Physiological Effects and Organ Distribution of *Bacillus amyloliquefaciens* AS385 Culture Broth Powder Containing 1-Deoxynojirimycin in C57BL/6J Mice

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(Received September 4, 2018)

**Summary** 1-Deoxynojirimycin (DNJ) has been known as a potent α-glucosidase inhibitor from mulberry leaves and considered beneficial in prevention of type 2 diabetes. Due to limited amount of DNJ in mulberry leaves, recent studies have focused on finding alternative source that can produce higher amount of DNJ. Previously, we produced a high DNJ-containing culture medium from *Bacillus amyloliquefaciens* AS385 and constructed a concentration method of bacterial culture medium using cation exchange column. However, less complicated concentration procedure is necessary to save time and cost during the large-scale production. Therefore, we developed a simpler concentration method using anion exchange resin to yield *B. amyloliquefaciens* AS385 culture broth powder (CBP; 1% DNJ) and evaluated the physiological effects of 5-wk dietary CBP intake in C57BL/6J mice. CBP intake tended to suppress the elevation of blood glucose level during oral glucose tolerance test. Moreover, CBP intake significantly lowered the fasting plasma glucose level and white adipose tissue mass. Next, we evaluated the absorption and distribution of DNJ in mice organs after daily CBP intake. We found detectable amount of DNJ in organs with intestine and kidney as the major targeted organs. We concluded that the DNJ content in CBP is absorbed from digestive tract, distributed and accumulated in organs, which most likely to contribute to the alteration of blood glucose regulation and adiposity in C57BL/6J mice. Our study was the first to report the physiological effects of CBP produced from *B. amyloliquefaciens* AS385 and the organ distribution of DNJ from CBP.

**Key Words** 1-deoxynojirimycin, *Bacillus amyloliquefaciens* AS385, physiological effects, organ distribution, blood glucose regulation

1-Deoxynojirimycin (DNJ), a glucose analogue, is a main alkaloid component in mulberry leaves (*Morus alba* L.) and potent competitive inhibitor of α-glucosidase (1–3). Mulberry leaves DNJ has been increasingly investigated for its potential therapeutic activity in prevention and improvement of diabetic conditions by delaying the absorption of sugar in intestinal brush border (3–5), leading to suppression of postprandial blood glucose (3) and increased insulin sensitivity (6). Other physiological properties of mulberry leaves DNJ includes the anti-obesity effect through suppression of hepatic lipid accumulation (7) and improvement of lipid metabolism (8). Nonetheless, DNJ content in mulberry leaves is as low as 0.1% (9), makes it inconvenient for industrial scale production. This has prompted the search for alternative source of DNJ.

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Past studies have reported the ability of some microorganism strains to produce DNJ, such as *Bacillus* sp. (10–12) and *Streptomyces* sp. (13, 14). The beneficial properties of microbial DNJ are comparable to its mulberry leaves counterpart, especially in terms of anti-hyperglycemic (15–17) and anti-hyperlipidemic effects (6, 18). While some microorganism can produce high amount of DNJ, most of the known DNJ-producing bacteria are isolated from soil which is not preferable to be applied in food product. Previously, we discovered that *B. amyloliquefaciens* AS385, a bacterial strain that is commonly found in fermented food products, can produce high amount of DNJ after being cultivated in the culture medium supplemented with sorbitol (12). Next, we developed the purification method for *B. amyloliquefaciens* AS385 culture medium to increase the recovery rate of microbial DNJ (19) by using ethanol precipitation followed by cation exchange absorption-desorption. However, considering the amount of time and cost that
are required for this method, simpler method is more preferable for large-scale production. Therefore, in the present study, we proposed an alternative concentration method of bacterial culture medium using batch mixing with anion exchange resin to yield *B. amyloliquefaciens* AS385 culture broth powder (CBP). We then evaluated the physiological effects of dietary CBP in C57BL/6J mice after daily consumption, specifically in relation to blood glucose regulation and adiposity.

Moreover, while numerous studies have reported the beneficial effects of DNJ (3, 5–8, 15–22), the information about its absorption and distribution in organs after daily consumption in diet was lacking. Understanding the distribution of DNJ in organs is crucial and may serve as a basis to find the underlying mechanism on how DNJ exerts its beneficial effects in the body. Therefore, in this study, we also evaluated the organ distribution profile of DNJ in C57BL/6J mice after daily consumption of DNJ-containing CBP in diet for 5 wk. To the best of our knowledge, our study was the first to evaluate the physiological changes and DNJ distribution in organs after daily consumption of microbial DNJ, specifically deriving from *B. amyloliquefaciens* AS385 strain.

