

Gut microbial phenol impairs energy metabolism and promotes sarcopenic obesity-like phenotypes in mice

Shiori Oishi

University of Shizuoka

Kisaki Ikeda

University of Shizuoka

Misaki Matsui

University of Shizuoka

Sae Yuyama

University of Shizuoka

Tomoki Sato

University of Shizuoka

Momoka Yamaguchi

University of Shizuoka

Takuya Yoshida

Prefectural University of Kumamoto

Tadashi Fujii

Fujita Health University

Takumi Tochio

Fujita Health University

Hisayoshi Hayashi

University of Shizuoka

Shinji Miura

University of Shizuoka

Takuma Kobayashi

University of Shizuoka

Noriyuki Miyoshi

`miyoshin@u-shizuoka-ken.ac.jp`

University of Shizuoka

Keywords: Gut microbiota, phenol, phenyl sulfate, energy metabolism, sarcopenic obesity, tyrosine phenol-lyase

Posted Date: June 15th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9765736/v1>

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Scientific Reports on September 7th, 2026.
See the published version at <https://doi.org/10.1038/s41598-026-70256-4>.

Abstract

Gut microbial phenol has been recognized as a uremic toxin associated with chronic kidney disease; however, its effects under preclinical conditions remain unclear. In this study, we established a mouse model with increased endogenous phenol production using a tyrosine-rich diet. High-tyrosine feeding markedly increased fecal phenol and circulating phenyl sulfate (PhS) levels without affecting body weight, food intake, or glucose tolerance. In contrast, respiratory gas analysis revealed reduced energy expenditure and carbohydrate oxidation during the dark phase. CT and histological analyses demonstrated increased adiposity, adipocyte hypertrophy, and reduced skeletal muscle fiber size in the tyrosine-fed group. Inflammatory cytokines in adipose tissue and liver were also elevated. These alterations were largely suppressed by administration of the tyrosine phenol-lyase inhibitor 3,5-dihydroxybenzoic acid (35DHBA), accompanied by a marked reduction in phenol production. Although plasma PhS levels were elevated, no apparent renal dysfunction was observed in the current experimental conditions. These results suggest that gut microbial phenol production may contribute to alterations in energy metabolism and body composition before the onset of overt kidney dysfunction. Our findings indicate that microbial phenol production is potentially involved in metabolic abnormalities associated with excess dietary tyrosine.

Introduction

The gut microbiota plays an important role in host metabolism through the production of a wide variety of metabolites derived from dietary components [1][2]. Among these metabolites, compounds generated from aromatic amino acids such as tyrosine and tryptophan have attracted attention because of their association with metabolic disorders and chronic kidney disease [3][4]. Phenol is one of the representative microbial metabolites produced from tyrosine mainly via bacterial tyrosine phenol-lyase (TPL) activity [5][6][7]. After intestinal absorption, phenol is metabolized in the liver to conjugated metabolites including phenyl sulfate (PhS), which is recognized as a representative protein-bound uremic toxin (PBUT) [8][9]. These metabolites are detected in plasma and urine, and elevated levels have been associated with chronic kidney disease and related pathological conditions [10][11][12].

Several studies have suggested that phenol and its conjugated metabolites exert cytotoxic and pro-inflammatory effects in multiple tissues. Experimental studies demonstrated that phenol-related metabolites induce oxidative stress and inflammatory responses in renal tubular cells and other cell types [8][13][14]. In particular, PhS has been reported to contribute to the progression of diabetic kidney disease and renal fibrosis [15][8]. Most previous studies, however, focused mainly on pathological conditions associated with advanced renal dysfunction or marked accumulation of uremic toxins [16][17][18]. Therefore, it remains unclear whether enhanced microbial phenol production influences host metabolism before the onset of overt kidney dysfunction. In contrast to severe uremic conditions, relatively little information is available regarding the physiological effects of sustained exposure to gut microbial phenol under preclinical or non-disease conditions. It is also unclear whether increased phenol

production itself contributes directly to metabolic alterations or simply reflects pathological progression associated with renal impairment.

Energy metabolism is regulated by the balance between carbohydrate and lipid utilization according to nutritional and physiological conditions [19][20]. Disturbance of this balance is closely associated with metabolic disorders including obesity and type 2 diabetes, and these alterations can occur before obvious impairment of glucose tolerance or insulin sensitivity becomes detectable [21][22]. In addition, mitochondrial dysfunction and chronic low-grade inflammation are thought to impair substrate utilization and energy expenditure independently of severe endocrine abnormalities [23][24][25]. Therefore, changes in energy metabolism may reflect an early stage of metabolic dysfunction. Since intestinal microbial metabolites are continuously produced, absorbed, and circulated throughout the body, they may affect systemic metabolic homeostasis. However, the contribution of individual microbial metabolites to these metabolic alterations remains unclear.

In our previous studies, we identified dietary polyphenols including quercetin and 3,5-dihydroxybenzoic acid (35DHBA) as inhibitors of bacterial tyrosine phenol-lyase [6][7]. Administration of these compounds suppressed fecal phenol production in mice [6][7], suggesting that microbial phenol production can be regulated by inhibition of TPL activity. In the current study, we established a mouse model of enhanced endogenous phenol production using a high-tyrosine diet. This dietary intervention increased intestinal phenol production and circulating PhS levels under chronic feeding conditions. To further evaluate the contribution of microbial phenol production, 35DHBA was administered together with the high-tyrosine diet. This approach enabled us to distinguish the effects associated with microbial phenol production from those caused directly by dietary tyrosine itself, and to evaluate the physiological effects of enhanced phenol production *in vivo*.

In the present study, we evaluated the effects of enhanced microbial phenol production on whole-body energy metabolism using respiratory gas analysis, with particular focus on carbohydrate and lipid utilization. In addition, adiposity, inflammatory responses, and tissue histology were analyzed to determine whether metabolic alterations associated with phenol production occur before overt renal dysfunction becomes apparent. Through these analyses, we examined the physiological effects associated with enhanced gut microbial phenol production in mice.

Materials and Methods

Reagents

L-Tyr, D₄-L-Tyr and 3,5-dihydroxybenzoic acid (3,5DHBA) was purchased from Tokyo Chemical Industry (Tokyo, Japan). All compounds are > 95% pure by HPLC analysis.

Animal experiments

All animal experiments were approved by the animal ethics committee of University of Shizuoka (approved number 245440 and 255487), and performed according to guidelines for the care and use of laboratory animals at the University of Shizuoka. C57BL/6J mice (5-week-old male) were purchased from CLEA Japan Inc. (Tokyo, Japan). All animals were housed individually in plastic cages and had free access to drinking water under controlled conditions of humidity ($50 \pm 10\%$), light (12/12-h light/dark cycle) and temperature ($23 \pm 2\text{ }^{\circ}\text{C}$). After a 1-week adaptation period with AIN-93G *ad libitum*, mice were divided into four experimental groups as shown in Supplemental Fig. 1. Mice were fed normal diet (AIN-93G, $n = 5$), L-Tyr diet (AIG-93G supplemented with 5% L-Tyr, $n = 17$), normal diet supplemented with 0.2% 3,5DHBA ($n = 5$), L-Tyr diet supplemented with 0.2% 3,5DHBA ($n = 9$) (Supplemental Table 1). Body weight and amounts of food intake were measured every 2–3 days. Feces, urine and blood samples were collected sequentially. At the end of the experimental period, mice were anesthetized with 2.5% isoflurane inhalation anesthesia using an inhalation anesthesia apparatus (Muromachi Kikai Co., Ltd., Tokyo, Japan) and euthanized by exsanguination. The 16s rRNA gene amplicon sequencing raw reads have been deposited in the NCBI BioProject database (BioProject accession: PRJNA1463006). Data were analyzed using Nephele (National Institutes of Health, Bethesda, MD, USA) and QIIME2. Abdominal CT images were obtained using COSMOScan FX (Rigaku, Tokyo, Japan). Blood glucose levels in an oral glucose tolerance test (OGTT) were monitored using ACCU-Chek Aviva (Roche). Respiration gas analysis was performed using mass spectrometer (model ARCO-2000, Arco System). Metabolic parameters were calculated as follows: $\text{RQ} = \text{VCO}_2 / \text{VO}_2$, $\text{FAT} = 1.695 \times \text{VO}_2 - 1.701 \times \text{VCO}_2$ (mg/min), $\text{CHO} = 4.585 \times \text{VCO}_2 - 3.226 \times \text{VO}_2$ (mg/min), $\text{energy} = 3.815 \times \text{VO}_2 + 1.232 \times \text{VCO}_2$ (cal/min). Locomotor activity was measured by counting the frequency of passes into the different regions in the cage every 5 min during the observation period. Frozen section of liver, white adipose (epididymal fat) and skeletal muscle (tibialis anterior) were subjected to HE staining for the histological analysis. Precision-cut liver slices (PCLSs) were prepared from mouse liver using a vibrating blade microtome. Briefly, freshly excised liver tissue was mounted onto the specimen holder using tissue adhesive and submerged in ice-cold Krebs–Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO_4 , 1.25 mM CaCl_2 , 1.2 mM KH_2PO_4 , 11 mM D-glucose, and 25 mM NaHCO_3). Liver tissue was sectioned into 250 μm -thick slices using a vibratome (Leica VT1200S, Leica Biosystems) at a speed of 1.0 mm/s and an amplitude of 1.0 mm. The slices were transferred into 6-well plates and cultured in 10% FBS DMEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37°C under 5% CO_2 . After equilibration, PCLSs were incubated with PhS (10 or 50 μM) or lipopolysaccharide (LPS, 100 ng/mL; positive control) for 6 h.

