian gene) can regulate glucose uptake of Caco-2 cells *in vitro* through changing the translation of *SGLT1*.

Per2 is a core circadian clock gene that regulates cell proliferation and apoptosis (Yang *et al*., 2009; Wang *et al*.,2016), lipid metabolism (Grimaldi *et al*., 2010), the expression of the glutamate transporter and uptake of glutamate by astrocytes (Spanagel *et al*., 2005). Recent research on the biologic relevance of a clock in ruminants has concentrated on the mammary gland of dairy cows, with data indicating that PER2 affects the metabolic rhythm of lactation and the synthesis of α-casein protein in bovine mammary epithelial cells (Wang *et al*., 2015; b; Hu *et al*., 2017). Casey and Plaut (2012) proposed that circadian clocks in rodents are involved in the homeorhetic regulation of lactation through altering hormonal profiles (e.g., prolactin) and metabolism. Ruminants ferment grain and forage in the rumen to provide most of the digestible energy (70%-80%) required by the host animal (Bannink *et al*., 2008). As the main product of ruminal fermentation and principal energy source for ruminants, volatile fatty acids (VFA) are mainly absorbed by ruminal epithelium through the action of transporters including PAT1 have (putative anion transporter 1), Dra, MCT have (mono-carboxylate transporter) and AE2 (Stumpff, 2018; Naeem *et al*., 2012).

In this current study, the general hypothesis was that the supplementation of short-chain fatty acids would change the utilization of VFA by affecting clock genes and VFA-related genes. To test our hypothesis, we measured pH to verify ruminal tissue culture conditions *in vitro* and used real-time qPCR assays to explore the relationship between circadian clock genes and VFA-related genes.

MATERIALS AND METHODS

Experimental design

In this experiment, 3-month-old Boer goats were selected for rumen tissue culture *in vitro*. The rumen epithelial tissue culture device was composed of an inner and outer part. The inner solution was maintained in an anaerobic state to simulate rumen environment, and the outer solution is medium for sustaining survival of tissue. The internal solution treatment included four groups: control group (20mL buffer solution+20mL tumor solution), acetic acid group (20mL buffer + 20mL tumor solution + 15mmol/L acetic acid), internal acid vitamin (20mL buffer + 20mL tumor fluid + 15mmol internal acid), acid group (20m buffer + 20m tumor fluid + 15mmol/L butyric acid), that is, adding 1:1 buffer and rumen fluid and 15mmol/L different acids to the inner solution. The outer culture medium was a 300mL complete medium. Placed in an incubator (38°C, 5% CO_2+95% O_2) raising for 24 h. The inner and outer culture media were collected for 0, 3, 6 and 24 h to measure pH and the VFA concentration. Tissues were collected at 0 and 24 h to measure the expression of circadian clock genes and VFA-related genes. Each treatment has 3 replicates.

Reagents

Reagent A: 382.51 mg of K_2HPO_4, 292 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 200 mg of NaCl, 100 mg of $MgSO_4$ $7H_2O$ and 4000 mg of Na_2CO_3 dissolved and mixed in distilled water to a volume of 1000 mL.

Reagent B: 500mg of EDTA, 200 mg of $FeSO_4$ $7H_2O$, 200 mg MnCl4 $4H_2O$, 10 mg $ZnSO_4$ $7H_2O$, 330 mg H_3BO_3, 20 mg $CoC1_2$: $6H_2O$, 1 mg $2H_2O$, 2 mg $NiCl_2$ $6H_2O$ and 3 mg $NaMoO_4$ were dissolved and mixed with distilled water to 1000 mL. After the continuous introduction of CO_2 for 18h, the bottle was tightly capped and kept in the refrigerator.

Reagent C: 25 g of Na_2S $9H_2O$, dissolved in distilled water and volume made upto 100 mL volumetric flask to 100 mL. The reagent was bubbled with CO_2 for 20 min, before keeping it in the refrigerator.

