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Soy yoghurts produced with efficient GABA (γ-aminobutyric acid)-producing *Lactiplantibacillus plantarum* ameliorate hyperglycaemia and re-establish gut microbiota in streptozotocin (STZ)-induced diabetic mice†

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Soy yogurt has been gaining popularity as a vegan food produced simply by soymilk fermentation with proper microbial manipulation. It is well known that soy containing rich isoflavones is beneficial for ameliorating hyperglycaemic disorders. Soy fermentation can improve the bioavailability of these precious nutrients. *Lactiplantibacillus plantarum* is one of the most abundant and frequently isolated species in soymilk manufacturing. Soy yogurts produced with efficient GABA (γ-aminobutyric acid)-producing *L. plantarum* and the deglycosylating activity of *L. plantarum* were functionally assessed in a STZ-induced hyperglycaemic mouse model. Hyperglycaemic mice were assigned into groups and treated with daily gavage of either dH_2O, soymilk, soy yoghurts produced with high GABA-producing *L. plantarum GA30* (LPGA30), low GABA-producing *L. plantarum PV30* (LPPV30) or the soy yoghurts fortified with additional 30 mg g^{-1} GABA counterparts (GA + GABA and PV + GABA groups). Except the dH_2O group, all soy yoghurt groups retained body weight with improved glucose homeostasis, glucose tolerance test results and renal tissue integrity, while the soymilk group shows partial benefits. Plasma GABA concentrations in the daily soy yoghurt-supplemented groups (LPGA30 and LPPV30) plateaued at 5 times higher than the average 0.5 μM in dH_2O and soymilk groups, and their GABA-fortified soy yoghurt counterparts (GA + GABA and PV + GABA) groups were accountable for the restored plasma insulin levels. Gut microbiome analysis revealed dysbiosis in STZ-induced hyperglycemic mice of the dH_2O group with breached out facultative anaerobic Proteobacteria over the normal phyla Firmicutes and Bacteroidetes. Restored gut microbiota with transitionally populated Actinobacteria was demonstrated in the LPGA30 group but not in the LPPV30 group. Soy yoghurts produced with efficient GABA-producing *L. plantarum GA30* showed exceptional benefits in modulating gut microbiota with dominant genera of *Enterococcus*, *Lactobacillus* and *Bifidobacterium*, and the presence of some minor beneficial microbial communities including *Akkermansia muciniphila*, *Butyricicoccus pullicaecorum*, *Corynebacterium* spp. and *Adlercreutzia* spp. Efficient GABA-producing *L. plantarum GA30* fermented soymilk to produce soy yoghurts that exhibit profound synergistic protections over rich soy isoflavones to restore pancreatic β-cell functions for insulin production in STZ-induced hyperglycaemic mice. Additionally, the probiotic role of GABA-producing *L. plantarum* in re-establishing healthy gut microbiota in hyperglycaemic mice implies a possible symbiotic relationship, awaiting further exploration.

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Introduction

Soybean consumption is associated with reduced risks of both type I and II diabetes, and it ameliorates diabetic symptoms.[1–3] Soymilk made by boiling soaked and ground soybean and filtering it is a traditional Chinese breakfast soup, and it can be developed into tofu and assorted soy-based products. Soymilk exhibits nutraceutical properties including skin protection, hypocholesterol, antihypertension, anti-dia-

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betes, anticancer and anti-aging properties.[4,5] The anti-diabetic health promoting functions are contributed by phytoestrogenic soy isoflavones including four chemical categories: aglycones, glucosides, acetylglucosides, and malonylglucosides.[6] Among these, aglycone genistein is well demonstrated to promote pancreatic β-cell turnover *via* the cAMP/PKA signalling pathway.[7] A higher therapeutic potential of daidzein has also been addressed.[8] Nevertheless, abundant soy isoflavones cannot be utilized directly as they naturally occur as glycosidic conjugates, often retained in soybean residues, and proper manipulated microbial fermentation can dramatically increase their bioavailability.[9] Furthermore, the glycosidic moiety of soy oligosaccharides resembles prebiotic constitutions. It has been demonstrated that soy oligosaccharides improve gut microbiota and boost immunity.[10]

Soy yoghurts are a novel soy-based vegan product gaining popularity nowadays. It is produced solely by soymilk fermentation with selected lactic acid bacteria. Acidification by microbial anaerobic metabolism assists in solidification of soymilk into soy yoghurts. Besides, soy fermentation has multiple nutritional benefits such as lower anti-nutrient factors, increased antioxidative capacity, and together with the microbial fermentation process for enteral digestion through gut microbiota it may help to enzymatically hydrolyze glycosidic bonds to optimize the bioavailability of nutrients such as isoflavones. Limited studies with soy yoghurts reported that consumption of soy yoghurts can prevent lipid accumulation in the rat liver.[11] Moreover, *Lactobacillus plantarum*-fermented soy products have been demonstrated to improve lipid homeostasis in rats fed with a high-cholesterol diet.[12] As probiotics, *Lactobacillus* spp. can influence enterobacteria by inhibiting pathogenic *E. coli*, *Salmonella typhimurium* and other Gram-negative bacteria *via* bacteriocin production.[13,14] These studies imply a potential role in gut health.

Insufficient GABA (γ-aminobutyric acid) intake from a regular diet has been cautioned.[15] Nevertheless, GABA can be indigenously acquired from lactic acid bacterial species in the intestinal tract.[16] Indeed, lactic acid bacteria-fermented foods are major exogenous sources of GABA. In addition to its well-known role as a neurochemical, recent findings have supported a key role of GABA in anti-diabetes. GABA exerts protective and regenerative effects on pancreatic β-cell mass.[17] Moreover, Ben-Othman *et al.* (2017)[18] have demonstrated GABA receptor-mediated pancreatic β-cell proliferation with sustained 5 μM to 1 mM GABA treatment in *ex vivo* cultures. In addition, GABA also has autocrine functions in the active regulation of pancreatic α- to β-cell conversion *via* insulin or glucagon secretion to maintain blood glucose homeostasis. We have recently demonstrated the increase in GABA concentration in a fermentation time-dependent manner in a soybean residue substrate fermented with co-inoculation of *Rhizopus oligosporus* and GABA-producing *L. plantarum*, and the fermented product exhibits advantages in reducing intracellular reactive oxygen species of canine kidney cells under high-glucose culture conditions, and improving glucose homeostasis in STZ-induced hyperglycaemic mice when supplemented daily.[9] Hence, a stable and sufficient GABA intake *via* exogenous fermented food sources or *via* prebiotics mediating the enhancement of indigenous gut microbial sources may pose a critical health impact.

