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Effects of brown rice on cellular growth and metabolic changes in mice

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ABSTRACT

The consumption of rice, especially brown rice, is known to promote cellular growth but the underlying mechanisms and key metabolites of this process are unclear. This study investigated the effects of brown rice on cellular growth and the underlying mechanisms using a metabolomics approach *in vivo*. C57BL/6J male mice were fed an isocaloric diet composed of control chow, Ilpum rice (IP; *Oryza sativa* L. cv. Ilpum), or Ilpum brown rice (IB) for 4 weeks, and then the metabolites of the liver were analyzed. The rice consumption reduced the liver enzyme levels in plasma and did not result in obesity. The plasma total-cholesterol levels were significantly reduced in the IB group compared with the IP group. The plasma concentrations of growth hormone and insulin growth factor-1 were significantly increased, along with increased thigh bone width in the IB group compared with the IP group, suggesting that the intake of brown rice promoted animal growth. Gas chromatography coupled to time-of-flight mass spectrometry (GC-TOF-MS) was used for the metabolic profiling of liver samples obtained from IP- and IB-fed mice. The obtained data sets were subjected to multivariate statistical analysis to investigate which metabolites contributed to the differences between the IP and IB groups. GC-TOF-MS analysis revealed the presence of 31 metabolites consisting of 8 lipids, 4 sugars and sugar alcohols, 15 amino acids, and 4 organic acids. Partial least-squares regression analysis demonstrated significant correlations between the data collected from GC-TOF-MS and the levels of growth hormone. The levels of L-valine, L-glutamine, and D-mannose were significantly higher in the IB group than the control and IP groups.

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1. Introduction

Rice (*Oryza sativa* L.) is used as a staple food and energy source in many Asian countries, mainly due to its high carbohydrate content. Two types of rice are generally consumed: brown and white rice (Lin, Singh, Chang, & Chang, 2011). Only the outer layer is removed from whole rice grain to produce brown rice, keeping the bran intact, and this represents an abundant source of diverse minerals, vitamins, fiber, and phenolic compounds. In contrast, white rice is produced after milling to remove the husk bran and most of the germ, which markedly reduces the content of fiber, cellulose, minerals, and vitamins. The various nutrients and phytochemical compounds present in brown rice produce a wide variety of biological effects, such as lowering plasma cholesterol concentrations and decreasing the risk for type 2 diabetes and cardiovascular disease (Panlasigui & Thompson, 2006). Brown rice also has a lower glycemic index (GI) than white rice, which contributes to its hypoglycemic and anti-diabetic effects (Miller, Pang, & Bramall, 1992).

Bone is a dynamic organ that serves as a reservoir for essential minerals and is critical for structural mobility, support, and protection in animals (HHS, 2004). Bone growth is regulated *via* the complex interaction of hormones and growth factors in children and adolescents. Growth hormone (GH) is considered to be the key hormone regulating bone linear growth in childhood (Juul, 2001). GH activates cellular and organismal growth largely by stimulating the hepatic secretion of insulin growth factor (IGF)-1, which plays a vital role in the development of the body composition and bone mineral acquisition by promoting the differentiation of bone cells, thus affecting skeletal growth (Venken et al., 2005). This means that GH deficiency can result in severe postnatal growth retardation as well as dwarfism in both humans and mice (Donahue & Beamer, 1993; Zhou et al., 1997) and can cause bone loss, decreased muscle mass, increased body fat, and erratic sleep patterns. On the other hand, GH administration has been reported to be an effective therapy for promoting bone-mass growth in young adults with a GH deficiency. GH treatment also reportedly increases cortical bone thickness, bone formation, and remodeling activity (Johannsson, Rosen, Bosaeus, Sjostrom, & Bengtsson, 1996).

IGF-1 is the main mediator of GH functions and also exerts important effects on bone growth (Thorsen, Nordström, Lorentzon, & Dahlén, 1999), with an increased plasma concentration of IGF-1 stimulating the proliferation of chondrocytes, which then results in bone growth.

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Guler, Zapf, Scheiwiller, and Froesch (1988) demonstrated that an injection of IGF-1 is controlled by a complex mechanism that is associated with the regulation of circulating IGF-binding proteins (IGFBPs) (Guler et al., 1988). IGFBP-1, which is mainly produced by hepatocytes, is the most important IGFBP for the dynamic regulation of cellular growth. Lee, Giudice, Conover, and Powell (1997) showed that the induction of IGFBP-1 can inhibit GH/IGF-1-dependent cellular growth and differentiation *in vitro* (Lee et al., 1997). Therefore, the overexpression of IGFBP-1 in mice could result in growth retardation (Schneider, Lahm, Wu, Hoeflich, & Wolf, 2000). Collectively, these findings suggest that the activation of the GH/IGF-1 axis can promote cellular growth, while the induction of IGFBP-1 can cancel these effects; it is therefore possible that brown rice exerts growth-promoting effects by regulating the GH/IGF-1 system and/or suppressing IGFBP-1.

Diverse analytical methods have been developed for performing metabolite profiling in biological fluids and tissues, such as with the combination of mass spectrometry (MS) and gas chromatography (GC) and liquid chromatography (LC) (Gowda et al., 2008). In particular, GC coupled to time-of-flight MS (GC-TOF-MS) has a high sensitivity and resolution and also can be used for deconvolution analysis due to its fast scan rate (Ma et al., 2011). Previous studies have investigated rice using metabolomics for rice quality control to evaluate genetically modified rice (Matsuda et al., 2010) and investigate the hypolipidemic effects of Goami-3 rice (Lee et al., 2013). Lee et al. (2013) demonstrated that consuming Goami-3 rice significantly reduced obesity by lowering plasma glucose, mannose, and cholesterol levels. In particular, the plasma concentrations of L-valine were inversely correlated with the high-density lipoprotein (HDL)-/non-HDL-cholesterol and HDL-/total cholesterol ratios in partial least-squares regression (PLSR) analysis.

