J. Appl. Glycosci., **71**, 73−80 (2024)
doi:10.5458/jag.jag.JAG-2023_0019
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Regular Paper

Metabolic Profiling of Rat Kidney Tissue Following Administration of D-Allulose

(Received December 25, 2023; Accepted April 9, 2024)
(J-STAGE Advance Published Date: June 22, 2024)

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Abstract: D-Allulose (D-psicose) is a rare sugar and a C-3 epimer of D-fructose. D-Allulose has been reported to have several health benefits via its alteration of both glucose and lipid metabolism. It was previously reported that D-allulose alters the hepatic metabolomic profile. Although the kidneys are crucial organs in metabolic regulation, the effects of D-allulose on renal metabolism have not yet been established. Therefore, this study was designed to capture the overall metabolic response in the kidneys to D-allulose. This was done by providing an AIN-93G diet to Wistar rats, with or without 3 % D-allulose, for four weeks. Renal tissue and blood samples were collected after a 3-hour fasting for evaluation of the renal metabolic profile and their related plasma parameters. D-Allulose increased renal weight without changes in the plasma indices associated with reduced renal function. Metabolic profiling identified a total of 264 peaks. As the contribution rate was too low in the principal component analysis results of the metabolic profiling results, we evaluated the metabolites that were significantly different between two groups and identified 23 up-regulated and 26 down-regulated metabolites in the D-allulose group. D-Allulose also had significant influence on several metabolites involved in glucose metabolism, amino acid metabolism, and purine metabolism. Moreover, the levels of trimethylamine N-oxide and symmetric dimethylarginine, which are associated with several diseases such as chronic kidney disease and cardiovascular disease decreased following D-allulose diets. This study showed that D-allulose affects the renal metabolic profile, and our findings will help elucidate the function of D-allulose.

Key words: D-allulose, D-psicose, kidney, metabolomics

INTRODUCTION

D-Allulose (D-psicose) is a rare sugar, meaning that there are less amounts of its monosaccharides in nature. D-Allulose is a C-3 epimer of D-fructose, with approximately zero caloric value despite having 70 % of the sweetness of sucrose [1]. The United States Food and Drug Administration has deemed D-allulose as "Generally Recognized as Safe," implying its potential use as a food or dietary supplement. In addition, D-allulose has been assigned as a food category in Japan, and several reports have shown that D-allulose supplementation provide a variety of health benefits. For instance, D-allulose has been reported to suppress the elevation of postprandial glucose levels in humans [2] and animals through the suppression of glucose absorption by α-glucosidase inhibition [3] and the enhancement of glucose uptake into the liver by glucokinase activation [4]. Other report has shown that D-allulose enhances postprandial fat oxidation in humans [5].

In addition, approximately 70 % of the ingested D-allulose in humans is absorbed via GLUT5 in the small intestine [6], while the remaining 30 % is not absorbed and slightly fermented by intestinal bacteria before being excreted through the feces [7]. The absorbed D-allulose is not metabolized and circulates in the body before eventually being excreted into the urine within 24 h of ingestion [8, 9]. D-Allulose has been reported to collect in the kidneys [10] and is thus expected to modulate renal metabolism.

Moreover, the previous report indicated that D-allulose induced specific changes in the hepatic metabolomics profile of rats [11]. The kidneys are also one of the most critical organs in the human body, regulating a variety of important metabolic pathways. However, the effect of D-allulose on renal metabolism remains largely unknown.

The aim of this study is to evaluate the changes in the overall metabolism of the kidney associated with D-allulose consumption, and identify several key factors for future evaluation of kidneys.

MATERIALS AND METHODS

Animal treatment. All experimental procedures were performed in accordance with the national ethical guidelines, and ethical approval was granted by the Animal Experiment Committee of Matsutani Chemical Industry Co., Ltd. (Approval No. 181112). Twelve 6-week-old male Wistar (Jcl: Wistar) rats were obtained from CLEA Japan Inc.

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Abbreviations: BUN, plasma urea nitrogen; CE, capillary electrophoresis; GFR, glomerular filtration rate; HCA, Hierarchical cluster analysis; HMT, Human Metabolome Technologies Inc.; MS, mass spectrometry; MT, migration time for CE-TOF-MS measurement; PCA, principal component analysis; PI3K, class III phosphatidylinositol 3-kinases; SDMA, symmetric dimethylarginine; TMAO, trimethylamine *N*-oxide; UDP-GlcNAc, UDP-*N*-acetylglucosamine.