Nevertheless, the significance of GABA uptake through fermented soy products awaits exploration, and the effects of soy fermented *via* microbial GABA on blood glucose homeostasis and modulating health gut microbiota are still not well understood. In the present study, the health promoting implications of the functional soy yoghurt produced by soymilk fermentation with selected *Lactiplantibacillus plantarum* spp. showing high efficiency in converting glutamate to GABA and deglycosylating activity were therefore investigated in the STZ-induced hyperglycaemic mouse model.

Materials and methods

Sources of *Lactiplantibacillus plantarum* strains and GABA synthesis

An efficient GABA producer, *L. plantarum GA30*, and a low producer, *L. plantarum PV30* strains, were isolated from traditional Chinese tofu manufacture. Both isolates were routinely cultured in a soya peptone–based medium (CAS Number 91079-38-8. Sigma, MO, USA) for laboratory characterization tests, and have been officially deposited in the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan, ROC). The *L. plantarum* strain BCRC 10069 was purchased as a referenced standard for the comparison and setting of optimal fermentation conditions to produce GABA from the two isolated strains.[19] The basal culture medium (specified as recommended by BCRC, Lactobacilli MRS broth, DIFCO 0881) was composed of initial 500 mM sodium L-glutamate for evaluating the GABA-producing efficacy. The analysis of GABA and dialysis purification are described in a later section. Purified and lyophilized bacterial produced GABA was used as the addition for the succeeding dietary GABA-fortified soy yoghurt groups.

Soy yoghurt fermentation

Soymilk was purchased from a commercial tofu producer (Chiayi, Taiwan), sterilized and subsequently cooled to room temperature under hygienic conditions. A near exponential-phase bacterial culture (10^9 CFU mL^{-1}) of *L. plantarum* strain was added to soymilk at a 1% (v/v) ratio in a 5 L anaerobic fermenter at 37 °C for 48 hours. During the subsequent overnight cooling process, the final fermented soymilk acidified spontaneously into coagulated soy yoghurts. For later animal experimentation, an aliquot of yoghurt was then freeze-dried and stored in a −80 °C freezer until the preparation for animal study.

Analysis of isoflavones in soy yoghurts

Isoflavones contents were detected by high-performance liquid chromatography (HPLC) according to our previous method.[9] Briefly, an aliquot of sample was weighed and placed in a Falcon tube for methanol extraction. The supernatant was collected after centrifugation. HPLC was performed using a Li Chrospher 100RP-18e column (4 mm × 250 mm, 5 μm), a mobile phase of 0.05% TFA : CH_3CN = 95 : 5_gradiant_60 : 66

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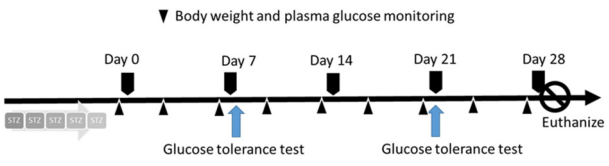
mm, and a UV detector of 280 nm, and the temperature was set at 40 °C. The specific setting of a reversed-phase column for a run time of 60 min at a flow rate 1.0 mL min^{-1} could detect peaks of current isoflavones. Internal standards were used upon assurance check. The quantification of specific peaks of the area under the curve was performed against a previously established individual chemical calibration curve. Six isoflavones, namely, daidzin, glycitin, genistin, daidzein, glycitein, and genistein were purchased from (Sigma, St Louis, MO, USA). The limit of detection (LOD) and limit of quantitation (LOQ) were evaluated by serial dilution of six standard solutions and then analysed by HPLC. The LOD and LOQ were 0.0305 μg mL^{-1} and 0.1221 μg mL^{-1}, respectively.

Animal experimentation

Animal experimentation protocols conducted in the current study were approved by the Institutional of Animal Care and Use Committee, National Chiayi University, Chiayi, Taiwan, ROC with approval no. 103015. Eight-week-old male C57/BL6 mice were purchased from the National Laboratory Animal Centre, Taiwan, ROC and housed in an environmental controlled, specific pathogen-free facility. Animals were ear-tagged with labels and fed *ad libitum* with a basal diet (LabDiet 5001, USA) and distilled water. The body weight was monitored twice a week throughout the entire study.

STZ-induced hyperglycaemia mouse model and treatment groups

Hyperglycaemia was induced and determined according to the descriptions by Hariyanto *et al.* (2022).[9] Briefly, intraperitoneal injection of a low-dose (40 mg per kg BW) streptozotocin (STZ; 10 mM sodium citrate, pH = 4.5) for 5 consecutive days was performed. Fasting blood glucose levels in successfully induced hyperglycaemic mice were determined to be not less than 250 mg dL^{-1}. Thirty hyperglycaemic mice were then randomly assigned into six experimental groups. Treatments and sampling schedules are illustrated in Scheme 1. All animals were daily gavage fed 100 μL dose in the morning for the entire experiment period. Except the mock control group was gavage 100 μL dH_2O only, the treatment groups were daily gavage fed 100 μL of 200 mg per kg BW soymilk, soy yoghurt produced with the efficient GABA producing *L. plantarum GA30* (LPGA30 group), low GABA producing *L. plantarum PV30* (LPPV30 group), and the respective soy yoghurts fortified with additional 30 mg g^{-1} (w/w at dry basis) GABA, the GA + GABA group and the PV + GABA group.



Treatment Groups	Soy yogurt produced with	GABA	Description
dH2O	–	–	Mock control
Soymilk	–	–	Vehicle
LPGA30	L. plantarum GA30	–	Efficient GABA producer
GA + GABA	L. plantarum GA30	30 mg/g soymilk	GABA fortification
LPPV30	L. Plantarum PV30	–	Poor GABA producer
PV + GABA	L. Plantarum PV30	30 mg/g soymilk	GABA fortification

Scheme 1 The experiment time line and treatment groups

Plasma glucose level measurement

About 50–70 μL blood sample was venepuncture collected twice a week *via* rotating side of saphenous veins using heparinized capillary tubes (Chase, no. 2501, USA). Plasma was obtained after centrifugation (Hettich Mikro 20 Centrifuge, Hettich, Germany). The plasma glucose level was immediately determined using a blood glucose monitoring system (Ascensia Elite® XL, Bayer, No. 1502, Basel, Switzerland).

