Effects of casein, chicken and pork proteins on regulation of body fat and blood inflammatory factors and metabolite patterns are largely dependent on protein level and less attributable to the protein source

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1 **Abstract:** The impact of meat protein on metabolic regulation is still disputed, and may
2 be influenced by protein level. This study aimed to explore the effects of casein, pork
3 and chicken proteins at different protein levels (40% E vs. 20% E) on body weight
4 regulation, body fat accumulation, serum hormone levels, and inflammatory
5 factors/metabolites in rats maintained on high-fat (45% E fat) diets for 84 d. Increased
6 protein-levels resulted in a significant reduction in body fat mass and an increase in
7 serum levels of the anti-inflammatory cytokine IL-10, independent of protein source.
8 Analysis of blood via untargeted metabolomics analysis identified 8, 4 and 4
9 metabolites significantly altered by protein level, protein source, and a protein
10 level*source interaction, respectively. Together, the effects of casein, chicken and pork
11 protein on the regulation of body fat accumulation and blood metabolite profile are
12 largely dependent on protein level, and less attributable to the protein source.

13

14 **Keywords:** meat protein, obesity, untargeted metabolomics, high-fat diet, rats

15 **Introduction**

16 With the globally increasing prevalence of obesity and metabolic syndrome,
17 dietary protein is regarded as the most promising macronutrient for improving body
18 composition and metabolic profile[1]. Meat protein is an important animal-derived
19 dietary protein consumed by humans. In 2015, the World Health Organization (WHO)
20 classified red and processed meat as “probably carcinogenic to humans” and
21 “carcinogenic to humans”, respectively[2], adding to a resurgence of interest in
22 understanding the biological functions of meat proteins and their relationships to human
23 health[3-8].

24 To date, studies exploring the biological effects of meat proteins have been
25 conducted by several research groups, including groups from China[3-8], Japan (mainly
26 on fish protein)[9], Norway and Denmark[10-11]. These studies have mainly focused on the
27 impact of meat proteins on energy regulation, glucose or lipid metabolism[3, 6-7, 9-11], and
28 gut microbiota[6, 8, 12]. Previously, we investigated the effect of different protein sources
29 (casein, soy, beef, pork, chicken and fish) at recommended normal protein levels (20%
30 E) on growth and metabolism in healthy young rats[3-5, 7]. Short-term (7 or 14 days)
31 feeding of the different protein sources resulted in distinct physiological, transcriptome
32 and proteome changes. Both red (beef and pork) and white (chicken and fish) meat
33 proteins displayed beneficial effects on growth and lipid metabolism when compared
34 to their casein and soy protein counterparts[7]. Conversely, intake of high-fat diet in
35 combination with normal level (about 18-20% E) beef protein for 12 weeks increased
36 dyslipidemia, hypercholesterolemia, and triglycerides accumulation in liver and led to
37 systemic inflammation, impaired glucose metabolism and insulin resistance, when
38 compared to casein and soy protein[6]. Thus, highlighting the discrepancies between
39 metabolic regulation and dietary meat proteins, which may be closely influenced by

40 dietary fat and protein levels.

41 It has been reported that in rats, increasing dietary protein levels reduces lipid 42 accumulation in the adipose tissue, attenuating the metabolic dysfunction associated 43 with high-fat or -sucrose induced obesity[13-14]. However, most of these studies have been 44 focused primarily on dairy proteins, like casein and whey[13]. The implications of high 45 level meat protein intake on metabolic health remains distinctively lacking. 46 Epidemiologic studies link high consumption of red or processed meat with increased 47 risk of obesity and diabetes[15-16], however such epidemiologic results still lack validation 48 from rigorous animal studies. Here, we provide high-fat diet maintained rats with one 49 of three protein sources (casein, pork or chicken) at either high (40% E) or normal (20% 50 E) protein levels, in order to clarify the effects of high level meat protein consumption 51 on body composition and metabolic health. To achieve this, we monitored body weight/ 52 body fat mass, and measured blood hormone/inflammatory factors as well as employing 53 a metabolomics approach.

54 **Materials & Methods**

55 **Protein Sources and Diets**

56 Detailed methods for preparation of chicken and pork protein sources have been 57 previously described[3]. Briefly, cooked meat was freeze-dried and broken into powder. 58 Dry meat powder was defatted with methylene chloride/methanol (2: 1, v: v). The final 59 meat protein powders consisted of more than 90% of protein.

60 Seven diets were prepared based on 3 main formulas of D12450H, D12451 and 61 D12451m (**Supplementary table 1**). Low fat diet with casein was prepared according 62 to formula of Research Diet D12450H (10% E fat, 20% E protein, 70% E carbohydrate) 63 and was used as low-fat control diet (LF group). High fat (HF) diet with casein was 64 prepared according to Research Diet D12451 (45% E fat, 20% E protein, 35% E

65 carbohydrate) and used as high-fat control diet (CS group). Other two HF diets with
66 chicken (CK group) or pork (PK group) protein sources were prepared according to the
67 same diet formula of CS diet (D12451) in which the protein source (casein) was fully
68 replaced by isolated proteins from pork or chicken. High-fat-high-protein (HFHP) diet
69 with casein (HCS group) was prepared by increasing protein but reducing carbohydrate
70 (only starch) in D12451 (45% E fat, 40% E protein, 15% E carbohydrate, D12451m).
71 Other two HFHP diets with chicken (HCK group) or pork (HPK group) protein sources
72 were prepared according to the same diet formula of HCS diet (D12451m) in which the
73 casein was fully replaced by isolated proteins from pork or chicken.

74 **Animals and Sample Collection**

75 All animals were handled in accordance with the guidelines of the Ethical
76 Committee of Experimental Animal Center of China Pharmaceutical University (the
77 license number is SYXK (Su) 2018-0019). The *Wistar* rat employed in this experiment
78 is considered the standard rodent for diet-induced obesity experiment due to their
79 susceptibility to diet induced obesity and insulin resistance[17]. After a one-week adaption
80 period, 49 male *Wistar* rats with initial body weight of 180-200g were randomly
81 assigned into the 7 groups (n=7 rats in each group). To test long-term effects of dietary
82 proteins on regulation of body weight and metabolic health, rats were fed for 12 weeks
83 diets of LF, CS, CK, PK, HCS, HCK or HPK, respectively. Feed intakes and body
84 weight was measured every 3 days. On day 84, rats were deprived of feed but were
85 given free access to water for 6 hours prior to sacrifice. Immediately following
86 euthanasia, blood was taken and serum was isolated. Perirenal and epididymis fat pad
87 were obtained and weighed. All samples were snap frozen in liquid nitrogen and stored
88 at -80 °C until analysis.