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(Tokyo, Japan). The animals were housed individually in steel cages with free access to a commercial diet (CE2: CLEA Japan Inc.) and purified water for one week to facilitate acclimatization to environmentally controlled room [temperature: 22−24 ˚C, light/dark cycle: 7:00−19:00 (JST)]. These animals were then weighed and divided into two groups (n = 6/group) according to their body weight. The rats were given an AIN-93G diet (the control group) or a modified AIN-93G diet, including 3 % D-allulose (the D-allulose group; Table 1). D-Allulose with a purity of more than 99 % was obtained from Matsutani Chemical Industry Co., Ltd. (Itami, Japan). The dose of D-allulose (3 %) was calculated using an animal to human conversion formula based on body surface [12] to correspond to approximate human intake amount [2, 13−15]. The rats were fed these diets *ad libitum* for four weeks. After the feeding periods, rats were euthanized under anesthesia with isoflurane after fasting for 3 h. The fasting time was set to 3 h to evaluate renal metabolites in the presence of sufficient D-allulose in the body [8]. Blood was collected from the abdominal aorta using a heparin-treated syringe and the rats were then perfused with saline to facilitate blood removal. Plasma samples were obtained from the whole blood by centrifugation at 1,693 × G for 15 min. Subsequently, the kidneys and the liver were removed, weighed, and immediately frozen in liquid nitrogen. All samples were stored at −80 ˚C until further analysis for biochemical and metabolomic profiling. The liver was used in a previous report [11].

Plasma biochemical parameters. Total protein and albumin levels in the plasma were evaluated using a Protein Assay BCA Kit and an A/G B-Test Wako, respectively (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan). The levels of plasma urea nitrogen (BUN) in each sample were measured using a Urea Nitrogen (BUN) Colorimetric Detection Kit (Arbor Assays Inc., Ann Arbor, MI, USA). The creatinine levels were measured using a Creatinine Colorimetric Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA), and urea acid was evaluated using a Urea acid C-Test Wako (FUJIFILM Wako Pure Chemical Corp.). Plasma osmotic pressure was measured using an Advanced Osmometer® 3250 (Advanced Instrument Inc., Norwood MA, USA).

Metabolomic profiling. Metabolome measurements were carried out using a facility service from Human Metabolome Technologies Inc. (HMT), Tsuruoka, Japan. Approximately 30 mg of the frozen kidney was plunged into 600−750 µL of 50 % acetonitrile/Milli-Q water containing 20 µM internal standards (Solution ID: 304-1002, HMT) at 0 ˚C to inactivate enzymes. The tissue was homogenized twice at 1,500 rpm for 120 s, and then the homogenate was centrifuged at 2,300 × G at 4 ˚C for 5 min. Then, 400 µL of the upper aqueous layer was filtered through a Millipore centrifugal filter with a 5-kDa cutoff at 9,100 × G at 4 ˚C for 120 min to remove the proteins. The filtrate was then concentrated and resuspended in 50 µL of Milli-Q water for capillary electrophoresis (CE) -mass spectrometry (MS) analysis.

CE-TOF-MS was performed using an Agilent CE Capillary Electrophoresis System equipped with an Agilent 6210 Time of Flight mass spectrometer, Agilent 1100 isocratic HPLC pump, Agilent G1603A CE-MS adapter kit, and Agilent G1607A CE-ESI-MS sprayer kit (Agilent Technologies, Waldbronn, Germany). These systems were controlled by Agilent G2201AA ChemStation software version B.03.01 for CE (Agilent Technologies). The metabolites were analyzed using a fused silica capillary (50 µm *i.d.* × 80 cm total length), with a commercial electrophoresis buffer (Solution ID: H3301-1001 for cation analysis and H3302-1023 for anion analysis, HMT), which acted as the electrolyte. The sample was injected at a pressure of 50 mbar for 10 s (approximately 10 nL) for cation analysis and 22 s (approximately 22 nL) for anion analysis. The spectrometer scanned from m/z 50 to 1,000 and all of the other conditions were applied as previously described [16−18].

Peaks were extracted using automatic integration software MasterHands (Keio University, Tsuruoka, Japan), which extracted critical peak information including m/z, migration time for CE-TOF-MS measurement (MT), and peak area [19]. Signal peaks corresponding to isotopomers, adduct ions, and other product ions of known metabolites were excluded. The remaining peaks were annotated as putative metabolites using the HMT metabolite database based on their MTs and m/z values. The tolerance range for peak annotation was configured at ±0.5 min for MT and ±10 ppm for m/z. In addition, peak areas were normalized against those of the internal standards, and the resultant relative area values were further normalized by sample amount.

Hierarchical cluster analysis (HCA) and principal component analysis (PCA) were performed by HMT proprietary software PeakStat and SampleStat, respectively.

Statistical analysis. All data are presented as the mean ± SE. The missing values in the metabolomic analysis were replaced by half of the minimum value. Data from the biochemical parameters and metabolomic profiles were statistically evaluated using Student's t-test and Welch's t-test, respectively, through SPSS S software (IBM SPSS Statistics version. 27, International Business Machines Corporation, Armonk, N.Y, USA). Values were considered statistically significant when the p-value was less than 0.05.

