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FULL PAPER

Functional papaya beverage increases healthy gut microbiota in Streptozotocin-induced diabetic Sprague Dawley rats

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Abstract

Diabetes mellitus is a silent killer illness, characterized by hyperglycaemia effect and can cause many health complications. A new functional papaya beverage was produced *via* selected pure symbiotic culture of bacteria and yeast (SCOBY) which offers an alternative food therapy in regulating blood glucose and its efficacy was evaluated using Streptozotocin (STZ)-induced diabetic Sprague Dawley rats for one month treatment. Under moderate diabetes condition, SCOBY papaya beverage was shown able to reduce high blood glucose in diabetic rats to normal level, comparably effective with commercial drug, Metformin. Not only that, it also helped restore body weight of diabetic rats to a healthier state with a gradual increment of body weight observed weekly. In contrast, untreated diabetic rats experienced a sharp rise in blood glucose with stunted body weight. Nutrigenomic studies were conducted to identify the mechanisms that support the effectiveness of SCOBY papaya beverage as an anti-diabetic therapy. Evidence from quantitative polymerase chain reaction (qPCR) analysis disclosed significant higher expression on gene markers related to insulin receptor substrate 1 (Irs1), glucose transporter (Slc2a8) and glutathione S-transferase mu 1 (Gstm1), but lower expression of gene markers indicative of diabetes complications and inflammation e.g., tissue inhibitors of metalloproteinase 1 (Timp1), nuclear factor kappa B subunit 1 (Nfkb1) and nitric oxide 2 (NOS2) in diabetic rats treated with SCOBY papaya beverage and Metformin (p<0.05). SCOBY papaya treated diabetic rats showed an increment of short chain fatty acids content and gut microbiota enriched with some beneficial microbes particularly for *Alloprevotella*, *Ruminococcus 1, Lachnospiraceae NK4A136, Prevotellaceae UCG-001* and *Prevotellaceae* NK3B31 compared to untreated diabetic rats. These data support the effectiveness of SCOBY papaya as a functional beverage in improving intestinal health by changing the environment of the microbiome of diabetic rats, in turn offering cost-effective food therapies in blood glucose regulation.

1. Introduction

Diabetes mellitus, one of the public concern health problems has affected about 463 million people worldwide and was projected to reach 700 million by the year 2045. The prevalence of diabetes has increased steadily over the last few decades with approximately 79% of diabetic adults living in low and middle-income countries. About 10% of global health expenditure is spent on diabetes with the amount approximately reaching USD 760 billion (International Diabetes Federation IDF report, 2019). Insulin is an important hormone produced in the pancreas to regulate glucose transportation into cells for growth and energy. Obesity was identified as one of the high-risk factors for the development of Type 2-diabetes. On the other hand, Type 1 diabetes is resulting from pancreas destruction, an autoimmune response toward insulin-making beta cells of the pancreas. As a consequence, insulin therapy is the key remedy for Type 1 diabetes. The primary goal of diabetes treatment is to constant monitoring and regulate the blood sugar level of diabetic patients within limits *via* diet, physical exercise, hypoglycaemia drugs and insulin therapy to prevent some diabetes-related health complications such as fatigue, excessive thirst,

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frequency of urination, delay healing of cuts and wounds. Serious diabetes complications may affect multiple organ systems such as nephropathy, retinopathy, neuropathy, and cardiovascular disease (Klein *et al*., 2007; Mohamed *et al*., 2016).

Antidiabetic drugs may produce a serious side effect. For example, Metformin may cause lactic acidosis, folate and B_{12} malabsorption; Thiazolidinediones cause weight gain and oedema (Campbell, 2007). The search for safer and more effective hypoglycaemic agents is still challenge. Globally, there are many medicinal plants extracts that have been explored and used traditionally to manage diabetes which is relatively less expensive. Even though these medicinal plants may contribute some cytotoxic effect but the side effect of these phytotherapeutic agents is less common than synthetic drugs. *Ajuga remota*, a traditional medicinal plant used for the treatment of various diseases including diabetes, Malaria, high blood pressure and swelling. Recent antidiabetic activity of *A. remota* on alloxan-induced diabetic mice revealed the ability of the aqueous extract (500 mg/kg) in reducing blood glucose levels, supporting traditional claims about the plant (Tafesse *et al*., 2017).

The papaya (*Carica papaya* L.) is a tropical commodity fruit, widely cultivated and consumed worldwide due to its high nutritional and pharmacological properties (de Oliveira and Vitória, 2011). This climacteric fruit is a low-calorie fruit with a good source of Vitamins A, C, and B complex (B_1, B_2, B_3, B_6 and B_9), dietary fibre and minerals like calcium, phosphorus and iron (Boshra and Tajul, 2013), making this fruit a good choice for obese people that are on weight management control regime. Chymopapain and papain are two potent active compounds of papaya which are known to aid in digesting protein food. Pectin from papaya helps to increase viscosity in the intestinal tract, reducing cholesterol absorption and providing prebiotic source for microorganisms in the large intestines and colon to release short-chain fatty acids to maintain gut health. Papaya leaf was known to have multiple pharmacological properties such as anti-inflammatory, wound healing effect (Mahmood *et al*., 2005), anti-dengue potency (So'aib *et al*., 2018) and analgetic activity (Hasimun *et al*., 2014).