**MATERIALS AND METHODS**

Preparation of *B. amyloliquefaciens* AS385 culture broth powder (CBP). CBP was prepared according to our previous study (19) with modifications. First, *B. amyloliquefaciens* AS385 was cultured in 100 mL of medium containing 4% soybean peptone and 5% sorbitol. Fermentation was carried out for 5 d in 500 mL tryspinizing flask at 37˚C with constant shaking at 120 rpm. In the current study, additional 2.5 g of sorbitol was incorporated into every 100 mL of culture medium at the third day of fermentation. At the end of fermentation period, the culture medium (pH 7.0) was collected from five Erlenmeyer flasks, centrifuged (10,000 rpm for 10 min) and the supernatant (pH 8.9) was collected. Next, instead of passing the supernatant through the cation exchange column as in the previous study, we mixed 1,860 mL of supernatant with 372 g of strong anion exchange resin (Amberlite IRA910CT; Dow Chemical Company, Michigan, USA) in a beaker glass and mixing the mixture periodically to improve the purity of the supernatant. After 4 h, the mixture was filtered (Whatman glass microfiber filter grade GF/A; Whatman, Maidstone, UK) and the solid content of the filtrate was measured. Dextrin was then added and the mixture was freeze-dried to yield *B. amyloliquefaciens* AS385 culture broth powder (CBP). The amount of added dextrin was adjusted to equal the solid content in the filtrate, thereby yielding the 50% dextrin content in CBP. The concentration of DNJ in CBP was found to be 1%, determined using hydrophilic interaction liquid chromatography with hybrid quadrupole/linear ion trap tandem mass spectrometry (HILIC-MS/MS) based on the method similar to determine the DNJ distribution in organs.

Animals and experimental protocols. Six-week old male C57BL/6J mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). After 1-wk acclimatization period, mice were randomly assigned to two groups: normal control diet (control group, n=8) and diet supplemented with 8 g CBP/kg diet (CBP group, n=8), equivalent to 80 mg DNJ/kg diet. The mice were group-housed (four mice per cage) and placed in the animal experimental room with 12 h light/dark cycle and free access to diet and distilled water for 5 wk. The temperature and relative humidity in the experimental room were maintained at 22˚C and 43% respectively. The diet composition was shown in Table 1. Feed intake and body weight were recorded twice a week throughout the experimental period. At the end of experimental period, mice were fasted overnight (12 h). Prior to dissection, mice were anesthetized with isoflurane and blood was collected in heparinized tube after decapitation. Liver, kidney, intestine, lung, heart, brain, spleen, pancreas, epididymal white adipose tissue (WAT), retroperitoneal WAT, mesenteric WAT were harvested, weighed and stored in −80˚C freezer until use. Plasma was obtained by centrifugation of collected blood at 1,000 ×g for 10 min at 4˚C, then stored at −80˚C until use. The animal study was performed according to the protocols approved by Institutional Committee for Use and Care of Laboratory Animals of Tohoku University, which were granted by the Tohoku University Ethics Review Board (Permission number: 2017AGA-043).

**Table 1. Composition of control and CBP group diet.**

| Ingredient                        | Control group (AIN93G) | CBP group (modified from AIN93G) |
|-----------------------------------|------------------------|----------------------------------|
| Cornstarch                        | 397.486                | 389.486                          |
| Casein (≥85% protein)             | 200.000                | 200.000                          |
| Dextrinized cornstarch (90–94% tetrasaccharides) | 132.000                | 132.000                          |
| Sucrose                           | 100.000                | 100.000                          |
| Soybean oil                       | 70.000                 | 70.000                           |
| Fiber                             | 50.000                 | 50.000                           |
| Mineral mix (AIN-9 3G-MX)         | 35.000                 | 35.000                           |
| Vitamin mix (AIN-93-VX)           | 10.000                 | 10.000                           |
| L-Cystine                         | 3.000                  | 3.000                            |
| Choline bitartrate                | 2.500                  | 2.500                            |
| tert-Butylhydroquinone            | 0.014                  | 0.014                            |
| Culture broth powder (CBP)        | —                      | 8.000                            |

Oral glucose tolerance test (OGTT). After 4 wk of experiment, four animals from each group underwent an oral glucose tolerance test. After overnight fasting (12 h), mice were orally administered glucose solution (2 g/kg body weight). Blood was collected from tail vein after 0, 15, 30, 60, 120, and 180 min of glucose loading and blood glucose level was measured using glucose meter StatStrip Xpress 900 (Nova Biomedical, Tokyo, Japan). The area under curve (AUC) of blood glucose levels over 180 min was calculated by trapezoid method.

