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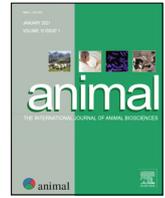
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Flash dietary methionine supply over growth requirements in pigs: Multi-faceted effects on skeletal muscle metabolism



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ABSTRACT

Dietary methionine affects protein metabolism, lean gain and growth performance and acts in the control of oxidative stress. When supplied in large excess relative to growth requirements in diets for pigs, positive effects on pork quality traits have been recently reported. This study aimed to decipher the molecular and biochemical mechanisms affected by a dietary methionine supply above growth requirements in the loin muscle of finishing pigs. During the last 14 days before slaughter, crossbred female pigs ($n = 15$ pigs/diet) were fed a diet supplemented with hydroxy-methionine (**Met5**; 1.1% of methionine) or not (**CONT**, 0.22% of methionine). Blood was sampled at slaughter to assess key metabolites. At the same time, free amino acid concentrations and expression or activity levels of genes involved in protein or energy metabolism were measured in the *longissimus lumborum* muscle (**LM**). The Met5 pigs exhibited a greater activity of creatine kinase in plasma when compared with CONT pigs. The concentrations of free methionine, alpha-aminobutyric acid, anserine, 3-methyl-histidine, lysine, and proline were greater in the LM of Met5 pigs than in CONT pigs. Expression levels of genes involved in protein synthesis, protein breakdown or autophagy were only scarcely affected by the diet. Among ubiquitin ligases, *MURF1*, a gene known to target creatine kinase and muscle contractile proteins, and *OTUD1* coding for a deubiquitinase protease, were up-regulated in the LM of Met5 pigs. A lower activity of citrate synthase, a reduced expression level of *ME1* acting in lipogenesis but a higher expression of *PPARD* regulating energy metabolism, were also observed in the LM of Met5 pigs compared with CONT pigs. Principal component analysis revealed that expression levels of many studied genes involved in protein and energy metabolism were correlated with meat quality traits across dietary treatments, suggesting that subtle modifications in expression of those genes had cumulative effects on the regulation of processes leading to the muscle transformation into meat. In conclusion, dietary methionine supplementation beyond nutritional requirements in pigs during the last days before slaughter modified the free amino acid profile in muscle and its redox capacities, and slightly affected molecular pathways related to protein breakdown and energy metabolism. These modifications were associated with benefits on pork quality traits.

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Implications

Nutritional recommendations consider the role of amino acids for growth and maintenance functions. Adding methionine in diet for pigs beyond these recommendations improves pork quality traits. The present study shows that this benefits could be related to subtle molecular modulations in muscle protein and energy metabolism as well as to changes in the muscle concentrations of free methionine and other amino acids affecting oxidative metabolism. This indicates that the functional roles of methionine in

different pathways should be considered when formulating diets for finishing pigs.

Introduction

Sulfur-containing amino acids methionine and cysteine are the first and third limiting factors for diets of chicken and pigs, respectively (NRC, 2012). As the building blocks of all proteins, the dietary supply of sulfur amino acids tightly regulates lean growth rate (Wu, 2009). Skeletal muscle is the body compartment mostly affected by a dietary deficiency in total sulfur amino acids, as compared to blood, intestine, liver and other internal organs (Conde-Aguilera et al., 2014). Reduced protein content, lower

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fractional rate of protein synthesis, decreased proteolytic enzyme activities but higher glycolytic potential have been reported in muscle of pigs or chicken fed diets deficient in total sulfur amino acids (Conde-Aguilera et al., 2010, 2014, 2016a; 2016b). In addition, sulfur-containing compounds such as glutathione and taurine, which are derived from methionine metabolism (Estévez et al., 2020 for a recent review), have antioxidant properties to scavenge reactive oxygen species and attenuate lipid peroxidation. Considering that exogenous dietary antioxidants retard and even prevent oxidation processes with benefits on the quality of animal-derived products (O'Grady and Kerry, 2009), few studies have examined the effects of methionine supplied beyond growth requirements on meat quality traits. Pigs fed L-methionine-supplemented diets from 40 kg up to 100 kg BW had lower lipid peroxidation, higher ultimate pH and lower drip loss in the loin muscle during meat storage (Li et al., 2017). Similarly, we demonstrated that pigs fed extra methionine supply to five-fold the requirements during the last 14 days before slaughter had lower thiobarbituric acid reactive substances levels in the loin muscle during a forced lipid oxidation challenge, and exhibited higher ultimate pH and lower drip loss during loin meat storage (Lebret et al., 2018). Dietary supplementations with DL-methionine, DL-hydroxy-methionine or combined methionine sources over nutritional requirements for chicken have also protected effects against oxidation with benefits on drip loss, pH and color of the meat (Estévez et al., 2020). However, the mechanisms underlying these effects of extra dietary methionine supply on muscle traits remain to be clarified. Considering data reported for a methionine dietary deficiency in pigs (Castellano et al., 2015; Conde-Aguilera et al., 2014; 2020), we suspected that changes in amino acid supply and a modulation of energy metabolism may occur in the muscle of living animals following the ingestion of a diet supplemented in methionine over the nutritional requirements for growth and maintenance. These changes could affect the composition of the muscle proteins, the intracellular turnover of proteins and the biochemical processes leading to the *postmortem* transformation of muscle into meat with effects on ultimate pH, drip loss and tenderization. Dietary methionine supply may also affect the body content in the sulfur-containing molecules and the equilibrium in amino acids having antioxidant or pro-oxidant properties, and thus affect the ability of the muscle tissue to modulate lipid and protein oxidation during meat storage. This study aimed at investigating the effects of dietary methionine provided in excess relative to requirements during the last 14 days before slaughter on free amino acids and molecular indicators of protein and energy metabolism in the loin muscle of finishing pigs.