Table 1. Food composition.

	Control (%)	D-Allulose (%)
α-Corn starch	52.9	49.9
Casein	20.0	20.0
Sucrose	10.0	10.0
Soybean oil	7.00	7.00
Cellulose	5.00	5.00
Mineral Mix (AIN-93G)	3.50	3.50
Vitamin Mix (AIN-93)	1.00	1.00
L-Cystine	0.300	0.300
Choline bitartrate	0.250	0.250
tert-Butylhydroquinone	0.00140	0.00140
D-Allulose	−	3.00
Total	100	100

RESULTS AND DISCUSSION

D-Allulose is absorbed via GLUT5 in the small intestine [6] and excreted into the urine via the kidneys, and thus is transiently present in kidney tissues. The GLUT5 protein is also present in the kidney [20] and may uptake D-allulose, implying its possible influence on renal metabolism. Therefore we evaluated the renal metabolomics profile of

Wistar rats in response to a D-allulose diet in this study.

D-Allulose significantly decreased final body weight and reduced total body weight gain in the treated animals without reducing food intake and calorie intake (data not shown). This suggested that the food efficacies in the D-allulose group were lower than those in the control group (Table 2). Several previous studies reported that D-allulose enhances energy expenditure [21, 22], suggesting that D-allulose decreases body weight by enhancing energy expenditure. Furthermore, an increase in the weight of the kidneys was observed in the D-allulose treated group compared to the control group (Table 2).

The biochemical parameters for each group are summarized in Table 3. Compared to the control group, the D-allulose group presented with increased plasma total protein and albumin but reduced plasma creatinine levels. There were no differences in BUN, uric acid, and osmotic pressure between the two groups.

Metabolomic analysis identified 264 unique peaks, and the results of both HCA and PCA are shown in Fig. 1. The PCA results show that PC1 and PC2 contributed 25.1 and 18.9 % to the overall clustering, respectively, and it seemed to characterize these groups at PC2. However, the contribution rate of PC2 (18.9 %) was too low to explain the changes in renal metabolism following D-allulose feeding. Thus, we then assesed the significantly different metabolites in these groups, identifying 23 upregulated and 26 downregulated metabolites in the D-allulose group (Table 4).

Our initial evaluations revealed increased total protein and albumin levels in the plasma samples of the animals in the D-allulose group when compared to those in the control group. Albumin is synthesized in the liver during periods of adequate nutrition, and its synthesis is suppressed under conditions of inadequate nutrition [23]. *De novo* synthesized albumin is not stored in hepatocytes but is rapidly secreted into the blood [24]. Thus, an adequate quantity of plasma albumin may indicate healthy liver function. A previous human trial reported that D-allulose decreases markers of liver function (γ-GTP and ALT) [13]. Thus, it is suggest that the higher plasma albumin levels are likely a result of increased albumin synthesis in the liver. If this was the case, it would be expected a concurrent increase in amino acid levels in the blood, which are in dynamic equilibrium to protein levels in organs [25]. Because almost all amino acids that move from the blood to the kidney are reabsorbed at the proximal convoluted tubule [26], the increased amino acids in the blood are thought to lead to an increase in amino acid reabsorption. Here, D-allulose provided the kidneys with high levels of essential amino acids (methionine and threonine) (Table 4), which are not synthesized in the body. This results supported our suggestion that D-allulose increases influx of amino acid into the kidney. It is reported that increased delivery of exogenous amino acids to the kidney increases the activity of class III phosphatidylinositol 3-kinases (PI3K), leading to an increase in kidney/body weight ratio in a unilateral renal mouse model [27]. PI3K is reported to be associated with both cell proliferation and growth. An increase in kidney/body weight ratio was observed in this experiment (Table 2), which was further validated by several reports describing asymptomatic increases in liver and kidney weight in D-allulose-fed rats [28, 29]. Given this information, it is suggested that this could have been caused by increased protein synthesis in the liver and the resulting increased influx of amino acids into the kidneys. The significant increase in various amino acids and their related metabolites in the kidney in response to D-allulose validates this suggestion.

Intake of D-allulose could accelarate amino acids metabolism in the kidney by increasing renal amino acid levels in response to increased inflow of amino acids into the kidney. Indeed, D-allulose increased several amino acids (glutamine, glycine, methionine, threonine, glutamic acid, and serine) and their related metabolites (cystine, creatine, putrescine, and 5-oxoproline), and reduced several amino acid-related

Table 2. Effect of D-allulose on body weight, food intake, and kidney weight.