For preparation of buffer 790.4 mL of A solution was mixed with 8 mL of B solution, CO_2 was passed through this mixture for 18 h, add 1.6 mL C solution water bath to preheat to 39°C for use.

Ruminal fluid: fresh ruminal fluid was obtained from the rumen of fistulated sheep (provided by the experimental farm of Yangzhou University) through a ruminal fistula, filtered through four layers of gauze and then packed into a thermos to keep warm.

Acetate acid, propionate acid, and butyrate acid were all from sigma.

HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) was supplied by Thermo Fisher (China), model 11330-BMEM/F-12.

Complete medium comprised 5% fetal bovine serum (fetal bovine seruM, FBS) 0.1 mg/mL sulfuric acid, gentamicin, 5 g/mL soluble amphotericin B (Solexpro, Beijing, China), 2% penicillin mix (100x), 1% insulin transfer selenium (ITS, Gibco, USA) and 10 ng/mL epidermis growth yinzi (Epidermal growth factor, EGF,

Peprotech, USA) in F11330- MEM/F-12 medium.

Trypsinol (100 mL) had 0.25 g trypsinol and 0.026 g disodium EDTA were dissoloved in 100 mL PBS solution with pH adjusted at 7.2-7.4. Finally, the solution was filtered through a 0.2 ml filter membrane to remove bacteria.

pH measurement

A Shanghai Leici PHS-3C precision pH meter was used to measure pH in ruminal fluid.

VFA concentration

The VFA concentration in the culture medium at each sampling time was determined by SPD-15C high-performance liquid chromatography (Shimadzu, Japan). Each treatment had 3 replicates and all samples were acidified with hydrochloric acid and then filtered with a 0.22 μm hydrophilic polyethersulfone syringe filter (ANPEL, China). The sample injection volume was 10 μL.

Real-time PCR

RNA was extracted by the Trizol method. The cDNA synthesis was conducted with a Quantscript RT kit (KR103, TIANGEN Biotech Co., Ltd, Beijing, China) and 1 μg RNA of sample was used for cDNA synthesis according to the manufacturer's instructions. The primer sequences for target genes and the internal control (ACTB) were designed with Oligo 6, listed in Table I, and synthetized by Invitrogen Trading (Shanghai) Co. Ltd. (China). The RT-PCR was conducted with Super Real PreMix Plus (SYBR Green) from TIANGEN Biotech (Beijing) Co. Ltd (No. FP215, China) in an Applied Biosystems 7500 Real-Time PCR System (ThermoFisher Scientific, USA). Each sample had 3 technical replicates for each target gene. The protocol was described in detail in a previous manuscript from our laboratory (Wang *et al*., 2014). The primer specificity was checked by dissociation curve and agarose gel electrophoresis of PCR products were also used to check the primer specificity. The relative gene expression was calculated with the $2^{-\Delta\Delta}$Ct method.

Statistical analysis

Results are presented as means±SD. We used the SPSS software (version 16.0) to conduct the statistical analysis. Significant differences between control and treatment were determined by Duncan's method, and the data were deemed significant if $P < 0.05$ and to have a tendency if $0.05 < P < 0.10$.

Table I. Primer sequences for target and internal control gene.

Gene function	Gene name	Primer sequence 5'→3'	Source	Product length (bp)
Internal control gene	*ACTB*	GCAGACAGGATGCAGAAAGA AGTCCGCCTAGAAGCATTTG	NM_173979.3	72
Circadian gene	*CLOCK*	TCATCGGCAACAAGAAGAAC TTGAACAACCTGGCTTTGC	XM_013964639.1	186
	PER2	AGCGTGAAGCAAGTGAAAGC ACAGCGGCCACAAACATATC	XM_013963061.1	160
	PPARα	GCACAGATCGTTTCCTCCTTTA TTGTAGGAAGTCTGCCGAGAGT	XM_005681211.2	249
	PPARγ	AAGCCCATTGAGGACATACAAG AGAAGTGGGTGGAGACTCATGT	NM_001285658.1	196
Cell cycle gene	*AE2*	CTTGGTCATCGGGGATCTGC TCCCATTCAGGGAGGTCACG	AY_324395.1	89
	MCT1	CTTGGCAGACCTTTATCCTC CTCCACAATGGTCACCAATCC	NM_001037319.1	163
	NHE1	TTACTACTTCCAGATCCATCCATTGCTT GAATGAGTAGGGTGACGGAAT	NM_174833.2	158
	NHE3	AAGAACCTGTTTGTCAGCACCAC TTCACTTCTCTTCACCTTCAGCC	NM_001192154.1	108