Determination of γ-aminobutyric acid (GABA)

The measurement of GABA concentrations was modified from previous methods.[9,20] An aliquot of plasma sample was pre-diluted 10-folds with PBS. Subsequently, dialysis of the diluted solution was performed using a high-performance dialysis membrane (MWCO3500, Servapor®, Kelowna International Scientific Inc., Taiwan) for 5 h according to the manufacturer's recommendation. The dialyzed solution was then reacted with *O*-phthalaldehyde (OPA, Sigma) to measure GABA. A 50 μL sample was used for HPLC analysis. The LOD and LOQ were evaluated by serial dilution of the standard. The LOD and LOQ were 33.7 nM and 112.6 nM, respectively.

Glucose tolerance test

The glucose tolerance tests were performed at week 1 and week 3 according to the previously reported method.[9,21] Mice were fasted overnight and administered a 100 μL single peritoneal injection of glucose solution (0.5 g per kg BW). Immediately, a drop of blood was collected as described previously, and the glucose level was measured at 0, 30, 60, 90 and 120 min after the glucose administration. The glucose level was immediately determined using a blood glucose monitoring system (Ascensia Elite® XL, Bayer, No. 1502, Basel, Switzerland).

Insulin measurement

The blood samples collected from a saphenous vein at day 7 and from heart puncture at the euthanasia endpoint at day 28 were measured for the plasma insulin level using an ELISA kit (Mercodia mouse insulin cat#10-1247-01, USA). Briefly, 10 μL plasma was added into an antibody-coated 96-well plate. ELISA procedures were conducted following the manufacturer's recommendations. Colorimetric analysis of the HRP-TMB reaction was performed at OD450 nm using an ELISA reader (Biotek Instruments, USA).

Metagenomics analysis of the microbiome in the intestine

Approximately 2 cm intestine was aseptically collected and immediately wrapped in an aluminium foil, and then preserved in a −80 °C freezer until further analysis. For micro-

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biota analysis, the defrost sample was flushed internal lumen with 2 mL of PBS to collect lavage. Three samples were pooled from mice of the same group. They were the STZ-induced diabetic mice in the dH_2O, LPGA30 and LPPV30 groups. The gut microbiome analysis were conducted by the Qiagen Co., Taiwan, ROC. Briefly, the genomic DNA of the pooled lavage sample was extracted using genomic DNA extraction kits, and profiling of the intestinal microbes was performed using next-generation sequencing technology (QIAseq and GeneRead Library Prep Kits, Qiagen, USA). The gut microbiome data of normal mice were adopted from a sibling experiment performed under the same condition.

Renal histopathological examination

Kidneys were collected and immediately soaked in 10% formalin for three days and then changed to 10% formalin with 4% formaldehyde solution for 5 hours prior to paraffin embedding. Vertical cross sections of paraffin-embedded kidney tissues were then de-waxed and stained with haematoxylin and eosin according to previous methods.[22] Microscopic assessments of the integrity of renal glomerular units and the features of chronic tubule-interstitial damage were performed using a CCD-equipped microscope.

Statistical analysis

Data are presented in the table, and graphical figures are expressed as mean ± standard deviation. Data were analysed by two-way ANOVA using the Prism, GraphPad Software, San Diego, CA, USA. Otherwise, treatment comparisons were performed by MS Excel Student's *t*-test. The significance between treatments was expressed as asterisk labels at significance level $\alpha = 0.05$.

Results and discussion

Soymilk fermented with selected *Lactiplantibacillus plantarum* strains to enriched isoflavone aglycones and GABA

Lactic acid bacteria are common sources of probiotics present in various fermented food. Some of them naturally produce GABA, while some are efficiently catalysing glycosylated phenolic compounds such as liberating glucosidic isoflavones into their aglycones. In Table 1, the glycosylated and deglycosylated isoflavones, total isoflavones, and GABA contents of soymilk and soy yoghurts produced by various *L. plantarum* strains are shown. Majority of isoflavones are naturally bound with polysaccharides, which limited their bioavailability. In soymilk, the sub-total of glycosylated isoflavones (3985 ± 7.6 μg g^{-1}) exceeded 6 times higher than aglycones (609 ± 8.1 μg g^{-1}). Fermentation with *L. plantarum* strains of either LPGA30, LPPV30 or BCRC10069 has obviously increased concentrations of isoflavone aglycones – daidzein, glycitein, and genistein, while reduced glycosylated isoflavones – daidzin, glycitin, and genistein. Nevertheless, the efficient GABA-producing strain LPGA30 is relatively inefficient as compared to the other. Moreover, slight reduction on the total isoflavones could be attributed to biodegradation during fermentation for 48 hours. Oligosaccharide-conjugated soy isoflavone utilization (absorption) relies primarily on microbial hydrolysable enzymes such as β-glucuronidase to free up into aglycones. It has been reported[23] that daidzin or genistin in human plasma post a soymilk ingestion was not detected by sensitive mass spectrometry; in contrast, daidzein and genistein peaked in plasma within an hour and remained at a high concentration for 12 hours. Similarly, when fermented soymilk was fed to mice, a preferential plasma uptake of daidzein and genistein was evident in the current mouse model, while the deglycosylated form glycitein was not detected (ESI data† with HPLC chromatography).

GABA is mainly dietary available from fermented food and also readily synthesized by GABA-producing microbes in the gut. It is drawing attention to the bioavailability of pre- and probiotic GABA sources in related health implications. Soymilk fermentation for 48 h had differentially resulted in the elevations of GABA content at the dry basis levels of 460 ± 2.8 μg g^{-1}, 530 ± 4.4 μg g^{-1}, and 67 ± 2.1 μg g^{-1} by the *L. plantarum* strains BCRC10069, GA30, and PV30 respectively (Table 1). In addition, GABA was not detectable in soymilk. Recent studies[24,25] have also demonstrated using *L. plantarum* spp. in producing GABA-rich products under manipulated culture conditions and a traditional fermented food practice. In dairy yoghurts, under optimized conditions (inclusion of L-monosodium glutamate and pyridoxal-5-phosphate), *L. plantarum NDC75017* had resulted in a high GABA content of 315 mg per 100 g milk.[26] Currently, the *L. plantarum strains*

Table 1 Changes of glycosylated, deglycosylated isoflavones, and GABA contents in soymilk and soy yoghurts produced by soymilk fermentation with selected *L. plantarum* strains