Innovative functional foods are the trend of popularity to meet consumer's demand that confers positive effects on health. Microbial fermentation offers a promising technique to develop new functional foods *via* microbiological action. Fermented papaya preparation – a yeast fermented papaya product was known favourably in modulating immunostimulatory, anti-inflammatory, protecting effect by preventing the progression of oxidative stress-induced cell damage and induction of antioxidant enzymes (Aruoma *et al*., 2010; Marotta *et al*., 2012; Barbagallo *et al*., 2015). Nevertheless, available information on other types of microbial fermentation on papaya fruit is limited. In MARDI, a functional papaya beverage was developed using selected symbiotic culture of bacteria and yeast (SCOBY) to explore its bioefficacy properties as new functional food with multiple health promoting properties. Our selected SCOBY strains have been confirmed to have potential probiotic characteristics (Sharifudin *et al*., 2021). The antimicrobial property of this SCOBY papaya beverage was reported earlier and exhibited strong antimicrobial activity against few selected foodborne pathogenic microorganisms (*Escherichia coli* O157, *Salmonella enterica* serovar Enteritidis (isolated from infected chicken) and *Salmonella enterica* serovar Typhimurium ATCC 53648 (Koh *et al*., 2017). In this study, Streptozotocin-induced Sprague Dawley rats were performed to investigate the hypoglycemia effect of SCOBY papaya beverage in regulating blood glucose of diabetic rats. The blood biochemistry targeted gene markers expressions and gut microbiome profile of all rats' group were examined to assess the potential of SCOBY papaya beverage as new effective food therapy for diabetes management control.

2. Materials and methods

2.1 Preparation of SCOBY papaya beverage

Papaya pulp (*Carica papaya*, Sekaki variety) was used as a substrate for the preparation of 5% pulp suspension before being inoculated with mixed SCOBY strains (colony-forming unit of 10^8/mL). The SCOBY strains consisting of *Dekkera* sp. and *Komagataiebacter* sp. were collected from MARDI's Collection of Functional Food Cultures and added into papaya pulp suspension at the ratio of 4:1. The SCOBY papaya fermentation was incubated at 37°C for 4 days with an agitation rate of 200 rpm. Following 4 days of the fermentation process, the papaya supernatant was collected by centrifugation at 10,000 rpm to remove residue biomass before being subjected to a pasteurization temperature of 90°C for a duration of 30 mins and keep in chill condition for further analysis.

2.2 Experimental design for Streptozotocin-induced diabetic rat study

The male Sprague Dawley rats (8 weeks old) were selected for the Streptozotocin-induced diabetic study. The rats were placed in the Animal Metabolism, Toxicology and Reproductive Centre (AMTREC MARDI, Serdang) and maintained under standard conditions: room temperature (25±2°C) with the humidity level (55%±2) and alternating light-dark cycle

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at the interval of 12 hrs. The rats have free access to standard rat pellets and water ad libitum for the entire experiment periods. All animals were acclimatised for two weeks in the cage before proceeding to anti-diabetic study. The experimental protocol was conducted according to the animal guidelines and was approved by the Animal Ethics Committee of MARDI (20180810/R/MAEC00025).

A total of 24 male Sprague Dawley rats were selected in the anti-diabetic study. Prior to 16 hrs fasting time with only water provided ad libitum, the rats were injected with Streptozotocin (STZ, 40 mg/kg) freshly prepared in 0.1 M sodium citrate buffer (pH 4.5) to induce diabetes mellitus symptoms. There are three groups of STZ-induced diabetic rats with each group contained 6 rats: a) diabetic rats treated with commercial drugs, Metformin [4.5 mg/kg, CTRL (+)]; diabetic rats treated with SCOBY papaya beverage [1.5 mL/kg, PP]; c) diabetic rats without treatment [CTRL (-)]. The rats in the normal group were injected intraperitoneal with sodium citrate buffer (pH 4.5) as control (Normal, n=6). Three days after STZ induction, sampling blood from the tail vein of each rat was analysed using a commercial glucometer (ACCU-CHECK Active Glucose Monitor, Roche, Germany) to confirm hyperglycemia condition for the diabetic rats' group. The fasting rats with a blood glucose level of more than 11 mmol/L after injection, were considered successfully established Type 2 diabetic rats. All treated diabetic rats were administered orally by oral gavage technique with respective treatment for 28 consecutive days. The body weight and blood glucose of each rat were recorded at the initiation of treatment and continued examination at an interval of a week until the day of necropsy. All rats were observed daily for abnormal behaviours and changes in hyperactivity, tremors, ataxia, salivation, diarrhoea, lethargy, and sleep of all rats were examined also throughout the experiment period.