LC-MS quantification analysis

Fecal phenol quantification was performed as previously reported [6][7]. Briefly, fecal samples (~ 100 mg) in 10 volumes of 50 mM potassium phosphate buffer (pH 8.0) were homogenized (5,000 rpm, 20 sec, twice at $4\text{ }^{\circ}\text{C}$) using a MicroSmash MS-100R (TOMY, Japan) with 5.0-mm zirconia beads (six beads/tube). After centrifugation at $20,800 \times g$ for 10 min at $4\text{ }^{\circ}\text{C}$, 400 μL of the supernatant was mixed with 40 μL of 50 μM 4-isopropyl phenol (internal standard) solution, 200 μL of 6 N HCl, and 600 μL of

ethyl acetate and then vortexed for 10 min. The mixture was centrifuged at 5,000 $\times g$ for 10 min at 4 °C, and the organic phase was collected. The solvent was evaporated, and the residue was reconstituted in 20% methanol for LC–MS analysis. LC-MS analysis was performed using an Agilent 1290 Infinity II LC System coupled with a G6410B MS. LC separation was carried out with an ODS-120H column (1.9 μm , 50 mm $\times$ 2.0 mm i.d.; TOSOH, Tokyo, Japan) at 40 °C using Milli-Q water (MQ water) as solvent A and methanol as solvent B. The gradient program for solvent B was as follows; 0–4 min = 5–47.5%, 4.01–6 min = 90%, 6.01–8 min = 90–5%, and 8.01–11 min = 5%. The flow rate of the mobile phase was 0.4 mL/min. Ion source parameters and method validation are shown in Supplemental Tables 2 and 3.

Fecal L-Tyr was quantified by LC-MS following derivatization with 3-aminopyridyl-*N*-hydroxysuccinimidyl carbamate (APDS) [26]. Briefly, collected fecal samples were ground into a fine powder. An aliquot of 20 mg of powdered feces was suspended in 1,000 μL of 40 μM D₄-L-Tyr (0.01 N HCl). L-Tyr was extracted from the fecal samples by agitation at 50 rpm for 1 h at 4 °C). The samples were then centrifuged at 20,800 $\times g$ for 15 min at 4 °C), and an aliquot (100 μL) of the supernatant was mixed with 400 μL of methanol in a new 1.5 mL tube, vortexed thoroughly, and centrifuged again at 20,800 $\times g$ for 15 min at 4 °C). The resulting supernatant was collected and evaporated to dryness. The dried residue was reconstituted with 80 μL of 0.2 M sodium borate buffer (pH 8.8) and 20 μL of 20 mg/mL APDS, vortexed, and incubated at 55 °C) for 10 min. After cooling to room temperature, the reaction was terminated by adding 100 μL of 0.2% acetic acid followed by vortex mixing. The prepared samples were injected into an Agilent 1290 Infinity II LC System coupled with a G6410B MS for quantitative LC-MS analysis. LC separation was carried out with an ODS-120H column (1.9 μm , 50 mm $\times$ 2.0 mm i.d.; TOSOH, Tokyo, Japan) at 40 °C using 25 mM formic acid (pH 6.0, adjusted with ammonium hydroxide) as solvent A and 60% MeCN in MQ water as solvent B. The gradient program for solvent B was as follows; 0 min = 0%, 1.0 min = 4%, 2.26 min = 4%, 6.0 min = 20%, 6.5 min = 50%, 7.5 min = 95%, and 7.51 min = 0%. The flow rate of the mobile phase was 0.4 mL/min. Ion source parameters and method validation are shown in Supplemental Tables 2 and 3.

Plasma and urine concentrations of PhS, phenyl glucuronide (PhG), *p*-cresyl sulfate (*p*CS), indoxyl sulfate (IndS), and creatinine (Cre) were quantified as follows: 10 μL of mouse plasma or urine was mixed with 90 μL methanol, vortexed, and centrifuged at 20,800 $\times g$ for 15 min at 4 °C, and the supernatant was transferred to a 1.5 mL tube. The supernatant was evaporated to dryness, and the residue was reconstituted in 50 μL of MQ water by vortex mixing. After a second centrifugation at 20,800 $\times g$ for 15 min at 4 °C, the resulting supernatant was injected into an Agilent 1290 Infinity II LC System coupled with a G6410B MS. LC separation was carried out with an ODS-120H column (1.9 μm , 50 mm $\times$ 2.0 mm i.d.; TOSOH, Tokyo, Japan) at 40 °C using 0.1% formic acid in MQ water as solvent A and 0.1% formic acid in MeCN as solvent B. The gradient program for solvent B was as follows; 0 min = 1.0%, 5 min = 40%, 5.01–8 min = 100%, and 8.01–11 min = 1%. The flow rate of the mobile phase was 0.4 mL/min. Ion source parameters and method validation are shown in Supplemental Tables 2 and 3.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA extracted using RNAiso Plus reagent (TaKaRa bio) from mice skeleton muscle, liver, WAT, and PCLSs were converted into cDNA using PrimeScript RT Master Mix (TaKaRa). To quantitatively estimate the level of each gene, quantitative PCR was performed using gene-specific primers, cDNA and SYBR Premix (TaKaRa). To quantitatively estimate the expression level of each gene, qPCR was performed using gene-specific primers, cDNA, and SYBR Premix (TaKaRa Bio). The sequences of the PCR primer pairs used were shown in Supplemental Table 4.

Statistical analysis

Statistical analyses were conducted using EZR (Saitama Medical Center, Jichi Medical University), a graphical user interface for R (The R Foundation for Statistical Computing), and the data were analyzed using multiway and one-way analysis of variance (ANOVA), followed by the Tukey honestly significant difference (HSD) post-hoc test and paired *t*-test. Differences were considered significant at $p < 0.05$.