	Glycosylated isoflavones (μg g^{-1})				Aglycones/deglycosylated isoflavones (μg g^{-1})						
Category	Daidzin	Glycitin	Genistin	Sub Total	Daidzein	Glycitein	Genistein	Sub total	Total isoflavones (μg g^{-1})	GABA (μg g^{-1})	Final Bacteria counts (10^8 CFU g^{-1})
Soymilk	1501 ± 2.5	202 ± 1.4	2282 ± 3.8	3985 ± 7.6	310 ± 6.7	25 ± 2.1	227 ± 4.2	609 ± 8.1	4319 ± 11.2	ND[a]	ND
Soy yoghurt											
LPGA30	1329 ± 4.1	209 ± 1.7	2058 ± 3.1	3595 ± 7.4	481 ± 3.6	101 ± 1.5	474 ± 2.3	1055 ± 4.7	4650 ± 11.7	530 ± 4.4	81 ± 2.3
LPPV30	**ND**	**157 ± 3.0**	**36 ± 0.7**	**193 ± 2.4**	**1397 ± 5.3**	**55 ± 1.6**	**1826 ± 3.4**	**3279 ± 1.3**	**3472 ± 1.1**	**67 ± 2.1**	**63 ± 2.1**
BCRC											
10 069	ND	ND	ND	NA[b]	1367 ± 1.7	16 ± 0.6	1815 ± 4.5	3198 ± 5.8	3198 ± 5.8	460 ± 2.8	76 ± 0.6

[a] ND = not detectable. [b] NA = not available; data are presented as unit of w/w at dry basis, and in mean ± SD, $n = 3$.

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GA30 and *PV30* were selected divergently exhibiting efficient GABA production or deglycosylation. Our previous results have demonstrated that the maximized conversion of glutamate into GABA by the selected *L. plantarum GA30* for 48 h had reached a final output of 8 log CFU mL^{-1} bacteria and over 5000 mM GABA production.[19] Under the current culture condition, soymilk fermentation with *L. plantarum GA30* produces (53 mg per 100 g fresh soy yoghurt) near 8 times higher GABA than the *L. plantarum PV30*, even though the dairy yoghurt is still superior to the vegan soy yoghurt. Nevertheless, *L. plantarum PV30* demonstrates a better efficiency in isoflavone deglycosylation than the *L. plantarum GA30* by contributing 3 times more isoflavone aglycones for fermentation. Tamang *et al.* (2016)[27] has reported that the protective functions of soymilk and its fermented products can be associated with the multiple practical properties of fermented food by enhancing nutrient utilization, promoting appetite, elevating antioxidant activity, and fortifying the gut microbiome. Previously, we have also demonstrated co-inoculating *Rhizopus oligosporus* and GABA-producing *Lactiplantibacillus plantarum* in soybean residues derived from soymilk manufacture can maximize the use of residual isoflavones, and health function-value added for recycling.[9] The current results indicated that soymilk fermented with the selected *L. plantarum* strains exhibited efficient production of GABA or increase in isoflavone aglycones contents. The anti-hyperglycaemic properties of the two soy yoghurts produced with selected *L. plantarum* spp. divergent exhibiting efficient GABA producing or deglycosylation deserve further investigations.

GABA-enriched soy yoghurts ameliorated disorders in STZ-induced hyperglycaemic mice

The impact of dysregulated glucose utilization can lead to oxidative damage, chronic inflammation and body weight loss with prolonged hyperglycaemia conditions. As shown in Fig. 1A, the dH_2O group had a body weight loss at the last experimental period (20 to 28 days) under a prolonged hyperglycaemic condition. In general, daily gavage supplementation with soymilk or soy yoghurts prevented the body weight loss, except that the LPPV30 group had reduced weight at the end of the study. Moreover, STZ-induced hyperglycaemia was evidenced in the elevating plasma glucose levels in all groups (Fig. 1B). While daily soymilk supplementation showed moderate benefits in blood glucose homeostasis as compared to the dH_2O group, the soy yoghurt groups and their GABA fortified counterparts had all significantly ($P < 0.05$) lowered the elevating plasma glucose as compared with soymilk and dH_2O groups. Interestingly, extra GABA fortification of the GA + GABA and PV + GABA groups exhibited negligible improvement over the LPGA30 and LPPV30 groups, and statistical significances were obtained. Nevertheless, this result demonstrates the critical role of soy isoflavones in blood glucose homeostasis. Moreover, microbial fermentation process can free up glycosidic phenolic components as well as aid isoflavone bioavailability, raised anti-oxidative status may facilitate first-line protection on the STZ caused oxidative burdens and damages associated with hyperglycaemic conditions.[9] Higher antioxidative status of the LPPV30 group was anticipated since the soy yoghurt produced with *L. plantarum PV30* had 3 times higher aglycones contents than that of *GA30* (Table 1).

Moreover, the glucose tolerance tests performed at week 1 and week 3 demonstrated that a single-dose glucose injection had resulted in blood glucose levels peaking at 30 min in the dH_2O group but not the other groups indicated poor control of blood glucose homeostasis (Fig. 1C). Surprisingly, soymilk supplementation was as effective as seen in the soy yoghurts and their extra GABA-added groups. Nevertheless, a delayed peaked blood glucose level at 60 min in week 1, and a prolonged peaked glucose levels at 30 to 90 min in week 3 of soymilk group implied extra benefits from soymilk fermentation with *L. plantarum* strains. Finally, the protective function was also assessed histopathologically on the renal glomeruli tissue sections, as shown in Fig. 1D. Chronic hyperglycaemia often leads to renal damages. Histopathological examinations on the focal area of renal cortex glomeruli found capsular basement membrane thickening and irregular edges (as indicated in arrow head) in the dH_2O group, and less prominent in the soymilk group as compared to the other treatment groups. Moreover, glomerular blood capillaries were compact and less dilated in the GA + GABA and PV + GABA groups. In general, chronological renal damages were less noticeable in the LPGA30 and LPPV30 groups, and their GABA fortified counterparts GA + GABA and PV + GABA groups seem to have even better protection with no obvious signs of enlarged capsular basement space and irregular edges. This is in agreement with our previous findings on fermented soybean residues enriched with GABA and isoflavone-protected renal integrity.[9] Soy yoghurts produced with selected *L. plantarum* strains demonstrate overall promised outcomes in protection of hyperglycaemic complications, and the presence of synergistic protection of GABA is recognized in addition to soy isoflavone benefits.