Material and methods

Pigs, diets and samples

The full experimental design was described elsewhere (Lebret et al., 2018) and is only briefly summarized here. At approximately 70 kg BW, 45 crossbred ((Large White × Landrace) × Piétrain) female pigs in 15 litters issued from four different sires (littermates or half-littermates) were chosen to have similar BW and backfat thickness, and distributed in three dietary groups. From 70 kg to 105 kg BW, all pigs were first fed a control diet (**CONT**) formulated to meet their nutritional requirements (0.22% methionine) based on NRC guidelines (2012). During the last 14 days before slaughter, pigs ($n = 15/\text{group}$) received either the CONT diet or a diet supplemented with DL-2-hydroxy-4-(methylthio)butanoic acid (**HMTBA**, also called hydroxy-DL-methionine) with a calculated methionine value being three times (**Met3** group: 0.66% methionine) or five

times greater (**Met5** group: 1.10% methionine) than that in the CONT diet. Diets were formulated to be iso-energetic and iso-nitrogenous. All pigs had daily free access to feed and water until slaughter. After an overnight fast, pigs were slaughtered by exsanguination following electronarcosis, alternating animals of the three dietary groups. Jugular blood was sampled on heparin, and plasma was immediately prepared by low speed centrifugation and stored at -20°C . Thirty minutes after slaughter, samples were taken from the *longissimus lumborum* muscle (**LM**) at the last rib level, cut into small pieces, and immediately frozen in liquid nitrogen. Muscle samples were stored at -76°C until performing the measures of amino acid concentrations, enzyme activities and expression levels of target genes. Other biochemical parameters (intramuscular fat, protein, and glutathione contents, glycolytic potential, thiobarbituric acid reactive substances) were measured on the same samples. Loin meat quality traits (pH, drip loss, and color parameters L^* , a^* , b^* and h°) and indicators of protein oxidation (carbonyls and free thiols) were also assessed at one and (or) four days after slaughter. All methods and results are presented in detail by Lebret et al. (2018). Since the responses on meat quality traits were mainly or only significant for the dietary treatment with the highest methionine supply, the 30 pigs fed either the Met5 diet or the CONT diet (Table 1) were considered in the present study aiming to decipher the muscle mechanisms associated

Table 1
Composition of diets with adequate or extra methionine concentration fed to pigs.

Items	Diet	
	CONT	Met5
Ingredients (g/kg as fed basis)		
Maize	661	661
Wheat	100	100
Soybean meal 48	160	160
Wheat middlings	10.0	0
Palm oil	30	30
Calcium carbonate	12.8	12.8
Dicalcium phosphate	9.8	9.8
Vitamin/mineral premix ¹	10	10
Salt	4.0	4.0
L-Lysine HCl	2.2	2.2
L-Threonine	0.1	0.1
L-Tryptophane	0.4	0.4
DL-HMTBA	0	10.0
Crude protein	13.7	13.6
Crude Fat	5.82	5.79
Ash	4.80	4.76
Cellulose	2.59	2.54
Calculated nutrient composition (%)		
Net energy (MJ/kg)	10.38	10.44
Methionine ²	0.20	1.02
Cysteine ²	0.25	0.25
Lysine ³	0.73	0.73
Threonine ³	0.46	0.46
Tryptophane ³	0.13	0.13
Arginine ³	0.80	0.79
Histidine ³	0.35	0.35
Isoleucine ³	0.54	0.53
Leucine ³	1.24	1.23
Phenyl-alanine ³	0.64	0.64
Phenyl-alanine + Tyrosine ³	1.11	1.10
Valine ³	0.61	0.61

Abbreviations: CONT = control diet; Met5 = methionine-supplemented diet; HMTBA = 2-hydroxy-4-(methylthio)butanoic acid.

¹ Vitamins (/kg diet) A (7 500 UI), D₃ (1 500 UI), E (20 UI), B₁ (0.5 mg), K₃ (1.0 mg), B₂ (3.0 mg), B₅ (10.0 mg), B₆ (1.0 mg), B₁₂ (0.015 mg), PP (1 500 mg) and minerals iron (80 mg), iodine (0.50 mg), copper (15 mg), manganese (40 mg), zinc oxide (80 mg) and sodium selenite (0.25 mg) together with phytase (500 FTU).

² Analyzed methionine equivalent [total methionine + 2-hydroxy-4-(methylthio)butanoic acid] and cystine levels.

³ Adjusted for 86.3% of DM.

to extra dietary methionine supply ($n = 15$ pigs/diet). Statistics for meat quality traits (ultimate pH, color, drip loss, intramuscular fat content) are provided in [Supplementary Table S1](#). Additional details about the methods used to determine biochemical and meat quality traits are given in [Supplementary Material S1](#).

Plasma metabolites, muscle free amino acids and peptides

Commercial kits were used to measure the plasma levels of glucose (Glucose GOD, Thermo Fisher Scientific, Waltham, MA, USA), lactate (lactate oxidase, Trinity Biotech, Jamestown, NY, USA), urea (urease, Thermo Fisher Scientific) and the activity of creatine kinase (CK IFCC, Thermo Fisher Scientific), using a spectrophotometric analyzer (Konelab20, Thermo Fischer Scientific). Details for quality assurance are available in [Supplementary Material S1](#).

The LM samples were ground in a 6N trichloro-acetic acid solution using a polytron (PT3100, Kinematica AG, Germany), and centrifuged at 10,000g for 15 min at 4 °C. Free amino acid and dipeptide concentrations were determined on supernatants after AccQ-Tag Ultra derivatization (MassTrak AAA Waters, Milford, MA) using a ultra high performance liquid chromatography system (Waters Acquity Ultra Performance LC system, Waters, Guyancourt, France) coupled to an Acquity tunable UV detector and a mass detector, as previously described ([Merlot et al., 2016](#)) and detailed in [Supplementary Material S1](#).

Metabolic enzyme activities in the longissimus lumborum muscle

Cathepsin activity was evaluated as a protease playing important role in intracellular protein catabolism. The first step in glucose use by the muscle cells was evaluated by measuring the hexokinase activity. Glycolysis was evaluated by measuring the lactate dehydrogenase (**LDH**) acting in terminal route. The oxidative pathway was assessed by measuring citrate synthase (**CS**), the first step in tricarboxylic acid cycle.