Notes: CLOCK, Circadian locomotor output cycles kaput; PER, Period circadian clock; PPARα, Peroxisome proliferator-activated receptor alpha; PPARγ, Peroxisome proliferator-activated receptor gamma; AE2, Anion exchanger 2; MCT1, Monocarboxylic acid transporter 1; NHE1, Na+/H+ exchanger 1; NHE 3, Na+/H+ exchanger 3. ACTB, β-actin.

RESULTS

pH changes in the inner and outer solution of in vitro *culture*

There was no significant difference in pH on the time axis of the inner solution (P <0.05; Table II), but in the inner solution, the control group had higher pH value compared with acetate, propionate and butyrate acid groups (P <0.05) and there was no significant difference between these acid groups (P >0.05). There was no difference in pH of the outer solution among treatments (P <0.05), but concerning pH in outer solution, it was significantly higher at 3 h than that at other different time, and pH at 24 h was lower than that in the other three groups (P <0.05).

Table II. Changes in pH of the inner and outer solution of rumen epithelial tissue culture.

Items	Time	Control	Acetate	Propionate	Butyrate	Mean	SEM	P value
pH in inner solution	0h	6.52	5.16	5.43	5.43	5.64	0.14	0.08
	3h	6.69	5.52	5.47	5.79	5.87		
	6h	6.45	5.83	5.53	5.52	5.83		
	24h	6.55	6.01	5.93	5.74	6.06		
	Mean	6.55^{a}	5.63^{b}	5.59^{b}	5.62^{b}		0.1	<0.01
pH in outer solution	0h	7.03	7.03	7.03	7.03	7.03^{b}	0.04	<0.01
	3h	7.24	7.34	7.07	7.08	7.18^{a}		
	6h	6.99	7.04	6.98	7.06	7.02^{b}		
	24h	6.75	6.91	6.88	6.76	6.83^{c}		
	Mean	7.00	7.08	6.99	6.98		0.05	0.3

Note: values with different superscripts in the same line differ significantly (P <0.05), and values with the same superscripts in the same line have no significance (P >0.05).

VFA in culture fluid after incubations

Table III shows that on the time axis, the molar concentration of acetic acid, propionic acid, butyric acid group was similar among different time points. (P>0.05). The molar concentration of acetic acid in the acetic acid group was extremely higher than that in the control, propionic and butyric acid groups (P<0.01); The molar concentration of propionic acid in the propionic acid group was higher than the other three groups (P<0.01), but there was no significant difference in propionic concentration among the control group, acetic acid group, the butyric acid group (P> 0.05); The control group, acetic acid group and propionic acid group had lower concentration of butyric acid group (P<0.01).

Relative gene expression of CLOCK and PPARs family in ruminal epithelial tissue

Figure 1 shows that the relative expression of *PER2* in the propionic acid group has significant higher than the other groups (P < 0.05). There was no significant difference in the relative expression of the *CLOCK* gene between the control group and acetic acid group (P >0.05).The relative expressions of *PPAR-α* and *PPAR-γ* were the highest in the propionic acid group and the lowest in the butyric acid group, but there was no differences in expression of *PPAR-α* and *PPAR-γ* in the control group, acetic acid group, propionic acid group and butyrate acid group (P >0.05).

Table III. Effects of different VFA on the molar concentration of acetic acid, propionic acid and butyric acid in culture fluid of rumen epithelial tissue.

