Animal (2020), **14:11,** pp 2315–2325
doi:10.1017/S1751731120001287

animal

Carbohydrate and amino acid metabolism and oxidative status in Holstein heifers precision-fed diets with different forage to concentrate ratios

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(Received 19 August 2019; Accepted 18 May 2020; First published online 30 June 2020)

*Previous work led to the proposal that the precision feeding of a high-concentrate diet may represent a potential method with which to enhance feed efficiency (**FE**) when rearing dairy heifers. However, the physiological and metabolic mechanisms underlying this approach remain unclear. This study used metabolomics analysis to investigate the changes in plasma metabolites of heifers precision-fed diets containing a wide range of forage to concentrate ratios. Twenty-four half-sib Holstein heifers, with a similar body condition, were randomly assigned into four groups and precision fed with diets containing different proportions of concentrate (20%, 40%, 60% and 80% based on DM). After 28 days of feeding, blood samples were collected 6 h after morning feeding and gas chromatography time-of-flight/MS was used to analyze the plasma samples. Parameters of oxidative status were also determined in the plasma. The FE (after being corrected for gut fill) increased linearly ($P < 0.01$) with increasing level of dietary concentrate. Significant changes were identified for 38 different metabolites in the plasma of heifers fed different dietary forage to concentrate ratios. The main pathways showing alterations were clustered into those relating to carbohydrate and amino acid metabolism; all of which have been previously associated with FE changes in ruminants. Heifers fed with a high-concentrate diet had higher ($P < 0.01$) plasma total antioxidant capacity and superoxide dismutase but lower ($P \leq 0.02$) hydroxyl radical and hydrogen peroxide than heifers fed with a low-concentrate diet, which might indicate a lower plasma oxidative status in the heifers fed a high-concentrate diet. Thus, heifers fed with a high-concentrate diet had higher FE and antioxidant capacity but a lower plasma oxidative status as well as changed carbohydrate and amino acid metabolism. Our findings provide a better understanding of how forage to concentrate ratios affect FE and metabolism in the precision-fed growing heifers.*

Keywords: metabolomics, high concentrate, feed efficiency, redox, heifer

amounts of ME calculated to meet, but not exceed, requirements for an appropriate average daily gain (**ADG**). Since the diets were designed and offered according to the nutritional requirements and all the heifers were metabolically satiated (NRC, 2001; Zhang *et al.*, 2018), this feeding strategy has been described as precision feeding in previous studies (Zanton and Heinrichs, 2016; Pino and Heinrichs, 2017).

Previous studies on precision feeding of heifers have focused mainly on nutrient intake and digestibility (Lascano *et al.*, 2009; Zanton and Heinrichs, 2016); however, metabolic changes in heifers may occur during and even after the precision-feeding period. Some studies have investigated blood parameters such as urea, glucose, insulin, cholesterol and/or triglyceride in precision-fed heifers (Lascano *et al.*, 2016; Zhang *et al.*, 2018). Further clarification on metabolic changes in precision-fed heifers is needed to have a better understanding while applying this feeding strategy on growing heifers.

As an emerging research area, high-throughput metabolomics can be used to identify and quantify small molecular metabolites in biological samples (biofluids or tissues) and represents a powerful tool for the characterization of metabolic pathways in ruminant research (Sun *et al.*, 2017; Luo *et al.*, 2019; Zhang *et al.*, 2019). Due to its high-throughput capability and high sensitivity, gas chromatography time-of-flight/MS (**GC-TOF/MS**) is widely used in the analysis of biofluids, such as plasma (Sun *et al.*, 2017; Luo *et al.*, 2019). The identification and integrative analysis of these metabolites provides us with a powerful means of characterizing a range of metabolic mechanisms at the molecular level.

Previous studies on precision feeding have used two or three levels of concentrate (55% *v.* 10%; 60% *v.* 20%; 75% *v.* 25%; 37% *v.* 20% *v.* 6%) (Hoffman *et al.*, 2007; Zanton and Heinrichs, 2007; Lascano *et al.*, 2009; Zanton and Heinrichs, 2016) and do not provide a sufficient data set with which to understand the physiological variation in metabolites in heifers fed different forage to concentrate ratio (**F : C**) diets. In the present study, we hypothesized that, in precision-fed Holstein heifers, a high-concentrate diet will increase FE compared with a low-concentrate diet, with associated differences in systemic metabolism. Therefore, the objective of this study was to investigate the effects of pre-