Results

High-tyrosine diet induces phenol production via TPL-dependent mechanisms without affecting body weight or food intake

During the experimental period, no significant differences in body weight or food intake were observed among groups (Fig. 1). In contrast, fecal phenol levels were markedly increased in mice fed a high-tyrosine diet (Fig. 2A). The fecal phenol levels in the control and Tyr groups were 455 ± 9 and 1751 ± 162 nmol/g feces, respectively. This increase was almost completely suppressed by supplementation with the TPL inhibitor 35DHBA (499 ± 12 nmol/g feces) (Fig. 2A). Fecal L-tyrosine levels were comparable between the Tyr and Tyr + 35DHBA groups (3.5 ± 0.6 vs. 3.9 ± 0.9 μ mol/g feces), indicating a similar substrate supply for TPL (Fig. 2B). Consistently, cecal phenol levels were significantly elevated in the Tyr group and strongly suppressed by 35DHBA, whereas cecal tyrosine levels did not differ among groups, showing patterns parallel to those observed in feces (Supplemental Fig. 2). Plasma and urinary phenol conjugates, including PhS and PhG, were markedly increased in the Tyr group and completely suppressed by 35DHBA (Fig. 2C). In contrast, *p*CS levels were modestly increased in the Tyr group (4.4-fold increase, $p = 0.78$) but were not reduced by 35DHBA, and were present at lower concentrations than PhS (Fig. 2C). IndS levels were not affected by either the high-tyrosine diet or 35DHBA treatment (Fig. 2D).

Microbiota analysis showed that alpha diversity (Chao1 index) was significantly reduced in the Tyr group compared with the control group, suggesting mild dysbiosis (Fig. 3A). Beta diversity analysis revealed

significant differences between the Tyr group and all other groups ($p < 0.001$) (Fig. 3B). However, the variance explained by the first principal coordinate (PCoA1, 20.918%) was relatively modest, indicating that the overall compositional shift was not substantial. No TPL-expressing bacterial species were identified among taxa increased in the Tyr group based on database annotation (Supplemental Table 5). Although alterations in microbiota composition may at least partly contribute to phenol production, the suppression of fecal phenol by 35DHBA is more likely attributable to direct inhibition of TPL activity rather than major microbiota changes.

Additionally, urinary cystatin C levels, a marker of renal function, were within the normal range and did not differ among groups (Fig. 4A), and no apparent glomerular abnormalities were observed histologically (Fig. 4B). These findings suggest that, despite elevated plasma PhS levels, renal function remained preserved, representing a preclinical or early-stage condition prior to overt kidney dysfunction.

High-tyrosine diet reduces energy expenditure and carbohydrate oxidation independent of locomotor activity

To evaluate how increased phenol production affects energy metabolism, respiratory gas analysis and locomotor activity were assessed (Fig. 5). During the dark phase, energy expenditure was significantly lower in the Tyr group compared with ND group, whereas no differences were observed during the light phase (Fig. 5A). Carbohydrate oxidation was also significantly reduced in the Tyr group compared with the control and 35DHBA groups during the dark phase, with no differences observed during the light phase (Fig. 5B). Fat oxidation did not differ among groups in either the light or dark phase (Fig. 5C). Locomotor activity, which typically increases during the dark phase in nocturnal animals, showed no significant differences among groups in either phase (Fig. 5D). These results indicate that phenol production induced by a high-tyrosine diet is associated with reduced energy expenditure and carbohydrate oxidation during the active (dark) phase, independent of changes in locomotor activity. Notably, no significant differences in glucose tolerance were observed among groups, as assessed by oral glucose tolerance testing (Supplemental Fig. 3), indicating that phenol-induced metabolic alterations occur independently of overt impairment in glucose homeostasis.

High-tyrosine diet promotes adiposity and adipocyte hypertrophy with increased inflammatory responses in adipose tissue

Given the reduction in energy metabolism observed in the Tyr group, the effect on adiposity was evaluated by CT analysis (Fig. 6). The Tyr group exhibited significantly higher body fat and visceral fat percentages compared with the other groups. Subcutaneous fat percentage was also significantly increased in the Tyr group compared with the control and 35DHBA groups (Fig. 6A). Histological analysis of epididymal white adipose tissue revealed a significant increase in adipocyte size in the Tyr group compared with the other groups (Fig. 6B). Furthermore, gene expression analysis demonstrated that the

levels of inflammatory cytokines TNF- α and IL-6 were significantly elevated in the Tyr group, whereas no significant differences were observed for IL-1 β and MCP-1 (Fig. 6C). These findings suggest that a high-tyrosine diet induces adipocyte hypertrophy and promotes adipose tissue inflammation, leading to increased fat accumulation.

High-tyrosine diet reduces muscle fiber size without inducing inflammatory or atrophy-related gene expression in skeletal muscle

Protein-bound uremic toxins (PBUTs) derived from gut microbial metabolism, such as IndS and pCS, have been implicated in systemic dysfunction, including skeletal muscle atrophy [27][28][29]. To assess the effect of elevated PhS levels, skeletal muscle was evaluated in mice fed a high-tyrosine diet (Fig. 7). Histological analysis of the tibialis anterior muscle revealed that muscle fiber cross-sectional area was significantly reduced in the Tyr group compared with the other groups (Fig. 7A). However, gene expression analysis showed no significant increases in inflammatory cytokines (TNF- α , IL-6, IL-1 β , and MCP-1) in the Tyr group (Fig. 7B). Similarly, the expression levels of muscle atrophy-related genes (Cbl-b, Atrogin-1, and MuRF-1) were not significantly altered among groups (Fig. 7C). These results indicate that a high-tyrosine diet induces morphological changes in skeletal muscle without accompanying activation of inflammatory or atrophy-related transcriptional pathways. Consistent with these findings, PhS did not induce the expression of atrophy-related genes in differentiated C2C12 myotubes (Supplemental Fig. 4), supporting the notion that muscle alterations occur independently of canonical atrophy signaling pathways.

High-tyrosine diet induces hepatic inflammatory responses with mild histological alterations without activation of lipogenic gene expression

Histological analysis of the liver revealed characteristic changes in the Tyr group, including macrophage infiltration and lipid droplet accumulation (Fig. 8A). However, no significant differences were observed in liver weight or liver weight normalized to body weight among groups (Supplemental Table 6), suggesting that these alterations were relatively mild. Gene expression analysis demonstrated that inflammatory cytokines (TNF- α , IL-6, IL-1 β , and MCP-1) were significantly upregulated in the Tyr group compared with the other groups (Fig. 8B). Consistent with these findings, PhS increased the expression of inflammatory cytokines in precision-cut liver slices (PCLSs) (Fig. 8C). In contrast, the expression levels of lipogenic genes (Fas, Acc1, Acc2, and SCD1) were not significantly different among groups (Fig. 8D). These findings indicate that a high-tyrosine diet induces hepatic inflammation accompanied by modest histological changes, without overt activation of de novo lipogenesis.

Discussion

In the present study, we showed that a high-tyrosine diet enhanced gut microbial phenol production and induced several metabolic alterations in mice. The Tyr group exhibited reduced energy expenditure and carbohydrate oxidation together with increased adiposity and reduced skeletal muscle fiber size, despite no marked differences in body weight. Fecal phenol and circulating PhS levels were markedly elevated in

the Tyr group, whereas these changes were largely suppressed by administration of the TPL inhibitor 35DHBA. These findings suggest that enhanced microbial phenol production is associated with alterations in energy metabolism and body composition. In the current experimental conditions, elevated PhS levels were detected without apparent renal dysfunction, indicating that phenol-related metabolites may affect systemic metabolism before severe kidney dysfunction becomes detectable.

The marked suppression of phenol production by 35DHBA is likely attributable mainly to inhibition of bacterial TPL activity rather than major alterations in microbiota composition. Although high-tyrosine feeding induced significant changes in microbial diversity (Fig. 3), the overall compositional shift was relatively modest. In addition, fecal L-tyrosine levels were comparable between the Tyr and Tyr + 35DHBA groups (Fig. 2), indicating that substrate availability itself was not markedly different between the groups. Other aromatic amino acid-derived metabolites, including pCS and IndS, were also not reduced by 35DHBA treatment (Fig. 2). These findings support the idea that 35DHBA selectively affected the TPL-mediated phenol production pathway. Microbiota analysis nevertheless provided some additional information regarding bacterial populations associated with phenol production. In the Tyr group, several bacterial taxa including members of the genus *Petralousia* were increased (Supplemental Fig. 5). Although the metabolic characteristics of *Petralousia* remain unclear, members of the family Lachnospiraceae have been associated with aromatic amino acid metabolism and microbial fermentation in the intestinal tract [30]. In contrast, several bacterial species including *Phocaeicola sartorii* were enriched in the Tyr + 35DHBA group (Supplemental Fig. 5). These bacterial changes may partly contribute to the individual variability of phenol production observed in the current model. However, the marked reduction of fecal phenol by 35DHBA is more consistent with direct inhibition of TPL activity. Further analyses using gnotobiotic animals or defined microbial systems will be necessary to clarify the contribution of individual bacterial species to phenol production.