2.3 Determination of blood haematology and serum biochemistry profile

At the end of experiment, the rats were sacrificed in the carbon dioxide chamber and blood was drawn from the brachial artery and stored in tubes containing anticoagulant EDTA-2K. The blood haematology profile (red blood cell, platelet, white blood cell, haemoglobin and hematocrit) were obtained by running an automated haematology analyser (Exigo Blood Haematology Analyzer, Sweden). Blood serum samples were obtained by centrifuging blood (Centrifuge S417R, Eppendorf, CA, USA) at 4,000 rpm for 10 mins under the controlled temperature of 4°C. Then, the collected serums samples were analysed using a clinical chemistry autoanalyzer (DIRUI CS-300, China) to identify the liver functional profile (alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), total protein, albumin and globulin) and kidney functional profile (creatinine, urea). The data obtained will be compared with the normal range of Sprague Dawley rats as reported by Petterino and Argentino-Storino (2006). The sacrificed rats were then dissected, and the organs (lung, liver, heart, kidney, and spleen) were harvested and weighed. The relative organ weight was calculated as (organ/body weight) × 100%.

2.4. Quantitative real-time polymerase chain reaction (qPCR) analysis

Liver samples of the treated and control rats' group were collected. Total RNAs were extracted from liver samples using RNeasy Lipid Tissue Mini kit (Qiagen, Germany). Complementary DNAs were synthesized using QuantiNova Reverse Transcriptase kit (Qiagen, Germany). Predesigned PrimeTime Assay Std Probes 5' FAM/ZEN/3' IBFQ were synthesized by Integrated DNA Technology, IDT, Singapore) as follows: Actin-b (Actb) (5'-/56-FAM/CCGCCACCA/ZEN/GTTCGCCATG/31ABkFQ/-3'), glyceraldehyde 3-phosphate dehydrogenase (GADPH) (5'-/56-FAM/CACACCGAC/ZEN/CTTCACCATCTTGTCT/31ABkFQ/-3'), insulin receptor substrates (Irs1) (5'-/56-FAM/ TTCATTCTG/ZEN/CCTGTGACGTCCAGTT/31ABkFQ/-3'), glucose transporter (Slc2a8) (5'-/56-FAM/CTGAGGATA/ZEN/CAGAAGGCAGCGGT/31ABkFQ/-3'), tissue inhibitors of metalloproteinase 1 (Timp1) (5'-/56-FAM/TCATCGAGA/ZEN/CCACCTTATACCAGCGT/31ABkFQ/-3'), glutathione S-transferase mu 1 (Gstm1) (5'-/56-FAM/CTTCAGGTT/ZEN/TGGGAAGGCGTCCA/31ABkFQ/-3'), nitric oxide synthase 2 (NOS2) (5'-/56-FAM/CAACTACAA/ZEN/GCCCCACGGAGAACA/31ABkFQ/-3') and nuclear factor kappa B subunit 1 (Nfkb1) (5'-/56-FAM/TACAGCGCC/ZEN/ATCTCACCGCG/31ABkFQ/-3'). The qPCR assay was performed using synthesized PrimeTime Assay Std Probe 5' FAM/ZEN/3' IBFQ in a StepOnePlus real-time PCR system (Applied Biosystems, USA). The PCR cycling conditions used for sample analysis were as follows: 1 cycle of 95°C/3 min for DNA polymerase activation, 40 cycles of 95°C/5 s for denaturation, and 60°C for 30 s for annealing and extension. The fluorescent dye ROX served as an internal reference for normalization of the FAM fluorescent signal. Gene expression was normalised to the average expression values of reference genes of Actb and GADPH and relative gene expression analysis was performed using 2–[delta][delta]Ct method.

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2.5 Gut microbiota analysis via 16S rRNA gene sequencing

The composition of gut microbiota in individual treated and control rat samples was evaluated *via* 16S rRNA sequencing on rat faecal samples. Bacterial genomic DNA was extracted from individual rat faecal samples of each group using NucleoSpin tissue mini kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. The 16S rRNA variable V3-V4 regions were amplified using 16S Amplicon PCR Forward Primer, 5'- CCTAYGGGRBGCASCAG-3' and 16S Amplicon PCR Reverse Primer 5'-GGACTACNNGGGTATCTAAT-3' and Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA). Sequencing libraries were constructed using NEBNext® Ultra™ DNA Library Prep Kit for Illumina® according to the manufacturer's instructions. Sequencing of 16S rRNA libraries was performed using lllumina HiSeq 2500 platform with 250 bp paired end reads generated. Paired-end reads were merged using FLASH (V1.2.7) (Magoč and Salzberg, 2011) to generate raw tags before the raw tags were subjected to quality filtering to obtain clean tags using QIIME (V1.7.0) (Caporaso *et al*., 2010). To detect chimera sequences, the tags were compared with the reference data namely Gold database using UCHIME algorithm (Edgar *et al*., 2011) for obtaining the effective tags. Sequences analysis were performed by Uparse software (V7.0.1001) using all the effective tag (Edgar *et al*., 2013). Sequences with ≥97% similarity were assigned to the same OTUs. Annotation of OTUs at each taxonomic rank was performed using Mothur software against the SSUrRNA database of SILVA Database (Wang *et al*., 2007). The evolutionary tree was generated with RAxML and viewed in ITOL (Letunic and Bork, 2007).