A detailed description of the experimental design has been provided previously (Zhang *et al.*, 2018). In brief, 24 half-sib Holstein heifers (8 to 10 months of age and 263 ± 30 kg in BW) of similar body conditions were selected from Sanyuan Dairy Group (Beijing, China). All experimental heifers were housed in the same tie-stall barn with rubber mattress bedding as well as proper light, ventilation and temperature conditions. The heifers were randomly assigned into four groups and fed diets containing different levels of concentrate (20%, 40%, 60% and 80%, based on DM; these groups were referred to as C20, C40, C60 and C80, respectively) under the precision-feeding system (Zhang *et al.*, 2018). Corn silage was used as the sole forage source (Table 1). All heifers were fed for 4 weeks with a pre-experimental diet containing 50% concentrate (based on DM; Table 1) and then transferred to the experimental diets for another 4 weeks. Diets were formulated to meet the requirements for the growth of dairy cattle (NRC, 2001) and provided similar intakes of ME. In addition, diets were formulated to maintain a similar CP : ME ratio in order to minimize the potential effects arising from the differences in CP intake across treatments. Heifers were individually fed with a total mixed ration twice daily at 12 h intervals (0700 and 1900 h). Heifers were weighed weekly, 2 h prior to the morning feeding (0500 h) on 2 consecutive days throughout the experiment. The amount of feed offered was adjusted weekly based on crude BW with gut fill, and the same diet compositions were used through the whole experimental period as described in previous studies (Lascano *et al.*, 2016; Zanton and Heinrichs, 2016). Water was available *ad libitum*, and individual DM intake (**DMI**) was recorded daily. Heifers had free access to an exercise lot for at least 2 h on days without sampling.

According to most limit-feeding studies in heifers, paunch girth was the only changed parameter among body measurements, and the greater paunch girth might indicate more digesta and fluid in the gastrointestinal tract (Zanton and Heinrichs, 2007 and 2016). For gut fill correction, the empty BW prediction equation of Williams *et al.* (1992), based on dietary neutral detergent fiber concentration, was used to calculate corrected ADG (**cADG**) and corrected FE (**cFE** = cADG/DMI).

Table 1 *Ingredients and chemical composition of the experimental diets for heifers*

Item	Dietary concentrate inclusion (%)				
	C20	C40	C60	C80	50[1]
Ingredients, % of DM					
Steam-flaked corn	5.76	22.97	40.26	57.06	31.50
Soybean meal	11.39	13.86	16.05	18.66	15.04
Corn silage	80.00	60.00	40.00	20.00	50.00
Mineral mix[2]	2.85	3.17	3.69	4.28	3.46
Chemical composition					
OM, % of DM	92.9	93.0	93.0	92.8	93.0
CP, % of DM	12.9	13.8	14.6	15.5	14.2
NDF, % of DM	45.6	36.7	27.8	19.0	32.3
NFC[3], % of DM	30.5	38.8	47.5	54.6	43.7
Starch, % of DM	26.3	32.3	38.2	43.8	35.1
ME[4], MJ/kg	10.1	10.9	11.7	12.1	11.3
CP : ME (g/MJ)	12.6	12.6	12.6	12.6	12.6

OM = organic matter; NFC = non-fibrous carbohydrate; ME = metabolizable energy.
The C20, C40, C60 and C80 diets contained 20%, 40%, 60% and 80% of concentrate, respectively.
[1]Pre-experimental diet.
[2]Contained 18.50% Ca, 6.00% P, 4.2% Mg, 1.4% K, 2.6% S, 7.5% Na, 12.0% Cl, 30 mg/kg of Se, 0.25% Zn, 0.25% Fe, 0.25% Mn, 1100 mg/kg of Cu, 15 mg/kg of I, 265 000 IU/kg of vitamin A, 110 200 IU/kg of vitamin D and 2300 IU/kg of vitamin E.
[3]NFC = 100 − (NDF + CP + ether extract + ash).
[4]Estimated as ME = total digestible nutrients × 0.04409 × 0.82, according to the Nutrient Requirements of Dairy Cattle (NRC, 2001).

internal standard) were added to a 100-μl sample of plasma and mixed by vortexing. This was then centrifuged at 4°C and 12 000×**g** for 15 min. Then 385 μl of the supernatant was transferred into a 2-ml GC/MS silylated vial. Next, 16 μl of the supernatant was taken from each sample and pooled to act as a quality control (**QC**) sample. The extracts were then dried using a vacuum concentrator without heating, and 60 μl of methoxyamine hydrochloride (20 mg/ml in pyridine) was added and mixed gently; the solution was then incubated at 80°C for 30 min. Subsequently, 80 μl of *N,O*-bis(trimethylsilyl)trifluoroacetamide reagent (containing 1% trimethylchlorosilane, v/v) was added to each sample and incubated at 70°C for 2 h with mixing. Subsequently, the samples were subjected to detection by GC-TOF/MS. Prior to analysis, 8 μl of fatty acid methyl esters (a standard mixture of FAMEs, C8–C16 : 1 mg/ml; C18–C24 : 0.5 mg/ml in chloroform) was added into the QC sample when it had cooled to room temperature.

1 min and then increased to 300°C at a rate of 20°C/min; the column was then maintained for 6.5 min. The energy provided was −70 eV in electron impact mode. The temperature of injection, transfer line and ion source was 280, 280 and 220°C, respectively. The MS data were acquired in full-scan mode with a mass to change ratio (m/z) range of 30 to 600 at a rate of 20 spectra per second after a solvent delay of 4.93 min.

Identification of compounds discovered by gas chromatography time-of-flight/MS

Chroma TOF4.3X software (LECO Corporation) and the LECO-Fiehn Rtx5 database were used to extract raw peaks, filtering and calibrating the data baseline, peak alignment, deconvolution analysis, peak identification and integration of the peak area. Missing values in the raw data were replaced by half of the minimum value. Noise was then removed by filtering the data with an interquartile range

and display similarities and differences. The OPLS-DA was used to obtain the maximal covariance between the measured data and the response variable. The data scale conversion mode used for processing plasma was UV formatted (unit variance scaling).