	Control	D-Allulose	*p*-Value
Initial body weight (g)	212 ± 4	213 ± 3	0.971
Final body weight (g)	360 ± 6	334 ± 6	0.013
Body weight gain (g)	147 ± 4	122 ± 5	0.003
Food intake (g/day)	21.6 ± 0.7	20.6 ± 0.2	0.203
Food efficacy (g BW gain/g diet)	0.245 ± 0.006	0.221 ± 0.008	0.009
Kidney weight (g)	2.60 ± 0.04	2.70 ± 0.04	0.093
(g/100g BW)	0.723 ± 0.010	0.807 ± 0.017	0.002

Data are presented as mean ± SE. The *p*-values were determined using Student's *t*-test. BW: body weight.

Table 3. Effect of D-allulose on plasma bio-parameters.

	Control	D-Allulose	*p*-Value
Plasma			
Total protein (g/dL)	6.46 ± 0.16	7.10 ± 0.05	0.006
Albumin (g/dL)	4.10 ± 0.07	4.29 ± 0.04	0.040
BUN (mg/dL)	14.3 ± 0.5	13.6 ± 1.1	0.551
Creatinine (mg/dL)	1.11 ± 0.05	0.779 ± 0.088	0.010
Uric acid (mg/dL)	1.27 ± 0.28	1.45 ± 0.31	0.667
Osmotic pressure (mOsm/kg · H_2O)	293 ± 1	293 ± 1	0.903

Data are presented as mean ± SE. The *p*-values were determined using Student's *t*-test.

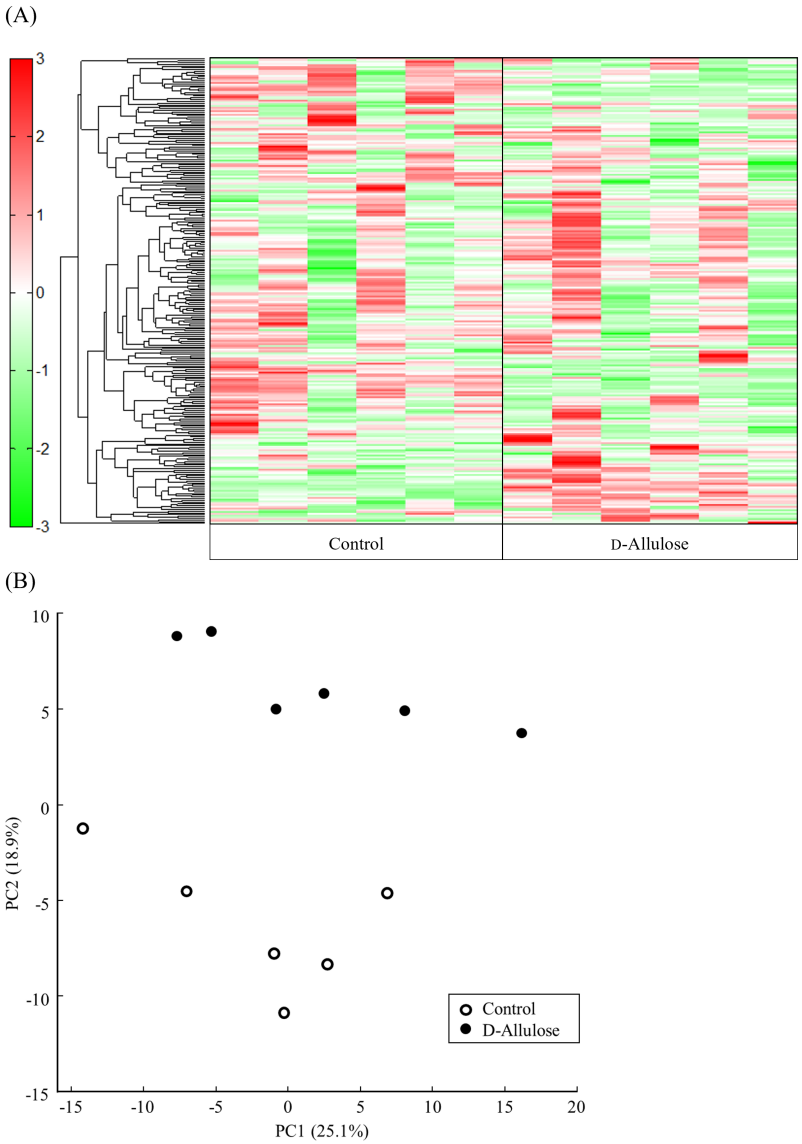

Fig. 1. Effects of D-allulose on renal metabolomic profile.
(A) Results of hierarchical cluster analysis. The deeper red and green indicate higher and lower than average levels, respectively. (B) Results of principal component analysis. Control and D-allulose groups are shown as white and black circles, respectively.