89 **Oral Glucose Tolerant Test (OGTT)**

90 OGTT was conducted on day 80. Rats were submitted to a 6-h fasting prior to
91 glucose administration. Each rat was given 2g glucose /kg body weight by gavage. Tail
92 vein blood drops were collected for glycemia measurement before (0 min) and after
93 glucose gavage (30min, 60min, 90min and 120min) through a glucometer (Jiangsu
94 Yuyue Medical Equipment & Supply Co. Ltd., Shanghai, China). The value of area
95 under curve (AUC) was calculated.

96 **Blood Hormone and Inflammatory Factors Detection**

97 Serum insulin concentrations were detected using ELISA kits (Elabscience
98 Biotechnology Co., Ltd., Wuhan, Hubei, China) according to the manufacturer's
99 instructions. Serum leptin concentration were detected using Bio-Plex Pro Rat Diabetes
100 Leptin kits (Bio-Rad Laboratories, Inc., Hercules, California, USA) according to the
101 manufacturer's instructions. Concentrations of serum inflammation factors of tumor
102 necrosis factor-α (TNF-α), monocyte chemoattractant protein-1(MCP1), interleukin-6
103 (IL-6) and interleukin-10 (IL10) were detected using Bio-Plex Cytokine Express 5-Plex
104 (Bio-Rad Laboratories, Inc., Hercules, California, USA) according to the
105 manufacturer's instructions.

106 **Blood Untargeted Metabolomics Detection**

107 **Metabolites Extraction.** All serum samples were pooled as QC (quality control)
108 sample. Serum (n=7 in each group) were extracted with methanol, with L-2-
109 Chlorophenylalanine (1mg/mL stock in dH_2O) as internal standard. After centrifuging
110 for 15min at 12000rpm at 4°C, the supernatant was taken and dried completely in a
111 vacuum concentrator without heating. Methoxy amination hydrochloride (20mg/mL in
112 pyridine) was added to the dried sample, and the solution was incubated at 80°C for
113 30min. Then, BSTFA regent (1% TMCS, v/v) was added to the sample aliquots, and it
114 was incubated at 70°C for 1.5h. FAMEs (in chloroform) was added to the QC sample

when cooling to the room temperature.

GC-TOF-MS Analysis. All samples were analyzed using Agilent 7890 gas chromatograph system coupled with a Pegasus HT time-of-flight mass spectrometer (GC-TOF-MS). The system utilized a DB-5MS capillary column coated with 5% diphenyl cross-linked with 95% dimethylpolysiloxane (30m×250μm inner diameter, 0.25μm film thickness; J&W Scientific, Folsom, CA, USA). A 1μL aliquot of the analyte was injected in splitless mode. Helium was used as the carrier gas, the front inlet purge flow was 3mL/min, and the gas flow rate through the column was 1mL/min. The initial temperature was kept at 50°C for 1min, then raised to 310°C at a rate of 20°C/min, then kept for 6min at 310°C. The injection, transfer line, and ion source temperatures were 280, 280, and 250°C, respectively. The energy was -70eV in electron impact mode. The mass spectrometry data were acquired in full-scan mode with the m/z range of 50-500 at a rate of 20 spectra per second after a solvent delay of 4.7min.

Data Preprocessing and Annotation. MS-DIAL software and FiehnBinbase database[18] were used for raw peaks exacting, the data baselines filtering and calibration of the baseline, peak alignment, deconvolution analysis, peak identification and integration of the peak area[19]. Both of mass spectrum match and retention index match were considered in metabolites identification. Remove peaks detected in ≤50% of QC samples, or <50% samples of every group (except QC group), or RSD > 30% in QC samples. Blood metabolomics data was analyzed by using the web tool of MetaboAnalyst 4.0 (https://www.metaboanalyst.ca/)[20]. Before comparison, metabolomics data was normalized by log-transforming and auto-scaling (i.e. mean-centered and divided by the standard deviation of each variable). KEGG pathway over representation analysis of significant metabolomics items were performed by using algorithms of hypergeometric test and relative-betweeness centrality topology analysis.

140 The overlaps of significant metabolomics items of different comparisons are shown in
141 Venn plots drawn by using a web tool on https://bioinfogp.cnb.csic.es/tools/venny/
142 index. html. All significant features were shown in a clustered heatmap, where
143 clustering was based on the Euclidean distance calculated using Ward algorithm.
144 Correlations of significant features were analyzed using Pearson method.

145 **Statistical Analysis**

146 Statistical analysis was performed using SPSS for Windows 16.0 software (SPSS
147 Inc., Chicago, Illinois, USA). For comparisons of effects of low fat (LF), high fat (HF)
148 and high fat high protein (HFHP) diets, the LF group was set as control. CS, CK and
149 PK groups were combined as a HF super-group. HCS, HCK and HPK groups were
150 combined as a HFHP super-group. The effects of LF, HF and HFHP diets were tested
151 by using one-way ANOVA and Duncan post hoc test. Without LF group, the other 6
152 groups (CS, CK, PK, HCS, HCK and HPK) were tested by using two-factor ANOVA
153 with dietary protein source and level as fixed factors. $P < 0{\cdot}05$ was considered
154 significant. When the main effects of protein source were significant, multiple
155 comparisons of HF groups (CS, CK and PK) and HFHP (HCS, HCK and HPK) groups
156 were done by using Duncan post hoc method. When the interaction effects of protein
157 level and source were significant, multiple comparisons of the 6 groups (CS, CK, PK,
158 HCS, HCK and HPK groups) were done by using Duncan post hoc method.