Identification of plasma metabolites showing significantly different expression between treatments

A loading plot was constructed based on the OPLS-DA to show the relative contribution of different variables to the differences observed between every two treatment groups of four. For further refinement, we obtained the first principal component of variable importance projection (**VIP**). The remaining variables were then evaluated using the Student's *t* test. The fold change for each metabolite was calculated by comparing the mean value of the peak area obtained from the two comparison groups. Thus, we used VIP > 1.0 and $P < 0.05$ to identify significant differentially expressed metabolites (**DEMs**) between the two dietary treatment groups (Luo *et al.*, 2019). In addition, commercial databases, including KEGG (http://www.genome.jp/kegg/) and NIST (http://www.nist.gov/index.html), were utilized to further identify and validate different metabolites.

Metabolic pathway analysis of differentially expressed metabolites between treatments

Metabolites showing differential expression when compared between heifers fed high and low concentrate were then analyzed by MetaboAnalyst 3.0 software (http://www.metaboanalyst.ca) (Xia *et al.*, 2015) in order to identify the metabolic pathways involved. For this, we used the *Bos taurus* (cow) pathway library along with global test pathway enrichment analysis and relative-betweenness centrality pathway topology analysis. All matched pathways, as determined by *P* values from pathway-enrichment analysis and pathway impact values from pathway topology analysis, were then shown in the metabolome view.

Oxidative status measurements

The enzyme activities of total superoxide dismutase (**T-SOD**), glutathione peroxidase (**GSH-Px**) and catalase (**CAT**); were reported, and significant differences were declared at $P < 0.05$; tendency was reported at $0.05 \leq P \leq 0.10$.

Results

Animal performance

Animal performance results can be found in our previous publication (Zhang *et al.*, 2018). The diets were designed to meet the nutritional requirements and were offered in limited amounts: no leftovers were observed. In brief, DMI (5.32, 4.97, 4.69 and 4.42 kg/day in the C20, C40, C60 and C80 groups, respectively) decreased significantly with a quadratic ($P = 0.02$) response with increasing levels of dietary concentrate. The measured ADG (with gut fill; 1.01, 0.89, 0.83 and 0.77 kg/day in the C20, C40, C60 and C80 groups, respectively) linearly decreased with increasing dietary concentrate levels, whereas the measured FE (with gut fill; 0.19, 0.18, 0.18 and 0.18 in the C20, C40, C60 and C80 groups, respectively) was similar among treatments. After being corrected for gut fill, both the cADG (0.38, 0.59, 0.83 and 1.07 kg/day in the C20, C40, C60 and C80 groups, respectively) and the cFE (0.07, 0.12, 0.18, and 0.24 in the C20, C40, C60 and C80 groups, respectively) increases linearly ($P < 0.01$) with increasing levels of dietary concentrate.

Identification and quantification of gas chromatography time-of-flight/MS compounds

The GC-TOF/MS total ion chromatograms of plasma from heifers fed with the four diets are shown in online Supplemental Figure S1. In total, 365 valid peaks were identified in plasma samples from the four groups. Based on the LECO/Fiehn Metabolomics Library, the majority of peaks were endogenous metabolites; some of these peaks may also represent derivatives of by-products. Next, we quantified 160 metabolites in plasma from the four groups, including amino acids (**AAs**), fatty acids, sugars and organic acids. Detailed information (peak name, similarity, RT, count and mass) relating to all of the metabolites identified in the four groups is given in online Supplemental Table S1.

