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Beneficial Effects of Macaroni Made with Resistant Starch Type 4 from Unripe Banana and Turmeric Extract on Blood Clinical Chemistry and Gut Microbiota of Healthy Rats

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Abstract Unripe banana is rich with resistant starch (RS) and this non-processed food confers many health benefits. However, unripe banana is not consumed directly and when cooked its native RS is rendered digestible. We have developed macaroni from chemically modified unripe banana flour (RS4), which maintains their content of resistant starch after being cooked, with and without the supplementation of turmeric extract. We hypothesized that consuming our banana RS4 macaroni would confer beneficial effects on metabolic profiles. Healthy Wistar rats were fed for 6 weeks with cooked banana RS4 macaroni, with and without the turmeric extract supplementation, and compared to rats fed with standard wheat macaroni. No significant physiological differences between groups were observed except rats that consumed banana RS4 macaroni had significantly smaller stomachs ($p<0.05$). Rats receiving banana RS4 macaroni had significantly lower triglyceride levels ($p<0.05$) and a trend for lower fasting blood glucose and increased expression of insulin-like growth factor-2 in theirs livers. Although it was not statistically significant, turmeric supplementation showed a trend of reducing serum total cholesterol and LDL-cholesterol as well as liver expression levels of genes involved in cholesterol metabolism, HMG CoA reductase (HMGR) and LDL-receptor related protein-1 (LRP-1). Banana RS4 macaroni did not significantly change fecal microbiota, nevertheless, addition of turmeric extract significantly increased alpha diversity and the relative abundance of *Lachnospiraceae Erysipelotrichaceae* and *Clostridiaceae*. In conclusion, the consumption of banana RS4 macaroni by healthy rats receiving a regular diet improved their blood chemistry profile associated with metabolic syndrome, and the addition of turmeric extract can significantly alter fecal microbiota.

Keywords: *metabolic syndrome, functional foods, Resistant Starch type 4 (RS4), unripe banana flour, gut microbiota, turmeric*

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1. Introduction

Changes in our environment and lifestyle have drastically raised the prevalence of metabolic syndrome (MetS) that increase the risk of an individual to develop type 2 diabetes and cardiovascular diseases (CVDs). Not only MetS reduces the patients'quality of life, it also has a significant economic impact on public health expenditure due to the high morbidity rates [1]. Therefore, there is a need to develop functional foods that reduce the incidence

of metabolic syndrome and reduce the risk of costly chronic diseases.

Starch constitutes about 25% of the calories in the typical human diet and humans have enzymes to break it down into glucose. However, some types of starch, termed resistant starch (RS), is able to escape digestion and passage through the stomach and small intestine to reach the colon where it can be fermented by the microbiota, ultimately modulating both microbial composition and activity [2,3]. Consequently, the gut microbiota has been proposed as a key component in mediating the metabolic benefits of RS [4]. Microbial metabolites that result from RS fermentation, especially short chain fatty acids (SCFAs), have been shown to have important biological functions associated with colon cancer prevention [5,6], hypoglycemic effects [7], hypolipemic effect [8] and immune modulation effect [9,10]. Although all RS share these properties, there are several types defined by what causes their resistance. Resistant starch are classified into five types: RS1 (physically inaccessible starches), RS2 (granular starches with B- or C-polymorph), RS3 (retrograded starches), RS4 (chemically modified starches), and RS5 (amylose-lipid complexes) [11]. Resistant starch type 4 is a unique class of resistant starch due to the diversity of chemical modifications that decrease digestibility [12]. Common chemical modifications include cross-linking, substitution, and pyrodextrinization [13]. Replacing the relative amount of refined digestable starch with resistant starch in a regualr diet may improve blood chemistry profiles and reduce the risk of metabolic syndrome.