Another important point in the current study is the contribution of other aromatic amino acid-derived metabolites to the observed metabolic alterations. In addition to PhS, plasma pCS levels were modestly increased in the Tyr group; however, these levels were not reduced by 35DHBA treatment (Fig. 2). In contrast, adiposity and skeletal muscle alterations were largely improved by suppression of phenol production with 35DHBA (Figs. 6 and 7). These findings suggest that pCS is less likely to be the major metabolite responsible for the observed phenotypes under the present experimental conditions. In addition, circulating PhS levels were substantially higher than those of pCS in this model (Fig. 2). Previous studies reported that p-cresol and its conjugated metabolites exhibit pro-inflammatory and cytotoxic activities [31][32][33]. Therefore, a possible contribution of pCS cannot be completely excluded. However, the current results suggest that PhS is more closely associated with the metabolic alterations observed in the Tyr group. Further studies will be necessary to determine the relative contribution of each aromatic metabolite to systemic metabolic dysfunction.

The metabolic alterations observed in the current study suggest a possible association between gut microbial phenol production and systemic energy metabolism. The high-tyrosine diet reduced energy expenditure and carbohydrate oxidation, especially during the dark phase, whereas lipid oxidation was

not markedly altered (Fig. 5). Since locomotor activity was comparable among the groups, these changes were unlikely to be caused simply by reduced physical activity. In addition, glucose tolerance was not significantly altered in oral glucose tolerance testing (Supplemental Fig. 3). Therefore, the alterations in energy metabolism observed in the Tyr group may precede overt glucose metabolic dysfunction [34][35][36]. In parallel with these metabolic changes, visceral and subcutaneous fat accumulation was increased in the Tyr group (Fig. 6). Reduced skeletal muscle fiber size was also observed (Fig. 7), although overall body weight was not significantly changed (Fig. 1). These features resemble metabolic characteristics associated with sarcopenic obesity-like conditions [37][38][39]. Since these phenotypes were largely improved by suppression of phenol production with 35DHBA, enhanced microbial phenol production may contribute to disruption of systemic energy balance. Inflammatory responses may also be involved in these metabolic alterations. Expression of inflammatory cytokines including TNF- α and IL-6 was significantly elevated in adipose tissue and liver of the Tyr group (Figs. 6 and 8). In addition, PhS increased expression of TNF- α and IL-6 in precision-cut liver slices (PCLSs) (Fig. 8), suggesting that circulating phenol metabolites directly affect hepatic inflammatory responses. Chronic low-grade inflammation is strongly associated with obesity and metabolic disorders [40][41]. Therefore, inflammatory responses induced by phenol-related metabolites may partly contribute to the altered energy metabolism observed in the current study.

Another characteristic finding in the current study was the discrepancy between morphological changes and transcriptional responses in skeletal muscle. Although muscle fiber size was reduced in the Tyr group, expression levels of inflammatory cytokines and classical atrophy-related genes were not significantly altered (Fig. 7). Similarly, PhS treatment did not induce atrophy-related gene expression in differentiated C2C12 myotubes (Supplemental Fig. 4). These findings suggest that the skeletal muscle alterations observed in the current model may not be mediated through canonical muscle atrophy pathways [42][43]. Phenol and PhS circulate as protein-bound metabolites [44][45][46], and therefore may affect multiple peripheral tissues including liver, adipose tissue, and skeletal muscle. Previous studies reported that protein-bound uremic toxins impair mitochondrial function and cellular metabolism [47][48]. Although the precise molecular targets of phenol metabolites remain unclear, impaired mitochondrial function and altered energy metabolism may partly contribute to the reduction in muscle fiber size observed in the Tyr group [49][42][50]. Further studies will be required to clarify how phenol-related metabolites influence skeletal muscle metabolism and tissue remodeling.

Consistent with our previous studies [6][7], considerable individual variability in fecal phenol production and circulating PhS levels was observed in the high-tyrosine diet model (Fig. 2). This variability may reflect differences in intestinal microbiota composition, metabolic activity, or absorption and excretion of phenol-related metabolites among animals. Nevertheless, mice with higher phenol production generally exhibited stronger metabolic alterations, suggesting that systemic exposure to phenol metabolites is associated with the observed phenotypes. An important feature of the current model is that elevated PhS levels and metabolic alterations were observed without apparent renal dysfunction. Urinary cystatin C levels and renal histology did not show obvious abnormalities under the present experimental conditions. These findings suggest that enhanced microbial phenol production may affect systemic

metabolism before severe kidney dysfunction develops. In the Tyr group, inflammatory responses, reduced energy expenditure, adiposity, and skeletal muscle alterations were observed together with elevated phenol production. Therefore, sustained exposure to phenol-related metabolites may contribute to early metabolic abnormalities associated with chronic low-grade inflammation. Further studies are necessary to clarify how long-term phenol exposure influences disease progression under pathological conditions.

Several limitations should be considered in the current study. Although suppression of phenol production by 35DHBA improved multiple metabolic phenotypes, additional studies using gnotobiotic animals or TPL-deficient bacterial strains will be necessary to establish the causal role of microbial phenol production more directly. The bacterial species mainly responsible for phenol production also remain unclear. In addition, relatively large individual variability was observed in phenol production and circulating PhS levels in this model. This variability may partly reflect differences in intestinal microbiota composition and metabolic activity among animals. Another limitation is that oral administration of PhS alone did not sufficiently elevate circulating PhS levels in our preliminary experiments (unpublished observations). Therefore, the direct physiological effects of circulating PhS could not be fully evaluated *in vivo* under the current experimental conditions. In addition, longer experimental periods may be necessary to determine whether sustained exposure to phenol-related metabolites contributes to progression of kidney dysfunction or other chronic pathological conditions. Despite these limitations, suppression of phenol production by inhibition of TPL activity consistently improved the metabolic alterations observed in the Tyr group. In summary, the current study demonstrated that enhanced gut microbial phenol production was associated with altered energy metabolism, adiposity, inflammatory responses, and skeletal muscle remodeling in mice. These changes occurred even in the absence of apparent renal dysfunction. Our findings suggest that microbial phenol production may contribute to metabolic abnormalities associated with excess dietary tyrosine. Further studies using controlled microbial systems and long-term experimental models will be necessary to clarify the underlying mechanisms and physiological significance of phenol-related metabolites.

Abbreviations

TPL

tyrosine phenol-lyase

PhS

phenyl sulfate

PBUT

protein-bound uremic toxin

PCLSs, precision-cut liver slices

APDS

3-aminopyridyl-*N*-hydroxysuccinimidyl carbamate

PhG

phenyl glucuronide

pCS
p-cresyl sulfate
IndS
indoxyl sulfate
Cre
creatinine.

Declarations

Ethics declaration

The authors declare no conflict of interest. All animal experiment methods were performed in accordance with the relevant guidelines and regulations. Authors complied with all the relevant guidelines and regulations and complied with ARRIVE guidelines.

Funding

This study was supported by Grant-in-Aid for Scientific Research (KAKENHI, number 26KJ1898 to SO, 25KJ0262 to TK and 26K01700 to NM) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology (MEXT, Tokyo).

Author Contribution

S. O., K. I., M. M., S. Y., T. S., M. M., T., Y., T. F., H. H., T. K. Validation, Formal analysis, Investigation; S. O., and N. M. Writing original draft; T. T., S. M., and N. M. Writing - Review & Editing; S. O. Visualization; N. M. Conceptualization, Supervision, Project administration; S. O., T. K., and N. M. Funding acquisition.