Statistical comparison of plasma metabolites between treatments

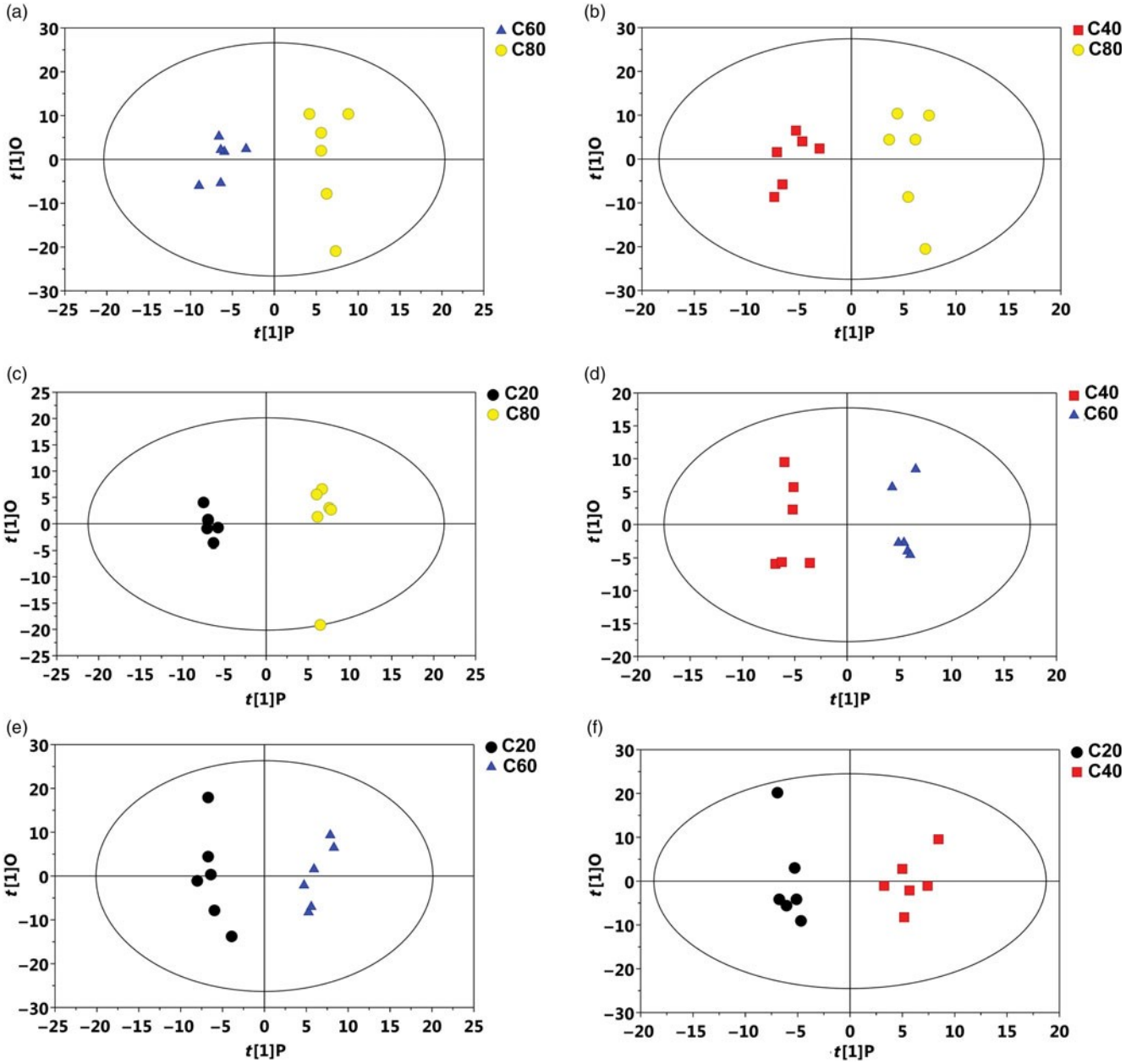

Figure 1 (colour online) Corresponding OPLS-DA score plots derived from the GC-TOF/MS metabolite profiles of plasma samples between heifers fed diets with different forage to concentrate ratios. Corresponding OPLS-DA score plots (respectively) for: (a) the C80 group *v.* C60 group; (b) the C80 group *v.* C40 group; (c) the C80 group *v.* C20 group; (d) the C60 group *v.* C40 group; (e) the C60 group *v.* C20 group; (f) the C40 group *v.* C20 group. OPLS-DA, orthogonal projections to