The aim of the current study was to determine the potential of a new rice variety [*O. sativa* L. cv. Ilpum] for promoting growth in childhood and/or preventing idiopathic short stature. The efficacy of IB for regulating the GH/IGF1 system was compared with the control using *in vitro* (HepG2 cell lines) and *in vivo* (C57BL/6 J mice) models. Metabolic profiling of liver samples from Ilpum rice (IP)- and Ilpum brown rice (IB)-fed mice was performed using GC-TOF-MS, and the resultant data sets were subjected to multivariate statistical analysis to determine the metabolites that contributed to the ability to discriminate between the IP and IB groups. This study also investigated the correlation between the levels of liver metabolites, GH, and IGF-1. To our knowledge, this is the first detailed study of the potential of rice to regulate growth.

2. Methods and materials

2.1. Rice

Ilpum rice (IP) and Ilpum brown rice (IB) were newly bred rice species by the National Institute of Crop Science of the Rural Development Administration in Korea (Suwon, Korea). Rice extracts (80%) were used for *in vitro* cell culture studies. IP and IB rice was ground using mills and the fine powder was stirred in an 80% ethanol solution overnight. The extracts were prepared in dimethyl sulfoxide (DMSO) for cell experiments. For animal feeding, an isocaloric diet was formulated with 20% (*v*/*w*) rice powder based on an AIN-93G standard chow diet. The composition of the diet is shown in Supplemental Table 1.

2.2. Cell culture and treatment

GH3 and HepG2 cells were grown in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37 °C in 5% CO_2. At confluency, cells were seeded in 6-well plates at a density of 5×10^5 cells/well and incubated for 24 h in serum-free medium containing IP, IB (10 μg/mL) or 1% DMSO as vehicle control.

2.3. Animals and diets

C57BL/6J male mice (8 weeks old) were purchased from Samtako (Seoul, Korea) and maintained in a temperature-controlled (25 °C) specific-pathogen-free facility on a 12 h light/dark cycle (Central Lab. Animal Inc., Seoul, Korea). The mice were divided into three groups (N = 7 per each group) and fed with either control diet, IP-isocaloric diet or IB-isocaloric diet. The diet composition of each group is shown in Supplemental Table 2. The body weight and food intake was measured twice per week. After 4 weeks of feeding, the mice were sacrificed according to a protocol approved by the Animal Experiment Committee of Korea University (Protocol No. KUIACUC-20,090,420-4). The blood was collected by cardiac puncture and tissue samples including liver, abdominal, intestinal and perirenal fat were collected. The organ weights were determined immediately prior to snap freezing and storage at −80 °C. The length and width (upper, middle and bottom region) of the thigh bones were measured using a digital caliper.

2.4. Plasma analysis

Plasma lipid analyses were performed after 4 weeks of feeding. Plasma total protein, C-reactive protein (CRP), triglycerides (TG), total cholesterol, high-density lipoprotein (HDL)-cholesterol and glucose concentrations as well as alanine aminotransferase (ALT) activities and aspartate aminotransferase (AST) activities were determined using Cobas111 (Roche, Basel, Switzerland). Plasma GH (Invitrogen, Carlsbad, CA, USA) and IGF-1 (Abcam, Cambridge, UK) were measured using an ELISA kit according to the manufacturer's instructions.

2.5. RNA preparation and quantitative PCR (qPCR)

Total RNA was isolated from the mouse liver and HepG2 cells using RNAiso Plus reagent (Takara Bio Inc., Otsu, Japan), and 2 μg of RNA from each sample was used to generate cDNA. Real-time qPCR was performed with the iQ™ SYBR® Green Supermix reagent and iQ5 Cycler System (Bio-Rad, Hercules, CA, USA). under the following conditions: initial denaturation at 95 °C for 3 min, followed by 35 cycles of amplification with denaturation at 95 °C for 20 s and annealing and extension at 60 °C for 20 s and 72 °C for 1 min. The data were calculated using IQ5 Optical System Software version 2 (Bio-Rad, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as a control. The primer sequences are shown in Supplemental Table 3.

2.6. Liver sample extraction

Approximately 100 mg of the liver sample was initially homogenized in 1 mL of 1 M NaCl using an IKA homogenizer (Wilmington, NC). 200 mL of the liver samples were mixed with methanol (0.5 mL) after being spiked with 0.03 mL of the each internal standard compounds (Sigma-Aldrich, St. Louis, MO, USA) *i.e.*, 2-deoxy-D-ribose [400 ppm (*w*/*v*) in water] for carbohydrates, heptadecanoic acid [200 ppm (*w*/*v*) in hexane] for lipids, tropic acid [100 ppm (*w*/*v*) in acetone] for organic acids and norleucine [200 ppm (*w*/*v*) in water] for amino acids. The sample was homogenized with a shaker vortex (IKA Genius 3, Germany) and incubated on ice for 1 min for protein removal and then centrifuged (Hanil Union 32R plus, Inchon, Korea) at 3857.1 ×*g* for 15 min at 4 °C.

2.7. Liver metabolite analysis using GC-TOF-MS

400 mL of the extract supernatant was dried using a Centri-Vap (Labconco Corp., Kansas City, MO, USA) for 10 h (Kim et al., 2013;