Acknowledgement

The authors are grateful to the members of the Laboratory of Biochemistry for their continuous support.

Data Availability

All raw sequences have been deposited in the NCBI BioProject database under accession number PRJNA1463006.

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Figures

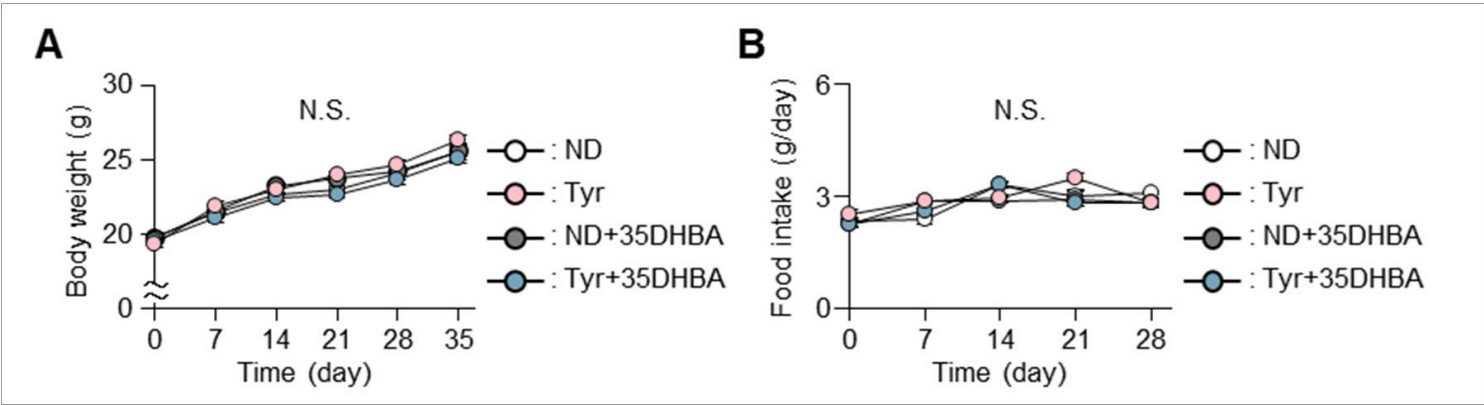


Figure 1

Body weight and food intake.

(A) Changes in body weight over 35 days. (B) Food intake over 28 days. Data are presented as means $\pm$ SE (n = 5–17). No significant differences were observed among groups (one-way ANOVA followed by Tukey’s post hoc test).

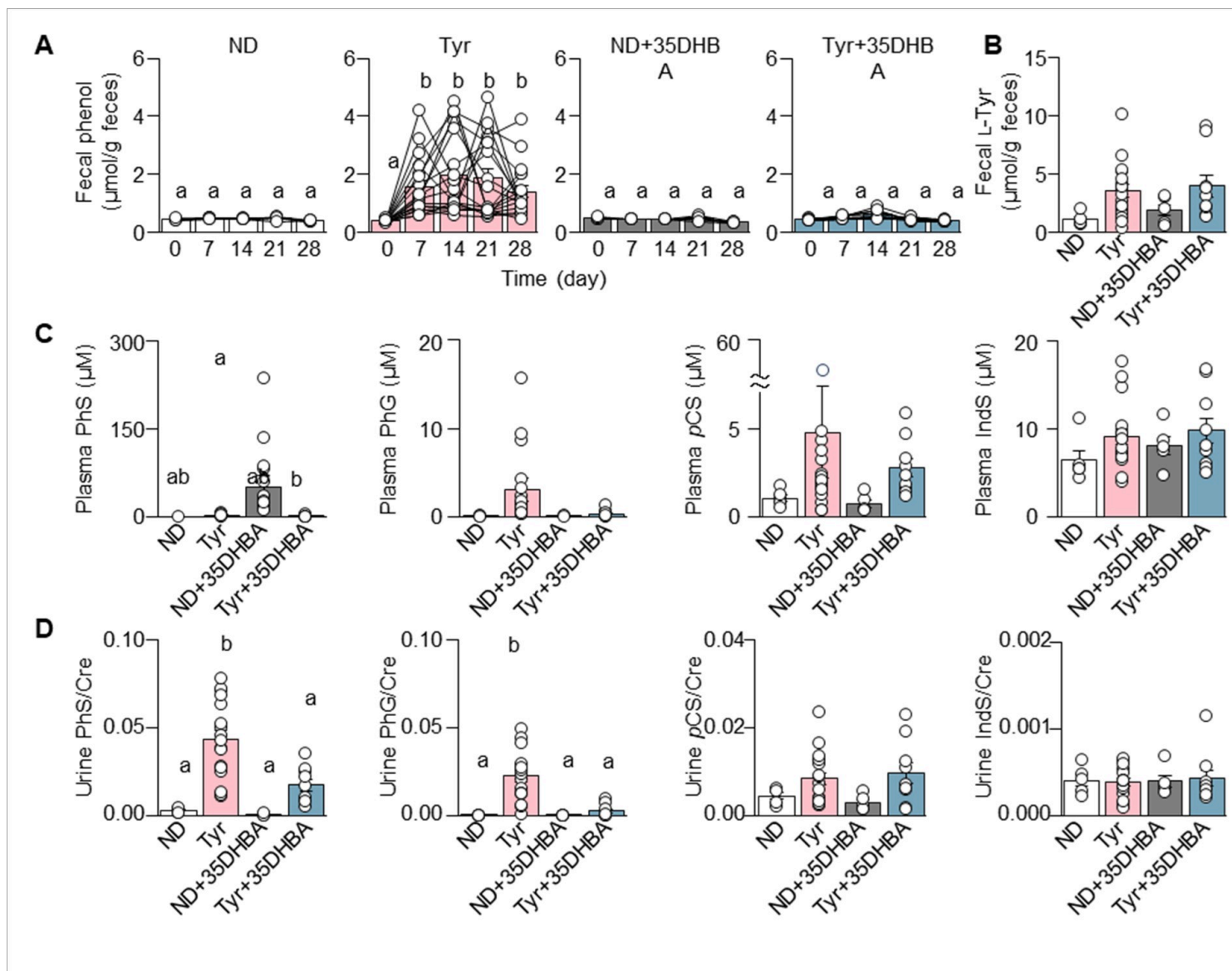


Figure 2

Quantitative analysis of uremic toxins and their precursors.

(A) Fecal phenol levels are shown for individual mice as a line graph, with mean values summarized as bar graphs. (B) Fecal L-Tyr levels at Day 35. (C) Plasma uremic toxin levels (PhS, PhG, pCS, and IndS) at Day 35. (D) Urinary uremic toxin levels (PhS, PhG, pCS, and IndS) at Day 28. Individual values are shown for each mouse, with mean values presented as bar graphs. Data are expressed as means $\pm$ SE ($n = 5-17$). Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test; groups not sharing a common letter are significantly different.