2.6 Quantification of short chain fatty acids using gas chromatography

The faecal samples from all diabetic rats' groups and normal healthy rats' groups were collected at the end of the experiment and the short chain fatty acids (SCFAs) will be extracted according to the method described in Zhao *et al*. (2006) with minor modification and quantified using gas chromatography. The frozen faecal samples (0.3 g) were smashed using mortar and pestle first before adding in 3 mL distiller water with the adjusted pH 2 using 10 M hydrochloric acid. Then, the solution was transferred onto the tube containing Lysing Matrix E and subjected to high speed homogenisation (10,000 rpm, 10 mins) using FastPrep-24 homogeniser (MP Biomedicals, Germany) to extract the SCFAs solution. The homogenate was transferred into new tubes and repeated centrifugation process at 10,000 rpm for 5 mins to remove unwanted residues. The collected supernatant needs to be filtered using a 0.22 µm syringe filter prior to GC injection using Agilent 6890N GC system equipped with a flame ionization detector (FID). The SCFAs analysis was carried out using a Zebron ZB-Waxplus capillary column (30 m × 0.25 mm internal diameter × 0.25 µm film thickness) to separate the SCFAs peaks. The injection port and FID temperature was set at 250°C. The flow rate of hydrogen, air and make up gas nitrogen were maintained at 40, 350 and 30 mL/min, respectively with a total run time of 10 mins. The nitrogen was used as carrier gas and the split ratio was set at 100:1 with the split flow of 99.4 mL/min. The SCFAs peaks were separated individually using gradient heating profile: The initial oven temperature was set to 50°C for 1 min before heating up the column to 200°C at 60°C/min and held for 1 min before further raised to 250°C with the heating rate of 20°C/min and then maintained at 250°C for 3 mins. The amount of SCFAs was quantified from external calibration curve of SCFAs standards (acetic acid, butyric acid and propionic acid) using HP Chemstation Plus software (Agilent, USA).

2.7 Statistical analysis

All data are presented as mean±standard deviation and subjected to one-way analysis of variance (ANOVA) and the multiple comparisons were performed by Duncan's test. All analyses were performed using statistical analysis software (IBM SPSS Statistic 22.0) with statistical significance set at the level of $p < 0.05$.

3. Results and discussion

The diabetic epidemic has increased steadily worldwide as a consequence of the exponentially increment of the obesity rate globally which has contributed to the rising prevalence of Type 2 diabetes mellitus. This growing metabolic disorder has caused a substantial impact and imposes a huge economic burden to the nation's healthcare system. In current practice, the cost of treating diabetes and its complications is incalculable. Therefore, an attempt at the development of cost-effective diabetes therapy with fewer side effect is highly demanded. In MARDI, a new functional papaya beverage was developed *via* selected starter cultures of SCOBY under controlled biofermentation process. The SCOBY starter cultures was confirmed to have potential probiotic characteristics and produced various bioactive metabolites which has been reported to have strong antimicrobial activity towards few selected foodborne pathogenic microorganisms (Koh *et al.,* 2017; Sharifudin *et al*., 2021).

In this study, the potential anti-diabetic effect of SCOBY papaya beverages was explored using

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Streptozotocin (STZ)-induced diabetic rats and treated for a duration of one month. The STZ is toxic to the insulin-producing pancreatic beta cells and was used to induce Type 2 diabetic rats as a model of human Type 2 diabetes mellitus in which peripheral insulin resistance. Three days after injecting 40 mg STZ/kg body weight into rats, it was successfully raised up blood glucose reading above 11 mmol/L. Then, the rats were observed for another 2 days to confirm hyperglycaemia condition before being divided into three groups and proceeding to respective treatment for a duration of 28 days.

As expected, STZ-induced diabetic rats without treatment, CTRL (-) group was experienced dramatically increment of blood glucose level after a week of single dose STZ injection and reached a severe diabetic state after 4 weeks without treatment (Figure 1a). As a consequence, this diabetic rats' group were observed very weak and the body weight of rats was stunted with no significant (p>0.05) in weight increment after being sick for a month without treatment (Figure 1b). On the contrary, the blood glucose level of diabetic rats treated with SCOBY papaya beverage (PP) was shown a significant reduction (p<0.05) blood glucose level and successfully restore to normal blood glucose range after a week of treatment and remained in the healthy range throughout 28 consecutive days of treatment. Promisingly, the body weight of PP-treated diabetic rats' group also observed a gradual increment, indicating health recovery signs with a range of body weight closer to the normal rats' group (Figure 1). In comparison, PP-treated diabetic rats' group showed a comparable effect as shown in Metformin-treated diabetic rats' group in regulating blood glucose levels and restoring body weight to a healthier state. Further investigation on various relative organs weight (liver, heart, lung, spleen and kidney) on treated and non-treated diabetic rats' group revealed minor differences in normal rats' group, indicating no severe sign of organ injury damage for all diabetic rats' group except for liver and kidney organ which experienced a minor significant difference (p<0.05) (Figure 1c).