Plasma biochemical parameters. By the end of the study, plasma from each group was collected and was
Examined for various biochemical parameters. Plasma triacylglycerol (TG), total cholesterol (TC) and phospholipid (PL) were measured using the commercial enzyme kits (Wako Pure Chemical Industries, Ltd., Tokyo, Japan). Plasma glucose and insulin levels were measured using Wako Glucose CII-Test Kit (Wako Pure Chemical Industries, Ltd.) and Mouse Insulin ELISA Kit (Morinaga Institute of Biological Science, Yokohama, Japan), respectively.

Hepatic lipid parameters. Liver homogenates (20%, w/v) were prepared using 0.9% saline solution containing 1 mM ethylenediamine tetracetic acid. Total lipids were extracted from liver homogenates according to method by Folch et al. (23–25). Hepatic TG and TC levels were measured using commercial enzyme kits (Wako Pure Chemical Industries, Ltd.) and hepatic PL levels were determined using the method of Rouser et al. (24, 26).

Distribution of DNJ in organs. To prepare the organ extract, 100 mg of organ was homogenized with 1 mL of saline water. The organ homogenate (500 μL) was mixed with acetonitrile containing 0.1% of formic acid (500 μL), sonicated for 1 min and vortexed for 30 s. Next, the mixture was centrifuged at 1,600 × g for 15 min at 4 °C and the supernatant was collected. The column was evaporated using spin dryer (42 °C, 1,500 rpm) and reconstituted in 80% acetonitrile solution (200 μL). DNJ concentration in the organ extract was measured using HILIC-MS/MS. In short, an aliquot of 10 μL was subjected to HILIC-MS/MS consisting of ExionLC HPLC/ UHPLC system and a 4000 QTRAP tandem mass spectrometer (SCIEX; Tokyo, Japan). Chromatographic separations were performed on a HILIC column (TSK gel Amide-80, 5 μm, 4.6 mm × 150 mm; Tosoh, Tokyo, Japan) at a flow rate of 0.2 mL/min with the temperature maintained at 40 °C. The mobile phase consisted of acetonitrile (mobile phase A) and distilled water (mobile phase B), both containing 0.1% formic acid. Gradient elution began at 20% mobile phase B, linearly increased to 60% in 2 min, held constant for 3.5 min, then linearly decreased to initial concentration after 0.1 min and equilibrated for 4.4 min. DNJ was detected using MS/MS under positive ion modes with multiple reaction monitoring for the transition of the parent ion to the product ion (27, 28).

Statistical analysis. Data are shown as mean±SE. Differences between groups were assessed using Student’s t-test for all parameters, except for body weight data that was assessed using two-factor repeated measure analysis of variance (ANOVA) followed by Bonferroni post-hoc test. All statistical analyses were performed with IBM-SPSS statistics version 25 (IBM SPSS Inc., Chicago, IL, United States). p value less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

As the result of the fermentation process, culture medium of B. amyloliquefaciens AS385 contains various compounds that contributed to its deep brown color and strong odor. As we are aiming to apply microbial DNJ in commercial food products, it is necessary to produce microbial DNJ with acceptable sensory properties and DNJ content. Ion exchange resin has been employed for purification of fermentation products, such as lactic acid (29, 30), peptides, and succinic acid (31, 32). In this study, the B. amyloliquefaciens AS385 culture medium containing DNJ was mixed with the anion exchange resin to remove undesired compounds, decolorize and deodorize the culture medium, thus improving its physical properties while maintaining the DNJ content. Using this method, we were able to prepare CBP containing 1% DNJ.