Plateau plasma GABA achieved with soy yoghurt supplementation disregarding efficient GABA-producing *L. plantarum* or GABA fortification

Dietary GABA from lactic acid bacteria-fermented food is important to supply ordinary food deficiency.[15] As an inhibitory neurotransmitter, plasma GABA cannot pass through the blood–brain barrier. Plasma GABA is maintained at a steady level in the sub-micromolar range, and is age-independent in responding to inflammation in humans.[28] Importantly, GABA administration has been demonstrated to assist pancreatic β-cell proliferation and downregulated autoimmune T cell-mediated cytotoxicity in islets of a non-obese diabetic mouse model.[29] Furthermore, GABA is present in various peripheral tissues, particularly in mediating pancreatic endocrine functions.[30] It is indeed indigenously synthesized in the pancreas correlated with plasma glucose, and modulating pancreatic α-cell and β-cell conversion.[18] As shown in Fig. 2, STZ-induced hyperglycaemic mice with daily gavage supplementation of 100 μL dH_2O or soymilk had average plasma GABA levels, 0.46 and 0.66 μM respectively, whereas soy yoghurts or GABA-fortified soy yogurt supplementation had significantly elevated

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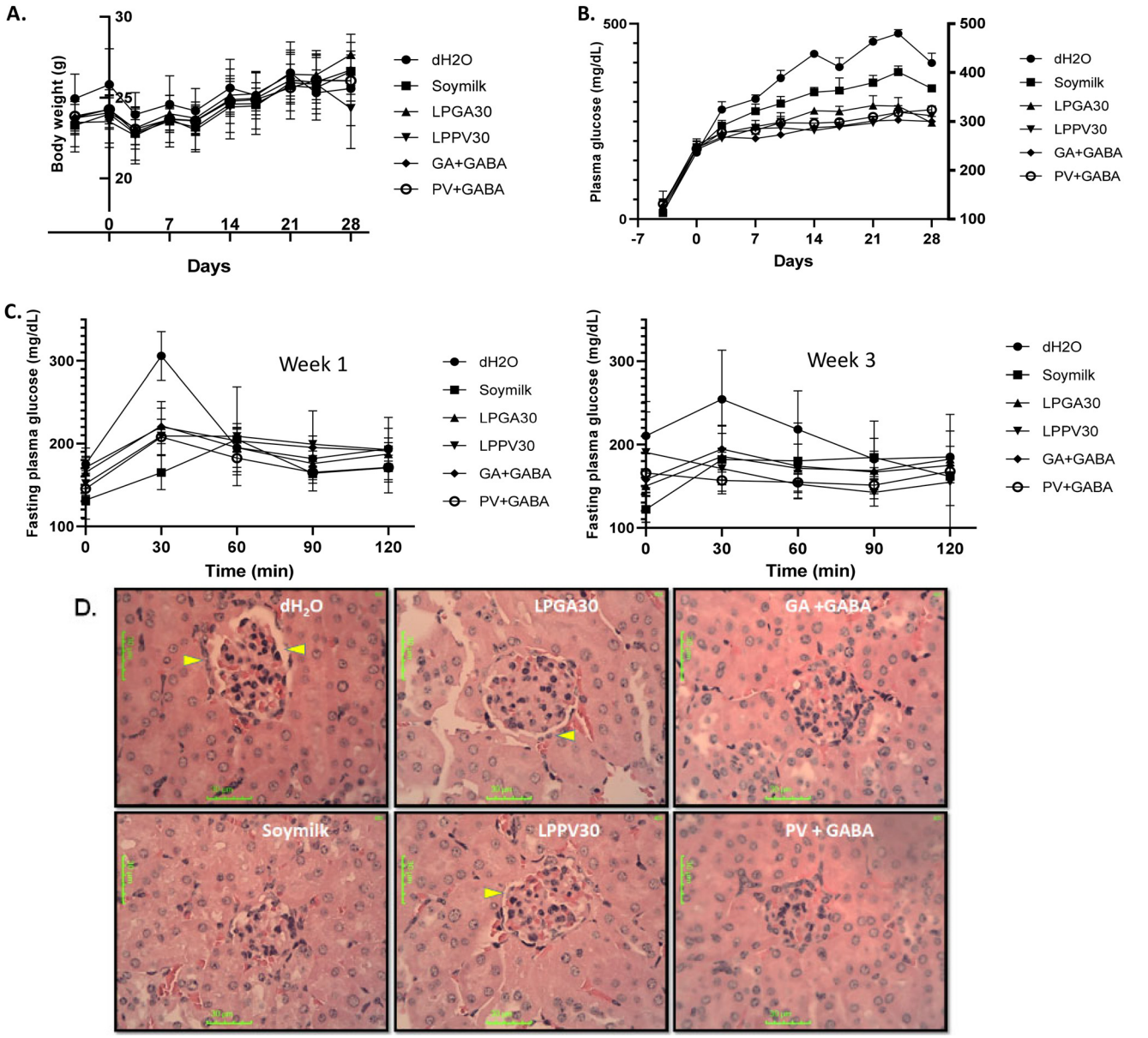

Fig. 1 Assessments on body weight changes (A), plasma glucose levels over entire experiment period (B), glucose tolerance tests at week 1 and week 3, and renal histopathology of glomerulus (D) of STZ-induced hyperglycemic mice daily supplemented with dH_2O, soymilk, soy yogurt groups of LPGA30 and LPPV30, their GABA fortified counterparts GA + GABA and PV + GABA groups.

plasma GABA levels to 5 times (around 2 to 3 μM) higher concentrations. Soy yoghurts produced by the efficient GABA producer *L. plantarum GA30* has only slightly higher plasma GABA levels than the low GABA producer *L. plantarum PV30* ($P > 0.05$). Moreover, the GA + GABA and PV + GABA groups supplied with extra GABA (30 mg g^{-1} soy yoghurt) showed no significant difference in plasma GABA levels in comparison with their soy yoghurt counterparts, the LPGA30 and LPPV30 groups. These results indicate that GABA is scarce in regular diets (mouse feed), and soymilk supplementation has no influence on plasma GABA levels. After daily supplementation for a 4-week experimental period, the soy yoghurt groups and their GABA-fortified counterparts seem to achieve plasma GABA in a plateau concentration. Gerber and Hare (1980)[30] have reported that the average plasma GABA was approximately 0.58 μM in healthy cannulated rodents, which is similar to current findings in mice of STZ-induced pancreatic β-cell destruction. It can be assumed that plasma GABA remains at a low level in normal and hyperglycaemic mice. Hence, the current GABA-enriched soy yoghurt with long-term supplementation may provide an optimal GABA uptake possibly reaching the physiological limitation. Li *et al.* (2015)[31] have reported that plasma GABA showed tightly controlled homeostasis, in correlation with increased circulating insulin levels in healthy human subjects under a pharmacokinetics and pharmacodynamics study. More in-depth studies are