Ku et al., 2009). The dried sample was derivatized using 0.05 mL of *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA + 1% TMCS, Supelco, Bellefonte, PA, USA) and 0.05 mL of acetonitrile. The samples were then reacted at 70 °C for 1 h and cooled for 10 min before GC-TOF-MS analysis. Subsequently, GC-TOF-MS analysis was performed on a 6890 N GC (Agilent Technologies, Palo Alto, CA, USA) connected to a Pegasus III TOF mass spectrometer (Leco, St. Joseph, MI, USA) using electron ionization (EI) at 70 eV. The GC was equipped with a DB-5MS column (30 m length × 0.25 mm i.d. × 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA). The flow rate of helium, a carrier gas, was 1.0 mL/min. GC syringe was cleaned three times, respectively, before and after injecting the derivatised samples into GC system, using dichloromethane. The derivatized sample (0.002 mL) was injected in splitless mode. The oven temperature program was as follows: initial temperature of 85 °C for 5 min raised to 205 °C at 8 °C/min, which was held for 5 min, elevated to 300 °C at 8 °C/min and then maintained for 5 min. The data acquisition rate was 20 scans/s in the mass range of 35 to 550 *m/z*. The injector and detector transfer line temperatures were 230 °C and 250 °C, respectively. Metabolites were positively identified by comparing their retention times and mass spectra with those of authentic reference compounds. The following pure standard chemicals were obtained commercially: Linoleic acid (CIL, Tewksbury, MA, USA), arachidonic acid, D-ribose, D-mannose, galactose, L-alanine, glycine, L-serine, L-aspartic acid, L-glutamine, GABA, acetic acid, oxalic acid, succinic acid, malic acid (Sigma Aldrich St. Louis, MO, USA), cholesterol, L-methionine, L-tryptophan (Yakuri, Kyoto, Japan), L-valine (BDH, Radnor, PA, USA), L-leucine (Kanto, Tokyo, Japan), L-proline (Samchun, Pyeongtaek-si, Republic of Korea), L-threonine, L-tyrosine (Junsei, Tokyo, Japan), and phenylalanine (Showa, Okayama, Japan).

When the authentic standard compounds were not available, metabolites were putatively annotated based on the Wiley 7n and 8n mass spectral database (Hewlett-Packard, Palo Alto, CA, USA; 1995), NIST05 MS Library and MS search Program V.2.0d (NIST, 2005). The relative levels of metabolites in the samples (N = 7 per each group) were determined by comparing their peak areas to those of the internal standard compounds.

2.8. Statistical analysis

In vitro and *in vivo* data were shown as the means ± standard error of the mean (SEM). Analysis of variance was performed to determine the statistical significance levels among groups ($p < 0.05$). Duncan's multirange test was employed when the samples exhibited significantly different peak areas for the metabolites at the significance level ($p < 0.05$). Multivariate statistical analyses, such as principal components analysis (PCA), partial least-square-discriminant analysis (PLS-DA) and partial least-squares regression (PLSR), were performed using SIMCA-P + 11.0 (Umetrics, Umeå, Sweden).

3. Results

3.1. IB consumption does not result in obesity in mice

Rice is a good source of dietary calories, but a high consumption of rice could cause obesity due to its high GI and nutrition value (Izadi & Azadbakht, 2015). To investigate the metabolic effects of new rice varieties in mice, the body weight and tissue weights, including the liver and adipose tissues (abdominal, intestinal, and perirenal fat), were assessed. The results of our study revealed that the body weights were unaltered in mice fed IP or IB compared with the control group (Fig. 1A), even though the masses of liver, intestinal, and perirenal fat were increased in IB-fed mice compared with the control-diet-fed mice The abdominal fat levels decreased significantly in IB-fed mice ($p < 0.05$), while their liver tissue weight increased ($p < 0.05$). These results demonstrate that consuming IB exerts a beneficial metabolic effect on weight management (Fig. 1B and C). These results demonstrate that the intake of IB does not result in obesity.

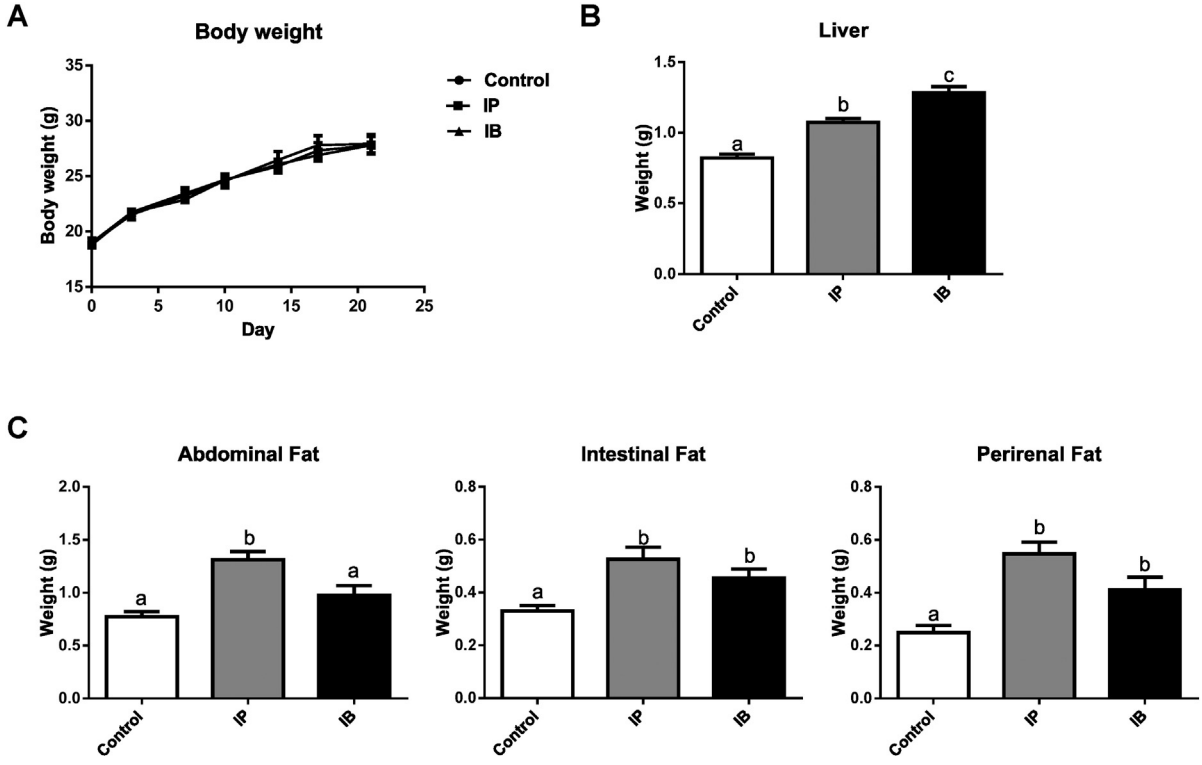

Fig. 1. Ilpum brown rice (IB) consumption did not result in obesity. (A) Body weights of mice fed a control diet, Ilpum rice (IP), and IB. (B) Quantification of the liver mass. (C) Quantification of the masses of abdominal, intestinal, and perirenal fat. The data are the mean and SEM values. Group comparisons (N = 9 or 10) were performed by one-way ANOVA, followed by Tukey's test ($p < 0.05$). Different letters indicate significant differences among the groups.