Five-week supplementation of dietary CBP did not induce any changes in body weight and food intake of C57BL/6J mice (Fig. 1A and B), which confirmed that any discrepancies in the observed parameters between control and CBP group were most likely resulted from

(continued)
the CBP intake rather than the difference in energy intake. CBP supplementation in diet tended to suppress the elevation of blood glucose level after an oral glucose load in OGTT, as indicated by lower peak of blood glucose level and area under curve (Fig. 2A and 2B). We found that CBP intake markedly reduced the plasma glucose level (Fig. 3A), while also induced the tendency to lower plasma insulin level (Fig. 3B). Recent studies have acknowledged the direct involvement of postprandial hyperglycemia in the development and pathogenesis of non-insulin-dependent (type 2) diabetes (33, 34) and since then, regulation and restoration of postprandial blood glucose response is considered vital in the treatment and prevention of type 2 diabetes. One of the therapeutic approaches to control the elevation of postprandial blood glucose is to reduce the absorption of glucose to bloodstream (35, 36), which can be achieved using α-glucosidase inhibitor. α-Glucosidase is one of the glucosidases located in the small intestine brush border and is a key enzyme in carbohydrate digestion. α-Glucosidase inhibitors block the activity of α-glucosidase in the small intestine, thus limit the conversion rate of oligosaccharides and disaccharides into monosaccharides, which necessary for absorption into the bloodstream (37). DNJ is as a potent inhibitor of α-glucosidase (1–3) and has demonstrated anti-hyperglycemic effect by delaying intestinal glucose absorption and accelerating hepatic glucose metabolism in study using streptozotocin-induced diabetic mice (38, 39). In the previous study, we found that single oral administration of DNJ-enriched mulberry leaves powder significantly suppressed the elevation of postprandial blood glucose in human (3). In the present study, we detected considerable amount of DNJ in the intestine, suggesting that the α-glucosidase inhibitory activity would be retained even after 12 h of fasting. Based on this finding and putting into consideration the role of α-glucosidase inhibitor in regulation of blood glucose profile, it is highly possible that DNJ content in CBP was accountable for the changes in blood glucose profile in the current study. Even so, we cannot dismiss the possibility that these changes may have occurred due to improvement of insulin sensitivity, as shown by the tendency of CBP group to have lower fasting plasma insulin (Fig. 3B). Previous study has also demonstrated the improvement of insulin resistance improvement of insulin resistance in ob/ob mice after intravenous administration of DNJ (40). There was however, notable difference in the significance level between the fasting blood glucose level (minute 0) during the OGTT (Fig. 2) and the fasting plasma glucose level (Fig. 3A). Firstly, it is important to take into consideration the effect of anesthesia on the changes of blood glucose level. Previously, Zuurbier et al. reported that using isoflurane as anesthetic induced hyperglycemia (41) and Constantinides et al. reported the significant increase of blood glucose level in C57BL/6J mice after inhalation of 1.5% and 2% isoflurane which may be associated with vasodilatory and increased blood-flow effects that also occurred during the study (42). In our study, isoflurane was used as
the anesthesia prior to the dissection and, therefore will affect the measurement of fasting plasma glucose. The measurement of blood glucose during the OGTT however, were carried out without any anesthesia, thus may explain the difference in both results.

In this study, CBP consumption induced significant loss of total WAT mass (Fig. 4). There are no significant changes, however, in plasma and hepatic lipid parameters (Supplemental Online Material, Fig. S1A–F). DNJ has been reported to exert anti-hyperlipidemic effect through regulation of adipokines. Intake of mulberry leaves DNJ was found to prevent diet-induced obesity by promoting an increase in plasma adiponectin and activating the β-oxidation in the liver (7, 8). DNJ also exhibited anorexigenic effect through modulation of leptin signaling in hypothalamus (43) and DNJ from mulberry leaves was found to decrease plasma leptin level in human study (44). Both leptin and adiponectin are adipokines that are exclusively produced by adipocyte tissues and assists in regulation of numerous endocrine functions in energy metabolism (45). The increasing level of adiponectin in obese subjects accompanied by declining level of endogenous leptin are commonly linked to suppression of lipogenesis and stimulation of lipolysis (46–48), which would explain the substantial loss of WAT that occurred in the present study.

While we were able to confirm the DNJ (1%) and dextrin content (50%) in the CBP, we were unable to specify the composition of other constituents in CBP. However, we predicted the presence of some intermediates from DNJ biosynthetic pathway, such as nojirimycin (NJ) and 2-amino-2-deoxy-β-D-mannitol (ADM) (49). NJ has been identified as iminosugar and is capable to exhibit an α-glucosidase inhibitory activity (50). Putting these factors into consideration, it is also necessary to investigate the contribution of the non-DNJ components in CBP towards the physiological changes that occur during the present study. In a separate study, we compared the effect of high purity DNJ (> 95% purity) and CBP containing equivalent amount of DNJ towards high-fat diet-induced impaired glucose tolerance and found that both treatments were able to suppress the elevation of blood glucose level in similar trend with no significant difference between both groups (unpublished data), thus confirming the role of DNJ as the major bioactive component in CBP with least or no effect from the non-DNJ components.