Unripe banana is considered the RS-richest non-processed food [14] and studies have suggested that consumption of unripe bananas confers beneficial effects for human health. Unripe bananas is a good source of fibers, vitamins [15], minerals [16] and resistant starch (RS) [17,18,19,20], potentially contributing to health benefits [21]. Economically, bananas are a major horticulture crop in tropical and subtropical areas. However, about one fifth of all bananas harvested are discarded due to the defect in their appearance thus resulted in economic loss and environmental hazard [22]. Utilisation of reject unripe bananas as a raw material for functional foods can provide both economical values as well as nutritional benefits. Nevertheless, fresh unripe banana is not usually consumed directly due to its hardness and high astringency but its native resistant starch is rendered digestible when cooked. Therefore, there is a need of modifying unripe banana flour to maintain their indigestibility after cooked in order to be used as a replacement of refine digesitible starch and formulating into functional food products like pasta or bekery.

Turmeric (*Curcuma longa L*) is a traditional medicinal plant that is used extensively in cooking due to its ability to improve the taste and color of foods and the therapeutic properties [23]. The result of a meta-analysis study showed that turmeric significantly improve fasting blood glucose, triglycerides, high-density lipoprotein cholesterol (HDL-C), and diastolic blood pressure levels althoguh no significant change in waist circumference measurement was observed [24].

We have develop RS-rich macaroni pasta from chemically modified unripe banana flour (RS4), which would not lose their indigestibility after cooked with or without the supplementation of tumeric extract. Although RS4 has been reported to reduce body fat and improve circulatory lipid profile in obese rodents [25], in this study we asked whether RS4 from banana could show beneficial effect even to a healthy rat consuming regular diet. We hypothesize that the unripe banana and turmeric derived food products may potentially be functional foods usable for improving blood glucose, lipid profile and insulin sensitivity as well as gut microbiota and provide better health even for a healthy individual.

2. Materials and Methods

2.1. Study Foods

Every 100 g of wheat-free banana RS4 macaroni contain 30 g of acid cross-linked banana starch and 70 g of a mixture of rice flours and freshly cooked rice. The resistant starch type 4 was prepared from unripe banana flour utilizing acid induced cross-linked reaction, which was the primary source of fiber in the macaroni. The raw flour is mixed with acid solution and heated at specific temperature to induce crosslinking of the starch afterward the starch is washed, dewatered, and dried to a moisture content not to exceed 18%. The wheat-free banana RS4 macaroni was also prepared in the form that supplemented with 0.15% (w/w) turmeric extract.

2.2. Measurement of Resistant Starch

Determination of resistant starch in the study foods was carried out using commercial kit Megazyme (Resistant starch assay procedure) according to AACC Method 32-40.01. Briefly 4 mL of α-amylase (10 mg/mL) containing amyloglucosidase (3 U/mL) was added to 100 mg of cooked macaroni, mixture was vortexed followed by incubation at 37 °C for 16 h to hydrolyze digestible starch. Then absolute ethanol (4 mL) was added to deactivate the enzymes, followed by centrifugation. The pellet obtained was washed twice with 50% ethanol and the sediment was dissolved in KOH (2 mL, 2 M) by vigorous stirring for 20 min. The solution was neutralized with sodium acetate buffer (8 mL, 1.2 M). Then 0.1 mL of amyloglucosise (3300 U/mL) was added followed by incubation for 30 min at 50°C. The samples were centrifuged at 3000×g for 10 min. GOPOD (3 mL) was added to aliquots (0.1 mL) of the supernatant and incubated at 50°C for 20 min for measuring D-glucose. Absorbance was measured at 510 nm using a spectrophotometer. RS was calculated as the amount of glucose × 0.9. Non-resistant starch is determined by pooling the original supernatant and the washings, adjusting the volume to 100 mL and measuring D-glucose.