metabolites (cysteinesulfinic acid, 2-metylserine, Glu-Glu, and *N*-acetyllysine) (Table 4). Amino acid-derived carbon skeletons are known to be metabolized to pyruvate and acetyl-CoA, which then enter the citric acid cycle to produce ATP or act as a substrate for gluconeogenesis. Our evaluations showed that both the pyruvate and acetyl-CoA levels were decreased in the D-allulose-fed animals compared to the control animals. In addition, the levels of thiamine, which functions in the production of acetyl-CoA from pyruvate, and thiamine diphosphate decreased after D-allulose feeding in this study (Table 4). These results suggest that D-allulose may affect the utilization of amino acids in ATP synthesis and/or gluconeogenesis. The AMP/ATP ratio can be used to evaluate the energy status of various cells and is associated with gluconeogenesis through fructose-1,6-biphosphatase [30]. This study showed that D-allulose decreased renal AMP levels without altering ATP levels (Table 4), suggesting that D-allulose modulates gluconeogenesis in the kidney. Decreases in AMP/ATP also facilitate glycogen synthesis by suppressing glycogen phosphorylase [30]. The kidneys are known to synthesize glycogen from glucose, and this study showed that D-allulose increases the levels of glucose-1-phosphate (Table 4), which acts as a substrate for glycogen synthesis. As these metabolic outcomes are also dependent on the energy demand of other organs, further investigation is needed to clarify the decrease in pyruvate and acetyl-CoA and the increase in amino acids related to these pathways. Our findings suggest that administration of D-allulose is likely to affect energy metabolic pathways associated with the metabolism of some amino acids. These changes may be related to the glycemic regulatory effect of D-allulose, as shown in several reports describing the importance of amino acids in renal gluconeogenesis to maintain blood glucose homeostasis [31, 32]. In addition, *N*-acetylglucosamine 1-phosphate, the intermediate product of the hexosamine pathway, and UDP-*N*-acetylglucosamine (UDP-GlcNAc), the final product of the hexosamine pathway, were significantly increased by D-allulose in this study (Table 4), suggesting that D-allulose upregulates hexosamine pathway. As glutamine is used as an amino donor in the first reaction in this pathway, the increase in glutamine by D-allulose (Table 4) was considered to be a

Table 4. The metabolites significantly altered by the D-allulose feeding.