159 **Results**

160 **Growth Performance and Body Fat Mass**

161 The initial body weight (IBW) of rats was constant across all 7 groups (**Figure 1**
162 **A**). The final body weight (FBW), body weight gain (BWG) and daily body weight
163 gain (DBWG) of rats fed HF diets was significantly higher (by 18.5%) than that of rats
164 fed LF diet. The FBW, BWG and DBWG of HFHP-fed rats was intermediate compared

with that of HF- and LF-fed rats, but was not significantly different from either ($P > 0.05$). The daily feed intake (DFI) of rats in the 7 groups did not differ. However, the daily energy intake (DEI) of rats fed HF and HFHP diets was significantly higher (by 21%) than that of rats fed LF diet. Changes in white adipose tissue mass of LF, HF and HFHP groups were similar to changes observed in BWG and DBWG (**Figure 1 B**). Perirenal (PATW), epididymal (EATW) and total (WATW = PATW + EATW) white adipose tissue weights of rats fed HF diets were significantly higher (by 31.5%) than that of rats fed LF diet, as to the relative percent content of perirenal (PATW/FBW), epididymal (EATW/FBW) and total (WATW/FBW) white adipose tissue to the final body weight. It is worth noting that the EATW of rats fed HFHP diets was significantly lower (by 15.2%) than that of rats fed HF diets ($P < 0.05$).

In order to test the effects of dietary protein source and level on growth and body fat mass of rats, six groups of CS, CK, PK, HCS, HCK and HPK (without LF) were compared using two-factor ANOVA (**Figure 1**). Increasing dietary protein levels (from 20% E to 40% E) significantly reduced EATW (protein level: F value = 6.476, P = 0.015) and EATW/FBW (protein level: F value = 5.552, P = 0.024) of rats fed HF diets whilst protein source had no effects on body weight or fat mass of rats.

Oral Glucose Tolerant Test (OGTT) and Serum Hormones

OGTT test was conducted on day 80 to measure the blood glucose clearance ability of rats fed different protein diets (**Figure 2 A**). Compared to the LF group, rats in HF and HFHP groups had significantly higher blood glucose levels 30 min after glucose gavage (glucose-30 min) and a significantly higher area under curve value (glucose-AUC, by 12% and 15% respectively). Two-factor ANOVA analysis showed significant effects of protein level on glucose-0 min (F value = 5.358, P = 0.026) and -60min (F value = 6.794, P = 0.013). HFHP significantly lowered glucose-0 min (by 9%) but led

190 to higher glucose-60 min (by 11%) than HF group ($P < 0.05$). Protein source had no
191 significant effects on glucose tolerance of rats. Similarly, no significant changes were
192 found for serum insulin and leptin between groups (**Figure 2 B**).

193 **Serum Inflammatory Factors**

194 Several serum inflammatory factors including TNF-α, MCP-1, IL-6 and IL-10
195 were measured (**Figure 2 B**). Compared with LF diet, concentrations of TNF-α and IL-
196 10 were increased significantly by HFHP diets ($P < 0.05$, by 94.7% and 118%
197 respectively), and concentrations of MCP-1 were increased significantly by HF diet (P
198 < 0.05, by 47.1%). For effects of protein source and level on blood inflammatory factors,
199 there were no significant effects of protein source on blood inflammatory factors.
200 However, the effects of protein level on IL-10 concentrations were significant (F value
201 = 4.498, $P = 0.042$). Compared to 20% E protein diet (HF group), 40% E protein diet
202 (HFHP group) increased IL-10 concentrations significantly (by 68.2%).

203 **Blood Untargeted Metabolome**

204 A total of 112 non-redundant metabolites were detected in the serum of the rats.
205 Fifteen metabolites were found significantly changed across LF, HF and HFHP groups
206 ($P < 0.05$). Two-factor ANOVA analysis revealed that 4 and 8 metabolites were
207 significantly altered by protein source and protein level respectively, a further 4
208 metabolite changes were associated with a protein-source*protein-level interaction.

209 When presented in a Venn plot (**Figure 3**), 24 unique metabolites with significant
210 changes were identified. Eight metabolites were specific for the comparison of LF, HF,
211 and HFHP groups, with both protein and non-protein related effects. Metabolites 2-
212 hydroxybutyric acid and palmitoleic acid were relatively high in LF group compared to
213 the HF and HFHP groups, and thus are likely regulated by lipid levels which
214 considerably differ across the LF and HF diets. Ketoleucine, L-proline and citric acid

215 levels were influenced by protein source. and were increased in casein groups (CS and
216 HCS) when compared to other meat protein groups (CK, PK, HCK and HPK) (**Figure**
217 **3**). Both lipid level and protein source were found to regulate myristic acid. L-tyrosine,
218 L-histidine, 5-aminopentanoic acid, N-acetylornithine and L-glutamine were regulated
219 by effects of protein level alone, and were particularly decreased in HF groups. In
220 contrast, xanthosine, D-fructose and turanose were increased in HF groups when
221 compared to HFHP. Thus, highlighting the importance of the protein: carbohydrate ratio
222 in regulating metabolite profile.

223 Utilizing a heatmap cluster analysis incorporating the 24 unique blood metabolites
224 together with the significantly altered parameters of body weight, body fat mass and
225 blood inflammatory factors (**Figure 4**). Three distinct clusters were observed, including
226 a HF cluster of CK, PK and CS, a HFHP cluster of HCK, HPK and HCS, and a LF
227 cluster.

228 Correlations of significant features were shown in **Figure 5**. See Pearson correlate
229 coefficient and P value in **Supplementary table 2 & 3**. DEI positively correlated with
230 body weight gain (FBW, BWG and DBWG) and body fat mass (Pearson correlate
231 coefficient > 0.4 & $P < 0.01$). The serum metabolites myristic acid, 2-hydroxybutyric
232 acid and palmitoleic acid negatively correlated with DEI, body weight and body fat
233 mass (Pearson correlate coefficient < -0.28 & $P < 0.05$). Additionally, the serum
234 metabolites 5-aminopentanoic acid, L-tyrosine, N-acetylornithine, L-glutamine and L-
235 proline negatively correlated with body weight and body fat mass (Pearson correlate
236 coefficient < -0.3 & $P < 0.05$). Serum IL-10 and TNF-α negatively correlated with
237 serum palmitoleic acid (Pearson correlate coefficient < -0.3 & $P < 0.05$).