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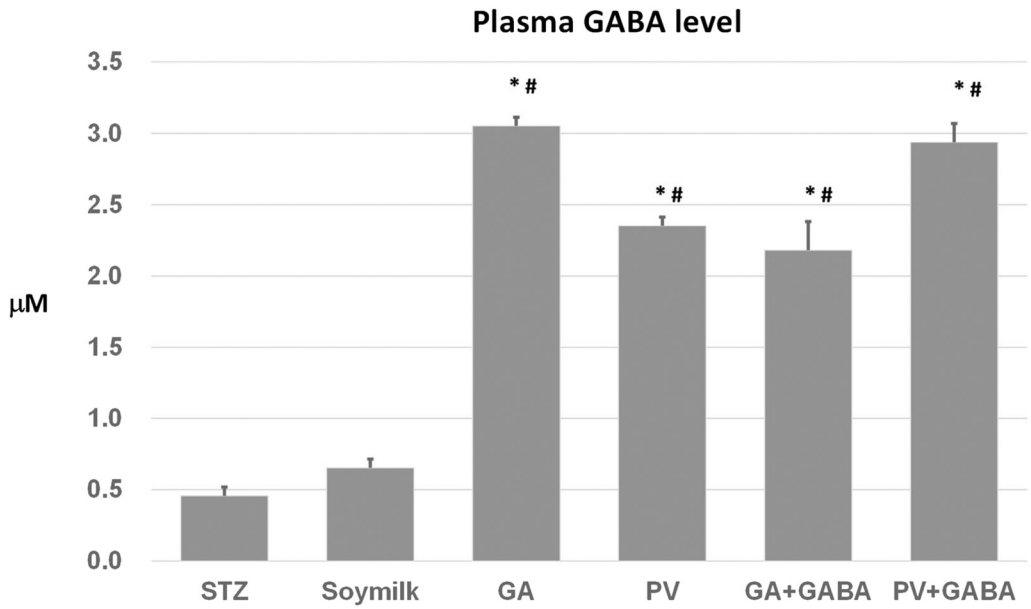

Fig. 2 Plasma GABA levels of STZ-induced hyperglycemic mice supplement with dH_2O, soymilk, soy yogurts and GABA fortified soy yogurts. Significance at $P = 0.05$, where * and # are presented in comparison with dH_2O and soymilk groups, respectively.

required for further elucidate the peripheral GABA uptake and its homeostasis in relating to the steady insulin level.

Nevertheless, it was not expected that soymilk fermentation with low GABA-producing *L. plantarum* spp. could contribute a plateau plasma GABA concentration, particularly, GABA synthesis efficacy of *L. plantarum PV30* is almost 8 times less than that of *L. plantarum GA30 in vitro* (Table 1) It may be easily reasonable to assume that the elevated plasma GABA is highly associated with soymilk fermentation by *L. plantarum* spp. Whether the silent GABA synthesis genes are reactivated through microbial communications or compensatory GABA absorption in the state of hyperglycaemia attributed to the plateau plasma GABA require further elucidation. In addition, the synbiotics (prebiotics + probiotics) property of soy yoghurts produced with the divergently selected *L. plantarum* strains is also of interest to be further investigated. Nonetheless, GABA has been found to exert regenerative effect on pancreatic β-cell mass[17] and in signalling pancreatic β-cell conversion.[32] In the present STZ-induced hyperglycaemic mouse study, GABA is readily taken up into plasma with a daily gavage of 200 mg per kg BW soy yoghurts disregarding the extra GABA (30 mg g^{-1} yoghurt) fortification. The GABA fortification has resulted in limited further augmentation on the plasma GABA level. Whether the plateau plasma GABA level would be critical in anticipating restored pancreatic β-cell functions in insulin production is conferred in followings.

GABA-fortified soy yoghurts best reserve pancreatic β-cell function in insulin production

Plasma insulin levels measured at the end of week 1 and week 4 are shown in Fig. 3. A general declined insulin level in all groups supported the dysregulation of blood glucose attributed to the streptozotocin-induced hyperglycaemia by destructed pancreatic β-cells. A dramatic reduction in blood insulin levels was obvious in the dH_2O group. In the first week, the LPPV30 and PV + GABA groups seem to have better preserved β-cell functions with a higher trend ($P < 0.1$) in insulin production than that of the dH_2O group. Although *L. plantarum PV30* has low efficacy in GABA synthesis, it is an efficient deglycosylator in producing isoflavone aglycones as compared with *L. plantarum GA30*. Glucosidic soy isoflavones are poorly absorbed until their glucose moiety is readily removed during the fermentation process or indigenously with the intestinal microbiota to become free isoflavone aglycones. Hence, liberation of glycosylated phenolic compounds by *L. plantarum PV30* means higher bioavailability of soy isoflavones and sufficient antioxidants to protect β-cells at the very beginning stage of the oxidative damage caused by STZ (streptozotocin). Indeed, intestinal absorption of aglycone isoflavones requires β-glucosidase activities because glycosylated isoflavones are not absorbed across the enterocytes of healthy adults, and intestinal microbiome plays an essential role in isoflavone bioavailability.[23,33] It has also been reported that β-glucosidic linkage in isoflavone glycosides from soymilk can be hydrolysed by β-glucosidase and β-galactosidase produced by *Bifidobacterium* of lactic acid bacterium.[34] Furthermore, plasma insulin levels of all treated groups were noticeably higher than that of the dH_2O group at the end of week 4. Surprisingly, soymilk group had a similar plasma insulin level to the LPGA30 and LPPV30 groups. Since plasma GABA was not affected in the soymilk group (Fig. 2), a prebiotic property of soymilk alone on gut microbiota, and the rich soy isoflavones in mediating protection in pancreatic β-cell insulin production is evidenced, which supports previous results of an intermediated controlling plasma glucose level in the soymilk group (Fig. 1A). Additionally, both GA + GABA and PV + GABA groups have averagely higher plasma insulin concentrations,