Metabolite	Control (C)	D-Allulose (A)	Ratio (A/C)	*p*-value
Cystine	$3.68 \times 10^{-2} \pm 1.07 \times 10^{-3}$	$5.53 \times 10^{-2} \pm 1.68 \times 10^{-3}$	1.5	< 0.001
Galacturonic acid-1	$7.68 \times 10^{-5} \pm 1.27 \times 10^{-5}$	$3.75 \times 10^{-4} \pm 3.58 \times 10^{-5}$	4.9	< 0.001
Glucosaminic acid	$7.41 \times 10^{-5} \pm 0.00$	$1.87 \times 10^{-4} \pm 1.17 \times 10^{-5}$	2.5	< 0.001
Cysteinesulfinic acid	$1.31 \times 10^{-4} \pm 6.45 \times 10^{-6}$	$8.88 \times 10^{-5} \pm 5.04 \times 10^{-6}$	0.7	< 0.001
UDP-*N*-acetylgalactosamine UDP-*N*-acetylglucosamine	$7.54 \times 10^{-3} \pm 1.57 \times 10^{-4}$	$9.33 \times 10^{-3} \pm 2.81 \times 10^{-4}$	1.2	< 0.001
2-Methylserine	$7.31 \times 10^{-4} \pm 6.39 \times 10^{-5}$	$3.54 \times 10^{-4} \pm 4.61 \times 10^{-5}$	0.5	< 0.001
4-(β-Acetylaminoethyl) imidazole	$1.42 \times 10^{-4} \pm 1.74 \times 10^{-5}$	$4.21 \times 10^{-5} \pm 8.18 \times 10^{-6}$	0.3	0.001
IMP	$4.76 \times 10^{-3} \pm 2.07 \times 10^{-4}$	$3.70 \times 10^{-3} \pm 1.24 \times 10^{-4}$	0.8	0.002
Ascorbate 2-sulfate	$1.58 \times 10^{-4} \pm 1.41 \times 10^{-5}$	$8.52 \times 10^{-5} \pm 1.08 \times 10^{-5}$	0.5	0.002
N-Acetylglucosamine 1-phosphate	$1.67 \times 10^{-3} \pm 6.81 \times 10^{-5}$	$2.01 \times 10^{-3} \pm 4.55 \times 10^{-5}$	1.2	0.003
Trimethylamine *N*-oxide (TMAO)	$1.46 \times 10^{-3} \pm 1.64 \times 10^{-4}$	$6.94 \times 10^{-4} \pm 7.82 \times 10^{-5}$	0.5	0.004
Creatine	$3.52 \times 10^{-2} \pm 1.79 \times 10^{-3}$	$4.86 \times 10^{-2} \pm 3.06 \times 10^{-3}$	1.4	0.005
Thiamine diphosphate	$2.44 \times 10^{-4} \pm 6.31 \times 10^{-6}$	$1.98 \times 10^{-4} \pm 1.08 \times 10^{-5}$	0.8	0.006
N-Acetylmuramic acid	$6.47 \times 10^{-5} \pm 7.40 \times 10^{-6}$	$3.10 \times 10^{-5} \pm 3.03 \times 10^{-21}$	0.5	0.006
Glucose 1-phosphate	$1.28 \times 10^{-3} \pm 4.20 \times 10^{-5}$	$4.77 \times 10^{-3} \pm 7.76 \times 10^{-4}$	3.7	0.006
Glutamine	$3.87 \times 10^{-2} \pm 1.06 \times 10^{-3}$	$4.64 \times 10^{-2} \pm 1.98 \times 10^{-3}$	1.2	0.009
Glycine	$1.97 \times 10^{-1} \pm 6.53 \times 10^{-3}$	$2.24 \times 10^{-1} \pm 4.72 \times 10^{-3}$	1.1	0.011
Methionine	$1.33 \times 10^{-2} \pm 8.94 \times 10^{-4}$	$1.66 \times 10^{-2} \pm 6.47 \times 10^{-4}$	1.3	0.014
Threonine	$4.80 \times 10^{-2} \pm 1.95 \times 10^{-3}$	$5.57 \times 10^{-2} \pm 1.69 \times 10^{-3}$	1.2	0.014
ADP-glucose GDP-fucose	$1.71 \times 10^{-4} \pm 6.86 \times 10^{-6}$	$1.98 \times 10^{-4} \pm 5.68 \times 10^{-6}$	1.2	0.014
Glutamic acid	$4.09 \times 10^{-1} \pm 4.33 \times 10^{-3}$	$4.62 \times 10^{-1} \pm 1.49 \times 10^{-2}$	1.1	0.015
Acetyl CoA_divalent	$2.88 \times 10^{-4} \pm 2.61 \times 10^{-5}$	$1.97 \times 10^{-4} \pm 1.25 \times 10^{-5}$	0.7	0.015
Taurocyamine	$4.44 \times 10^{-4} \pm 1.76 \times 10^{-5}$	$5.61 \times 10^{-4} \pm 3.36 \times 10^{-5}$	1.3	0.016
CMP-*N*-acetylneuraminate	$1.37 \times 10^{-3} \pm 4.70 \times 10^{-5}$	$1.57 \times 10^{-3} \pm 5.60 \times 10^{-5}$	1.2	0.017
Glu-Glu	$4.80 \times 10^{-4} \pm 4.50 \times 10^{-5}$	$3.28 \times 10^{-4} \pm 1.73 \times 10^{-5}$	0.7	0.018
Ribose 5-phosphate	$2.69 \times 10^{-4} \pm 1.16 \times 10^{-5}$	$2.29 \times 10^{-4} \pm 7.83 \times 10^{-6}$	0.8	0.018
3-Guanidinopropionic acid	$2.44 \times 10^{-4} \pm 3.57 \times 10^{-5}$	$3.64 \times 10^{-4} \pm 1.97 \times 10^{-5}$	1.5	0.019
2-Hydroxyvaleric acid	$8.50 \times 10^{-5} \pm 1.41 \times 10^{-5}$	$3.83 \times 10^{-5} \pm 9.02 \times 10^{-6}$	0.5	0.022
N-Acetyllysine	$1.07 \times 10^{-2} \pm 3.73 \times 10^{-4}$	$8.87 \times 10^{-3} \pm 5.27 \times 10^{-4}$	0.8	0.022
1-Methylhistamine	$2.72 \times 10^{-4} \pm 2.91 \times 10^{-5}$	$1.75 \times 10^{-4} \pm 2.01 \times 10^{-5}$	0.6	0.023
Symmetric dimetylarginine (SDMA)	$1.05 \times 10^{-3} \pm 1.13 \times 10^{-4}$	$7.12 \times 10^{-4} \pm 3.33 \times 10^{-5}$	0.7	0.029
Putrescine	$4.77 \times 10^{-4} \pm 2.21 \times 10^{-5}$	$6.12 \times 10^{-4} \pm 4.49 \times 10^{-5}$	1.3	0.029
Pyruvic acid	$5.76 \times 10^{-4} \pm 9.20 \times 10^{-5}$	$2.99 \times 10^{-4} \pm 0.00$	0.5	0.030
Adenosine	$1.11 \times 10^{-2} \pm 4.74 \times 10^{-4}$	$9.61 \times 10^{-3} \pm 3.51 \times 10^{-4}$	0.9	0.030
Thiamine	$1.73 \times 10^{-4} \pm 9.79 \times 10^{-6}$	$1.42 \times 10^{-4} \pm 6.21 \times 10^{-6}$	0.8	0.031
myo-Inositol 2-phosphate	$6.35 \times 10^{-4} \pm 2.57 \times 10^{-5}$	$5.15 \times 10^{-4} \pm 3.94 \times 10^{-5}$	0.8	0.033
β-Ala-Lys	$2.32 \times 10^{-4} \pm 3.65 \times 10^{-5}$	$3.44 \times 10^{-4} \pm 2.50 \times 10^{-5}$	1.5	0.033
AMP	$1.45 \times 10^{-2} \pm 1.49 \times 10^{-3}$	$1.01 \times 10^{-2} \pm 6.00 \times 10^{-4}$	0.7	0.033
Ascorbic acid	$2.30 \times 10^{-4} \pm 7.30 \times 10^{-5}$	$2.13 \times 10^{-5} \pm 0.00$	0.1	0.036
Isovalerylcarnitine	$6.46 \times 10^{-4} \pm 7.72 \times 10^{-4}$	$9.46 \times 10^{-4} \pm 9.62 \times 10^{-5}$	1.5	0.036
Serine	$4.45 \times 10^{-2} \pm 9.40 \times 10^{-4}$	$5.05 \times 10^{-2} \pm 2.11 \times 10^{-3}$	1.1	0.037
2-Deoxyglucose 6-phosphate	$5.33 \times 10^{-5} \pm 8.02 \times 10^{-6}$	$8.12 \times 10^{-5} \pm 8.50 \times 10^{-6}$	1.5	0.038
O-Acetylcarnitine	$2.21 \times 10^{-2} \pm 1.42 \times 10^{-3}$	$2.77 \times 10^{-2} \pm 1.89 \times 10^{-3}$	1.3	0.040
5-Oxoproline	$8.73 \times 10^{-3} \pm 2.51 \times 10^{-3}$	$1.08 \times 10^{-2} \pm 7.67 \times 10^{-4}$	1.2	0.041
Glucuronic acid Galacturonic acid-2	$6.38 \times 10^{-4} \pm 3.64 \times 10^{-5}$	$5.34 \times 10^{-4} \pm 2.38 \times 10^{-5}$	0.8	0.042
Tartaric acid	$2.55 \times 10^{-3} \pm 5.07 \times 10^{-4}$	$1.16 \times 10^{-3} \pm 2.43 \times 10^{-4}$	0.5	0.043
Acetylcholine	$8.94 \times 10^{-5} \pm 1.29 \times 10^{-5}$	$5.02 \times 10^{-5} \pm 1.12 \times 10^{-5}$	0.6	0.045
Phosphorylcholine	$4.97 \times 10^{-2} \pm 1.73 \times 10^{-3}$	$4.41 \times 10^{-2} \pm 1.76 \times 10^{-3}$	0.9	0.047
Pyridoxamine	$1.60 \times 10^{-4} \pm 1.46 \times 10^{-5}$	$1.22 \times 10^{-4} \pm 6.55 \times 10^{-6}$	0.8	0.049