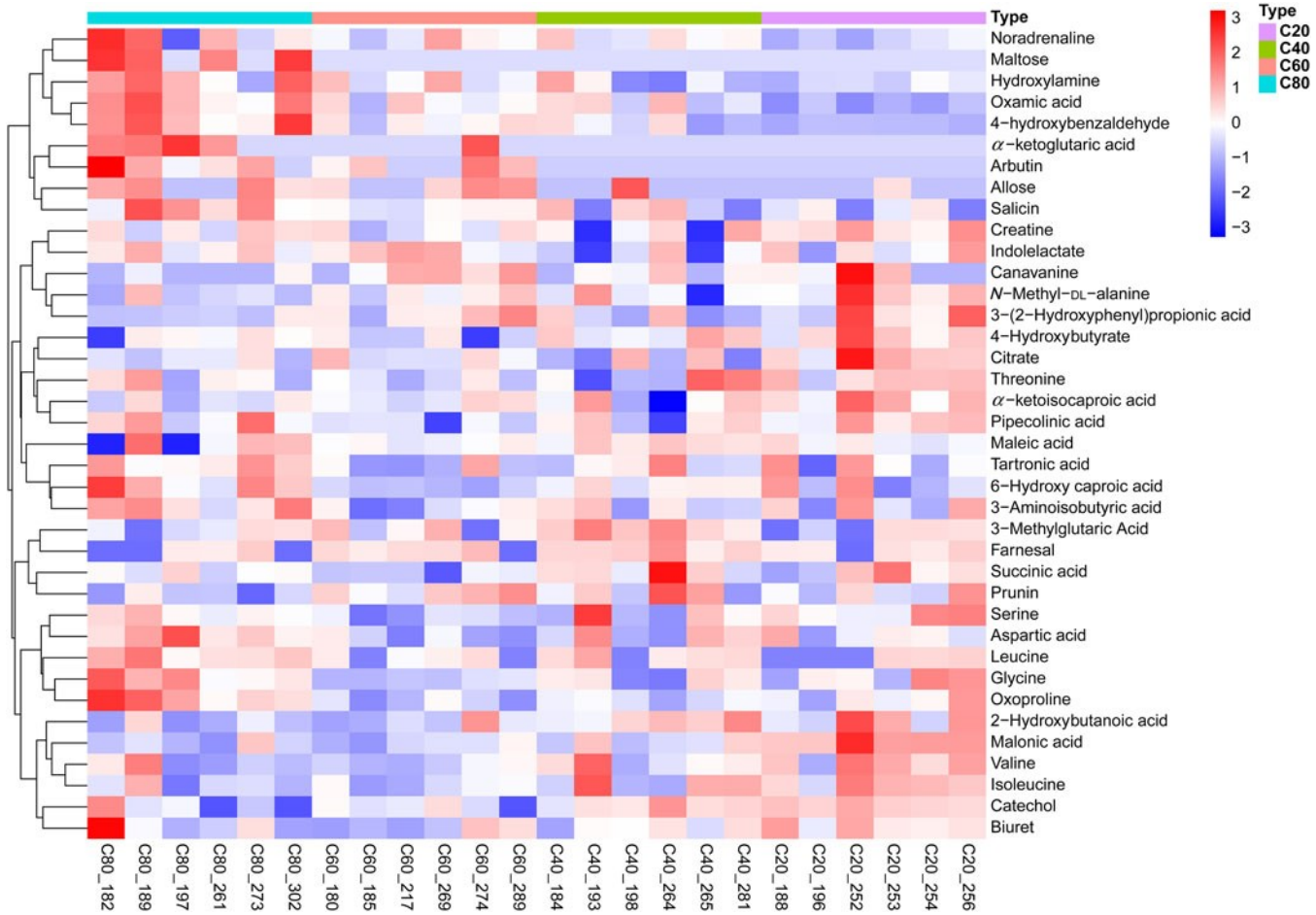

Figure 2 (colour online) Heat map of different metabolites in plasma samples between heifers fed diets with different forage to concentrate ratios. Cells are colored based on the signal intensity of metabolites measured in plasma. Light red cells represent high intensity, while light blue cells represent low signal intensity and white cells represent intermediate levels of intensity. The C20, C40, C60 and C80 diets contained 20%, 40%, 60% and 80% of concentrate, respectively.

Metabolic pathways associated with the differentially expressed metabolites identified in plasma between treatments

Differential metabolites in plasma samples from the treatment groups were analyzed by MetaboAnalyst 3.0 software to reveal their association with metabolic pathways. Nine pathways (tricarboxylic acid cycle (**TCA cycle**); starch and

Oxidative status

Plasma T-AOC and T-SOD increased significantly ($P < 0.01$) in a quadratic manner with increasing dietary concentrate, while the concentration of H_2O_2 and GSH decreased significantly with a quadratic response ($P = 0.02$) (Table 3). The plasma concentration of OH^- decreased linearly ($P = 0.01$) with increasing levels of dietary concentrate, while the

Table 2 *Significantly changed plasma metabolites between heifers fed diets with different forage to concentrate ratios*