Item	Time	Control	Acetate	Propionate	Butyrate	Mean	SEM	P value
Acetate/ mM	0h	31.10	56.39	30.31	28.64	36.61	3.08	0.33
	3h	28.29	34.33	28.62	28.41	29.91		
	6h	31.57	41.23	30.28	29.69	33.19		
	24h	29.98	26.95	27.02	31.23	28.80		
	Mean	30.24^{b}	39.73^{a}	29.06^{b}	29.49^{b}		4.36	<0.01
Propionate/ mM	0h	7.37	7.76	23.40	7.06	11.40	0.83	0.25
	3h	7.12	6.13	22.07	7.05	10.59		
	6h	7.48	7.14	22.01	7.11	10.94		
	24h	7.28	5.16	15.84	7.54	8.96		
	Mean	7.31^{b}	6.55^{b}	20.83^{a}	7.19^{b}		1.12	<0.01
Butyrate/ mM	0h	2.82	2.76	2.94	16.57	6.27	0.42	0.48
	3h	2.80	2.41	2.73	16.74	6.17		
	6h	2.90	2.82	2.79	15.70	6.05		
	24h	3.25	2.28	2.80	13.17	5.38		
	Mean	2.94^{b}	2.57^{b}	2.82^{b}	15.55^{a}		0.60	<0.01

Note: values with different superscripts in the same line differ significantly (P <0.05), and values with the same superscripts in the same line have no significance (P >0.05).

Relative expression of genes related to VFA transport and absorption in rumen epithelial tissues

As shown in Figure 2, there was no significant difference between the acetic acid group, the butyric acid group and the propionic acid group in the relative expression level of *MCT1*, but the three groups were extremely significantly lower than the control group (P <0.01). In expression value of *AE2*, there was no significant difference between the acetic acid group, the propionic acid group and the control group (P >0.05). There was no significant difference between the acetic acid group and the butyric acid group (P <0.05), but the butyric acid group was significantly lower than the control group. Concerning the relative expression levels of NHE1 and NHE3, the control group was numerically lower than the other three groups, but there was no significant difference (P >0.05).

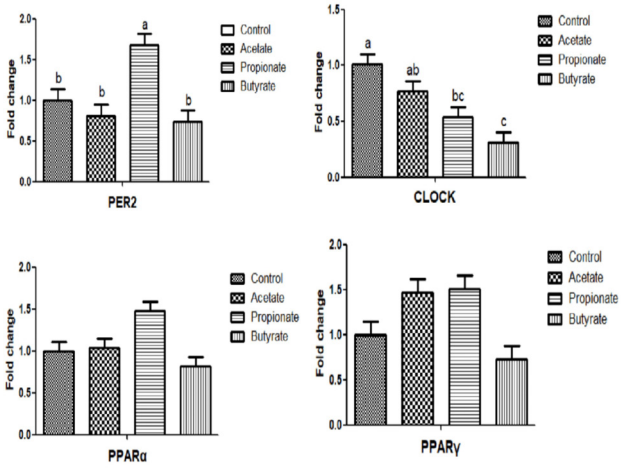

Fig. 1. Effect of different VFAs on relative gene expression of CLOCK and PPARs family in ruminal epithelial tissue. Note: PER, period circadian clock; CLOCK, clock circadian regulator; PPARα, peroxisome proliferator-activated receptor alpha; PPARγ, peroxisome proliferator-activated receptor gamma. Values with different superscripts on the bars differ significantly (P<0.05), and values with the same superscripts on the bars have no significance (P>0.05).