2.3. *In vitro* Digestion

The *in vitro* digestion of macaroni was carried out according to method described by Dhital *et al* [26]. Macaroni pasta after cooking, cooling and pre-warmed at 37°C was mixed with 1 ml of artificial saliva containing α-amylase (250 U/ mL carbonate buffer, pH 7.0) for 45

seconds followed by 2 mL of pepsin (1 mg/mL in 0.02M HCl, pH 2.0) and incubated for another 30 minutes to mimic gastric digestion. The time gap between cooking, cooling and enzyme treatment was kept to a minimum (10 minutes) to avoid retrogradation of starch molecule. The acidified mixture was then neutralized with 2 ml of 0.02M NaOH followed by the addition of 4 mL sodium acetate buffer (pH 6.0, 0.2M) and 1 mL of a mixture of pancreatin (2mg/ mL) and amyloglucosidase (28U / mL). The mixture was incubated at 37°C with continuous mixing. At each time interval (5, 10, 15, 20, 30, 40, 50, 60, 90, 120, 260, and 360 min.), 100 µL aliquots were taken into a fresh tube and mixed with 300 uL of stop solution to prevent further analysis. After centrifugation, glucose concentration in the supernatant was determined using a glucose oxidase colorimetric analysis kit (Megazyme)

2.4. Animal Treatment

Twenty-one female Wistar rats (*Rattus norvegicus*) aged 6–8 weeks with an average weight of 200 g were house in filtered- top cage with controlled environment at temperature of 21 ± 1°C, humidity of 50 ± 10%, light of 325 lux and noise ≤ 85 dB with a 12 h light/dark cycle. The experimental animals were handled under ethical consideration and the experimental protocol was approved by Institutional Ethic Committee of Chiang Mai University (Protocol Number 2563/RT-0003). After acclimating to the facility for one week, the Wistar rats were randomly assigned into three groups, with seven rats per group and administrated different pasta at the daily dose of 4 g (dried weight)/kg body weight as follows: (1) wheat macaroni (control) (2) macaroni prepared from RS4 banana flour and (3) macaroni prepared from RS4 flour supplemented with turmeric 0.15% (weight/weight).

Cooked macaroni was prepared and blended with water (1:2 (w/v)) prior to feeding to the animals by oral gavage every morning for 6 weeks. Animals were provided with Diet CE-2 diet (Nomura Siam International Co., Ltd) *at libitum* as a basic diet. Animals were weighed and recorded weekly. After 6 weeks, rats were sacrificed using thipental (short acting barbiturates) and their internal organs and tissues were collected.

2.5. Measurement of Animal Characteristics

The body weight of rats was measured daily and volume of diet and water consumed were measured every week. After 6 weeks, rats were sacrificed and internal organs, which include stomach, liver, kidney, lung, spleen, brain, gastrointestinal tract (GI tract) and heart will be subjected to size and weight measurement. The tissue sections were prepared, stained with hematoxylin & eosin (H&E staining) and subjected to histopathological examination for any tissue lesions by the pathologists.

2.6. Measurement of Blood Chemistry

Serum was collected from the saphenous vein after fasting overnight and the blood clinical chemistry testing was performed at baseline and after 6 weeks. The blood clinical chemistry tests included liver function test (Aspartate aminotransferase; AST, Alanine aminotransferase; ALT), renal function test (blood urea nitrogen; BUN, creatinine), fasting blood glucose, insulin and lipid profile (total cholesterol, HDL-cholesterol, LDL-cholesterol and triglyceride) were performed using Dimension® RxL Max®clinical chemistry system (Siemens Healthcare Diagnostics Inc. Newark, U.S.A.). Fasting blood level of insulin was measured using Human Insulin ELISA Kit (ab100578) (Abcam). The concentration of fasting blood glucose and insulin were used to calculate homeostasis model assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI), which have been used to quantify degrees of insulin resistance and β-cell secretory capacity. [27]

2.7. Measurement of Expression of Genes Involved in Cholesterol Metabolism and Metabolic Syndrome

Total RNA was isolated from liver tissues of the experimental animals using NucleoSpin RNA, Mini kit for RNA purification (Machrey-Nagel). cDNA synthesis was performed on 1 µg of total RNA using the Tetro cDNA synthesis kit (Meridian Bioscience). The PCR reactions were performed using cDNA from each sample, and primer sequences and the cycling conditions previously described as followed: HMG CoA reductase (HMGR) [28], LDL receptor-related protein-1 (LRP-1) [29], insulin-like growth factor-1 (IGF-1) [30], insulin-like growth factor-1 (IGF-2) [31], 18s rRNA [29].