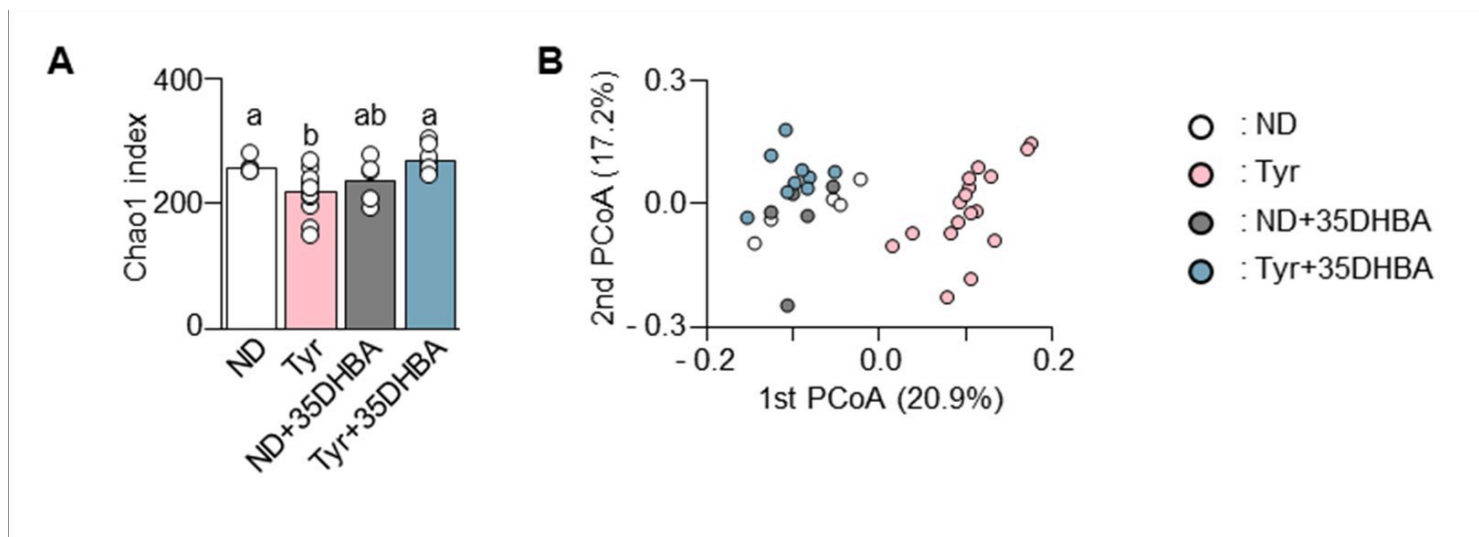


Figure 3

Gut microbiota diversity analysis based on 16S rRNA sequencing.

(A) Alpha diversity assessed by the Chao1 index. (B) Principal coordinate analysis (PCoA) of microbial community structure based on β -diversity. The percentage of variance explained by PC1 and PC2 is indicated. Groups: ND (n = 5), Tyr (n = 17), ND + 35DHBA (n = 5), and Tyr + 35DHBA (n = 9). Data are expressed as means $\pm$ SE. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test; groups not sharing a common letter are significantly different.

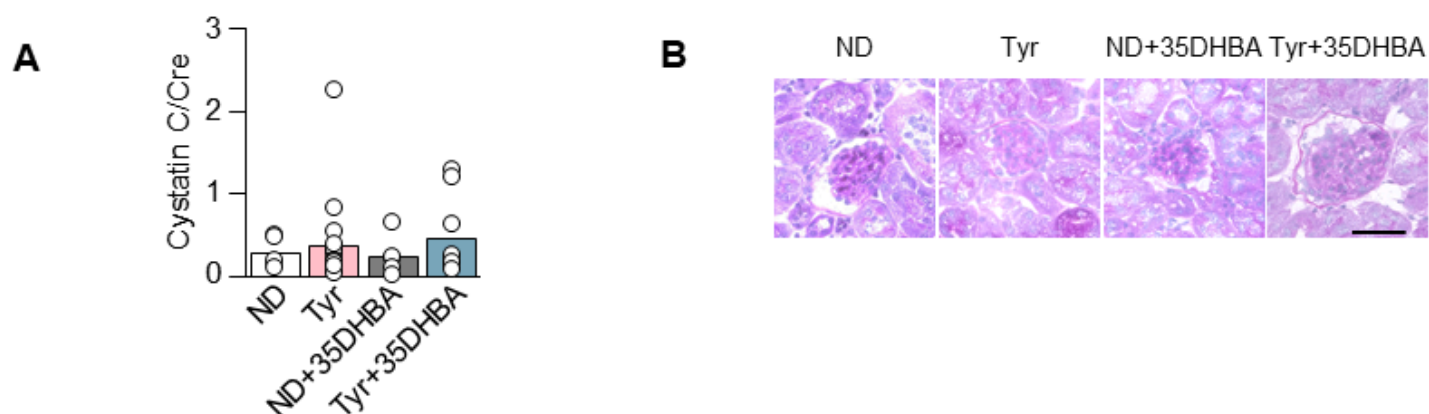


Figure 4

Assessment of renal function and kidney histology.

(A) Urinary cystatin C levels as a marker of renal function. Individual values are shown as plots, with mean values presented as bar graphs (ND, n = 5; Tyr, n = 17; ND + 35DHBA, n = 5; Tyr + 35DHBA, n = 9). Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test; no

significant differences were observed. (B) Representative periodic acid–Schiff (PAS)-stained kidney sections from each group (ND, Tyr, ND + 35DHBA, and Tyr + 35DHBA).

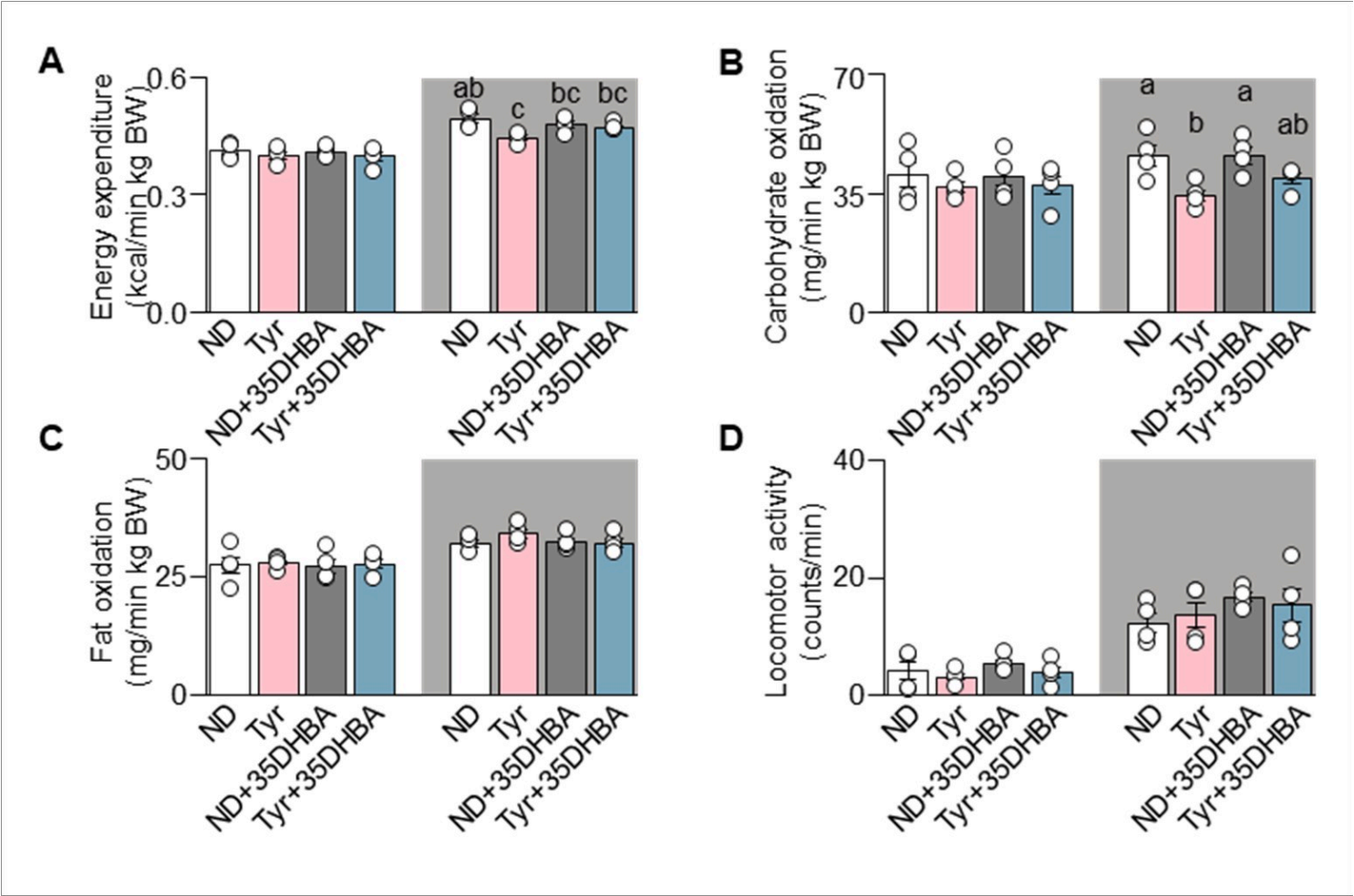


Figure 5

Respiratory gas analysis and locomotor activity.