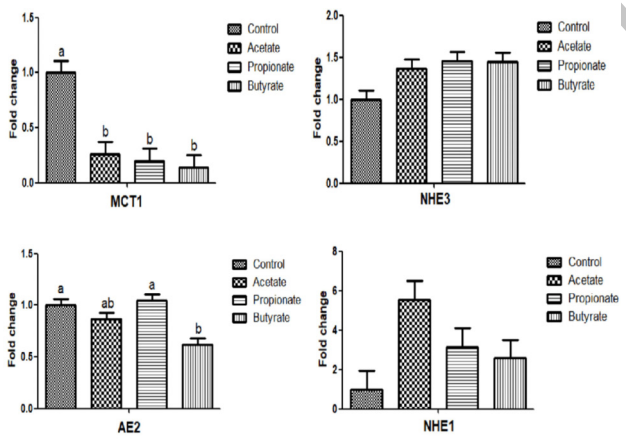

Fig. 2. Effect of different VFAs on relative gene expression of VFA transport and absorption in rumen epithelial tissues. Note: NHE, Na+/H+ exchange; AE2, anion exchanger 2; MCT1, monocarboxylic acid transporter 1. Values with different superscripts on the bars differ significantly (P<0.05), and values with the same superscripts on the bars have no significance (P>0.05).

Correlation between VFA uptake-related genes and PER2 gene expression

As shown in Figure 3, the relative expression of PER2 and AE2 is positively correlated. The relative expression of PER2 and MCT1 is negatively correlated.

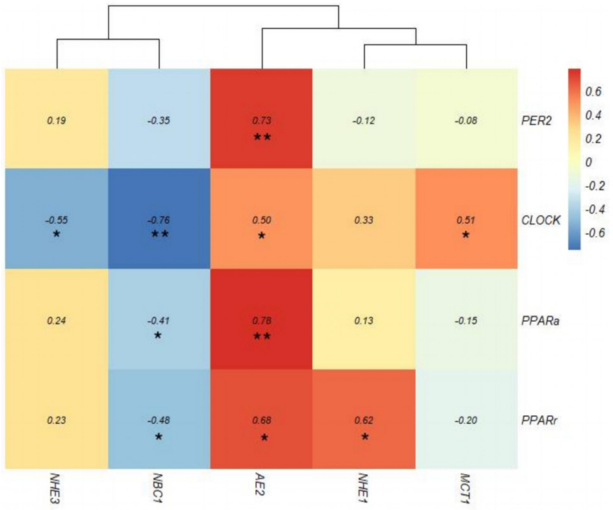

Fig. 3. Effect of different VFAs on relative gene expression of VFA transport and absorption in rumen epithelial tissues. Note: NHE, Na+/H+ exchange; AE2, Anion exchanger 2; MCT1, Monocarboxylic acid transporter 1. Values with different superscripts on the bars differ significantly (P<0.05), and values with the same superscripts on the bars have no significance (P>0.05).

DISCUSSION

Changes in the pH of the inner and outer solution in vitro

Ruminal pH is the acid-base value resulting from the combined effect of surimi VFA, saliva buffer salts, and VFA efflux with surimi, and it is one of the important indicators of the rumen internal environment (Aschenbach *et al*., 2011). The optimum pH for protein synthesis by microorganisms in the rumen is 6.3 to 7.4 (Calsamiglia *et al*.,2008). The results of the present study showed that the pH of the internal fluid varied between 5.64 and 6.06 over time, and the external fluid varied between 7.03 and 6.83 over time, with significant difference between the internal and external fluids. The differences between the pH values of the internal solution at 0 h and 24 h were similar, with higher values in the control group and lower values in the acetic acid, propionic acid and butyric acid groups. While in the external solution, there were no significant differences between the pH values at 0 h and 24 h and no significant differences between the groups. In addition, it was shown that acetic acid, propionic acid and butyric acid account for 95% of the VFA produced by rumen fermentation (Dijkstra, 2012). Thus, it is speculated that the fluctuation of pH values of the external solution may be related to the acid production by the rumen epithelial tissue in the culture medium. Collectively, the pH values

of both internal and external solutions fluctuated within a certain range, with little crossover between internal and external.