238 Pathway analysis (**Table 1**) showed that L-glutamine, N-acetylornithine, creatine,
239 spermine, L-proline were involved in arginine and proline metabolism. L-glutamine, L-

240 histidine, L-tyrosine and L-proline were involved in aminoacyl-tRNA biosynthesis.
241 These pathways were regulated by both protein level and protein source. Pathways of
242 glyoxylate and dicarboxylate metabolism were regulated by both protein source and fat
243 level. Pathways of nitrogen metabolism and beta-Alanine metabolism was regulated by
244 protein level only.

245 **Discussion**

246 In this study we evaluate the effects of protein source (casein, pork and chicken)
247 and protein level (normal and high) on body fat accumulation and metabolic function
248 of healthy rats maintained on a high-fat diet. As expected, HF diet induced body weight
249 gain, increasing 18.5% ($P < 0.05$), compared to the LF diet. A subsequent 31.5%
250 increase ($P < 0.05$) in white adipose tissue weight was identified in HF maintained rats,
251 indicating that body fat was more sensitive measure for assessing high-fat induced
252 obesity in animals than body weight, as described by Woods et al. (2003)[21]. The body
253 weight gain of HF maintained rats relates to the higher energy intake from the HF diets
254 (21% higher), and further supported by correlation analysis showing a positive
255 correlation between DEI, body weight and fat mass (Pearson correlate coefficient > 0.4
256 & $P < 0.01$). When increasing protein levels in the HF diets compensated by reducing
257 carbohydrate (starch) levels (i.e. HFHP diets), the EATW and EATW/BW of rats
258 provided with HFHP was decreased 15.2% ($P < 0.05$) and 11.0% ($P < 0.05$),
259 respectively. However, DFI, DEI and body weight gain of HFHP rats (although
260 nominally reduced by 8.8%) was not significantly different from the HF groups ($P >$
261 0.05), suggesting that increased dietary protein content may reduce high-fat induced
262 body fat accumulation without reducing energy intake and body weight gain. This was
263 similarly observed by Chaumontet et al. (2015), who purported that high protein diets
264 may reduce adiposity by inhibiting lipogenesis in the liver[14]. Interestingly, in the present

265 study, the body fat reducing effects of high protein diets appeared to be independent of
266 protein sources. Therefore, chicken, pork and casein were equally effective at reducing
267 body fat mass associated with HF diet.

268 Insulin resistance accompanies increased body fat accumulation associated with
269 high-fat induced obesity[21]. The oral glucose tolerance test (OGTT) is often employed
270 to evaluate insulin resistance[22]. Compared to the LF diet, HF and HFHP diets reduced
271 glucose tolerance (i.e. increased glucose-AUC) of rats independent of differences in
272 protein level and source. However, the serum insulin concentrations did not differ
273 between groups ($P > 0.05$). These findings were not in agreement with our previous
274 study[7], which suggests that red meat may increase risk of insulin resistance in rats.
275 However, Myrmel et al[11] also found that intake of pork protein based HFHP diet
276 reduced glucose tolerance and insulin sensitivity of rats, compared to casein based
277 HFHP diets. Chicken protein was not compared in their study. Previously, we have also
278 identified that a cluster of gene sets involved in the insulin signaling pathway was
279 mostly inhibited by chicken protein when compared to casein and other meat proteins[4].
280 The heterogeneity of these findings highlights the need for further studies to verify the
281 effects of meat proteins on insulin resistance.

282 Obesity is closely associated with a state of 'low-grade' chronic inflammation,
283 shown by increased levels of inflammatory markers[23]. Obese people and animals often
284 show a higher level of serum pro-inflammatory cytokines, such as TNF-α and IL-6[24].
285 In this study, compared to the LF diet, both HF and HFHP diets increased serum
286 concentrations of TNF-α (by 57.9% and 94.7%), IL-6 (by 18.2% and 47.7%) and MCP-
287 1 (by 47.1% and 13.9%) of rats. However, these pro-inflammatory cytokines were not
288 different between HF and HFHP groups ($P > 0.05$). Therefore, these increased pro-
289 inflammatory cytokines were a result of HF diet and were not attenuated by HFHP diet.

290 However, compared to the HF diet, the HFHP diet resulted in a significant increase of
291 serum anti-inflammatory cytokine IL-10 (by 68.2%, $P < 0.05$). These increased anti-
292 inflammatory cytokine IL-10 was supposed to be stimulated by the effects of higher
293 protein level in HFHP diet than in HF diet. Previous studies have shown a protective
294 role of IL-10 in regulation of metabolic inflammation and insulin sensitivity[25]. In
295 addition, Clement et al. (2004) found that IL-10 expression was increased following
296 weight loss in obese patients[26], as observed to some extent in this study, with HFHP fed
297 rats displaying increased serum IL-10 following body fat mass loss. Interestingly,
298 protein source had no effect on serum IL-10, and thus a high protein intake in general
299 could be beneficial for protecting high-fat induced metabolic inflammation.

300 Obesity-related chronic inflammation is principally triggered by nutrients and
301 metabolic surplus and therefore has close interface with metabolic responses[27]. Blood
302 metabolite profiles shifted significantly in response to the protein diets, with protein
303 level (8 metabolites) resulting in greater effects on the regulation of blood metabolome
304 than protein source (4 metabolites). Moreover, protein source*level interaction was also
305 evident.

306 At the individual metabolites level, HF and HFHP resulted in lower palmitoleic
307 acid and 2-hydroxybutyrate compared to LF fed rats, suggesting that lipid mobilization
308 and fatty acid oxidation was reduced by HF and HFHP diets. Palmitoleic acid
309 (palmitoleate) is a monounsaturated fatty acid most abundant in serum, adipose tissue
310 and liver[28]. Endogenous palmitoleic acid mainly originates from de novo lipogenesis in
311 the adipose tissue and liver[28]. Increased circulating concentrations of free palmitoleic
312 acid in the blood suggests increased lipid mobilization from adipose tissue. Therefore,
313 palmitoleic acid was referred to as a “lipokine” and has been associated with increased
314 lipolysis by activation of peroxisome proliferator-activated receptor-α (PPARα)[29].