(A) Energy expenditure (kcal/min/kg body weight), (B) carbohydrate oxidation (mg/min/kg body weight), (C) fat oxidation (mg/min/kg body weight), and (D) locomotor activity (counts/min) measured during the respiratory gas analysis period (Days 28–30). Individual values are shown as plots, with mean values presented as bar graphs. Groups: ND, Tyr, ND + 35DHBA, and Tyr + 35DHBA (n = 4 per group). Data are expressed as means $\pm$ SE. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test; groups not sharing a common letter are significantly different.

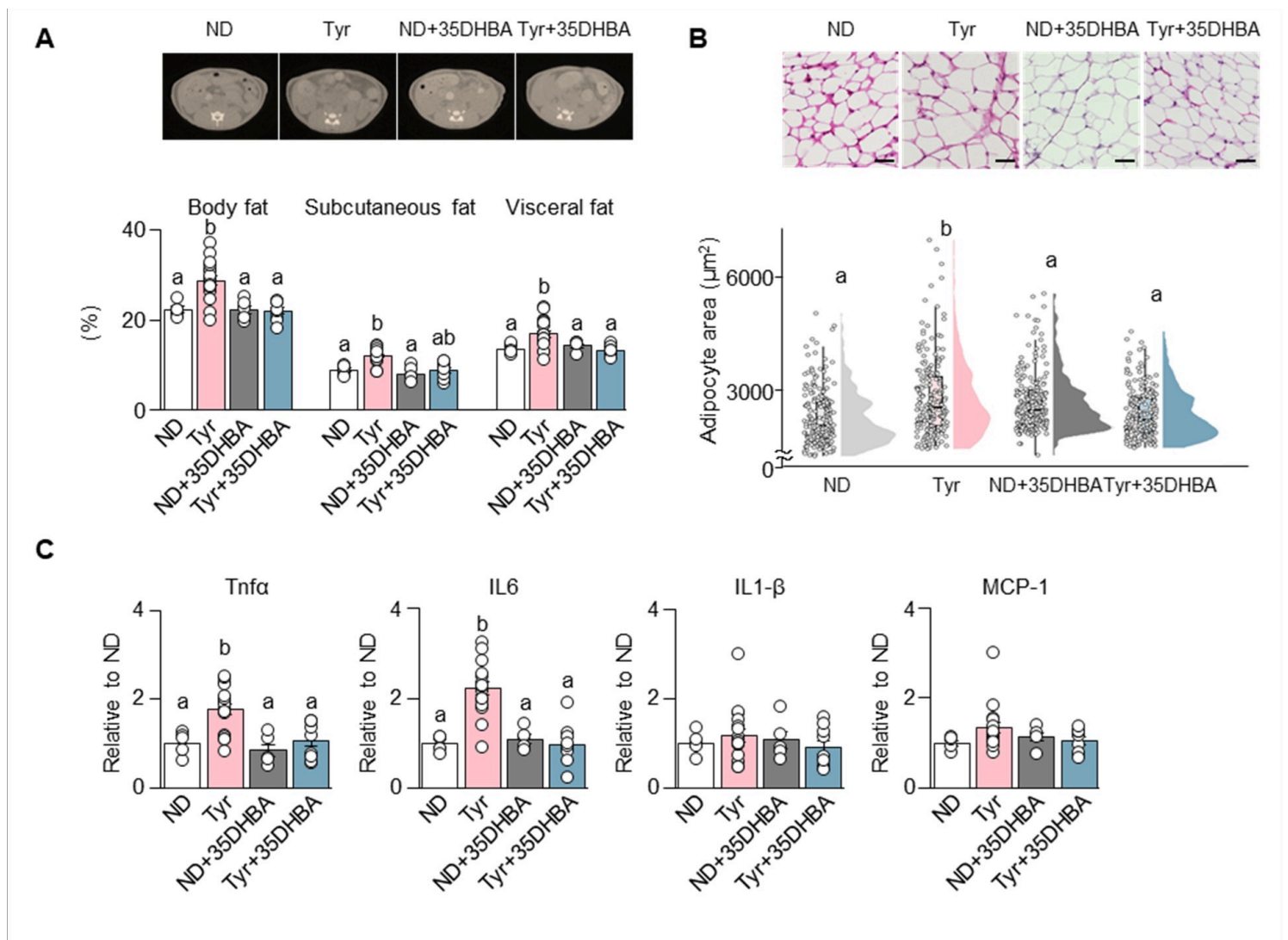


Figure 6

Effects of a high-tyrosine diet on adiposity and inflammation in adipose tissue.

(A) Representative CT images at the level of the fifth lumbar vertebra from each group (ND, Tyr, ND + 35DHBA, and Tyr + 35DHBA), with quantitative analysis of body fat (%), subcutaneous fat (%), and visceral fat (%) shown below. Fat volumes were calculated from the first to fifth lumbar vertebrae. Individual values are shown as plots, with mean values presented as bar graphs (ND, $n = 5$; Tyr, $n = 17$; ND + 35DHBA, $n = 5$; Tyr + 35DHBA, $n = 9$). (B) Representative hematoxylin and eosin (H&E)-stained images of epididymal adipose tissue from each group. Adipocyte area (μm^2) is shown as individual measurements with box-and-whisker and violin plots. 200 adipocytes per mice were quantified. Scale bar = 50 μm . (C) Gene expression analysis of inflammatory cytokines (TNF- α , IL-6, IL-1 β , and MCP-1) in adipose tissue assessed by RT-qPCR. Data are expressed as means $\pm$ SE. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test; groups not sharing a common letter are significantly different.