The blood haematology analysis was conducted to monitor health condition of red blood cells, white blood cells, haemoglobin, platelet and haematocrit for all diabetic rats and normal rats' group. In general, all rat blood samples showed a healthy range of haematology

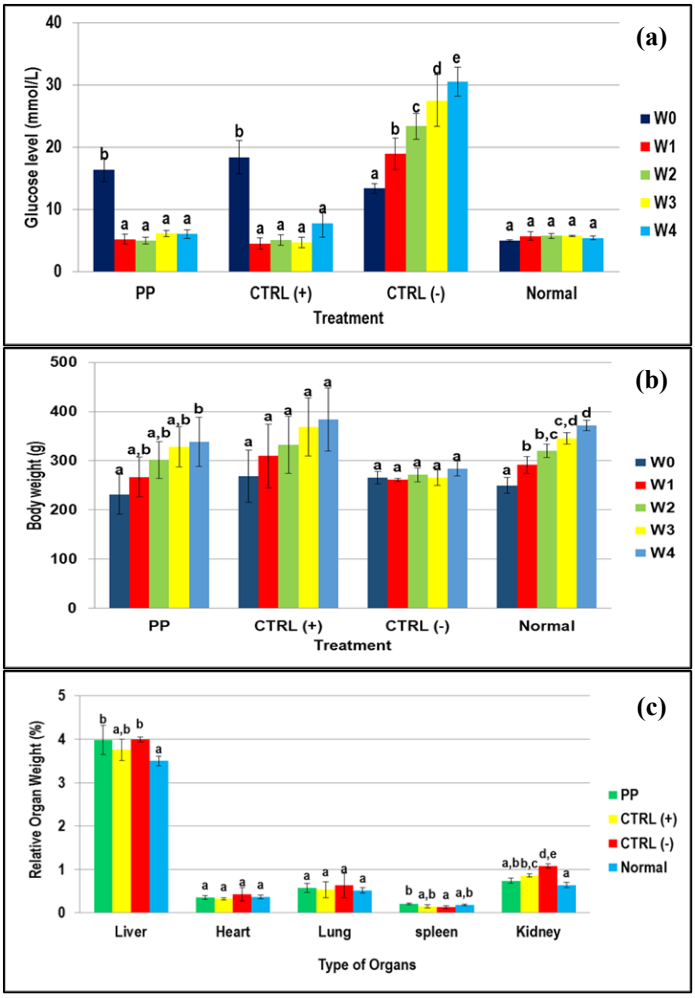

Figure 1. Comparison study between treated and non-treated diabetic – induced rats with normal healthy rat in term of a) blood glucose level; b) body weight; c) relative organ weight after one month treatment. Bars of the same colour with different notations are significantly different between samples (p<0.05).

profile (Table 1). However, the liver functional enzymes profile (alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and kidney functional profile (urea) of the diabetic rats' group revealed significant higher value (p<0.05) than normal rats' group, particularly for STZ-induced diabetic rats' group without treatment, CTRL (-) (Table 2). This phenomenon indicating that STZ injection has cause an injury damage to liver and kidney organ as a result of hyperglycaemia condition in accordance with the liver abnormalities occurs with diabetic mellitus complications as reported earlier (Mohamed *et al.*, 2016). Surprisingly, PP-treated diabetic rats' group showed a recovery sign with a lower reading of alkaline

Table 1. Blood haematology profile of diabetic-induce rats and normal rats

Treatment	Red blood cell (10^{12}/L)	White blood cell (10^{9}/L)	Platelet (10^{9}/L)	Hemoglobin (g/dL)	Hematocrit (%)
PP	11.22±0.38^{a}	9.23±3.38^{ab}	633.67±503.50^{a}	20.93±0.83^{a}	62.47±2.58^{a}
CTRL (+)	10.62±0.57^{a}	8.30±1.17^{ab}	959.00±203.66^{a}	19.57±0.80^{a}	55.97±3.39^{a}
CTRL (-)	10.70±1.09^{a}	7.18±3.93^{b}	498.25±217.07^{a}	20.30±1.96^{a}	60.53±6.85^{a}
Normal	10.91±1.64^{a}	13.48±3.13^{a}	1175.75±402.50^{a}	21.13±3.02^{a}	60.95±9.34^{a}

Values with different superscript within the same column are significantly different p<0.05.

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Table 2. Liver and kidney function profile of diabetic-induce rats and normal rats

Treatment	Liver Function Analysis						Kidney Function Analysis	
	Alanine aminotransferase (U/l)	Aminoaspartate transferase (U/l)	Alkaline phosphatase (U/l)	Total protein (g/l)	Albumin (g/l)	Globulin (g/l)	Urea (mmol/l)	Creatinine (µmol/l)
PP	378.50±331.63^{a}	317.67±293.91^{b}	201.00±74.81^{a}	65.20±1.97^{a}	37.45±1.32^{a}	27.77±3.29^{abc}	6.14±1.46^{b}	31.00±8.72^{a}
CTRL (+)	142.29±110.38^{bc}	226.00±156.47^{b}	224.33±34.31^{a}	64.23±3.25^{ab}	36.36±1.69^{a}	27.90±1.67^{abc}	7.72±1.02^{b}	22.33±4.51^{a}
CTRL (-)	296.33±130.30^{ab}	745.00±0.00^{a}	488.33±134.88^{a}	54.58±3.63^{c}	31.88±1.72^{b}	22.70±3.67^{c}	22.02±4.34^{a}	24.50±9.75^{a}
Normal	43.67±3.10^{c}	90.33±12.34^{b}	169.75±27.94^{a}	68.80±3.45^{a}	39.70±1.40^{a}	29.10±2.30^{ab}	5.97±0.79^{b}	24.25±2.22^{a}