Protein catabolism

The LM samples (50 mg) were homogenized using the polytron (PT3100, Kinematica AG, Germany) in a 20 mmol/L Tris-HCl buffer (pH = 7.2) containing dithiothreitol (DTT, 1 mmol/L) and Triton X100 (1%; Sigma, Steinheim, Germany). Mixtures were sonicated (Labsonic 2000, Braun, Germany), and then centrifuged for 15 min at 12 000g at 4 °C. The cathepsin activity was assayed on the LM homogenates (1:4 dilution) using a commercial kit (Cliniscience K142-100, Biovisio, Mountain View, CA, USA), including a synthetic substrate (amino-4-trifluoromethyl coumarin), an inhibitor and the reaction buffer. Activity was measured by fluorometry at 355 nm for excitation and 460 nm for emission during 90 min, respectively, and using a microplate reader (Thermo-Labsystems, Franklin, MA, USA).

Energy metabolism

The hexokinase activity was assessed in LM samples (500 mg) homogenized (dilution 1:9) in ice-cold buffer [50 mM Tris, 5 mM EDTA, 2 mM 1,4-dithiothreitol; pH 7.4] containing protease inhibitors cocktail (Complete, Roche Diagnostic, Meylan, France) as previously described ([Gondret et al., 2014](#)) and using a Cryolys Precellys (Ozyme, France). Homogenates were sonicated, and then centrifuged at 4 °C for 10 min at 900g. The hexokinase activity was measured spectrophotometrically on the Konelab at 340 nm absorbance, by coupling ribulose-5-phosphate formation from glucose-6-phosphate to the reduction of NADP, and using purified glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Sigma, Steinheim, Germany) as coupling enzymes.

Activities of LDH and CS were assayed as detailed by [Lefaucheur and Lebret \(2020\)](#), using the Konelab spectrophotometric analyzer. Details and quality assurance are available in [Supplementary Material S1](#).

Soluble protein concentration

Protein concentration was measured using the Bradford reagent (Sigma Aldrich, St-Quentin Fallavier, France) in the different homogenates to express activity of enzymes per mg of proteins.

Expression levels of target genes in the longissimus lumborum muscle

Total RNA was extracted from frozen LM samples using methods described previously ([Vincent et al., 2015](#)). First-strand cDNA was synthesized from 1 µg of total RNA using High Capacity RNA to cDNA Kit (Applied Biosystems, Foster City, USA). Primers were designed from porcine sequences available in Ensembl or NCBI databases using Primer Express® v3.0 software (Applied Biosystems). Information on the selected genes is provided in [Supplementary Table S2](#). Amplification reactions were carried out using the LightCycler 480 SYBR Green 1 Master (Roche Diagnostics, Meylan, France) with a final cDNA concentration of 1 ng/µL and a primer concentration of 500 nM dispensed using the WaferGene SmartChip Multisample Nanodispenser available at the EcogenO Platform (GEH, Rennes, France). Amplification conditions were as follows: 5 min at 95 °C followed by 45 cycles of 10 sec at 95 °C, 30 s at 60 °C and 30 sec at 72 °C, followed by 30 sec at 95 °C and 30 sec at 60 °C. Specificity of the amplification products was checked by dissociation curve analysis. As stated by the GeNorm algorithm, the most stable genes among 10 tested housekeeping genes were *B2M*, *PPIA* and *POLR2A*, and these three genes were used to calculate the normalization factor (**NF**). For each gene, the normalized expression level N of each gene was calculated according to the formula ([Vandesompele et al., 2002](#)): $N = E - \Delta Cq(-\text{sample-calibrator})/NF$ where E (efficiency) is calculated from the slope of calibration curve, Cq is the quantification cycle, and the calibrator is a pool of all samples. For the studied genes, E was between 1.60 and 2.23 (1.78 on average).

Statistics

Data were analyzed by ANOVA using GLM procedure of the SAS software (SAS Cary NC, NY, USA). Diet was considered as a fixed effect in the model. A P -value <0.05 was considered significant, and $0.05 < P < 0.10$ was discussed as a trend. In the Tables, data are presented as mean for each dietary group and pooled standard error of the means. A principal component analysis was performed using the PRINCOMP procedure of SAS, to provide an overview of the correlation matrix and facilitate graphical interpretation of the relationships between gene expression levels and pork quality traits. Each data point was projected onto the first two principal components defined as the eigenvectors obtained by a linear combination of the original variables that maximized the variance of the projected data. Details about the statistical models are available in [Supplementary Material S1](#).

Results

Performance and muscle biochemical composition at slaughter

Growth performance and carcass composition were previously published for the full animal design ([Lebret et al., 2018](#)). The data obtained in the Met5 and CONT groups are only briefly summarized here. No death nor impairment of growth performance and

feed intake were reported during the test period for the Met5 pigs, suggesting no toxic effects of the extra dietary methionine supply in those pigs. There were no dietary-associated differences in BW at slaughter and in carcass composition (Table 2), especially regarding lean meat percentage, weight of the loin primal cut (relative to chilled half-carcass weight) and raw weight of the LM. Protein concentration in the LM was similar in the two dietary groups, but total glutathione concentration was greater ($P < 0.01$) in Met5 pigs compared with CONT pigs. Conversely, the intramuscular fat content tended to be lower ($P < 0.10$) in the Met5 pigs than in CONT pigs.

Free amino acids and dipeptides in the longissimus lumborum muscle

Concentrations in free methionine and free lysine were greater ($P < 0.01$) in the LM of Met5 pigs than in CONT pigs (Table 3). Concentration in free 3-methyl-histidine (or π -methyl-L-histidine) was also 23% higher ($P < 0.01$) in the LM of Met5 pigs. Free anserine (or β -alanyl- π -methyl-L-histidine) exhibited a similar tendency ($P < 0.10$) for a greater concentration in this group as compared with control pigs. The concentrations of free proline and of free alpha-aminobutyric acid were greater in the LM of Met5 pigs than in CONT pigs. Conversely, free glutamine concentration was lower in Met5 pigs compared with controls.