315 PPAR-α is a transcription factor which plays a central role in controlling the fatty acid
316 β-oxidation[30]. It has been shown that increased fatty acid oxidation contributes to
317 elevated 2-hydroxybutyrate[31]. In contrast, the reduced serum palmitoleic acid and 2-
318 hydroxybutyrate in HF and HFHP groups reflected a suppressed lipid mobilization and
319 fatty acid oxidation, compared to the LF fed rats. The reduced lipid catabolism in HF
320 fed rats is likely associated with the increased body weight and fat mass observed. This
321 is supported by the correlation analysis, showing a negative correlation of blood
322 palmitoleic acid and 2-hydroxybutyric acid with body weight and fat mass. At the same
323 time, blood palmitoleic acid negatively correlated with glucose-AUC and blood TNF-
324 a, suggesting that palmitoleic acid is associated with decreased insulin resistance and
325 expression of proinflammatory markers, and is consistent with Frigolet et al. (2017)[28].
326 Notably, serum creatine, L-proline and ketoleucine were different between casein
327 and meat proteins. Creatine is a nitrogenous organic compound found in muscle and is
328 available in the diet through consumption of meat[32]. Therefore, the higher serum
329 creatine in rats fed meat proteins was expected. Creatine is a tripeptide compound
330 composed of arginine, methionine and glycine, and thus is involved in metabolism of
331 these amino acids[32]. Ketoleucine is an intermediate product of leucine metabolism[33]. In
332 this study, blood L-proline and ketoleucine were relatively high in casein groups, but
333 low in chicken and pork protein groups. These differences may be partly related to the
334 amino acid compositions of different dietary proteins. It has been shown that casein has
335 higher contents of proline and leucine than chicken and pork proteins[10]. Based on this,
336 pathway analysis showed that arginine and proline metabolism were the main metabolic
337 processes regulated differently by casein and meat proteins. In addition, serum L-
338 proline was negatively correlated with body weight and body fat mass. Therefore,

339 proline and its metabolism might contribute largely to the differences of anti-obesity
340 effects of casein and meat proteins.

341 On the other hand, serum L-histidine, 5-aminopentanoic acid, L-tyrosine, N-
342 acetylornithine and L-glutamine were mainly different between high and low protein
343 diets. They were relatively high in HFHP fed rats but low in HF fed rats. Pathway
344 analysis showed that nitrogen metabolism and aminoacyl-tRNA biosynthesis were the
345 main metabolic processes regulated differently by protein levels in diets. In addition,
346 blood metabolites 5-aminopentanoic acid, L-tyrosine, N-acetylornithine and L-
347 glutamine were negatively correlated with body weight and body fat mass. Therefore,
348 compared to low protein diets, the anti-obesity effects of high protein diets could mainly
349 attribute to the increased blood L-histidine, L-tyrosine and L-glutamine and their
350 related nitrogen metabolism. These ATP-consuming metabolic processes could be
351 associated with the augmented energy expenditure in rats fed high protein diet.

352 Taken together, the effects of dietary proteins on body fat accumulation and
353 metabolic health were dependent largely on protein level, but less on protein source.
354 Intake of high content of chicken and pork proteins had similar effects with casein on
355 reducing body fat mass of rats fed HF diets. These anti-obesity effects of dietary
356 proteins were closely related to the changes of blood metabolome. The effects of protein
357 levels were mainly related to changes of blood L-histidine, L-tyrosine, L-glutamine, 5-
358 aminopentanoic acid and N-acetylornithine. While, the effects of protein sources were
359 mainly related to changes of blood L-proline, ketoleucine, creatine and citric acid.
360 Further studies are still needed to expound the molecular mechanism behind the diverse
361 metabolic regulation effects of different protein sources and levels.

362 **Abbreviations Used**

363 AUC: area under curve; BWG: the body weight gain of rats from day 0 to day 84;
364 CK: high fat 20% E chicken protein group; CS: high fat 20% E casein group; DBWG:
365 the daily body weight gain of rat during 84-day feeding; DEI: the daily energy intake
366 of rats during 84-day feeding; DFI: the daily feed intake of rats during 84-day feeding;
367 EATW: epididymal adipose tissue weight; EATW/FBW: relative percent content of
368 epididymal adipose tissue to the final body weight of rats; FBW: the final body weight
369 of rats on day 84; HCK: high fat 40% E chicken protein group; HCS: high fat 40% E
370 casein group; HF: high fat; HFHP: high fat high protein; HPK: high fat 40% E pork
371 protein group; IBW: the initial body weight of rats on day 0; IL-6: interleukin 6; IL-10:
372 interleukin 10; LF: low fat; MCP-1: monocyte chemoattractant protein-1; OGTT: oral
373 glucose tolerant test; PATW: perirenal adipose tissue weight; PATW/FBW: relative
374 percent content of perirenal adipose tissue to the final body weight of rats; PK: high fat
375 20% E pork protein group; TNF-α: tumor necrosis factor α; WATW: total white adipose
376 tissue weights (WATW = PATW + EATW); WATW/FBW: relative percent content of
377 total white adipose tissue to the final body weight of rats.

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384 **Supporting Information**

385 Supplementary Table 1: Diet formulas of D12450H, D12451 and D12451m,
386 Supplementary Table 2: Pearson correlate coefficient of correlations of significant

387 features, and Supplementary Table 3: P value of correlations of significant features
388 (XLSX).

389 **References**

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Figure Captions

Figure 1 (A) the body weight, feed intake and (B) body fat mass of rats fed different diets.

Note: IBW: the initial body weight of rats on day 0; FBW: the final body weight of rats on day 84; BWG: the body weight gain of rats from day 0 to day 84; DBWG: the daily body weight gain of rat during 84-day feeding; DFI: the daily feed intake of rats during 84-day feeding; DEI: the daily energy intake of rats during 84-day feeding; PATW: perirenal adipose tissue weight; EATW: epididymal adipose tissue weight; WATW: total white adipose tissue weights (WATW = PATW + EATW); PATW/FBW: relative percent content of perirenal adipose tissue to the final body weight of rats; EATW/FBW: relative percent content of epididymal adipose tissue to the final body weight of rats; WATW/FBW: relative percent content of total white adipose tissue to the final body weight of rats. LF: low fat group; HF: high fat super group = CS + CK + PK; HFHP: high fat high protein super group = HCS + HCK + HPK; CS: high fat 20% E casein group; CK: high fat 20% E chicken protein group; PK: high fat 20% E pork protein group; HCS: high fat 40% E casein group; HCK: high fat 40% E chicken protein group; HPK: high fat 40% E pork protein group; Results are mean ± SD. The number of replications of LF, HF and HFHP groups were 7, 21 and 21, respectively. The number of replications of CS, CK, PK, HCS, HCK and HPK groups were 7. Different letters above bars mean significant different ($P < 0.05$) tested by one-way ANOVA and Duncan post hoc analysis. NS: no significant effects of protein source or protein level by two-factor ANOVA ($P > 0.05$). L: significant effect of protein level (L) by two-factor ANOVA ($P < 0.05$).