2.8. 16S rRNA Sequencing-based Gut Microbiome Profiling

16S rRNA based sequencing enables identification of the entire microbial community within a sample up to the species level. Genomic DNA (gDNA) extracted from animal's feces at baseline and before being euthanized were submitted to 16S rRNA amplicon sequencing (NovogeneAIT Genomics Singapore Pte Ltd, Singapore). Sequences are clustered into phenotypes termed "operational taxonomic units" or OTUs which is a definition used to classify groups of closely related organisms. DNA sequences can be clustered according to their similarity to one another, and OTUs are defined based on the similarity threshold (usually 97% similarity) set by the researcher. Representative sequences of each OTUs are then interrogated against a reference database of validated 16S rRNA gene sequences. Relative abundances of bacterial taxonomy can then be calculated and presented in graphical representation.

2.9. Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 15.0 (IBM, Chicago, IL, United States). Differences in blood chemistry parameters between groups and were analyzed using One-Way Analysis of Variance Kruskal-Wallis ANOVA. Alpha-diversity levels were determined by observed abundances and by estimators, which includes Chao1, Shannon and abundance-based coverage estimator (ACE). Beta diversity was measured using both

weighted and unweighted unifrac distance metrics. $\rho<0.05$ was taken as statistical significance.

3. Results

3.1. Resistant Starch Content and *in vitro* Digestion Characteristic of Wheat and Banana RS4 Macaroni

The resistant starch content of banana RS4 macaroni after cooking was determined in comparison to two the commercially available wheat macaroni. The result showed that after cooking the RS4 banana macaroni contains the resistant starch that resist digestion by amylase and amyloglucosidase for 16 hours at 37°C up to about 40% (w/w total starch), whereas the commercially available brands contains only 1-2 % (w/w total starch) respectively (Figure 1a). The *in vitro* digestion demonstrated almost half reduction of glucose hydrolyzed from RS4 banana macaroni compared to commercially available macaroni (Figure 1b), which is a good indication that a significant undigested proportion of banana RS4 macaroni would reach and being fermented by normal flora bacteria in the large intestine.

Table 1. Nutrition composition of banana RS4 macaroni in comparison to wheat macaroni

Nutrition composition/ kg	Wheat macaroni (Control)	Banana RS4 macarroni
Ash (g)	10.02	10.96
Total carbohydrate (g)	720.17	820.11
Available Carbohydrate (g)	700.7	490.2
Fat (g)	20.88	6.90
Protein(g)	131.1	48.9
Calories (kcal)	3682	2122
Dietary fiber (g)	64.5	356.7

3.2. Body and Gastro-intestinal Weight and Blood Chemistry Variables in Rats Fed with Wheat or Banana RS4 Macaroni

Rats were in good health and there were no significant growth differences at any interval throughout the study. All biochemical markers for liver functions (AST, ALT) and kidney functions (BUN, creatinine) were within normal range (Table 2). Interestingly, rats receiving banana RS4 macaroni showed significantly lower weight of an overnight fasted stomach and total GI tract compared to wheat macaroni control group.