Super class	Metabolite names	Treatments[1]				SEM	*P* values			
		C20	C40	C60	C80		Treatment	Linear	Quadratic	Cubic
Aliphatic heteromonocyclic compounds	Oxoproline	1.79E+00	1.63E+00	1.48E+00	2.23E+00	8.095E-02	<0.01	0.04	<0.01	0.11
Amino acids, peptides, and analogues	Valine	9.72E-01	8.92E-01	7.63E-01	7.91E-01	3.474E-02	0.12	0.03	0.41	0.48
	Leucine	2.91E-01	4.88E-01	3.29E-01	6.35E-01	5.614E-02	0.11	0.07	0.61	0.09
	Isoleucine	4.30E-01	3.81E-01	2.94E-01	2.94E-01	2.327E-02	0.09	0.02	0.58	0.52
	Glycine	7.16E-01	6.48E-01	5.95E-01	8.03E-01	2.822E-02	0.04	0.36	0.01	0.29
	Serine	1.29E-01	1.15E-01	9.43E-02	1.21E-01	5.146E-03	0.09	0.30	0.05	0.21
	Pipecolinic acid	2.33E-03	1.75E-03	1.37E-03	2.32E-03	1.658E-04	0.10	0.77	0.02	0.42
	Threonine	5.97E-02	4.70E-02	3.94E-02	4.76E-02	4.488E-03	0.48	0.29	0.27	0.80
	3-Aminoisobutyric acid	1.30E-02	1.27E-02	1.21E-02	1.41E-02	2.942E-04	0.12	0.30	0.06	0.25
	Aspartic acid	7.23E-03	7.44E-03	5.59E-03	9.34E-03	4.832E-04	0.04	0.25	0.05	0.06
	Creatine	6.19E-01	3.83E-01	4.71E-01	5.21E-01	3.845E-02	0.18	0.53	0.07	0.28
	Canavanine	7.74E-04	4.64E-04	8.55E-04	1.85E-04	1.220E-04	0.19	0.20	0.45	0.11
Aromatic heteropolycyclic compounds	Indolelactate	1.97E-03	1.22E-03	2.25E-03	2.10E-03	1.571E-04	0.08	0.28	0.31	0.03
	Catechol	9.59E-04	8.83E-04	5.76E-04	5.07E-04	6.906E-05	0.04	0.01	0.98	0.39
	4-hydroxybenzaldehyde	1.58E-03	1.91E-03	2.10E-03	2.72E-03	1.111E-04	<0.01	<0.01	0.33	0.40
	3-(2-hydroxyphenyl)propionic acid	1.80E-02	1.31E-02	1.66E-02	1.24E-02	9.583E-04	0.10	0.11	0.83	0.05
	Noradrenaline	1.06E-03	1.61E-03	1.56E-03	1.96E-03	1.546E-04	0.23	0.06	0.80	0.45
Carbohydrates and carbohydrate conjugates	Allose	1.76E-04	4.33E-04	1.02E-03	1.11E-03	1.816E-04	0.20	0.04	0.82	0.60
	Salicin	2.61E-04	3.35E-04	3.80E-04	6.13E-04	4.939E-05	0.06	0.01	0.38	0.59
	Arbutin	3.16E-06	3.01E-06	1.32E-03	1.98E-03	2.730E-04	0.01	<0.01	0.47	0.33
	Maltose	3.16E-06	3.01E-06	3.03E-06	2.08E-03	2.463 E-04	<0.01	<0.01	0.01	0.19
Homogeneous non-metal compounds	Hydroxylamine	5.47 E-02	5.47 E-02	6.17 E-02	6.97 E-02	2.306 E-03	0.05	0.01	0.34	0.74
Lipids	α-Ketoisocaproic acid	9.58 E-03	6.85 E-03	7.75 E-03	7.08 E-03	4.993 E-04	0.21	0.14	0.30	0.24
	Maleic acid	1.91 E-03	2.19 E-03	1.88 E-03	1.67 E-03	1.387E-04	0.65	0.43	0.40	0.61
Organic acids and derivatives	2-hydroxybutanoic acid	3.49E-02	3.48E-02	2.67E-02	2.39E-02	1.814E-03	0.05	0.01	0.69	0.36
	Malonic acid	2.06E-03	1.43E-03	1.25E-03	1.27E-03	8.720E-05	<0.01	<0.01	0.01	0.62
	Succinic acid	1.65E-03	1.76E-03	1.40E-03	1.59E-03	5.296E-05	0.10	0.22	0.72	0.03
	Tartronic acid	8.10E-04	8.21E-04	7.26E-04	9.22E-04	3.482E-05	0.27	0.43	0.19	0.20
	6-hydroxy caproic acid	3.21E-04	3.39E-04	2.77E-04	3.95E-04	1.431E-05	0.02	0.16	0.05	0.03
	α-Ketoglutaric acid	3.16E-06	3.01E-06	5.88E-04	2.01E-03	2.693E-04	0.01	<0.01	0.13	0.90
	Citric acid	1.22E-01	5.04E-02	7.73E-02	5.94E-02	9.854E-03	0.04	0.05	0.13	0.08
–	*N*-methyl-DL-alanine	6.49E-02	4.48E-02	5.33E-02	4.31E-02	3.835E-03	0.17	0.10	0.50	0.16
	4-Hydroxybutyrate	4.03E-03	3.56E-03	2.32E-03	2.79E-03	2.638E-04	0.09	0.03	0.35	0.26
	Oxamic acid	1.28E + 00	1.88E + 00	1.86E + 00	2.42E + 00	1.076E-01	<0.01	<0.01	0.90	0.08
	3-Methylglutaric acid	2.24E-04	4.53E-04	2.92E-04	2.53E-04	3.430E-05	0.07	0.79	0.04	0.08
	Biuret	7.86E-03	6.26E-03	5.35E-03	6.90E-03	5.279E-04	0.41	0.43	0.15	0.71
	Farnesal	1.30E-02	1.77E-02	1.39E-02	7.91E-03	1.450E-03	0.12	0.13	0.06	0.62
	Prunin	8.79E-03	1.03E-02	1.13E-02	5.07E-03	9.357E-04	0.08	0.20	0.03	0.38

The C20, C40, C60, and C80 diets contained 20%, 40%, 60%, and 80% of concentrate, respectively.
[1]The values represent the relative abundance (or signal intensity in MS) of the corresponding metabolites and are represented by scientific notation.