Plasma metabolites and enzyme activities in the longissimus lumborum muscle

There was no dietary-associated difference in the plasma concentration of urea (Table 4). The activity level of creatine kinase in plasma, assayed as a marker of damage of creatine kinase-rich tissues such as skeletal muscle, was higher for the Met5 pigs than for the CONT pigs ($P < 0.05$). Plasma concentrations of glucose and lactate assessed as indicators of the balance between the use and mobilization of energy at the body level, were not modified by diets.

In the LM, the specific activities of hexokinase and LDH did not differ between Met5 pigs and CONT pigs (Table 5). On the opposite, the activity of CS was lower in the LM of Met5 pigs than in CONT pigs. Consequently, the ratio of LDH to CS was greater ($P < 0.05$) in the LM of Met5 pigs than in the control group. The activity level of cathepsin in the LM did not differ between Met5 pigs and CONT pigs.

Table 2
Effects of extra methionine supply on body composition and muscle biochemical traits in pigs.

Items	Diet ¹		SEM	P-value
	CONT	Met5		
BW (kg)	124.7	123.0	1.8	0.53
Carcass composition (%)				
Lean meat content ²	58.9	58.9	0.5	0.91
Loin percentage ³	26.3	26.2	0.3	0.82
Longissimus lumborum muscle				
Weight ⁴ (g)	911	879	33	0.47
Protein (g/100 g)	18.4	18.5	1.1	0.88
Intramuscular fat (g/100 g)	1.67	1.44	0.09	0.07
Glutathione (nmol/g)	385	447	15	0.009

Abbreviations: CONT = control diet; Met5 = methionine-supplemented diet.

¹ Data were published in Le Bret et al. (2018) with $N = 15$ pigs in each diet.

² Calculated from the proportions of retail cuts with lean meat content (%) = 25.08 + 0.73% ham + 0.87% loin – 1.23% belly (Daumas, 2008).

³ Expressed relative to chilled half-carcass weight, 24 h postmortem.

⁴ Dissected muscle from the chilled half carcass.

Gene expression levels in the longissimus lumborum muscle

Expression levels of genes acting in protein synthesis, proteolysis or autophagy were measured in the LM of pigs at slaughter (Table 6). There were dietary-associated differences in expression levels of only few genes. Among genes encoding translation initiation factors of protein synthesis, the expression level of *EIF3A* tended ($P < 0.10$) to be up-regulated in the Met5 pigs. Two genes involved in the protein ubiquitination pathway, i.e., the E3 ubiquitin-protein ligase *TRIM63/MURF1* and the OTU domain-containing protein *OTUD1*, were also significantly up-regulated in the LM of the Met5 pigs as compared with CONT pigs. Conversely, the ubiquitin conjugating enzyme *UBE2M* and two genes acting in autophagy, *TFEB* and *ATG16*, tended to have a lower expression level in the LM of the Met5 pigs as compared with CONT pigs.

Expression levels of genes involved in energy metabolism, regulating cell development or involved in stress response were also assessed in the LM of pigs (Table 7). Expression level of *HK2* coding for the hexokinase was greater ($P < 0.05$) in the LM of Met5 pigs than CONT pigs, whereas phosphoglycerate mutase 2 (*PGAM2*) involved in glycolysis tended ($P < 0.10$) to be downregulated in the Met5 pigs as compared with controls. The transcription factor peroxisome proliferator-activated receptor delta (*PPARD*) tended to be up-regulated in the LM of Met5 pigs, whereas expression level of malic enzyme (*ME1*) was lower ($P < 0.05$) in those pigs than in CONT pigs. Expression levels of other studied genes did not significantly differ between the two dietary groups.

Gene expression levels in the longissimus lumborum muscle and pork quality traits

A principal component analysis was performed to represent the various correlations between gene expression levels and pork quality traits across dietary groups in a graphical way. The first three principal components explained 58% of the total variance, with the first one explaining 38% of the variance. Variables were projected on the coordinated system represented by the two first principal components. Ultimate pH was positively correlated with principal component 1 ($r = 0.77$, $P < 0.001$), whereas drip loss ($r = -0.77$, $P < 0.001$), h° ($r = -0.67$, $P < 0.001$), L^* ($r = -0.52$, $P < 0.01$), and a^* ($r = -0.44$, $P = 0.02$) to a lower extent, were negatively correlated with this principal component (Fig. 1). The intramuscular fat content tended to be associated with principal component 1 ($r = -0.35$, $P = 0.08$) and principal component 3 ($r = 0.34$, $P = 0.09$; data not shown). On the right quadrant, the expression levels of a number of genes participating to protein synthesis (*EIF3A*, *EIF1*, *EIF4B*), proteolysis (*PSMD1*, *TRIM63*, *LC3B*, *CTSD*,

Table 3
Effects of extra dietary methionine supply on free amino acids and small peptides in pig muscle.

Items	Diet		SEM	P-value
	CONT	Met5		
Amino acid concentration ($\mu\text{g/g}$ muscle) ¹				
Alpha-amino-n-butyric Acid	36.9	45.9	2.7	0.03
Alanine	95.9	106.7	5.2	0.16
Anserine	204	224	7	0.09
Arginine	15.4	15.4	1.7	0.99
Beta-Alanine	36.1	38.7	3.2	0.57
Carnosine	9 030	9 056	182	0.92
Citrulline	12.8	12.1	0.4	0.20
Glutamine	180	161.5	6.1	0.04
Glutamate	47.2	49.3	3.1	0.62
Glycine	83.7	81.8	4.3	0.76
Histidine	14.5	16.4	1.4	0.34
Isoleucine	17.8	20.0	1.2	0.19
Leucine	28.7	31.1	1.5	0.27
Lysine	17.9	24.2	1.4	0.003
Methionine	5.8	7.7	0.4	0.004
1-Methyl-Histidine	1.60	1.8	0.2	0.35
3-Methyl-Histidine	0.57	0.70	0.03	0.002
Ornithine	7.4	10.0	1.1	0.11
Phenyl-alanine	16.5	18.4	0.8	0.10
Proline	23.1	27.6	0.8	<0.001
Serine	23.9	18.4	2.2	0.10
Taurine	231	243	20	0.68
Threonine	21.5	24.1	1.1	0.10
Tyrosine	21.6	23.5	1.0	0.18
Valine	38.2	40.3	1.8	0.43

Abbreviations: CONT = control diet; Met5 = methionine-supplemented diet.