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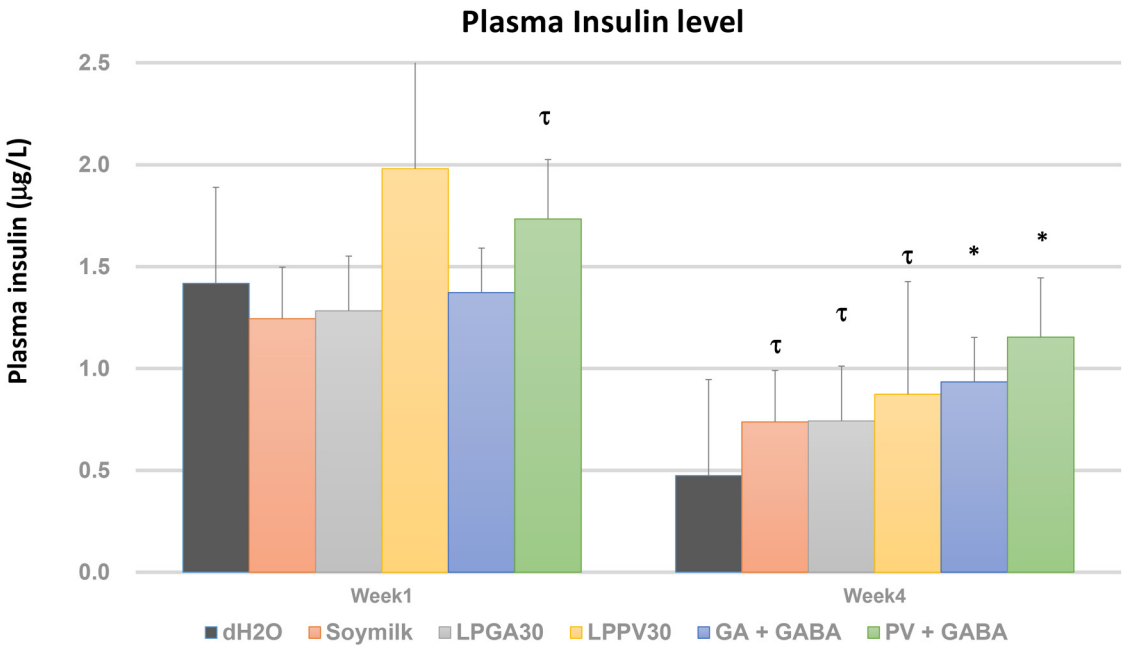

Fig. 3 Insulin levels measured at week 1 and week 4. Significances were obtained in comparison with the dH_2O group at *, $P < 0.05$, and $^{\tau}$, $P < 0.1$.

which are significantly ($P < 0.05$) higher than that of the dH_2O group. GABA derived from soy yoghurts may play a critical role in conferring protective and regenerative effects on pancreatic β-cells. Soltani *et al.*, (2011)[17] revealed GABA can react with the GABA receptor of the α-cells, leading to membrane hyperpolarization in suppressing glucagon secretion; in addition, they demonstrated a therapeutic effect by daily intraperitoneal (20 μmol per mouse) GABA injection continuously for 80 days, showing restored β-cell mass, improved blood glucose homeostasis, and reverted diabetes in STZ-induced diabetic mice. Hence, a long-term steady and sustaining GABA status is crucial to maintain glucose homeostasis and even to cure diabetes. Soy yoghurt supplementation with GABA fortification shall be a promise functional food in improving hyperglycaemic symptoms of diabetes. In conclusion, soy yoghurts produced by soymilk fermentation with selected *L. plantarum* strains being proficient in deglycosylation and GABA synthesis pose synergistic reimbursements in pancreatic β-cell insulin production, ameliorating diabetes.

Implications of re-establishing gut microbiota diversity and abundance in LPGA30 but LPPV30 groups

Gut microbiome analysis revealed the phylogenetic profiles of dominant bacterial populations in the intestine of healthy normal C57/BL6 mice, the STZ-induced hyperglycaemic mice and mice from the LPGA30 and LPPV30 groups supplemented with different soy yogurts produced with *L. plantarum GA30* or *L. plantarum PV30* respectively. Groups which differed at the phylum level of the gut microbiome are shown in Fig. 4. In general, *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* accounted for over 99% of bacteria in current animals fed on the standard LabDiet chow. While phyla *Firmicutes* (90.3%) and *Bacteroidetes* (8.1%) predominated in the normal healthy mice of the housekeeping control colony, the STZ-induced hyperglycaemic mice daily gavage dH_2O (dH_2O group) or the soy yoghurt produced with *L. plantarum PV30* (LPPV30 group) had overpopulated by *Proteobacteria* (94.4% and 98.2%, respectively). Besides, the Firmicutes and *Bacteroidetes* were dramatically decreased to only 5% and 0.3% in the dH_2O group, and 1.4% and 0.4% in the LPPV30 group. Expansion of *Proteobacteria* is a hallmark of gut dysbiosis;[35] changes in oxygen availability and pH levels in the upper intestine mediated by disrupted intestinal cell integrity facilitate an environment for facultative anaerobic Proteobacteria to replace obligated anaerobic Firmicutes. However, daily gavage with the soy yoghurt produced by the efficient GABA producer *L. plantarum GA30* (LPGA30 group) has been demonstrated to rescue the diminished Firmicutes and Bacteroidetes back to 66.2% and 1.2%, respectively. In addition, the Proteobacteria population appeared to be only 0.6% in conjunction with a pronounced abundance of 31.7% Actinobacteria (Fig. 4). Dysbiosis in the gut microbiota can shift host metabolism in lipids and glucose.[36] Indeed, gut microbiota plays an important role in host energy allocations and utilizations, and the dynamic changes of gut microbiota may be reflections of host glucose homeostasis. Nevertheless, Patterson *et al.*, (2015)[37] reported that single high-dose STZ-induced diabetic rats showed a dramatic impact on the gut microbiome for 5 weeks with a shift in the Bacteroidetes/Firmicutes ratio, reduced overall gut microbial diversity, and increases in the proportions of *Lactobacillus* and *Bifidobacterium* genera. The current study demonstrates that hyperglycaemia-induced mice with daily low-dose STZ for 5 consecutive days euthanized after 4 weeks has gut microbiome diversity with dominant genera