3.2. IB does not cause toxicity or inflammation

The plasma protein levels and lipid profiles were also analyzed. After 4 weeks of feeding, the levels of total protein and glucose were unaltered in IP- and IB-fed mice compared with the control-diet-fed mice (Fig. 2A). The plasma cholesterol level was significantly lower in IB-fed mice compared with IP-fed mice ($p < 0.05$), while the HDL-cholesterol concentration was significantly higher in IP- and IB-fed mice ($p < 0.05$). Triglyceride concentrations were increased in rice-fed groups compared with controls (Fig. 2B).

The concentrations of ALT and AST, two common clinical markers for liver health, were tended to reduce in IB-fed mice, while the concentration of CRP was unaltered (Fig. 2C). These results demonstrate that consuming IB does not cause significant organ toxicity or inflammation.

3.3. IB induces cellular bone growth

Consumption of a nutrient-rich diet could induce cellular bone growth by modifying the bone mineral density and cortical thickness. IB contains large amounts of beneficial components, so we also investigated the effect of IB on bone growth by measuring the length and width of the thigh bones in mice in regions 1, 2, and 3, as shown in Supplemental Fig. 1. As shown in Fig. 3A, the bone width in region 2 was significantly increased in IB-fed mice compared with control-diet-fed mice ($p < 0.05$), which demonstrates that IB promotes cellular bone growth.

3.4. IB activates the GH/IGF-1 axis and inhibits IGFBP-1

Bone linear growth is tightly regulated by multiple systemic hormones, including GH, IGF-1, and IGFBP-1. GH and IGF-1 are two key hormones that stimulate cellular growth, while the IGFBP-1 level is inversely associated with IGF-1 activity. We investigated the effects of IB on the plasma concentrations of GH and IGF-1 using ELISA kits. The results showed that the plasma concentrations of GH and IGF-1 were dramatically increased in IB-fed mice (Fig. 3B, $p < 0.05$). Moreover, we assessed the expression of IGF-1 and IGFBP-1 genes in livers and HepG2 cells by qPCR analysis. The expression levels of IGF-1 genes in livers were significantly increased ($p < 0.05$) in IB group compared with controls, while IGFBP-1 gene expression was significantly reduced in IB-fed mouse livers (Fig. 3C, $p < 0.05$). HepG2 cells stimulated with IB showed similar effects of IGF-1 induction and IGFBP-1 suppression (Fig. 3D). The expression of IGF-1 was increased in IP thus the induction of IGF-1 may be a common effect of rice at least in cultured cells. In addition, the mRNA expression of GH were dramatically increased in GH3 cells (Fig. 3D). Together, these results suggest that IB promotes cellular bone growth and organismal growth, possibly by activating the GH/IGF-1 axis and inhibiting IGFBP-1.

3.5. Profiling of liver metabolites by GC-TOF-MS

GC-TOF-MS analysis revealed the presence of the following 31 metabolites in the livers of mice fed a control diet, IP, or IB: 8 lipids (propanoic acid, butanoic acid, hexadecanoic acid, linoleic acid,