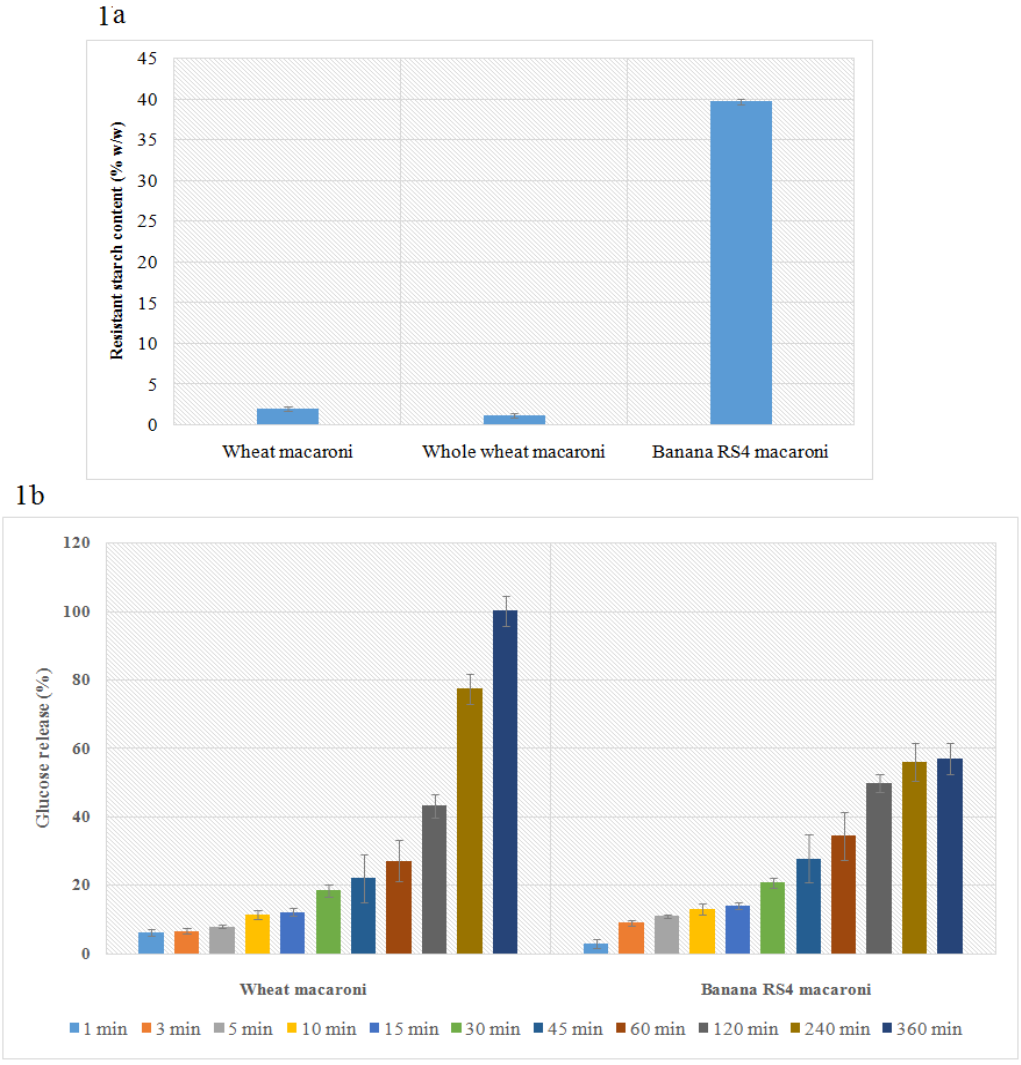

Figure 1. Characteristic of cooked banana RS4 macaroni in comparison to wheat macaroni, resistant starch content (1a) the amount of glucose hydrolyzed from *in vitro* digestion (1b)

Table 2. Effect of banana RS4 macaroni with and without turmeric extract on internal organ weights and bodyweights compared to wheat macaroni controls