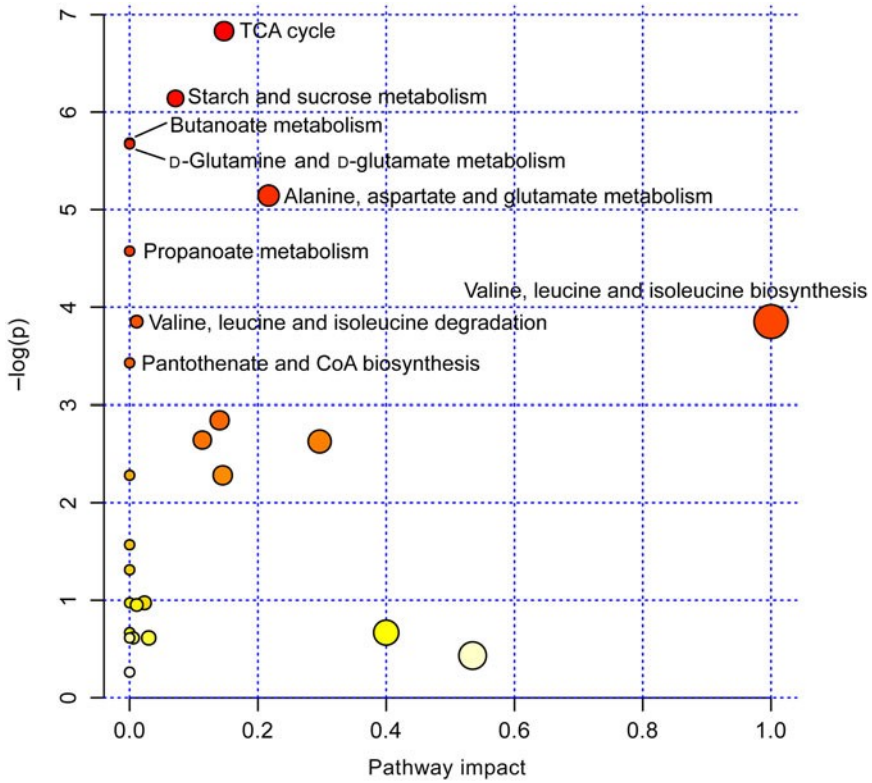

Figure 3 (colour online) Pathway analysis of differential metabolites in plasma between heifers fed diets with different forage to concentrate ratios using MetaboAnalyst 3.0. The color of the circles, from white to yellow to red, denotes incremental fold change (−log(p)). The size of the circles, from small to large, indicates an increment in pathway impact. TCA cycle, tricarboxylic acid cycle.

large amount of fermentable carbohydrate (Zhang *et al.*, 2018). This indicated that all of the heifers used in this study had a healthy internal environment in the rumen (Zhang *et al.*, 2017).

The DMI decreased with increasing dietary concentrate levels, in accordance with previous studies (Lascano *et al.*, 2009; Zanton and Heinrichs, 2009; Lascano *et al.*, 2016). This was because intakes of ME and CP were controlled in a similar manner across our treatment groups by precision feeding, and because high-concentrate diets contain higher energy levels. Other studies found that heifers fed low levels of concentrate had a greater amount of gastrointestinal tract digesta (Hoffman *et al.*, 2007; Zanton and Heinrichs, 2007). Thus, in the present study, we used cADG (after being corrected for gut fill) to calculate cFE (Williams *et al.*, 1992). We found that the high-concentrate diet feeding led to an increase in cFE, as reported by other studies (Hoffman *et al.*, 2007; Lascano *et al.*, 2009).

Systemic metabolites

Succinate, α-ketoglutarate and citrate have been identified as key players in the TCA cycle (Da Poian and Castanho,

Table 3 *Plasma oxidants and antioxidants of heifers fed diets with different forage to concentrate ratios*

2015). As a rate-determining intermediate in the TCA cycle, α-ketoglutarate plays a crucial role in cellular energy metabolism (Da Poian and Castanho, 2015). The increased levels of α-ketoglutarate in heifers fed high levels of concentrate not only suggest the increased availability of substrate but also a higher conversion rate in the TCA cycle. The cubically or tended to be cubically changed succinate and citrate among treatments may indicate that they were undergoing rapid generation and utilization, which may further promote the TCA cycle as well as energy turnover of the body (Zhang *et al.*, 2019). Allose, arbutin and maltose could be generated from dietary fructose, benzoate and starch, respectively (Bhuiyan *et al.*, 1998; Jiang *et al.*, 2017). After being converted to glucose through gluconeogenesis, allose, arbutin and maltose can enter the TCA cycle in the form of acetyl-CoA (Bhuiyan *et al.*, 1998; Jiang *et al.*, 2017). As a β-glucosyl ester derived from salicylate, salicin can be converted into succinate which then enters into the TCA cycle (Song and Lee, 2006). Thus, the increased abundance of allose, salicin, arbutin and maltose in heifers fed high-concentrate diet may provide abundant precursors for the TCA cycle.

According to our previous data, ruminal content of propionate increased linearly ($P = 0.01$) with increasing amounts of dietary concentrate (Zhang *et al.*, 2018). A previous study showed that 27% to 50% of glucose turnover originates from propionate (Young, 1977). In the present study, most of the DEMs were classified as AAs, peptides and analogues. Although some of these DEMS, such as valine, isoleucine, glycine, serine, threonine and aspartate can be used as glucogenic substrates, it is reasonable to infer that a propionate-induced abundance of precursors for gluconeogenesis can allow AAs to be utilized predominantly in protein synthesis. Previous research showed that leucine can not only serve as a substrate for protein synthesis but also act as a signal to increase the rate of protein synthesis in the skeletal muscle of dairy calves (Zheng *et al.*, 2019). In the present study, we found that the greater cADG in heifers fed high levels of concentrate was consistent with the tendency for a linear increase in leucine levels. As ketogenesis was downregulated in the C80 group (Zhang *et al.*, 2019), the ketogenic AAs were expected to increase. However, the presence of antagonisms among branched-