Values with different superscript within the same column are significantly different $p<0.05$.

phosphatase, aspartate aminotransferase and urea towards a healthier range after 28 days treatment when compared to diabetic rats without treatment (Table 2). Even though total protein, albumin, globulin and creatinine level of diabetic rats' group, CTRL (-) were higher than normal rats' group, but it was fall within the acceptable healthy range and was not affected much by the hyperglycaemia condition (Petterino and Argentino-Storino, 2006). Evidence findings from blood haematology and serum biochemistry profile support the potential of SCOBY papaya beverage as a promising effective food therapy in regulating blood glucose and restoring diabetic condition to a healthier state.

In order to investigate the underlying molecular mechanisms of SCOBY papaya beverage, the gene expression study was conducted using the liver tissue sample of treated and non-treated rats' group. During the progress of STZ-induced Type 2 diabetes mellitus, the concentration of reactive oxygen species in the liver tissue of treated groups was expected to increase and trigger oxidative stress (Lucchesi *et al*., 2013). The prolonged diabetes mellitus in turn causes inflammation to liver tissue and alters the activity of glucose transporter (Gorovits *et al*., 2003) as well as the level of insulin secretion (Rehman *et al*., 2016). Therefore, the gene expression of nitric oxide (NOS2), nuclear factor kappa B subunit 1 (Nfkb1), insulin receptor substrate 1 (Irs1) and glucose transporter (Slc2a8) were measured. It was shown that the expressions of NOS2 and Nfkb 1 were significantly reduced ($p<0.05$) in PP-treated group compared to the non-treated CTRL (-) group (Figure 2). Moreover, there were significant increases ($p<0.05$) in the gene expression levels of Irs1 and Slc2a8 in PP-treated group compared to the non-treated CTRL (-) group. Worth mentioning that those gene expression levels in the PP-treated group were found to respond in a similar trend as the CTRL (+) group which has been treated with the commercial drug, Metformin. Besides, during the development of Type 2 diabetes, inflammation of liver tissue could initiate another protective mechanism by glutathione S-transferase against oxidative stress (Allocati *et al*., 2018). The current gene expression analysis results showed that the gene expression levels of glutathione S-transferase mu 1 (Gstm1) increased significantly ($p<0.05$) to almost 3-fold in PP-treated group compared to CTRL (-) group. This indicated that the PP-treated rats significantly modulated the oxidative stress induced by diabetes. In addition, the development of Type 2 diabetes could cause liver complication such as enhanced liver fibrosis. The tissue inhibitors of metalloproteinase 1 (Timp 1) have anti-apoptotic function, acting as natural inhibitors for matrix metalloproteinases, which plays an important role in degrading extracellular matrix and process bioactive molecules including cytokines, chemokines and growth factors (Yabluchanskiy *et al*., 2013). In a recent study conducted by Wang *et al.* (2020), it was observed that a higher Timp1 level was associated with increased Type 2 diabetes risk in Chinese adults. In the current study, the increased gene expression of Timp1 in CTRL (-) group was found to be restored to a normal level in PP-treated group ($p<0.05$), indicating PP is able to reduce the risk of developing Type 2 diabetes. The overall gene expression analysis results indicated that the consumption of SCOBY papaya beverage was able to reduce oxidative stress, and inflammation in the liver tissue due to the diabetes mellitus induced by STZ, help in restoring the molecular activities of glucose transporter and insulin secretion of the liver. This phenomenon indicated that the SCOBY papaya beverage also plays a role in preventing the pathogenesis of

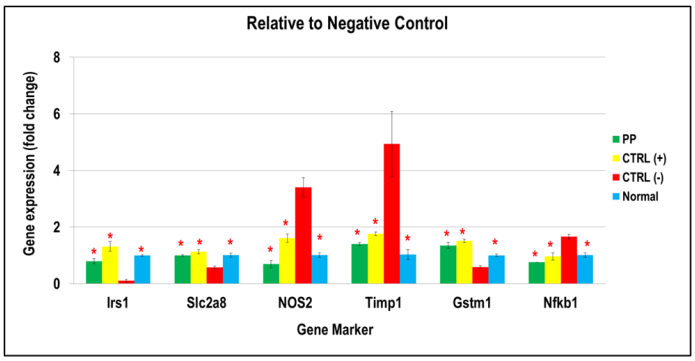

Figure 2. Gene expression levels measurement. Gene expression of Irs1, Slc2a8, NOS2, Timp1, Gstm1 and Nfkb 1 in positive control (CTRL(+)], negative control [CTRL(-)] and SCOBY papaya beverage treated groups [PP] relative to negative control. Significant differences between individual group and CRTL (-) are marked with * ($P<0.05$).