Effects of different VFAs on factors associated with CLOCK and PPARs

Circadian rhythms are involved in lipid homeostasis in the body and many metabolic factors (enzymes, transporter proteins and hormones) also show circadian rhythmicity. It has been shown that mutations in the *CLOCK* gene attenuate the circadian rhythm of feeding behavior, increasing energy intake and body mass thereby contributing to obesity symptoms (Turek *et al*., 2005). CLOCK gene expression is downregulated in mice with diet-induced obesity and such a result may be due to the fact that CLOCK may regulate leptin resistance and the operation of cytokine signaling inhibitor 3 in the ARC (Xie *et al*., 2013). It has been reported that leptin resistance has an important role in circadian dysfunction-induced obesity and metabolic syndrome, and that BMAL1/CLOCK can regulate the circadian rhythm of leptin transcription by generating C/EBPα in adipose tissue, and that mutations in PER and CRY in animals show similar disruption of the peripheral clock and leptin dysregulation (Gomezabellan *et al*., 2012; Sari *et al*., 2015). The results showed that different VFA treatments inhibited *CLOCK* gene expression, with more pronounced results with propionic acid and butyric acid treatments, which may be due to the increase in the relative expression of VFA uptake related proteins with the increase in the concentration of different volatile fatty acids and the feedback of the organism to inhibit *CLOCK* gene expression.

Peroxisome proliferators activated receptors (PPARs) are a class of nuclear transcription factors that are activated by ligands (Kettner *et al*., 2015). In the study of *PER2, BMAL1, CRY* and other clock genes showing correlation with *PPAR-γ* and genes related to glucocorticoid metabolism on the time axis, it was found that over time, clock genes regulate the clock and *PPAR-γ* is involved in adipose tissue metabolism. There was also a direct correlation between *CLOCK* gene expression and *PPAR-α*, a gene involved in adipose tissue metabolism. The expression of *PPAR-α* rises, the SFA content decreases, with a concomitant increase in UFA/SFA content, thus, it was hypothesized that the relative expression of *PPAR-α* gene is significantly correlated with fatty acid composition. The results of this experiment showed that the relative expression of *PPAR-α* and *PPAR-γ* genes in the acetic acid and propionic acid groups were numerically higher than the other groups, but the relative expression of *PPAR-α* and *PPAR-γ* genes in the butyric acid group was numerically smaller than the other groups, suggesting that there may be a correlation between different fatty acids and the expression of *PPAR-α* and *PPAR-γ* genes in the rumen epithelial tissue. In addition, propionic acid was able to induce *PPAR-α* gene expression in colonic epithelial cells, and butyric acid was able to increase *PPAR-α* and *PPAR-γ* relative expression and expression at the protein level in intestinal epithelial cells (Higashimura *et al*., 2016). These results are consistent with the results of the present experiment; therefore, different VFAs do regulate the expression of *PPAR-α* and *PPAR-γ* genes in intestinal epithelial cells to some extent, but whether the specific regulatory mechanism is related to VFA absorption-related genes remains to be further investigated.

Correlation of rumen epithelial PER2 *gene with VFA absorption-related genes*

VFA and its products provide important raw materials for lactation in ruminants, and studies have shown that BHBA (β-hydroxybutyric acid) and acetic acid in the blood can synthesize about 50% of milk fat (Wachtershauser *et al*., 2000). The synthesis of VFA from the mammary gland through rumen fermentation via fatty acid synthase (FAS) and acetyl coenzyme A carboxylase (ACC) is one of the important pathways of milk fat synthesis, the *PER2* gene is a core component of the biological clock rhythm oscillator, which is associated with fat metabolism, operation, and synthesis (Kiratl and Kato, 2009). The liver is the main site of lipid metabolism, and most metabolic diseases are associated with disorders of lipid metabolism and are accompanied by changes in certain biological rhythms, for example, in obese mice, the phase advance of *PER2* in the liver reduces the rhythmicity of CLOCK and BMAL1 mRNA in adipose tissue (Jing *et al*., 2021). In the study of screening and identification of mammary tissue clock genes in mice, (Kohsaka *et al*., 2007) found that *PER2* and *BMAL1* genes have a greater effect on mammary gland development and that PER2 gene expression is highest in mammary tissue of mice at puberty as well as mid-gestation. MCT is a proton-coupled transporter protein that has an important role in cellular metabolism (Metz *et al*., 2006). In addition, it has also been shown that MCT is not only present in the rumen epithelium involved in VFA transport, but also MCT1 and MCT2 on the basolateral membrane of mammary epithelial cells can transport acetic acid and BHBA through the basolateral membrane of epithelial cells into mammary epithelial cells, and then transported by MCT4 at the apical membrane into the lumen of the mammary vesicles thereby synthesizing fatty acids, which are ultimately used for the synthesis of milk fat in ruminants. Thus, MCT1 has the function of transporting volatile fatty acids. It has been reported that the expression of MCT1 and MCT4 in the gastrointestinal tract of calves