¹ Cysteine was not detected.**Table 4**
Plasma concentrations of metabolites in pigs according to diet.

Items	Diet		SEM	P-value
	CONT	Met5		
Urea (mmol/L)	4.37	4.40	0.21	0.93
Lactate (mmol/L)	7.21	8.23	1.63	0.66
Glucose (mmol/L)	6.18	6.34	0.26	0.67
Creatine kinase (U/mL)	9.19	16.04	1.73	0.01

Abbreviations: CONT = control diet; Met5 = methionine-supplemented diet.

SQSTM1; *ATG5*, *FBXO32*; *UBE2QL*; *UVRAG*; *ZNF24*; *ATG16*; *BNIP3*; *BECN1*; *OTUD1*; *BCN2*) and energy-regulation pathway (*PPARD*; *HK2*; *FOS*; *LIPE*; *MCAT*) were positively associated with ultimate pH. On the left quadrant, positive relationships were enlightened between L^* , drip loss, h° , a^* and *PRKAG3* expression level.

Discussion

Skeletal muscle is the largest pool of total amino acids in the body, including protein-bound amino acids and free amino acids

Table 5
Effect of excess dietary methionine supply on muscle enzyme activities in pigs.

Items	Diet		SEM	P-value
	CONT	Met5		
Enzyme activity				
Hexokinase (nmol/min/mg protein)	654	728	110	0.64
Lactate dehydrogenase ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	26.6	26.0	1.7	0.80
Citrate Synthase (nmol/min/mg protein)	69.3	55.6	3.5	0.01
Lactate dehydrogenase/Citrate synthase	396	472	25	0.04
Cathepsin (mRFU/min/mg protein)	413	446	39	0.55

Abbreviations: CONT = control diet; Met5 = methionine-supplemented diet; RFU = relative fluorescent unit.

(Bergström et al., 1974). Concentrations of free amino acids in skeletal muscle, although representing less than 3% of total amino acids, are considered as good indicators of amino acid intake and availability of specific amino acids for protein (re)synthesis (Ferna). In the growing chicken, supplementation of diet with DL-methionine gave rise to an increase in the concentration of methionine in skeletal muscle. The measurement of the changes in tissue concentrations of free methionine was thus proposed as a sensitive test to evaluate this amino acid in the diet (Fernandez Figares et al., 1997). In the current study, the free methionine concentration was 30% greater in the LM of pigs fed the Met5 diet during the last 14 days before slaughter as compared with controls. The efficiency of methionine metabolism process, the accumulation of methionine in various tissues, and a possible limited absorption of the methionine excess by the muscle may explain the differences in methionine concentrations between the diet and the LM of pigs. Nevertheless, changes in the concentration of free methionine in the LM of the Met5 pigs may affect the amino acid profile of the retained proteins (Conde-Aguilera et al., 2016a). Moreover, the methionine can be converted through the trans-methylation/trans-sulfuration pathway into cysteine (Estévez et al., 2020 for a recent review), a constitutive amino acid of glutathione, and this could explain the greater glutathione con-

Table 6
Effect of excess methionine supply on molecular indicators of protein metabolism in pig muscle.

Items	Diet		SEM	P-value
	CONT	Met5		
Gene expression level (arbitrary unit) ¹				
Protein synthesis				
Insulin Like Growth Factor 1 (<i>IGF1</i>)	0.82	0.74	0.05	0.21
Eukaryotic translation initiation factor-1 (<i>EIF1</i>)	0.74	0.70	0.03	0.43
Eukaryotic translation initiation factor 3 subunit A (<i>EIF3A</i>)	0.81	0.90	0.04	0.09
Eukaryotic translation initiation factor 4B (<i>EIF4B</i>)	0.84	0.84	0.03	0.89
Proteolysis				
Cathepsin D (<i>CTSD</i>)	0.83	0.87	0.06	0.67
Calpain-1 micro-molar (<i>CAPN1</i>)	0.63	0.60	0.03	0.42
Calpain-2 milli-molar (<i>CAPN2</i>)	0.66	0.63	0.04	0.66
Calpain-3 micro-molar (<i>CAPN3</i>)	0.80	0.78	0.03	0.50
26S proteasome non-ATPase regulatory subunit 1 (<i>PSMD1</i>)	0.78	0.89	0.08	0.22
E3 ubiquitin-protein ligase (<i>TRIM63/MURF1</i>)	0.79	1.15	0.07	0.008
OTU domain-containing protein 1 (<i>OTUD1</i>)	0.53	1.08	0.16	0.03
NEDD8-conjugating enzyme Ubc12 (<i>UBE2M</i>)	0.81	0.74	0.02	0.07
Ubiquitin-conjugating enzyme E2 Q1 (<i>UBE2Q1</i>)	0.63	0.68	0.04	0.33
Mitochondrial ubiquitin ligase activator of NFKB 1 (<i>MUL1</i>)	0.76	0.79	0.04	0.64
F-box only protein 32 (<i>FBXO32/Atrogin</i>)	0.95	1.33	0.15	0.12
Autophagy				
Transcription factor EB (<i>TFEB</i>)	0.87	0.73	0.05	0.07
Sequestosome-1 (<i>SQSTM1</i>)	0.88	1.03	0.07	0.19
Microtubule-associated proteins light chain 3A (<i>LC3A</i>)	0.72	0.65	0.04	0.23
Microtubule-associated proteins light chain 3B (<i>LC3B</i>)	0.83	0.94	0.05	0.18
Beclin-1 (<i>BECN1</i>)	0.65	0.59	0.04	0.32
Cysteine protease ATG4B (<i>ATG4B</i>)	0.62	0.52	0.06	0.26
Autophagy protein 5 (<i>ATG5</i>)	0.43	0.43	0.03	0.99
Ubiquitin-like modifier-activating enzyme ATG7 (<i>ATG7</i>)	0.87	0.79	0.07	0.43
Ubiquitin-like protein ATG12 (<i>ATG12</i>)	0.81	0.82	0.05	0.88
Autophagy protein 16 (<i>ATG16</i>)	1.09	0.86	0.09	0.07
UV radiation resistance-associated gene protein (<i>UVRAG</i>)	0.64	0.65	0.07	0.92
Cell death				
Bcl2 apoptosis regulator (<i>BCL2</i>)	0.73	0.80	0.08	0.55
Bcl2/E1B 19 kDa protein-interacting protein 3 (<i>BNIP3</i>)	0.89	0.86	0.05	0.70

Abbreviations: CONT = control diet; Met5 = methionine-supplemented diet.