Data are presented as Mean ± SE.The *p*-values were determined using Welch's *t*-test.

cause of this pathway upregulation. It is reported that the promotion of this pathway is associated with renal mesangial cell hypertrophy [33]. On the other hand, the downregulation of this pathway induces the depletion of *O*-linked *N*-acetylglucosamine modification, which uses UDP-GlcNAc as a sugar donor, and causes proteinuria through changes in the shape of podocytes [34]. Therefore, the alteration of these metabolites may lead to the elucidation of the mechanisms of the previously reported positive effects of D-allulose on glucose homeostasis [4, 35, 36] and diabetic nephropathy [37].

Methionine and cysteine are well known substrates of taurine and glutathione synthesis. Our data showed that D-allulose increased the levels of methionine and cystine

(Table 4), which is an oxidized dimeric form of cysteine, and decreased the levels of cysteinesulfinic acid (an intermediate metabolite in the conversion of cysteine to taurine). Thus, although D-allulose has been speculated to regulate taurine, the findings of this study did not demonstrate any changes in taurine levels (data not shown). Taurine has been reported to perform several important physiological functions, including osmoregulation [38]. The presence of D-allulose in the blood is thought to affect osmotic pressure, however, D-allulose had no effect on plasma osmotic pressure in this study (Table 3). It cannot be ruled out that D-allulose might modify the taurine synthesis pathway to help maintain osmotic pressure homeostasis. Glutathione is also an important antioxidant in the kidneys [39, 40]. To play a role in minimizing oxidative stress, the cystine/glutamate antiporter (system x_c^-), which exchanges extracellular cystine for intracellular glutamate, has been shown to play a role in minimizing oxidative stress by procuring intracellular cysteine for the synthesis of glutathione. Renal levels of glycine and L-glutamic acid, which are constituents of glutathione, were increased in D-allulose-fed rats (Table 4). Although the level of glutathione was below the level of detection in the metabolomic assay, the increase in these constituents may be associated with changes in the renal antioxidant response.