Figure 2 (A) Oral glucose tolerant test results, and (B) serum hormones and inflammatory factors of rats fed different diets.

Note: LF: low fat group; HF: high fat super group = CS + CK + PK; HFHP: high fat high protein super group = HCS + HCK + HPK; CS: high fat 20% E casein group; CK: high fat 20% E chicken protein group; PK: high fat 20% E pork protein group; HCS: high fat 40% E casein group; HCK: high fat 40% E chicken protein group; HPK: high fat 40% E pork protein group; TNF-α: tumor necrosis factor α; IL-6: interleukin 6; MCP-1: monocyte chemoattractant protein-1; IL-10: interleukin 10. Results are mean ± SD. The number of replications of LF, HF and HFHP groups were 7, 21 and 21, respectively. The number of replications of CS, CK, PK, HCS, HCK and HPK groups were 7. Different letters above bars mean significant differences ($P < 0.05$) tested by one-way ANOVA and Duncan post hoc analysis. NS: no significant effects of protein source or protein level tested by two-factor ANOVA ($P > 0.05$). L: significant effect of protein level (L) tested by two-factor ANOVA ($P < 0.05$).

Figure 3 Venn plot of serum metabolites with significant differences.

Note: LF_HF_HFHP: comparison of LF, HF and HFHP groups; prot_source: effects of protein source tested by two-factor ANOVA; prot_level: effects of protein level tested by two-factor ANOVA; interaction: interaction effects of protein source and level tested by two-factor ANOVA; Different letters in each row of heatmaps mean significant differences ($P < 0.05$) tested by Duncan post hoc analysis

Figure 4 Clustered heatmap of regulations of significant features.

Note: E: color of effects. Pink means effects of protein source. Green means effects of protein level, i.e. ratio of protein to carbohydrate. Yellow means interaction effects of

protein level and protein source. Blue means effects of fat level. Black means none-blood metabolomics items.

Figure 5 Clustered heatmap of correlations of significant features.

Note: E: color of effects. Pink means effects of protein source. Green means effects of protein level, i.e. ratio of protein to carbohydrate. Yellow means interaction effects of protein level and protein source. Blue means effects of fat level. Black means none-blood metabolomics items.

Table 1. Pathway analysis of 24 blood metabolites with significant changes

Pathways	Hits	P value	Effects
Arginine and proline metabolism	L-glutamine, N-acetylornithine, creatine, spermine, L-proline	0.0016	PL, PS
Aminoacyl-tRNA biosynthesis	L-glutamine, L-histidine, L-tyrosine, L-proline	0.0060	PL, PS
Glyoxylate and dicarboxylate metabolism	citric acid, glyceric acid, L-glutamine	0.013	PS, FL
Nitrogen metabolism	L-glutamine, L-histidine	0.014	PL
Pentose phosphate pathway	glyceric acid, gluconolactone	0.042	FL
beta-Alanine metabolism	spermine, L-histidine	0.042	PL

Note: PL: protein level; PS: protein source; FL: fat level.