This paper also provides significant evidence that DNJ, the constituent of interest in CBP, is absorbed from the digestive tract and widely distributed into various organs of mouse. Almost all organs contained detectable amount of DNJ (Table 2) after 5-wk intake of DNJ-containing CBP, with concentration ranging from 0.7–119 ng/g. We found substantial disposition of intact DNJ in intestine, kidney, liver, brain, lung and epididymal WAT with highest level of DNJ in intestine (119.0 ± 37.6 ng/g), followed by kidney (102.7 ± 16.7 ng/g) and liver (63.2 ± 10.6 ng/g). Moderate amount of DNJ were detected in the brain (17.0 ± 2.5 ng/g), lung (17.0 ± 3.8 ng/g), mesenteric WAT (7.5 ± 3.7 ng/g), epididymal WAT (14.2 ± 4.2 ng/g), spleen (9.3 ± 2.4 ng/g) and heart (4.2 ± 1.0 ng/g), while only trace amount of DNJ can be detected in retroperitoneal WAT and pancreas. Meanwhile, DNJ was not detected in the organs of mice in the control group. Kidney and intestine have been identified as the major targets of DNJ and accordingly, the synergistic effects that arise as the result of DNJ activity within these major targeted organs are presumably responsible for the therapeutic properties of DNJ, especially in regard to delaying the onset and pathogenesis of type 2 diabetes and its complications. In the animal study using streptozotocin-induced diabetic rats, DNJ consumption ameliorates diabetic nephropathy, a widely known diabetic complication that is characterized by the loss of kidney function (22). While the responsible mechanism behind this effect has not been investigated yet, it is highly possible that the uptake of DNJ by kidney induce the recovery process of damaged renal cells. In addition, renal excretion has been regarded as the major route of DNJ elimination from the body (15, 27), and therefore might as well explain the substantial accumulation of DNJ in the kidney. Interestingly, the accumulation of DNJ in brain was detected after daily consumption of CPB-supplemented diet. To the best of our knowledge, our study is the first that reveals the localization of DNJ in brain after daily consumption. This finding suggests the DNJ ability to cross the blood-brain barrier (BBB), a highly selective semipermeable membrane that regulates the transport of substances into and out of the brain. In the meantime, only small information can be gained concerning the effect of dietary DNJ on brain region. One of the notable studies by Chen et al. (51) has shown the amelioration of the age-related behavioral decline in SAMP8 mice after long-term DNJ supplementation in drinking water (10 and 20 mg/kg body weight/d). While this study linked the elevation of hippocampal insulin receptor level after DNJ intake with the improvement of brain degeneration, the underlying mechanism for this alteration has

| Organ                  | DNJ concentration (ng/g organ) |
|------------------------|--------------------------------|
| Intestine              | 119.0 ± 37.6                   |
| Kidney                 | 102.7 ± 16.7                   |
| Liver                  | 63.2 ± 10.6                    |
| Brain                  | 17.0 ± 2.5                     |
| Lung                   | 17.0 ± 3.8                     |
| Spleen                 | 9.3 ± 2.4                      |
| Heart                  | 4.2 ± 1.0                      |
| Pancreas               | trace                          |
| Mesenteric WAT         | 7.5 ± 3.7                      |
| Epididymal WAT         | 14.2 ± 4.2                     |
| Retroperitoneal WAT    | trace                          |

DNJ, 1-deoxynojirimycin; CBP, culture broth powder; WAT, white adipose tissue. Values are expressed as mean ± SE, n=8.
remained unknown. The ability of DNJ to be distributed into brain may offer a reasonable explanation on how this alteration can occur in the first place.

In conclusion, this study demonstrated that diet-supplemented with CBP reduces the fasting blood glucose level and WAT mass in C57BL/6J mice. Furthermore, after daily intake of DNJ-containing CBP for 5 wk, DNJ supplemented with CBP reduces the fasting blood glucose.

**Acknowledgments**

This work was supported in part by JSBBA Innovative Research Program Award (for KN) and KAKENHI (Grant Number 16K07750 to TK) of Japan Society for the Promotion of Science, Japan.

**Supporting Information**

Supplemental Online Material is available on J-STAGE.

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