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diabetic complications such as enhanced liver fibrosis. The efficacy of SCOBY papaya beverage was found to be comparable to the commercial drug (Metformin) used for treating diabetes mellitus.

In a recent finding, it was demonstrated that dysbiosis of gut microbiota significantly affects the progression of diabetes mellitus patients (Lu *et al*. 2019). Exploring the gut microbiota profile has been a focus nowadays as it has emerged as an essential mediator in the pathophysiology of obesity and related metabolic disorders studies. In our case study, the metagenomics analysis on rat faecal samples provided us insights into the alteration of gut microbiota under different diets and treatments. A heatmap dendrogram illustrating the relative abundance of the gut microbiome in STZ-induced diabetes mellitus treated and non-treated diabetic groups is shown in Figure 3. Results show that STZ-induced diabetes mellitus non-treated diabetic group, CTRL (-) was abundantly high with harmful pathogenic bacteria such as *Turicibacter* and *Treponema* (Allen *et al*., 2015; Radolf *et al*., 2016), however the abundance of these bacteria were relatively low in both PP- and Metformin treated STZ-induced diabetic group as well as in normal group. On the other hand, it was noticed that PP-treated diabetic group were highly enriched with bacteria from the Phylum of *Prevotellaceae, Alloprevotella and Lachnospiraceae, Ruminococcaceae, Phascolarctobacterium* and *Allobaculum*. Previous study has shown that *Alloprevotella, Lachnospiraceae and Prevotellaceae* were negatively associated with the Type 2 diabetes mellitus related biomarkers (Wei *et al*., 2018). In addition, the abundance of *Lachnospiraceae* was reported to be inversely correlated with the blood glucose response after a glucose intervention (Zhang *et al*., 2018). Likewise, similar to a study by Zhang *et al.* (2017), there was a decreased in the abundance levels of *Phascolarctobacterium,* and *Ruminococcus* in diabetes patients, however, following the consumption of SCOBY papaya beverage, it was able to enrich the gut composition of both these bacterial groups. Interestingly, a higher abundance of *Phascolarctobacterium* was linked with insulin sensitivity as reported in Naderpoor *et al.* (2019).

The diet is the major substrate for the production of small molecular metabolite by the gut microbiota, of which acetate, propionate and butyrate are the most important SCFAs. To date, SCFAs and their receptors are known to be an essential mediator that links diet and the gut microbiota to host physiology by modulating endocrine response, development and functional of leukocytes, enzymes activity and transcription factors (Li *et al.,* 2017). On the whole, it was noted that PP-treated

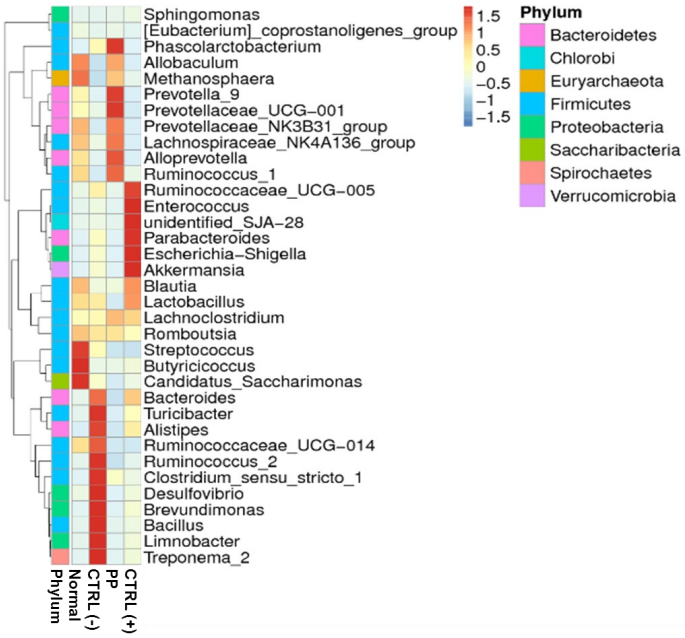

Figure 3. Relative abundance heatmap dendrogram on gut microbiome of STZ-induced diabetes mellitus rat fed with SCOBY PP diet. (Abbreviations: SCOBY PP diet, PP; STZ-induced DM non-treated, C(-); STZ-induced DM treated with Metformin, C(+); Normal diet mice, (Normal)