¹ Expression level of target gene was corrected by a normalization factor.**Table 7**
Effect of extra methionine supply on molecular indicators of energy metabolism and cell development in pig muscle.

Items	Diet		SEM	P-value
	CONT	Met5		
Gene expression level (arbitrary unit) ¹				
Glycolysis				
Hexokinase, muscle form (<i>HK2</i>)	0.59	0.90	0.08	0.01
Phosphoglycerate Mutase 1 (<i>PGAM1</i>)	0.69	0.71	0.04	0.71
Phosphoglycerate Mutase 2 (<i>PGAM2</i>)	0.78	0.70	0.03	0.06
Phosphofructokinase, Muscle (<i>PFKM</i>)	0.87	0.82	0.05	0.45
Aldolase, Fructose-Bisphosphate A (<i>ALDOA</i>)	0.76	0.74	0.03	0.55
Protein kinase AMP-activated non-catalytic subunit gamma 3 (<i>PRKAG3</i>)	0.75	0.66	0.04	0.12
Fatty acid metabolism				
Malic enzyme (<i>ME1</i>)	0.77	0.61	0.05	0.02
Malonyl-CoA-Acyl Carrier Protein Transacylase (<i>MCAT</i>)	0.72	0.67	0.06	0.69
Lipase E, Hormone Sensitive Type (<i>LIPE</i>)	0.81	1.12	0.17	0.23
Peroxisome proliferator-activated receptor delta (<i>PPARD</i>)	0.74	0.94	0.07	0.06
Fatty Acid Binding Protein 3 (<i>FABP3</i>)	0.94	0.97	0.11	0.85
Fatty Acid Binding Protein 4 (<i>FABP4</i>)	0.50	0.58	0.06	0.32
Cell development and stress response				
Fos proto-oncogene AP-1 transcription factor subunit (<i>FOS</i>)	1.09	2.77	0.67	0.11
Ankyrin Repeat Domain (<i>ANKRD</i>)	0.59	0.81	0.09	0.11
Heat Shock Protein Family D (Hsp60) Member 1 (<i>HSPD1</i>)	0.80	0.85	0.05	0.42
Receptor Transporter Protein 4 (<i>RTP4</i>)	1.03	1.03	0.18	0.99
Zinc Finger Protein 24 (<i>ZNF24</i>)	0.76	0.75	0.05	0.97

Abbreviations: CONT = control diet; Met5 = methionine-supplemented diet.

¹ Expression level of target gene was corrected by a normalization factor.

centration in the LM of Met5 pigs. However, the fact that free methionine was accumulated in the LM of Met5 pigs suggests that the capacity of this pathway remains quantitatively limited (Baker, 2004). Moreover, the two other amino acids constitutive of glu-

tathione, i.e., glycine and glutamate, had similar free concentrations in the LM of Met5 and control pigs. Glycine and glutamate are among the most abundant free amino acids in the plasma of pigs and they are extensively *de novo* synthesized. Free glycine