Our results revealed that D-allulose lowers the levels of glucuronate pathway-related metabolites (glucuronic acid, ascorbate 2-sulfate, and ascorbic acid) (Table 4). Rats can synthesize ascorbic acid within their cells via the glucuronic acid pathway [41]. The substrate of this pathway is reported to be provided by glycogenolysis [42]. As mentioned above, D-allulose may facilitate glycogen synthesis, meaning that D-allulose can act in the direction of glycogen accumulation. Therefore, the downregulation of the glucuronic acid pathway by D-allulose is regulated by glycogen metabolism. Ascorbic acid is reported to be a multifunctional nutrient, playing a role as an antioxidant and as a coenzyme for the synthesis of collagen [41, 43]. In a rat toxicity study, no abnormalities associated with ascorbic acid deficiency were observed in response to long-term D-allulose feeding [44]. The low levels of ascorbic acid in D-allulose-fed rats (Table 4) may be the result of an adaptation and interaction with other metabolic changes. In addition, since D-allulose was reported to be a free radical scavenger with the ability to ameliorate endoplasmic reticulum stress in human coronary artery endothelial cells, D-allulose can substitute ascorbic acid as an antioxidant.

Glutamine and glycine are also consumed during purine synthesis. While these amino acids were increased by D-allulose feeding, D-allulose significantly decreased several metabolites associated with purine metabolism (IMP, ribose 5-phosphate, adenosine, and AMP) (Table 4). Therefore, D-allulose may contribute to reduced purine metabolism. In addition, although renal uric acid, which is the end product of purine metabolism, was not significantly different between the two groups in this experiment. The result of a previous study showed a decrease in serum uric acid by D-allulose [28] and might reflect this suggestion. However, this needs further evaluation.

Increased levels of adenosine activate the adenosine A1 receptors, leading to sustained afferent arteriolar constriction and a reduction in glomerular filtration rate (GFR) [45]. Therefore, the observed decrease in adenosine levels after D-allulose feeding (Table 4) may implicate the role of D-allulose in the improvement of GFR. This suggestion is further supported by the decrease in several metabolites that are excreted in the urine following D-allulose feeding. For example, D-allulose decreased the levels of trimethylamine *N*-oxide (TMAO) (Table 4), a metabolite with a reported urinary excretion of 94.5 % within 24 h of administration [46]. Plasma creatinine levels were also significantly decreased by D-allulose (Table 3), further supporting this suggestion. This study did not evaluate kidney functions such as the GFR following D-allulose diet, therefore, further studies are needed to verify the effects of D-allulose on the kidneys.

In this study D-allulose reduced renal TMAO levels (Table 4) and a previous study showed that D-allulose also reduces hepatic TMAO level [11]. These findings suggest that, D-allulose may decrease TMAO levels throughout the body. TMAO is produced from trimethylamine, which is a product of the fermentation of dietary carnitine, betaine, choline, or choline-containing compounds by intestinal bacteria. Furthermore, TMAO levels are associated with cardiovascular diseases, kidney disease, diabetes, and cancer [46]. Symmetric dimethylarginine (SDMA) is also reported to be associated with chronic kidney disease [47], and D-allulose significantly reduced its levels in the kidneys of treated animals (Table 4). Taken together, these data suggest that D-allulose may be a useful food ingredient for the improvement or prevention of some diseases, such as chronic kidney disease.

In conclusion, D-allulose modulated the renal metabolomic profile, particularly of metabolites associated with both amino acid and purine metabolism in rats. Although D-allulose increased the renal weight with increasing amino acid influx, there were no changes in the plasma indices associated with renal dysfunction. Moreover, D-allulose reduced the levels of both TMAO and SDMA, which are associated with a variety of diseases, including chronic kidney and cardiovascular diseases. It should be noted that these results were obtained under limited condition of rats fasted for 3 h after a continuous intake of D-allulose. Furthermore, this study is the first study of D-allulose on renal metabolic profiling, and further studies such as measuring metabolic enzymes may further elucidate metabolic changes.

CONFLICT OF INTERESTS

This study was funded by Matsutani Chemical Industry Co., Ltd. (Itami, Japan). A.K., M.N., and T.I. are employees of this company.

ACKNOWLEDGMENTS

We would like to thank Editage (www.editage.com) for English language editing.

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