diabetic group showed a significant increment (p<0.05) of SCFAs (acetic, propionic and butyric acid) after 28 days treatment, more effective than Metformin-treated diabetic group (Figure 4). Inversely, STZ-induced diabetic group without treatment was observed to have significant lower content (p>0.05) of SCFAs. The SCFAS, a subset of key gut microbial metabolites contributes approximately 5-10% of body energy source. Particularly, butyrate is one of the key energy substrates for both coloncytes and enterocytes which play crucial role in energy expenditure *via* stimulating mitochondrial respiration and fatty acid oxidation (Gao *et al*., 2009; Donohoe *et al*., 2011). *Ruminococcus* is a butyric acid producing bacteria, its higher abundance in gut microbiota of PP-treated group was well corresponded to a higher butyric acid content found in PP-treated group (Figures 3 and 4). Butyric acid also plays an important role in ameliorating the development of diabetes. Lower abundance of butyric acid producing bacteria including *Ruminococcus* was found to contribute to the outcome of Type 2 diabetes (Kulkarni *et al*., 2021). Moreover, *Prevotellaceae*, was known to have the ability to ferment carbohydrates and produce SCFAs (acetate and butyrate), and increased abundance of this bacteria gave positive impacts to several non-communicable diseases including diabetes as demonstrated in PP-treated diabetic group which displayed significant increment of acetic and butyric acid after 28 days of treatment (Wei *et al*., 2018).

The increased production of propionic acid by the gut microbiota of PP-treated diabetic group might be

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considered beneficial in the prevention of Type-2 diabetes as propionic acid helps in lowering fatty acids content in liver and plasma, reduces food intake by increasing the duration of satiety, exerts immunosuppressive actions and improves tissue insulin sensitivity as reported earlier (Al-Lahham *et al*. 2010). Fatty acids and inflammatory factors cause insulin resistance (Permana *et al*., 2006; Kennedy *et al*., 2009). Propionic acid assists in lowering effects on fatty acids and inflammation might lead to the improvement in insulin sensitivity as high plasma fatty acids could cause inflammation. Taken together, SCFAs emerges as a major mediator in the link between nutrition, gut microbiota and physiology. In this study, the gene expression and gut microbiome analysis findings collectively suggest that PP helps in restoring altered blood glucose level *via* modulation of gut microbiota and SCFAs production.

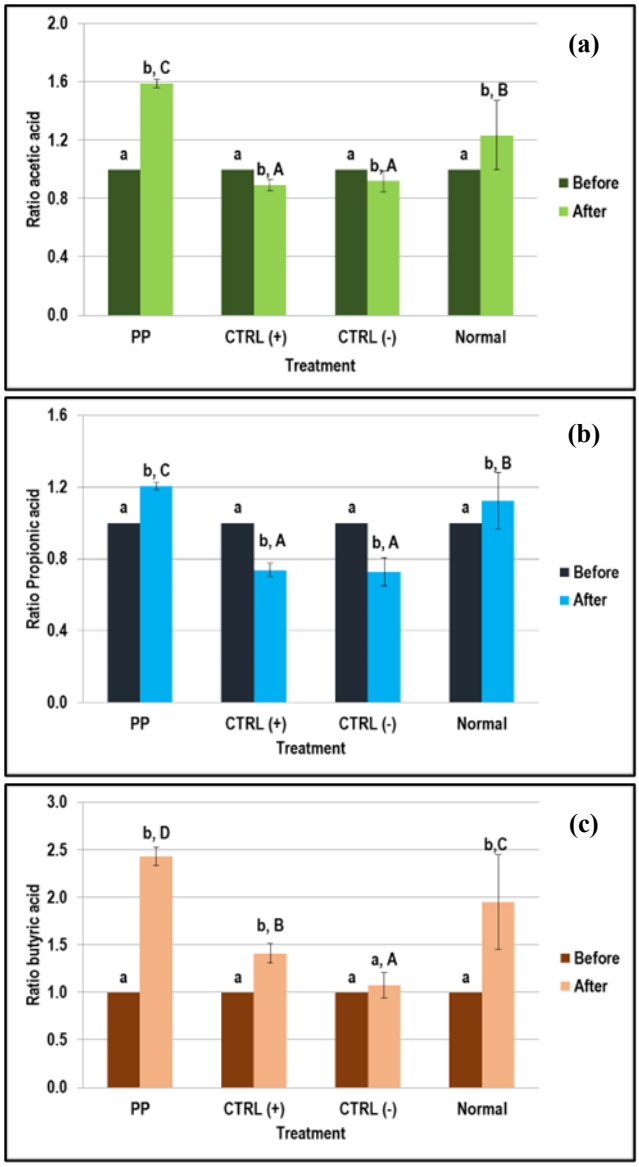

Figure 4. Short chain fatty acids comparison study between treated and non-treated diabetic –induced rats with normal healthy rat in term of: a) acetic acid; b) propionic acid; c) butyric acid after one month treatment. Bars of the same colour with different notations are significantly different between samples (p<0.05).

4. Conclusion

This study provides insights into the supplementation of SCOBY papaya beverage that is able to regulate blood glucose, restore body weight, improve liver insulin resistance, ameliorate liver inflammation as well as in modulating the composition of gut microbiota in STZ-induced rat diabetes model. The overall findings here supported the potential of SCOBY papaya beverage as an alternative and inexpensive intervention for Type-2 diabetes prevention and treatment. Further study will be focused on the human model to collect more evidence and information on clinical relevance to support the potential of SCOBY papaya beverage as a novel therapeutic target for diet-induced diabetes mellitus treatment.

Conflict of interest

The authors declare no conflict of interest

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