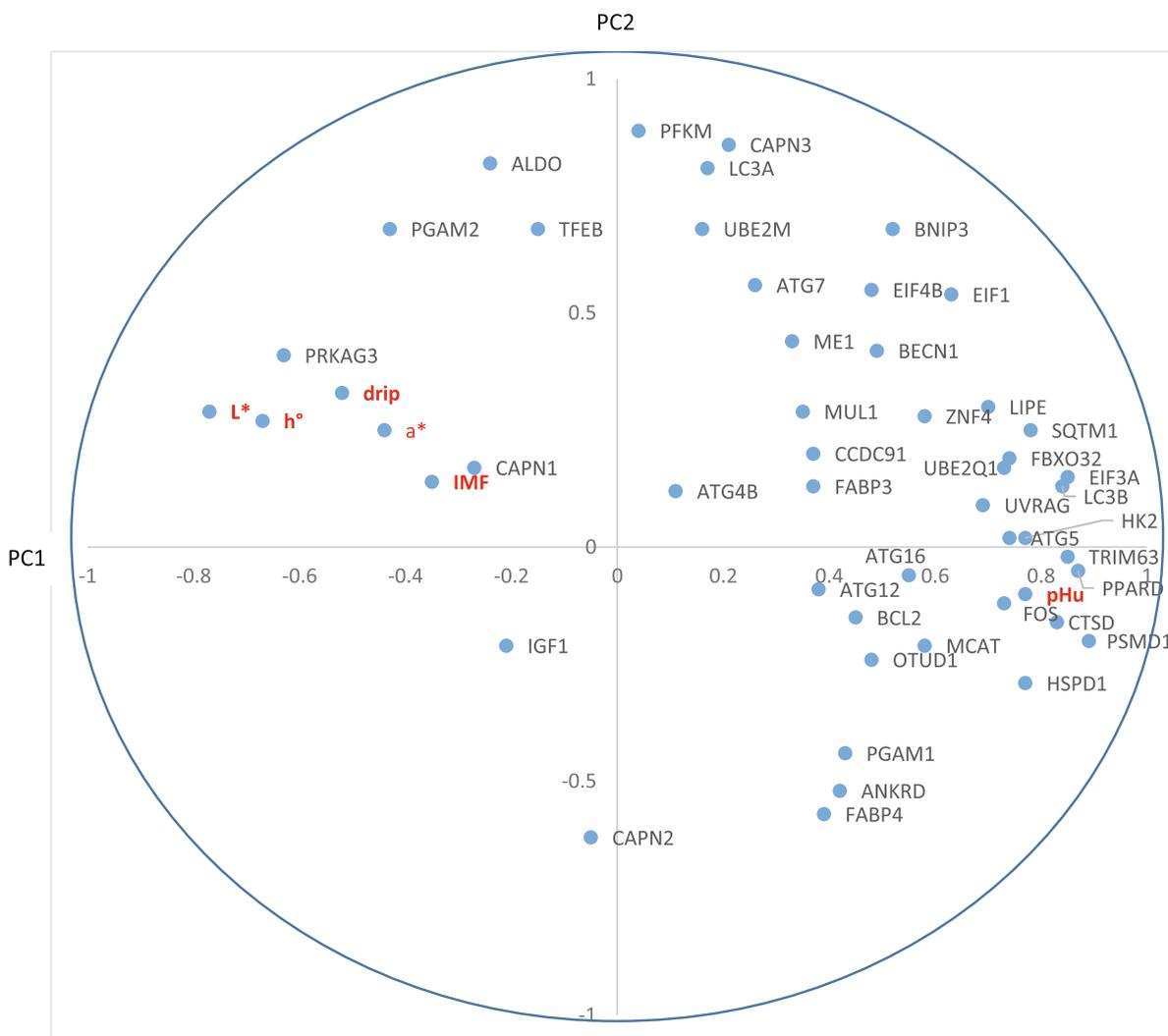


Fig. 1. Principal component analysis between expression levels of genes involved in protein and energy metabolism in the *longissimus lumborum* muscle and pork quality traits. The correlation matrix between mRNA levels of the studied genes in the *longissimus lumborum* muscle (LM) and meat quality traits was calculated using a principal component analysis. After mean-centering and scaling to unit variance, the data set was summarized by the two first principal components. Each observation (blue dot) was projected on the plane represented by the two first principal components to get a coordinate value along each principal component line. The figure displays the score plot of the first two principal components (PC1 and PC2 for axis 1 and axis 2, respectively) mapping the gene variables and meat quality traits (ultimate pH = pHu, intramuscular fat content = IMF, lightness = L*, redness = a*, hue = h°, drip loss from 1 to 4 days postmortem = drip, all indicated in red font on the principal component analysis plan). Positively correlated variables are grouped together, whereas variables positioned on diagonally opposed quadrants are negatively correlated.

and glutamate pools can be also exchanged between liver, plasma and muscle (Fernandez Figares et al., 1997) and thus contribute to carbon and nitrogen inter-organ fluxes. This may explain the lack of differences between dietary groups in free concentrations of glycine and glutamate in the LM of pigs. Another way for the muscle cells to cope with the oversupply in dietary methionine is the formation of homoserine and α -aminobutyric acid. This mechanism described in mice tissues after injection of DL-methionine (Matsuo and Greenberg, 1955) may explain the rise in free α -aminobutyric acid concentration reported in the LM of Met5 pigs as compared with CONT pigs. The concentration of free anserine (i.e., β -alanyl-3-methyl-L-histidine) also tended to be greater in the LM of Met5 pigs than in controls, and this change is in agreement with the increased anserine concentration reported in the muscle of rats when fed a methionine excess diet (Tamaki et al., 1978). In this pathway, methionine may efficiently serve as a methyl donor for the synthesis of the methyl group of anserine. Because methionine, glutathione, α -aminobutyric acid and anserine have protective capacities against oxidative stress, osmotic stress and acid stress (Wu, 2020), their greater concentrations in

the LM of Met5 pigs may then contribute to the better stability of the meat color during storage and lower drip loss reported as compared with controls by Lebreton et al. (2018). Among other amino acids containing a methyl group, a greater concentration in free 3-methyl-histidine in the LM was observed in Met5 pigs compared with controls. In most mammals, 3-methyl-histidine concentration in urine is used as a marker of myofibrillar protein degradation (Rathmacher and Nissen, 1998). However in pigs, 3-methyl-histidine is mainly retained in muscle as anserine (Boldyrev et al., 2013). Conversely, free glutamine concentration was lower in the LM of Met5 pigs compared with CONT pigs. A reduced intramuscular glutamine concentration correlates with an increased net protein breakdown in the muscle of rats (Heenenman and Deutz, 1993). Importantly, creatine kinase activity in plasma, considered an indirect marker of muscle damage and scaled to muscle disturbance (Baird et al., 2012), was increased in Met5 pigs compared with controls. Free lysine and proline concentrations were greater in the LM of Met5 pigs as compared to control pigs, but whether this may sign for increased proteolysis activity and (or) reduced protein synthesis remains to be evaluated. Although protein degra-

dation in tissues can represent more than half of the free amino acids released (Riis, 1983), the extent of this phenomena in Met5 and control pigs remains to be quantified by a direct measurement of protein breakdown. Circulating concentration of urea, the principal nitrogenous end product of AA catabolism, and protein content in the LM were similar in the two treatments. Concentrations in amino acids such as leucine, valine, and isoleucine were also unchanged between groups, but this lack of change may be due to the specific metabolism of branched chain amino acids (Kwon et al., 2020) rather than be interpreted as a sign of unaffected protein breakdown. Taken together, these data suggest that protein turnover, i.e., both synthesis and catabolism, could have been stimulated in the LM by the extra dietary Met supply. An increased protein turn-over may explain the tendency for a decreased meat shear force reported in the LM of pigs when fed a methionine-supplemented diet beyond requirements during the whole growth period (Yuan et al., 2020).