that have not started to ruminate is low relative to that of adult cows and that the VFA concentration corresponds to the amount of MCT1 and MCT4 expression levels in the gastrointestinal tract (Halestrap And Meredeth, 2004). In studying the effect of different VFA on VFA transporter protein-related genes, Jian Gao (Castells *et al*., 2013) found that different VFA treatments significantly down-regulated the relative expression of MCT1 and MCT4, this is consistent with the results of this experiment. In this experiment, 15 mmol/L propionic acid promoted the expression of PER2, but different acids inhibited the expression of MCT1. This may be due to the fact that MCT1 and MCT4 can only transport VFA properly with the assistance of the auxin CD147, which is lacking in *in vitro* culture (Bedford *et al*., 2020). It was shown that the expression of AE2 gene was not significantly different from the VFA concentration when rumen epithelial cells were cultured *in vitro*, but it decreased significantly when the culture medium pH was 6.8.

The results of this experiment showed a significant decrease in AE2 expression in the butyric acid group, which may be related to the pH in rumen tissue. the AE2 protein can regulate the intra- and extracellular pH and the balance of cellular Cl-, HC03- and H+ through Cl-/HC03- exchange (Enerson and Drewes, 2003). Xu Qing (Zhan *et al*., 2018) found that the addition of sodium butyrate significantly increased the relative expression of NHE1 in goat peripheral blood lymphocytes, but it was also demonstrated (Fransen *et al*., 2004; Laarman *et al*., 2013) that the addition of butyrate had no significant effect on the relative expression of *NHE1* gene, but increased the relative expression of *NHE3* gene. NHE is also a multifunctional transmembrane protein, which can regulate cellular NHE is also a multifunctional transmembrane protein that regulates cellular pH by regulating Na+/H+ exchange to maintain cellular stability. The results of this experiment showed that although there was no significant difference between different VFA treatments on NHE1 and NHE3, there was a significant difference in the values, while different VFA treatments had a significant effect on MCT1 and AE2. This may be because the pH in the culture medium fluctuated after adding different VFA, and NHE1, NHE3 and AE2 were involved in regulating the pH, or it may be that different VFA treatments affected the changes of master clock genes in the tissues and thus changed other genes. The results of the group's previous *in vitro* culture experiments on rumen cells confirmed that PER2 has some correlation with VFA uptake related genes and regulates VFA uptake, but the regulatory role needs to be studied in the next step.

CONCLUSION

The acetic acid, the propionic acid, and the butyrate acid inhibited *CLOCK* and *MCT1* genes, the acetate acid and the butyrate acid inhibited *AE2* genes, the propionic acid enhance *PER2* genes, and in the correlation analysis, PER2 was positively correlated with AE2, PER2 was negatively correlated with MCT1, CLOCK was positively correlated with MCT1 and AE2.

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IRB approval

IRB of Yangzhou University, Jiangsu Province, China approved the study (approval number: 202103202).

Ethical statement

The use of animals and experimental procedures were approved by the Animal Care and Use Committee of Yangzhou University, Jiangsu Province, China (No. 201406018).

Statement of conflict of interest

The authors have declared no conflict of interest.

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