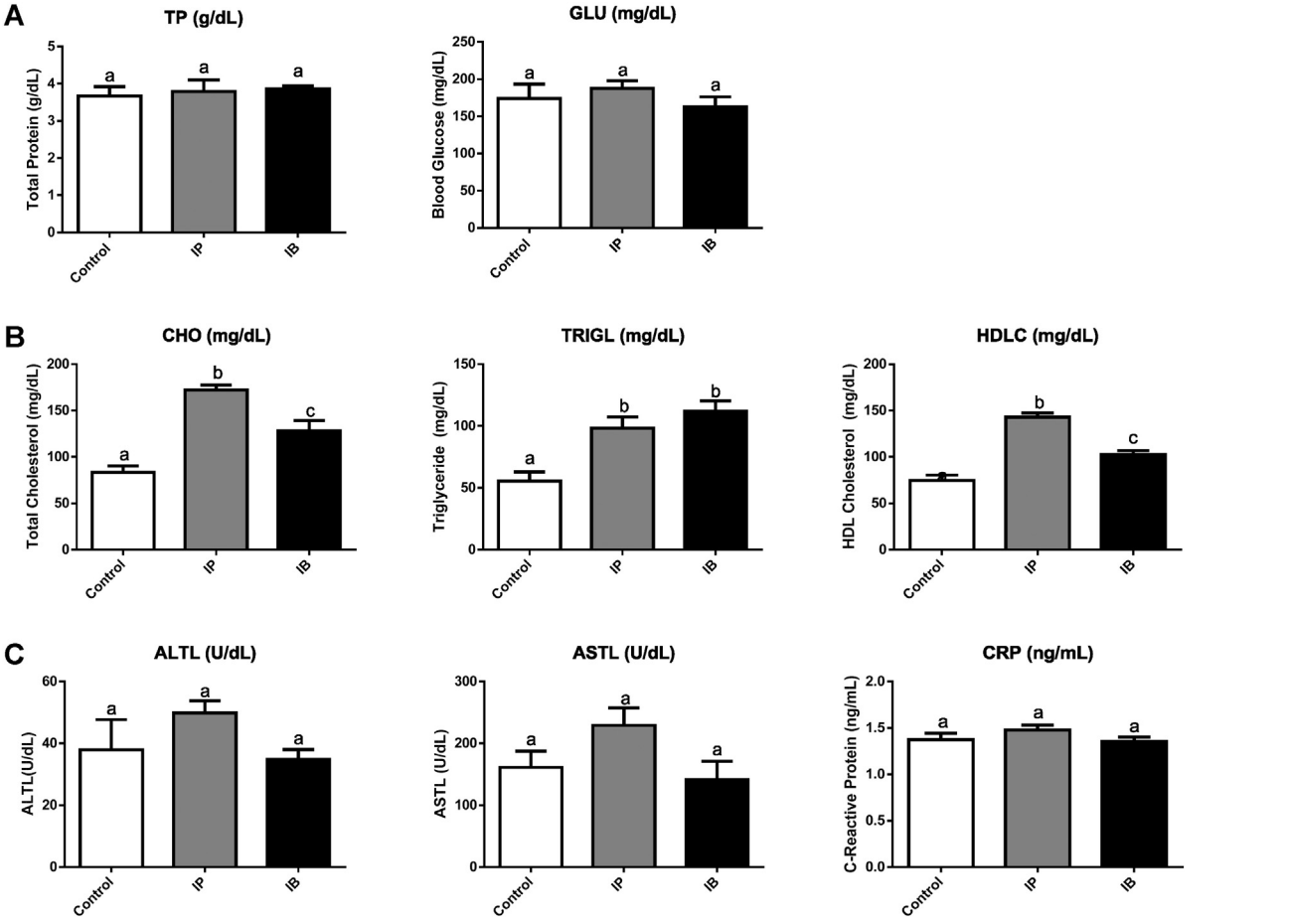

Fig. 2. Ilpum brown rice (IB) consumption did not cause significant organ toxicity or inflammation. (A) Plasma total protein and glucose concentrations in control-diet-, IP-, and IB-fed mice. (B) Plasma total-cholesterol, triglycerides (TG), and high-density lipoprotein (HDL) cholesterol levels. (C) Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and C-reactive protein (CRP) concentrations. The data are the mean and SEM values. Group comparisons (N = 9 or 10) were performed by one-way ANOVA followed by Tukey's test ($p < 0.05$). Different letters indicate significant differences among the groups.

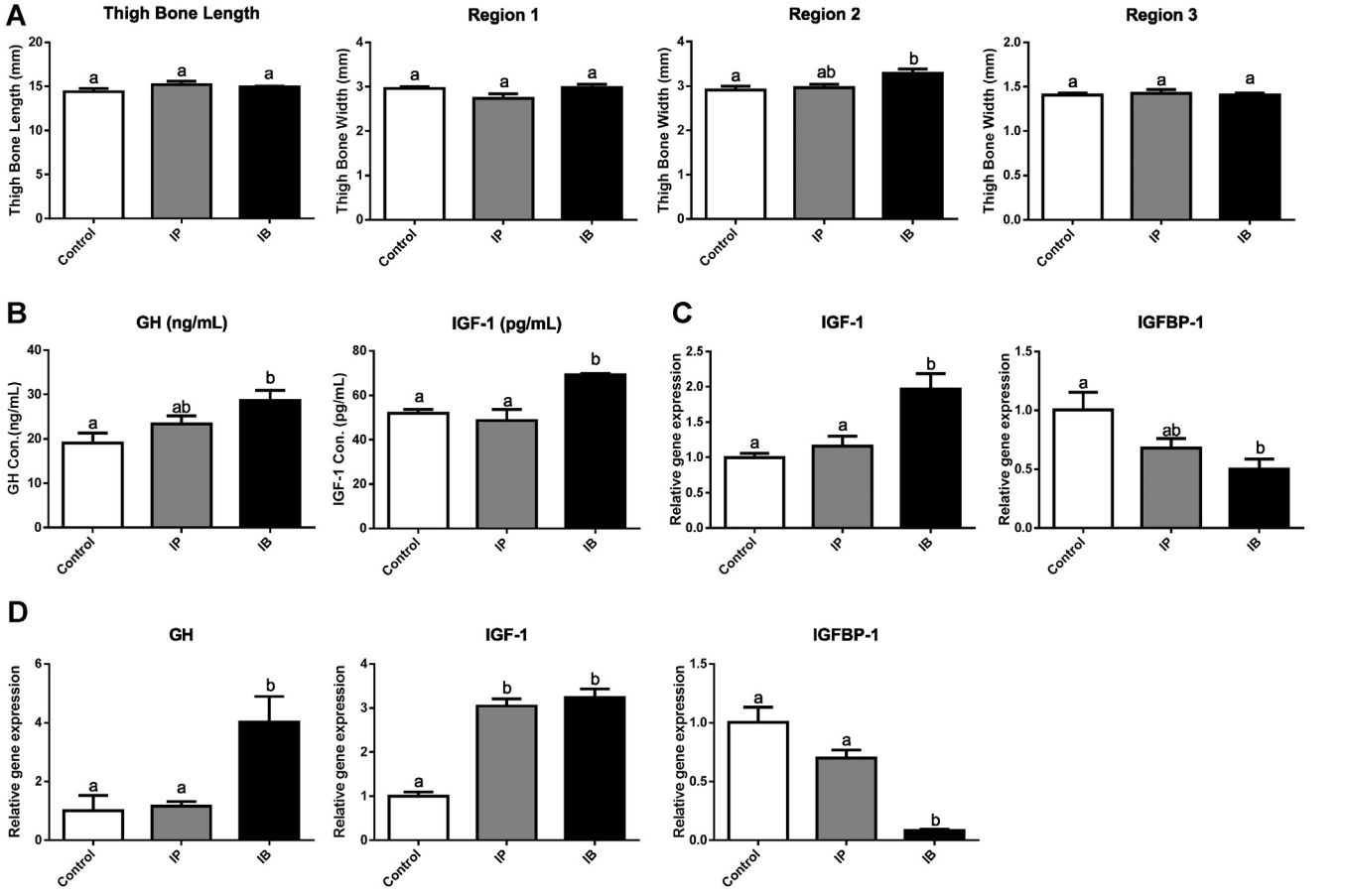

Fig. 3. Ilpum brown rice (IB) promotes cellular bone growth by activating the growth hormone (GH)/insulin growth factor (IGF)-1 axis. (A) Length and width (in regions 1, 2, and 3) of the thigh bone in control-diet-, IP-, and IB-fed mice (N = 4). (B) Plasma GH and IGF-1 concentrations by ELISA (N = 6). (C) Expression levels of IGF-1 and IGF-binding protein (IGFBP)-1 genes in the liver by qPCR (N = 3). (D) Expression levels of GH in GH3 cells and the expression of IGF-1, and IGFBP-1 genes in HepG2 cellscultured cells by qPCR (N = 4). The data are the mean and SEM values. Group comparisons were performed by one-way ANOVA, followed by Tukey's test ($p < 0.05$). Different letters indicate significant differences among the groups.