To further examine these possibilities, the expression levels of genes involved in protein proteolysis, protein synthesis or autophagy were measured in the LM of the pigs at the end of the dietary trial. Only scarce modulations in the mRNA abundances of some genes participating to the eukaryotic translation initiation factor pathway (*EIF3A*), ubiquitin-proteasomal process (*TRIM63/MURF1*, *UBE2M*, *OTUD1*) and autophagy-lysosomal pathway (*TFEB*, *ATG16*) were observed between Met5 and CONT pigs. Similarly, few molecular changes in these pathways have been reported in breast muscle of chicken after 14 days of feeding with diets supplemented with leucine above growth requirements, whereas more changes were observed at the initiation of the feeding challenge (Zeit et al., 2019a). Among affected genes by the diet, we reported a greater expression level of the muscle-specific RING finger-1 E3 *TRIM63/MURF1* coding for an ubiquitin ligase, and of *OTUD1*, a gene coding for a deubiquitinase that negates the action of ubiquitin ligases, in the LM of Met5 pigs compared with CONT pigs. The two ubiquitin-dependent metabolic processes may have functioned in synergy to adjust the intermediary sulfur metabolism to growth and conditions (Menant et al., 2006) when pigs were facing extra methionine dietary supply. In Met5 pigs, the greater mRNA level of *TRIM63/MURF1* coding for an enzyme known to target creatine kinase and muscle contractile proteins, argues for a shift in the protein balance from net synthesis to net degradation (Bodine and Baehr, 2014). In cows and chicks, studies have rather reported an up-regulation of *MURF1* in muscle of animals when fed a Met-deficient diet (Sadri et al., 2016; Zeit et al., 2019b). However, the expression levels of the majority of the studied genes involved in protein metabolism, including *OTUD1* previously proposed by Damon et al. (2013) as a biomarker of technological pork quality, were also positively associated to satisfactory pork quality (i.e., high ultimate pH, low pork drip and lightness) across dietary treatments. This suggests that subtle modulations in expression levels of these genes had cumulative effects on a panel of meat quality traits in those pigs.

Indicators of energy metabolism in the LM were also modified by the extra dietary methionine supply received by the pigs. Especially, a reduced activity of CS was measured in the Met5 pigs compared with controls. In line, increased CS activity (Conde-Aguilera et al., 2014; 2016a) and a greater mitochondrial oxidation of pyruvate through succinate dehydrogenase and respiratory chain enzymes (Conde-Aguilera et al., 2020) were observed in the LM of young piglets facing a dietary methionine deficiency. The citrate cycle was similarly identified as differentially regulated in chicken fed high vs. low methionine diets (Zhai et al., 2012). Importantly, CS activity is required to burst the tricarboxylic acid cycle rate for energetic or other anabolic purposes in cells, such as an increase in *de novo* fatty acid biosynthesis (Ciccarone et al., 2019 for a recent review). Therefore, the down-regulation of *ME1* in

the LM of Met5 pigs, the gene encoding the main NADPH supplier for lipogenesis in pig muscle, is in agreement with the dietary-associated changes in CS activity observed in those pigs as compared with controls. This could contribute to the reduction in intramuscular fat content in Met5 compared with CONT pigs. Taken together, these data suggest a shift toward a lower oxidative metabolism in the muscle of pigs fed an extra Met supply. These responses were opposite to those observed when growing pigs received diets supplemented with other amino acids such as arginine and glutamine (Hu et al., 2017). In addition, *PPARD* was up-regulated in the LM of Met5 pigs as compared with CONT pigs. The isotype delta in the family of the peroxisome proliferator-activated receptors is considered as having pleiotropic effects on glucose metabolism, fatty acid oxidation, oxidative stress and apoptotic process. Finally, the expression levels of *HK2*, regulating glucose use in muscle cells, and of *PPARD* and *LIPE*, involved in lipid metabolism, were positively correlated with ultimate pH across pigs, whereas *PRKAG3* encoding the regulatory subunit of the AMP-activated protein kinase (AMPK) as an important energy sensing enzyme, was positively associated with meat color (L^* , h^* , a^*) and drip loss, and to intramuscular fat content to a lower extent.

Conclusion

Extra dietary methionine supply during 14 days before slaughter affected free amino acid concentrations, glutathione content and citrate synthase activity in the loin muscle of finishing pigs. The expression levels of genes acting in protein and energy metabolism were little affected when measured at the end of this dietary trial. However, when considering all experimental pigs together, the modulations of gene expression profiles were associated with variations of pork quality traits in those pigs. Results presented by Lebret et al. (2018) in our companion paper demonstrated an improvement in several indicators of the technological quality of pork associated to the distribution of the Met5 diet, such as higher pH in the loin and ham meat, better stability of meat color along storage, and higher Meat Quality Index of fresh ham. The Met5 diet also induced a slight reduction in IMF content as compared with the control diet, but it is probably too small to affect the perceived sensory quality of meat. The improvements in the technological quality and appearance of pork are important for both meat processors and consumers and may be valued by higher prices of carcass, fresh ham (to processors) or fresh loin (to consumers). Altogether, methionine content and the ratio of methionine to lysine in diets for finishing pigs should thus be reconsidered for their roles in modulating muscle traits and pork quality.

Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.animal.2021.100268>.

Ethics approval

The experiment was performed at the INRAE pig experimental facility (UE3P, 35590 Saint-Gilles, France; <https://doi.org/10.15454/1.5573932732039927E12>) with the agreement for animal housing number C-35-275-32. Animals were cared in compliance with the French directive on animal experimentation (<https://www.legifrance.gouv.fr/eli/decret/2013/2/1/2013-118/jo/texte>). Collection of blood and muscle samples was carried out by an authorized staff adhering to the regulations and guidelines on ani-

mal husbandry and welfare, and having agreements for experimentation on living animals.

Data and model availability

None of the data were deposited in an official repository. The data that support the study findings are available upon reasonable request.

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F. Gondret, Y. Mercier and D. Batonon-Alavo: conceived and designed the experiments.

F. Gondret, B. Le Bret, N. Le Floch and M-H. Perruchot: performed the experiments.

All authors: analyzed and discussed the data. The first draft of the manuscript was written by **F. Gondret**. All authors commented on previous versions of the manuscript. All authors read, commented and approved the final manuscript.

Declaration of interest

The study was supported by Research Grants from ADISSEO and INRAE, and both have contributed to the experimental design. The funder INRAE also contributed to the conduct of the study, analysis of the samples and data, and interpretation of the findings. Funders did not interfere with the full presentation of this research, objective interpretation of the data and decision to submit for publication. Y. Mercier and D. Batonon-Alavo are employed by Adisseo France SAS. This does not alter the authors' adherence to policies on sharing data and materials.

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