Type of macaroni given / Weight (g)	Wheat (control)	Banana RS4	Banana RS4 ± Turmeric
body weight	240.1 ± 5.28	237.3 ± 5.89	243.0 ± 10.00
kidneys	1.6 ± 0.10	1.6 ± 0.12	2.1 ± 0.65
lung	1.9 ± 0.26	1.8 ± 0.37	2.1 ± 0.36
liver	7.3 ± 0.23	6.0 ± 2.21	7.5 ± 1.00
heart	0.77 ± 0.05	0.91 ± 0.25	0.98 ± 0.34
brain	1.7 ± 0.27	1.7 ± 0.06	2.0 ± 0.36
spleen	0.55 ± 0.05	0.49 ± 0.05	0.72 ± 0.29
Gastrointestinal (GI) track			
○ stomach	2.2 ± 0.09	**1.8 ± 0.15***	2.2 ± 0.43
○ jejunum	3.0 ± 1.07	2.69 ± 0.70	3.0 ± 0.31
○ ileum	2.2 ± 1.17	1.7 ± 0.71	2.1 ± 0.52
○ colon	1.7 ± 0.66	1.9 ± 0.82	1.8 ± 0.72
• total caecum	6.5 ± 0.88	5.3 ± 0.72	6.1 ± 0.80
○ caecum • empty	2.9 ± 0.50	2.1 ± 0.49	2.7 ± 0.64
• content	3.5 ± 0.77	3.18 ± 1.02	3.4 ± 0.59
total GI tract	18.1 ± 4.11	**15.9 ± 3.68***	18.1 ± 3.59

Note: * significant different from control group at p<0.05 by Kruskal-Wallis rank-sum test.

3.3. Influence of Banana RS4 Macaroni on Glucose Homeostasis and Lipid Profile

After 6th week, blood were collected after an overnight fast and subjected to glucose and insulin measurement, which were then utilized for the calculation of the homeostasis model assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI). Serum lipid profile, which includes total cholesterol, HDL-cholesterol, LDL-cholesterol and triglyceride were also measured. Although it was not statistically significant, the results showed that rats received banana RS4 macaroni had lower fasting blood glucose and HOMA-IR in comparison to control group (Table 3). Levels of blood triglyceride were significantly decreased in rats received banana RS4 macaroni and banana RS4 macaroni supplemented with turmeric extract (Figure 2a). The addition of turmeric extract did not show any significant impact on fasting blood glucose or triglyceride lowering effect of banana RS4 macaroni. In comparison to wheat control group, levels of total cholesterol (Figure 2b) and LDL-cholesterol (Figure 2c) in rats received banana RS4 macaroni showed the tendency to be reduced and further reduction was seen with turmeric extract supplementation, nevertheless it was not statistically significant.