oleic acid, octadecanoic acid, arachidonic acid, and cholesterol), 4 sugars and sugar alcohols (D-ribose, D-mannose, ribitol, and galactose), 15 amino acids [L-alanine, glycine, L-valine, L-leucine, L-proline, L-serine, L-threonine, L-methionine, L-aspartic acid, L-glutamine, γ-amino butyric acid (GABA), L-phenylalanine, L-ornithine, L-tyrosine, and L-tryptophan], and 4 organic acids (acetic acid, oxalic acid, succinic acid, and malic acid) (Table 1). One-way ANOVA ($p < 0.05$) was performed to demonstrate significant difference among experiment groups. Metabolites such as propanoic acid, D-mannose, L-valine, L-serine, L-aspartic acid, L-glutamine, oxalic acid, and succinic acid were significantly increased in IB group compared to Control and IP groups.

3.6. PLS-DA analysis

The complex GC-TOF-MS data sets that included all of the metabolite data collected from the liver samples of mice fed either the control diet, IP, or IB were assessed using PCA (Supplemental Fig. 2) and PLS-DA (Fig. 4A) score plotting. As an unsupervised method, PCA enables recognition of any inherent sample clustering without any bias because it does not require any information on the data sets (Carraro, Giordano, Reniero, Perilongo, & Baraldi, 2009). Therefore, it was applied to confirm the clustering prior to analysis by supervised methods such as PLS-DA. A supervised PLS-DA is one of the classification methods where the response variable is a 'dummy' Y matrix expressing an orthogonal unit vector of each class (Keun et al., 2003; Barker & Rayens, 2003).

Significant differences were observed among the three groups. The IB group was clearly separated from the control and IP groups (PCA and PLS-DA score plots). When internal cross-validation on PLS-DA was performed, component 5 and parameters, such as $R^2X = 0.96$, $R^2Y = 0.795$, and $Q^2Y = 0.822$, which showed its predictive power and degree of fit to the data, were obtained. A permutation test was also proceeded in order to further validate the model (Singletary et al., 2002). The R^2 and Q^2 intercept values were 0.636 and −0.587 after 100 permutations (Supplemental Fig. 3). The major metabolites involved in the separation along PLS1 (positive: control and IP) were butanoic acid, arachidonic acid, acetic acid, L-methionine, L-aspartic acid, L-phenylalanine, L-ornithine, L-tyrosine, and D-ribose (Fig. 4B), while propanoic acid, hexadecanoic, linoleic acid, oleic acid, octadecanoic acid, cholesterol, oxalic acid, succinic acid, L-alanine, glycine, L-valine, L-leucine, L-proline, L-serine, L-threonine, L-glutamine, GABA, L-tryptophan, D-mannose, and ribitol were the major compounds contributing to the separation along PLS1 (negative: IB) (Fig. 4B).

3.7. PLSR analysis

The PLSR analysis demonstrated the correlations between data collected from GC-TOF-MS and growth hormone markers (*e.g.*, GH and IGF-1). PLSR can be used to assess the relationship between a descriptor matrix **x** (metabolites of GC-TOF-MS data) and a response matrix **y** (GH and IGF-1). The PLSR loading biplot in Fig. 4C explained parameters of $R^2X = 0.687$, $R^2Y = 0.524$, $Q^2Y = 0.496$, and component 2. Metabolites with VIP values exceeding 1.0 were considered to be strong contributors to explain the selection of **y**; these metabolites included L-valine, L-glutamine, D-mannose, L-phenylanalnine, L-aspartic acid, L-methionine, L-ornithine, L-tyrosine, L-tryptophan,

Table 1
Metabolites detected in liver samples obtained from mice fed a control diet, Ilpum rice (IP), and Ilpum brown rice (IB).

Compounds	Control	IP	IB	ID
Lipids				
Propanoic acid	4.66[1] ± 1.07a[2]	5.77 ± 0.86ab	6.69 ± 1.68b	B[3]
Butanoic acid	0.26 ± 0.09b	0.46 ± 0.20c	0.09 ± 0.03a	B
Hexadecanoic acid	2.22 ± 0.72b	1.26 ± 0.35a	2.86 ± 0.62b	B
Linoleic acid	1.01 ± 0.36b	0.41 ± 0.18a	0.77 ± 0.17b	A
Oleic acid	0.88 ± 0.27b	0.52 ± 0.18a	1.13 ± 0.34b	B
Octadecanoic acid	0.59 ± 0.18a	0.78 ± 0.12a	1.04 ± 0.58a	B
Arachidonic acid	0.14 ± 0.08a	0.10 ± 0.06a	0.11 ± 0.05a	A
Cholesterol	0.41 ± 0.12a	0.56 ± 0.14a	0.54 ± 0.22a	A
Sugars and sugar alcohols				
D-Ribose	3.03 ± 0.81b	3.54 ± 0.87b	1.68 ± 0.69a	A
D-Mannose	2.62 ± 1.33a	3.55 ± 1.83a	8.41 ± 4.33b	A
Ribitol	0.16 ± 0.11a	0.16 ± 0.06a	0.27 ± 0.16a	B
Galactose	0.34 ± 0.17a	0.29 ± 0.07a	0.29 ± 0.10a	A
Amino acids				
L-Alanine	6.46 ± 0.78b	3.77 ± 0.39a	6.84 ± 1.54b	A
Glycine	0.84 ± 0.17a	2.12 ± 0.40b	2.28 ± 0.67b	A
L-Valine	0.60 ± 0.06a	0.41 ± 0.11a	2.33 ± 0.96b	A
L-Leucine	0.75 ± 0.27a	0.61 ± 0.13a	0.71 ± 0.21a	A
L-Proline	2.95 ± 0.17a	2.46 ± 0.54a	3.00 ± 0.72a	A
L-Serine	0.72 ± 0.10a	0.63 ± 0.07a	0.86 ± 0.15b	A
L-Threonine	0.62 ± 0.05b	0.44 ± 0.08a	0.54 ± 0.09b	A
L-Methionine	0.68 ± 0.13b	0.41 ± 0.14a	0.32 ± 0.08a	A
L-Aspartic acid	0.44 ± 0.13a	0.34 ± 0.11a	0.19 ± 0.09b	A
L-Glutamine	2.71 ± 0.22a	2.30 ± 0.42a	3.63 ± 0.58b	A
GABA	0.09 ± 0.05a	0.10 ± 0.04a	0.10 ± 0.05a	A
Phenylalanine	1.00 ± 0.14c	0.51 ± 0.23b	0.25 ± 0.07a	A
L-Ornithine	1.31 ± 0.39b	0.55 ± 0.21a	0.39 ± 0.18a	B
L-Tyrosine	0.51 ± 0.31b	0.25 ± 0.19a	0.05 ± 0.03a	A
L-Tryptophan	0.25 ± 0.05b	0.15 ± 0.09a	0.22 ± 0.04ab	A
Organic acids				
Acetic acid	0.63 ± 0.18b	0.33 ± 0.10a	0.43 ± 0.05a	A
Oxalic acid	0.20 ± 0.10ab	0.14 ± 0.06a	0.28 ± 0.07b	A
Succinic acid	1.14 ± 0.43ab	0.77 ± 0.57a	1.75 ± 0.48b	A
Malic acid	0.51 ± 0.18a	0.69 ± 0.22a	0.53 ± 0.20a	A