Figure 1

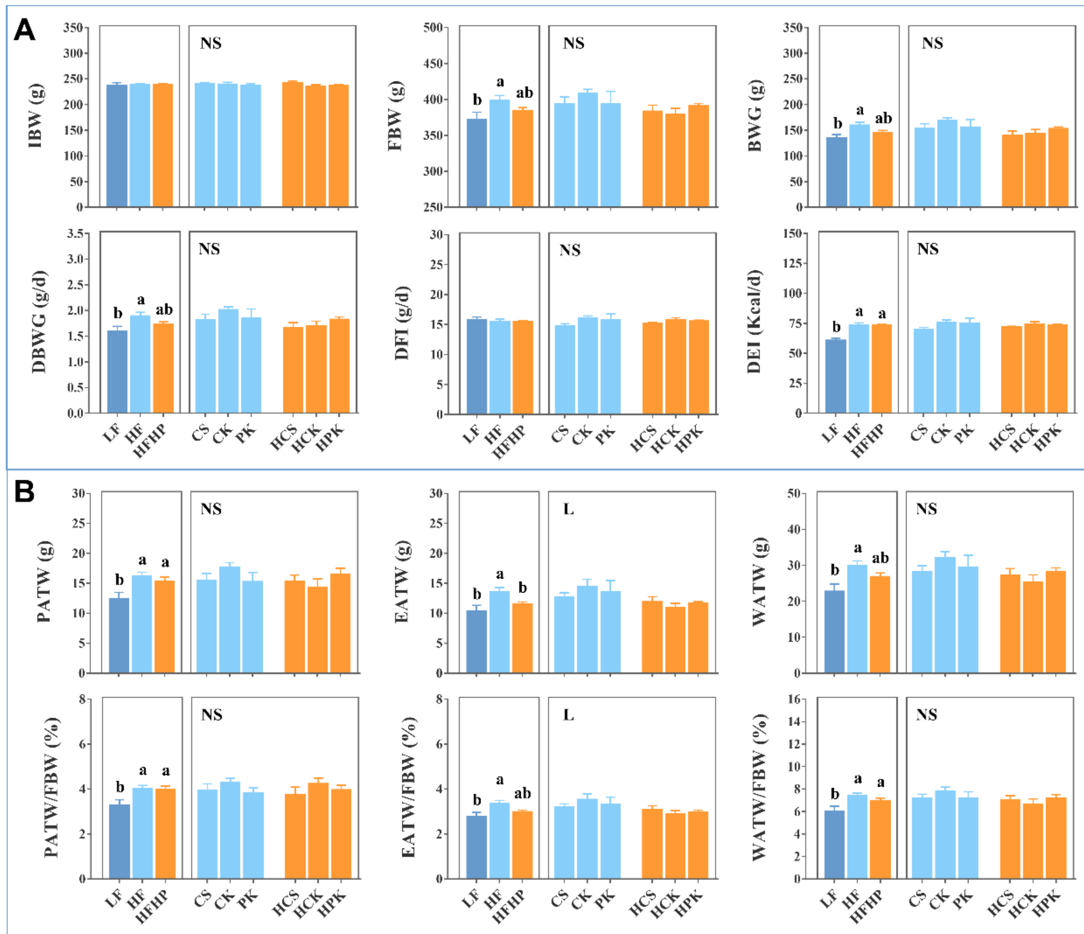


Figure 2

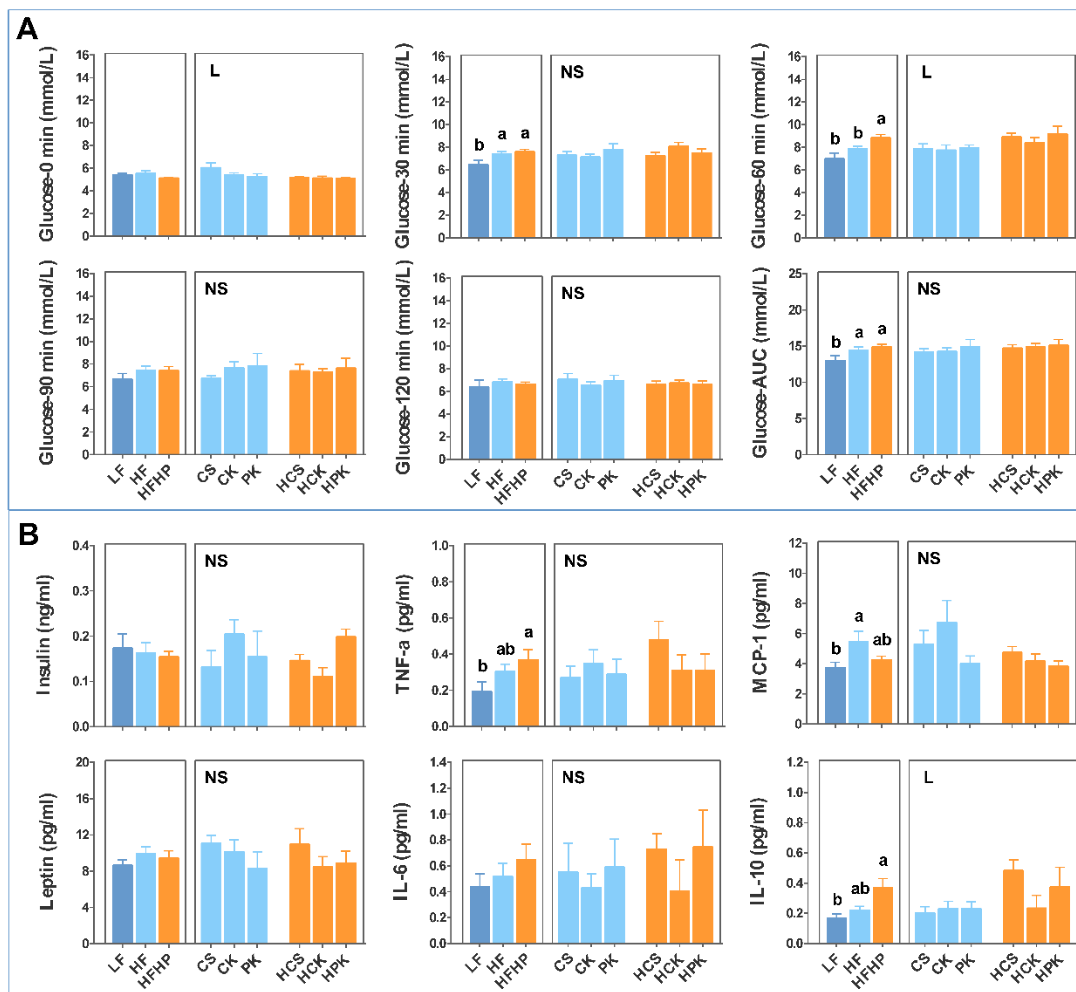


Figure 3

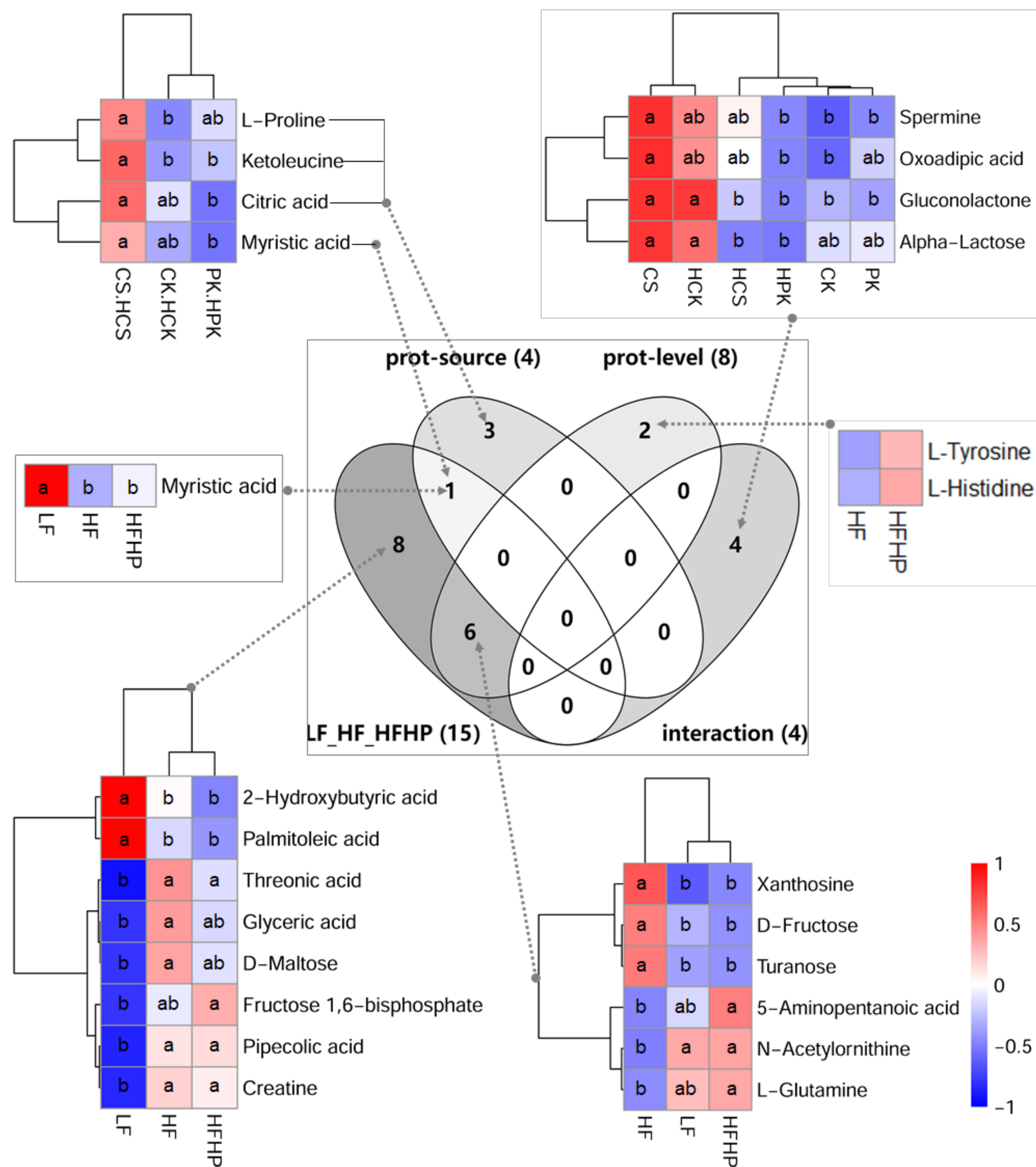


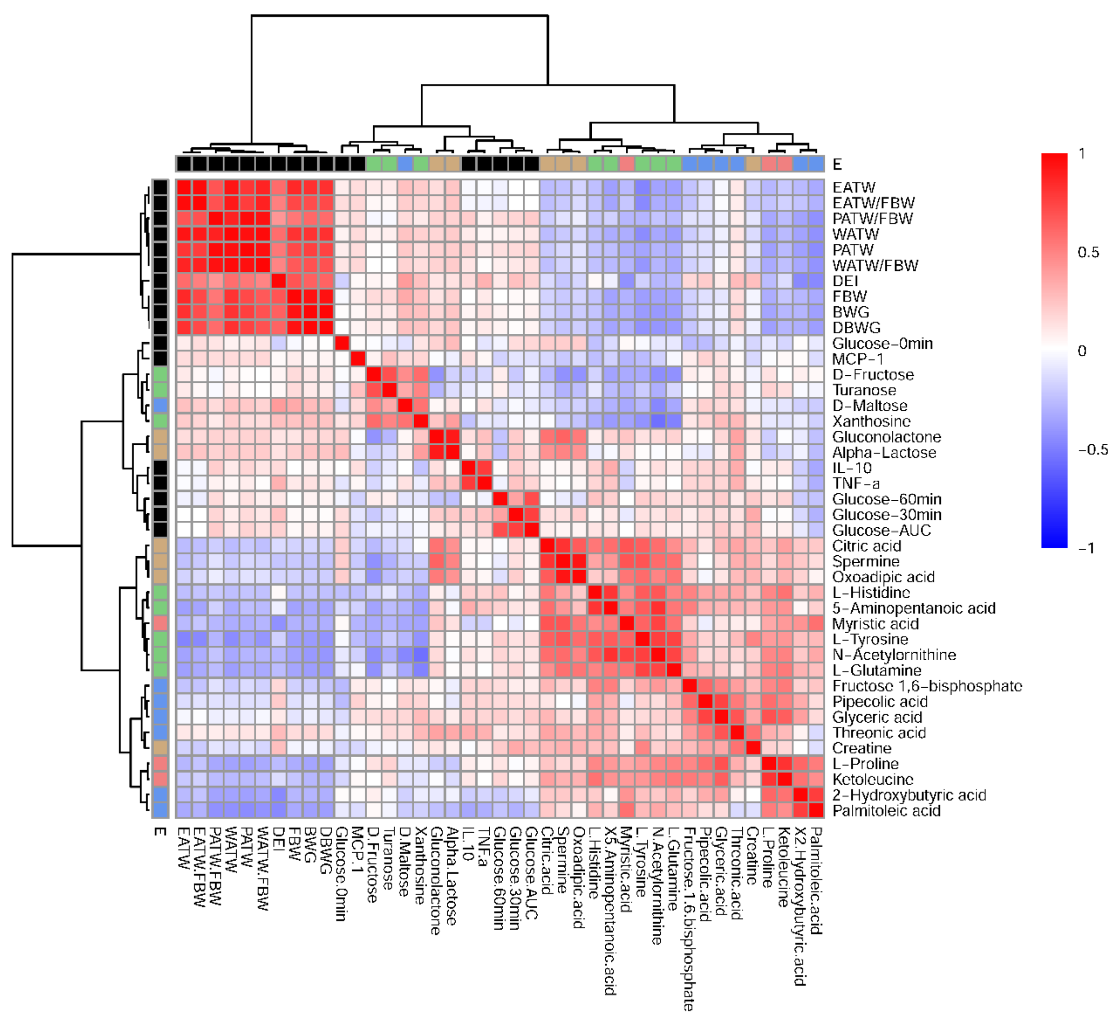
Figure 4

L−Histidine
5−Aminopentanoic acid
L−Tyrosine
N−Acetylornithine
L−Glutamine
Myristic acid
2−Hydroxybutyric acid
Palmitoleic acid
Oxoadipic acid
Citric acid
Spermine
Gluconolactone
Alpha−Lactose
Glucose−0min
L−Proline
Ketoleucine
DEI
Creatine
Glucose−30min
Glucose−AUC
Pipecolic acid
TNF−a
Glucose−60min
Fructose 1,6−bisphosphate
IL−10
D−Fructose
Turanose
Threonic acid
Glyceric acid
D−Maltose
Xanthosine
MCP−1
EATW
EATW/FBW
FBW
BWG
DBWG
WATW
WATW/FBW
PATW
PATW/FBW

E
LF
HCS
HCK
HPK
CS
CK
PK

1
0.5
0
−0.5
−1

Figure 5



Graphic for Table of Contents