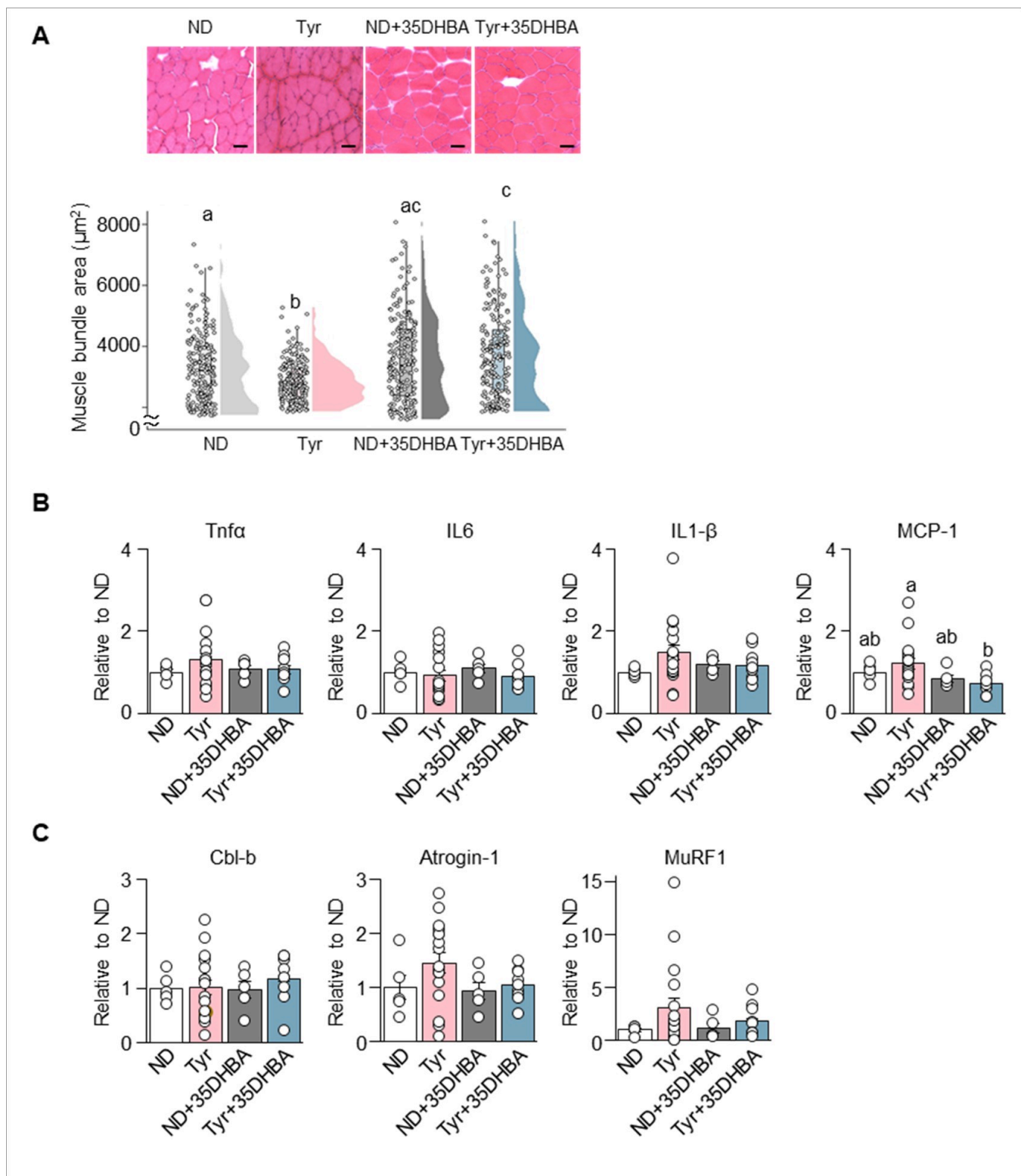


Figure 7

Effects of a high-tyrosine diet on skeletal muscle morphology and gene expression.

(A) Representative hematoxylin and eosin (H&E)-stained cross-sections of the tibialis anterior muscle from each group (ND, Tyr, ND + 35DHBA, and Tyr + 35DHBA), with quantitative analysis of muscle bundle area (μm^2) shown below. Individual values are presented with box-and-whisker and violin plots. 200

muscle fibers per mice were quantified. Scale bar = 50 μ m. (B) Gene expression analysis of inflammatory cytokines (TNF- α , IL-6, IL-1 β , and MCP-1) in the tibialis anterior muscle assessed by RT-qPCR. (C) Gene expression analysis of muscle atrophy-related genes (Cbl-b, Atrogin-1, and MuRF-1) in the tibialis anterior muscle assessed by RT-qPCR. Data are expressed as means $\pm$ SE (n = 5–17). Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test; groups not sharing a common letter are significantly different.

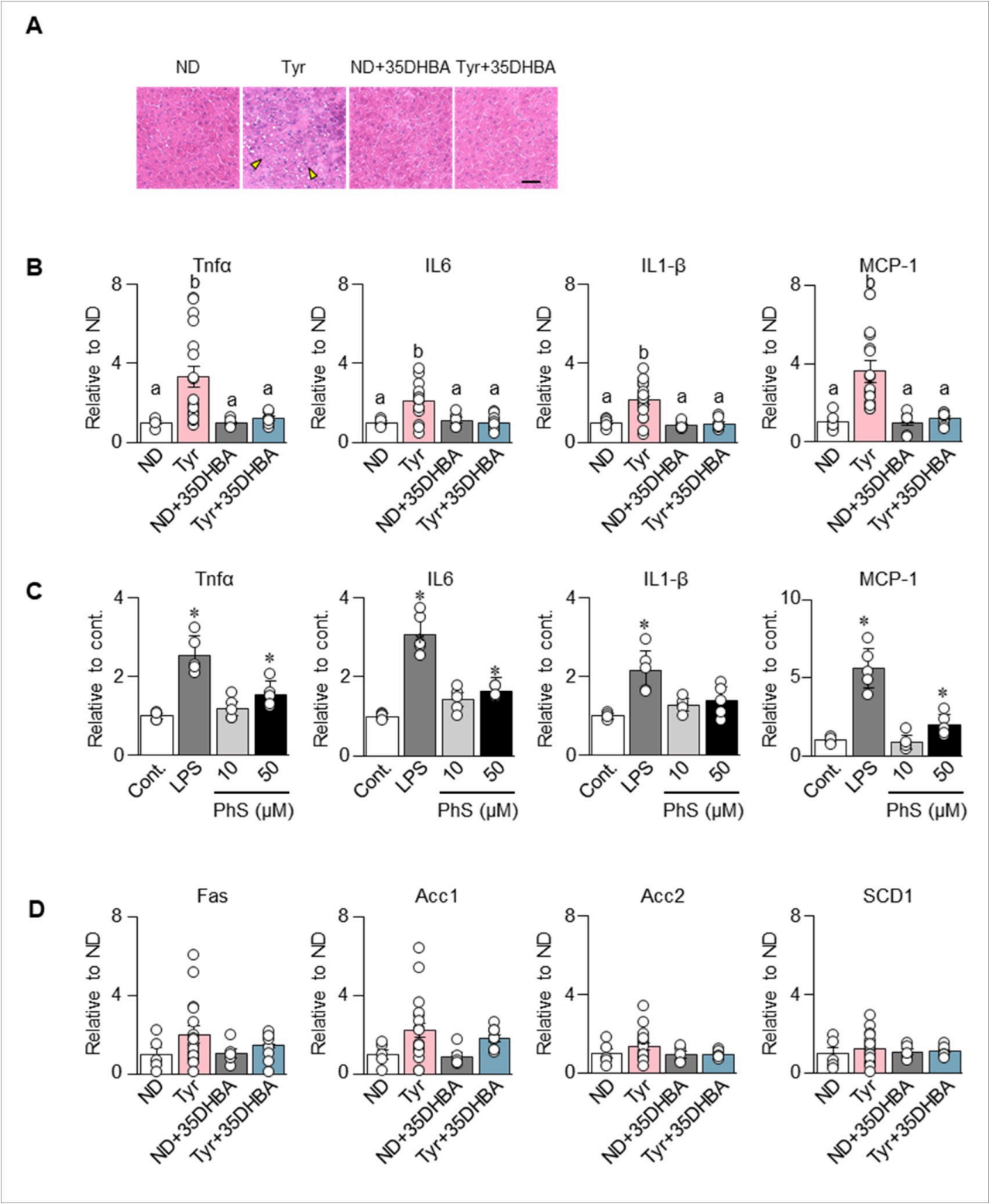


Figure 8

Effects of a high-tyrosine diet and phenyl sulfate on liver histology and gene expression.

(A) Representative hematoxylin and eosin (H&E)-stained liver sections from each group (ND, Tyr, ND + 35DHBA, and Tyr + 35DHBA). Arrowheads indicate macrophage infiltration and lipid droplet formation. Scale bar = 50 μ m. (B) Gene expression analysis of inflammatory cytokines (TNF- α , IL-6, IL-1 β , and MCP-1) in the liver assessed by RT-qPCR. (C) Effects of phenyl sulfate on inflammatory gene expression in precision-cut liver slices (PCLSs). PCLSs were prepared from mouse liver using a vibrating blade microtome. PCLSs were incubated with phenyl sulfate (10 or 50 μ M) or lipopolysaccharide (100 ng/mL; positive control) for 6 h. Total RNA was then extracted, and the expression levels of inflammatory cytokines were analyzed by RT-qPCR. Data are expressed as means $\pm$ SE (n = 5). (D) Gene expression analysis of lipogenic genes (Fas, Acc1, Acc2, and SCD1) in the liver assessed by RT-qPCR. Data are expressed as means $\pm$ SE (n = 5–17). Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test; groups not sharing a common letter are significantly different.

Supplementary Files

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