[1] Relative peak area was calculated by [(peak area of metabolite)/(peak area of internal standard)] in GC-TOF-MS total ion chromatogram.
[2] Different letters mean significant difference ($p < 0.05$) among samples using Duncan's multiple comparison test between samples having different letters in a row (N = 7).
[3] A: Identified compounds, mass spectrum and retention time were matched with those of authentic standard. B: Putatively annotated compounds, mass spectrum was identical with that of wiley 9 n mass spectral database.

butanoic acid, hexadecanoic acid, oleic acid, glycine, octadecanoic acid, and L-proline (Table 2). In particular, the levels of L-valine, L-glutamine, and D-mannose were significantly higher in the IB group than in the control and IP groups ($p < 0.05$).

4. Discussion

Brown rice contains large amounts of beneficial components including dietary fiber, amino acids, minerals, vitamins, and phytochemicals. It has a higher nutritional value and a lower GI compared with normal white rice (Hodge, English, O'Dea, & Giles, 2004). In particular, previous studies have suggested that consuming brown rice as a replacement for white rice could be associated with a lower risk of type 2 diabetes (*e.g.*, Sun et al., 2010).

A low GH level is known to influence increases in body fat (Brummer, 1998; Møller, Gjedsted, Gormsen, Fuglsang, & Djurhuus, 2003). The GH level was lower in IP-fed mice than in IB-fed mice in the present study. The abdominal fat levels decreased significantly in IB-fed mice, while their liver tissue weight increased. These results demonstrate that consuming IB exerts a beneficial metabolic effect on weight management. The effects of IB on the plasma protein levels and lipid profiles were also evaluated in mice. Feeding mice with IB for 4 weeks resulted in a significant increase in the HDL-cholesterol concentrations, although the total-cholesterol and TG concentrations also increased. No increases were found in the concentrations of ALT, AST, and CRP markers for liver health and inflammation in IB-fed mice compared with the control-diet-fed mice. These results demonstrate that IB consumption does not cause organ toxicity or inflammation.

In this study, we investigated the biological mechanisms of IB underlying cellular bone growth. Bone is a mineralized connective tissue that is vital in many important body functions including structural support for muscles, organs, and movement; protecting vital organs; and storing minerals. Long bones have a tubular bone shaft, the center section of which is called the diaphysis, which contains a medullar cavity filled with bone marrow. The ends of the bone are called the epiphyses, which are mostly cancellous bone covered by an outer layer of compact bone. In general, the linear growth of long bones occurs at the ossification zone, while increases in width or thickness occur through appositional growth. This study found that the width of region 2 was increased in IB-fed mice compared with the control-diet- and IP-fed mice. These results demonstrate that consuming IB could promote cellular bone growth. Two key hormones in bone are GH and IGF-1, both of which are essential for the development and the growth of the skeleton. IGF-1 mediates most of the effects of GH on the skeletal metabolism and increases bone formation by regulating the functions of the differentiated osteoblasts.

The bioavailability of IGF-1 is regulated by IGFBPs, which have different affinities for IGFs. IGFBP-1, which is produced mainly in the liver, can bind to IGF-1 and inhibits IGF-1-dependent cellular growth and differentiation. We found that the plasma concentrations of GH and IGF-1 were significantly increased in IB-fed mice. Moreover, the expression levels of the GH and IGF-1 genes were dramatically increased and IGFBP-1 gene expression was significantly reduced in IB-fed mouse livers as well as in HepG2 cells. Together, these results demonstrate that IB promotes cellular bone growth by regulating the expression of the GH, IGF-1, and IGFBP-1 genes.

The PLSR results showed that L-valine, L-glutamine, D-mannose and L-tryptophan are strongly associated with GH or IGF-1. In particular, the IB group had high levels of L-valine and L-glutamine, which were strongly correlated with IGF-1 in PLSR. Branched-chain amino acids (BCAAs), such as L-isoleucine, L-leucine, and L-valine, are necessary to humans and are catabolized primarily by skeletal muscle (Brosnan & Brosnan, 2006). Kawanago, Takemura, Ishizuka, and Shioya (2015) demonstrated that dietary supplementation with BCAAs increased body weight, growth, and the hepatic expression of IGF-1 and IGF-2. The production of IGF-1 and its effects are regulated by free amino acids in rat primary hepatocytes (Kawanago et al., 2015). L-Valine plays important roles in the repair, growth, and metabolism of muscle tissue (Wu, 2009). On the other hand, L-glutamine is the most abundant amino acid in human muscle and plasma, and it directly regulates both the production and removal of proteins and immune-cell activity. In addition, L-glutamine is converted into glutamate, which can directly enhance growth hormone secretion (Wu, 2009).