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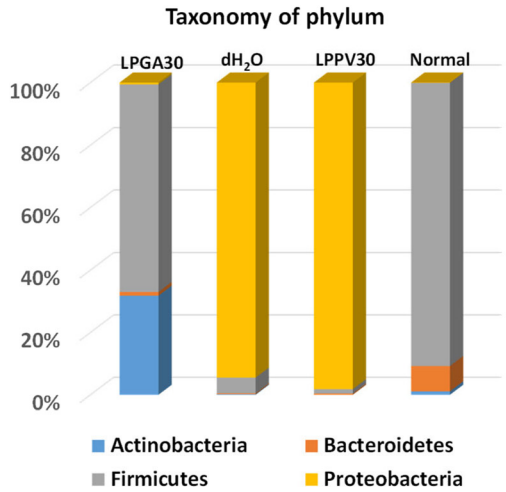
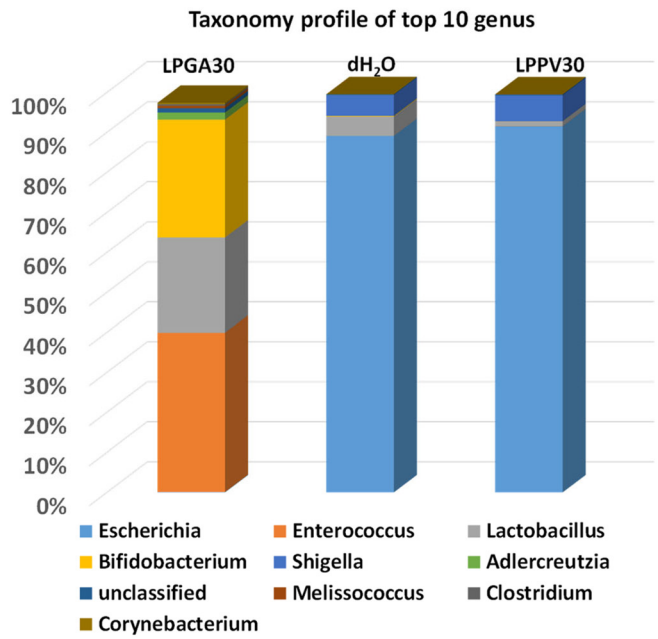


Taxonomy	LPGA30		dH_2O		LPPV30		Normal	
	count	%	count	%	count	%	count	%
Actinobacteria	12946	31.7%	72	0.2%	13	0.0%	950	1%
Bacteroidetes	510	1.2%	129	0.3%	152	0.4%	6933	8.1%
Firmicutes	27034	66.2%	2061	5.0%	554	1.4%	77083	90.3%
Proteobacteria	240	0.6%	38589	94.4%	40116	98.20	117	0.1%
Sum		99.7%		99.9%		100.0%		99.6%

Fig. 4 The taxonomy of phylum in the gut microbiome analysis of different treatment groups and house keeping control normal mice.

being *Escherichia* and *Shigella* (dH_2O group), while for the LPGA30 group the dominant genera being transitional populations of *Enterococcus*, *Lactobacillus* and *Bifidobacterium* of the phyla Firmicutes and Actinobacteria (Fig. 5). It has been reported that some *L. plantarum* strains can interact with some host factors in their residence by regulating their gene expressions for sugar transport and utilization.[38] Moreover, in a recent study in a high-fat diet setting, oral administration of GABA-producing *L. brevis* for 8 to 10 weeks improved metabolic syndrome and depression.[39] It is worth to mention that their GABA-producing *L. brevis* did not alter the abundance or ratio of the phyla Firmicutes and Bacteroidetes (cecum content).[39] This disagreement with the current study might be because current microbiome analysis was performed in the upper intestinal tract (duodenum). The gut microbiota composition occurs in a gut region-specific manner, with patterns of microbiota changes in the duodenum, ileum and colon of STZ-induced diabetic rats.[40] Moreover, it is worth mentioning than plateau plasma GABA levels were found in both LPGA30 and LPPV30 groups, but only efficient GABA-producing *L. plantarum GA30* produced soy yoghurt administered to STZ-induced hyperglycaemic mice showed transitionally rebalanced microbiota with phylum Actinobacteria (primary *Bifidobacterium* genera) being the second major population after Firmicutes in upper intestine. Indeed, the different inhibitory or excitatory tones of the upper intestinal contract-



Taxonomy profile of top 10 genus	LPGA30	dH_2O	LPPV30
Escherichia	0.10%	89.12%	91.55%
Enterococcus	39.81%	0.01%	0.05%
Lactobacillus	23.83%	4.84%	1.13%
Bifidobacterium	29.47%	0.16%	0.03%
Shigella	0.02%	5.21%	6.49%
Adlercreutzia	1.74%	0.01%	0.00%
unclassified	1.09%	0.16%	0.20%
Melissococcus	0.62%	0.00%	0.00%
Clostridium	0.37%	0.04%	0.07%
Corynebacterium	0.37%	0.00%	0.00%
SUM	97.41%	99.56%	99.54%

Fig. 5 The taxonomy profiles of top 10 genus in the gut microbiome analysis of different treatment groups.

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ibility depend on the distribution of different GABA receptor isoforms. GABA presence may affect GI tract motility, leading to microbial colonization with the diversity and abundance of gut microbiota. Auteri *et al.*, (2015)[41] described that GABA actions *via* enteric GABA receptor-mediated signalling affect gastrointestinal tract motility and anti-inflammatory activity. Finally, some minor and beneficial gut microbial populations including genera *Akkermansia muciniphila*, *Butyricicoccus pullicaecorum*, *Corynebacterium* spp. and *Adlercreutzia* spp. were scarcely observed in mice fed with *L. plantarum GA30*-produced soy yoghurts but not in those LPPV30 or dH_2O groups (data not shown). These minor bacteria may alter the gut pH value, sources of carbon and nitrogen and other growth factors in affecting symbiotic relationships among the microbiota members. It would be interesting to further assess host energy utilization in mutual aid with host digestive physiology and dynamic changes in gut microbiota with full consideration in attaining orchestration with GABA.

Conclusions

Soy yoghurts have gained popularity as a fermented soy product with health function promoted by a simple process of soymilk fermentation *via* manipulating selected *L. plantarum* spp. bearing potency in GABA synthesis and deglycosylation. As demonstrated in current hyperglycaemic mice fed with soy yoghurts produced with either *L. plantarum GA30* or *L. plantarum PV30*, multiple advantages are evidenced with optimized plasma GABA level and improved bioavailability of soy isoflavones. Synergistically, soy yoghurts enriched with GABA and isoflavones are promising to improve blood glucose homeostasis *via* restored pancreatic β-cell function in ameliorating hyperglycaemia in STZ-induced diabetic mice. In addition, the impact of hyperglycaemia in dysbiosis of gut microbiota is revealed. The soy yoghurt produced with efficient GABA-producing *L. plantarum GA30* supplementation can re-establish gut microbiota with a transitional presence of phylum *Actinobacteria*, which renders additional health benefits of soy yogurts. More in-depth investigations are demanded for further elucidation of prebiotic and probiotic interaction toward a host-microbial symbiotic orchestration.

Author contributions

Conceptualization, CWH and BCW; methodology, CWH, HDY, LGC, BCW; validation, CWH, LGC, CSC; data curation, HDY, BCW; writing – original draft preparation, BCW; writing – review and editing, CSC, BCW; supervision, CSC, BCW; project administration, CWH, LGC, BCW, CSC; funding acquisition, CSC. All authors have read and agreed to the published version of manuscript.

Conflicts of interest

There are no conflicts to declare.

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