(Kuhla *et al.*, 2009). Creatine has also been identified as a potential biomarker for diagnosing heat stress in dairy cows; increased levels of creatine in the blood may indicate extensive mobilization of phosphocreatine in the muscle tissue to supply energy (Tian *et al.*, 2015). Arginine, glycine and methionine are the three main precursors for creatine synthesis in the liver (Sun *et al.*, 2017). Higher concentrations of blood creatine were detected in dairy cows during early lactation after calving or with severe negative energy balance (Wang *et al.*, 2016; Luo *et al.*, 2019). A previous study, which ranked residual feed intake (**RFI**) in two populations of beef steers, found that there is a negative correlation between plasma creatine and FE and that creatine with other metabolites can account for 32% to 74% of phenotypic variation in RFI (Karisa *et al.*, 2014). Thus, the tendency for lower levels of creatine in heifers fed high levels of concentrate may not only be a signal of less muscle mobilization and protein turnover but also be a potential biomarker of higher FE (Karisa *et al.*, 2014). Moreover, in agreement with our present study, previous transcriptomic studies have also shown that most protein metabolism pathways related to protein turnover may play a major role in the regulation of FE in ruminants (de Almeida Santana *et al.*, 2016; Salleh *et al.*, 2017).

Oxidative status

Compared with heifers fed a low level of concentrate, those fed high levels showed lower levels of OH^- and H_2O_2 and higher levels of T-AOC and T-SOD. These data indicate that heifers fed high levels of concentrate exhibit a lower oxidative status (Li *et al.*, 2016). It is not clear as to why the levels of CAT and GSH-Px were so similar across different treatment groups, although the increased levels of T-SOD in heifers fed a high level of concentrate suggested that enzymatic scavengers were mainly responsible for defense against oxidative status. The cubically influenced MDA might indicate that the oxidative stress was not high enough to induce serious damage to the host. The low-concentrate diet fed heifers had lower ruminal propionate ($P = 0.04$) compared to the high-concentrate diet fed heifers, indicating fewer precursors for gluconeogenesis and/or TCA cycle (Zhang *et al.*, 2018). In order to produce enough energy for the whole body, the low-concentrate fed heifer has to

peroxisomal β-oxidation) and oxidative phosphorylation (Schaff *et al.*, 2012; Sejersen *et al.*, 2012). As the same dietary ingredients were used among treatments and no specific antioxidant compounds were added in high-concentrate diets, the altered metabolism might be related to the changed oxidative status.

As an organic acid, 2-hydroxybutanoic acid participates in several important metabolic pathways such as AA degradation and GSH synthesis (Wang *et al.*, 2018). The 2-hydroxybutanoic acid is also a by-product of the conversion of cystathionine to cysteine and GSH. Thus, changing the levels of 2-hydroxybutanoic acid may be associated with changes in GSH and redox state in the body (Cao *et al.*, 2017). An earlier study reported higher levels of 2-hydroxybutanoic acid in myocardial tissues of patients living in high-altitude pikas with activated oxidative stress (Cao *et al.*, 2017). In accordance with our previous findings (Zhang *et al.*, 2019), the linear reduction of 2-hydroxybutanoic acid indicates a lower oxidative status in heifers fed high levels of concentrate. Neutralizing ROS and degrading/replacing oxidatively damaged cells or biomolecules is an energy-consuming process (Nordberg and Arner, 2001; Russell, 2015). Even though the higher oxidative status in heifers fed with low levels of concentrate diet did not induce profound damage, the increase in oxidative defense might require more energy to protect the body from higher oxidative status, which may contribute to the decreased energetic utilization efficiency (Russell, 2015). Similarly, previous studies have also reported that inefficient energy utilization was primarily associated with increased oxidative metabolism that was potentially stimulated by increased oxidative status (Alexandre *et al.*, 2015; Russell, 2015; Zhang *et al.*, 2019).

In the present study, we used a metabolomics approach to investigate the plasma samples for evidence of alterations in key biological processes in Holstein heifers precision fed with a wide range of dietary F : C. Heifers fed high levels of concentrate had higher cADG and cFE values than heifers fed low levels of concentrate. Based on the plasma metabolomics analysis, we found that the main biological pathways affected were those related to carbohydrate and AA metabolism. Plasma oxidative status indicated a lower oxidative

and Development Program of China (2018YFD0501603-06-2 and 2018BBF33006). The funding bodies did not play a role in research direction. We would particularly like to acknowledge the staff of Beijing Jinxing Dairy Farm, and members of Dr. Li's laboratory, for their assistance with housing and sampling the animals.

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Declaration of interest

None of the authors has any conflicts of interest to declare.

Ethics statement

This study was carried out in accordance with the recommendations of Instructive Notions with Respect to Caring for Experimental Animals, Ministry of Science and Technology of China. The protocol was approved by the Ethical Committee of the College of Animal Science and Technology of China Agricultural University.

Software and data repository resources

The data were totally shown in the manuscript. The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731120001287

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