L-tryptophan is the precursor of serotonin, which regulates the secretion of pituitary growth hormone that, in turn, stimulates the production of IGFs (Musumeci, Trovato, Avola, Imbesi, & Castrogiovanni, 2013). IFG-1 is known to stimulate the uptake of amino acids and protein synthesis in bone (Copeland & Nair, 1994). The content of IGF-1 and its gene expression were higher in the IB samples than in the control and IP samples in the present study. Furthermore, the husk of brown rice contains fatty acids, vitamins, minerals, and phenolic compounds that could be related to growth-improving activities (Esa, Ling, & Peng, 2013).

5. Conclusions

The consumption of an IB-containing diet for 4 weeks elevated the plasma concentrations of both GH and IGF-1, which reduced

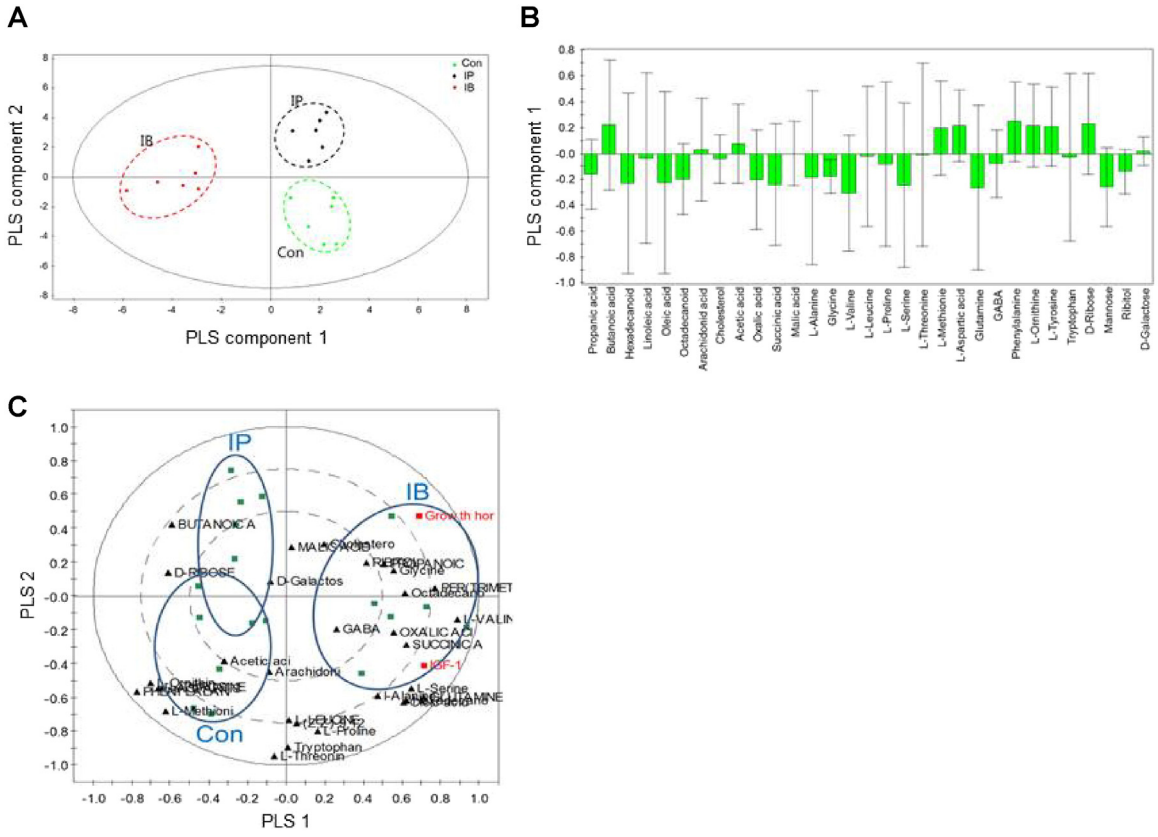

Fig. 4. Effects of IB on liver metabolites. (A) The score plot of partial least-squares discriminant analysis (PLS-DA) of liver metabolites from control-diet-, IP-, and IB-fed mice. (B) Column loading plot of PLS-DA for liver metabolites. (C) Correlation biplot of the relationship between liver metabolites (▲), as determined from GC-TOF-MS data, and the parameters involved in GH and IGF-1 activities (■). The control-diet-, IP-, and IB-fed mice groups examined using GC-TOF-MS are indicated by green symbols (■).

the IGFBP-1 levels and significantly increased thigh bone width in C57BL/6J mice. In metabolomics analysis, the levels of certain amino acids (*e.g.*, L-valine, L-glutamine, D-mannose and L-tryptophan) were strongly correlated with the plasma IGF-1 concentration in the IB group. These amino acids could play an important role in growth-promoting activity, affecting IGF-1 synthesis to promote cellular and bone growth in mice. Further studies in humans are needed to confirm the findings of this study.

Table 2
VIP (variable importance in the projection) values of the major liver metabolites for correlations between the data collected from GC-TOF-MS and growth hormone separation of control, IP, and IB samples in the PLSR-derived biplot.

No.	Compounds	VIP	Correlation
1	L-Valine	1.40	IB (IGF-1)
2	L-Glutamine	1.39	IB (IGF-1)
3	D-Mannose	1.33	IB (GH)
4	L-Phenylalanine	1.31	Con
5	L-Aspartic acid	1.19	Con
6	L-Methionine	1.19	Con
7	L-Ornithine	1.17	Con
8	L-Tyrosine	1.14	Con
9	L-Tryptophan	1.13	IB (IGF-1)
10	Butanoic acid	1.11	IP
11	Hexadecanoic acid	1.09	IB (IGF-1)
12	Oleic acid	1.07	IB (IGF-1)
13	Glycine	1.03	IB (GH)
14	Octadecanoic acid	1.01	IB (IGF-1)
15	L-Proline	1.01	IB (IGF-1)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2016.03.009.

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