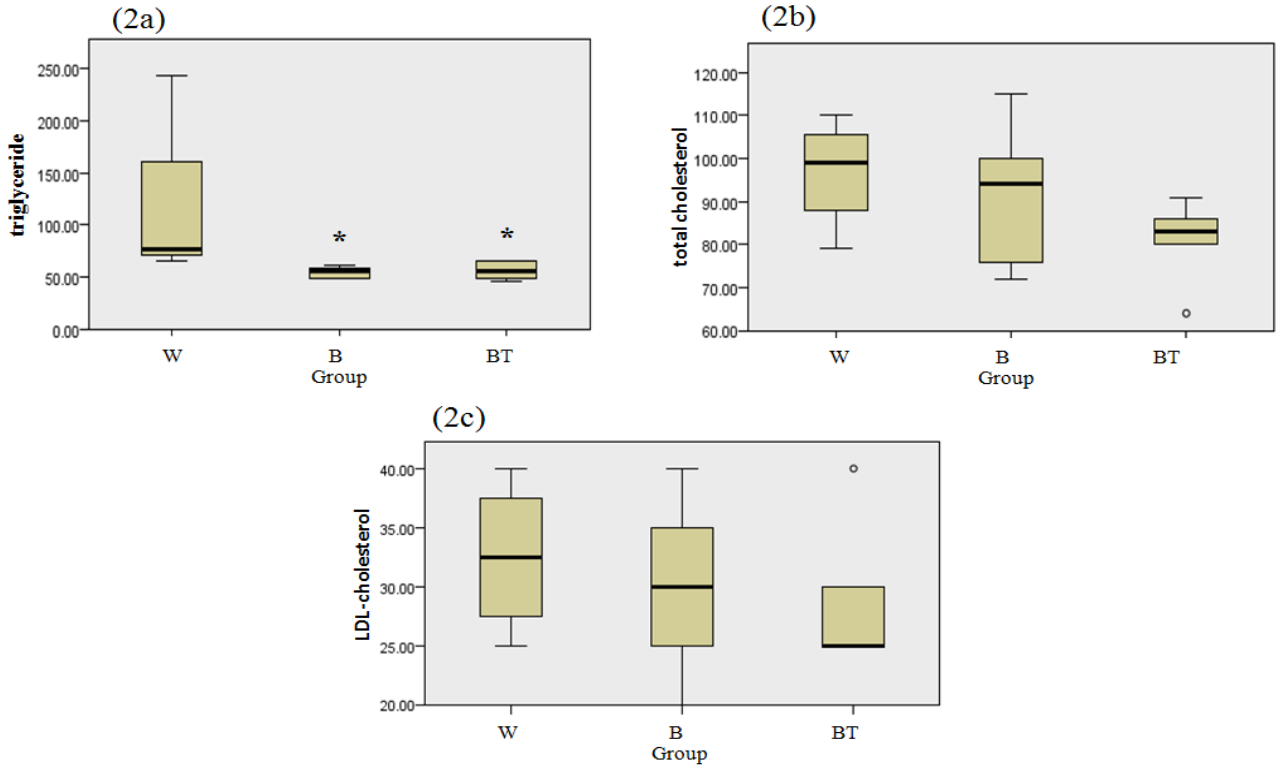

Figure 2. Boxplots showing serum levels of triglyceride (2a) total cholesterol (2b) and LDL-cholesterol (2c) in rats fed with wheat macaroni (W) with banana RS4 macaroni (B) or with banana RS4 supplemented with turmeric extract macaroni (BT) (Note: significant different from control group at p<0.05 by Kruskal-Wallis rank-sum test)

Table 3. Results of liver function tests and renal function tests of Wistar rats after 6 weeks of macaroni consumption

Type of macaroni given / Tests	Wheat (control)	Banana RS4	Banana RS4 + Turmeric	*Reference range*[a]	*Reference range*[b]
AST (U/L)	197.8 ± 24.3	202 ± 17.7	161 ± 53.4	*65 - 203*	*44 - 153.5*
ALT (U/L)	58.5 ± 9.3	61.8 ± 18.4	47.5 ± 14.1	*16 - 48*	*24.1 - 113.9*
BUN (mg/dL)	17.5 ± 3.7	18.1 ± 3.2	18.3 ± 4.3	*13.2 - 27.1*	*17.6 - 24.4*
Creatinine (mg/dL)	0.63 ± 0.05	0.64 ± 0.05	0.64 ± 0.05	*0.2 - 0.6*	*0.57 - 0.83*

[a] Giknis Mary L.A., Clifford Charles B. Clinical Laboratory Parameters for Crl:W I(Han), Charles river: 2008.
[b] Whary Mark T. et al, Laboratory Animal Medicine (BOOK 1), American College of Laboratory Animal Medicine Series.

3.4. Influence of Banana RS4 Macaroni on Expression of Genes Involved in Cholesterol Metabolism and Metabolic Syndrome

Total RNA were extracted from liver tissues and subjected to determination of expression of genes involved in cholesterol metabolism and metabolic syndrome by RT-PCR. The expression levels of gene related to cholesterol metabolism, HMGR an LRP1, were not significantly changed in comparison to control group (Figure 3, Figure 4). Nevertheless, rats given banana RS4 macaroni supplemented with turmeric extract showed the tendency to have decreased levels of HMGR an LRP1 expression (Figure 4a-b), which is consistent with the reduction of serum cholesterol in this group (Figure 2b).

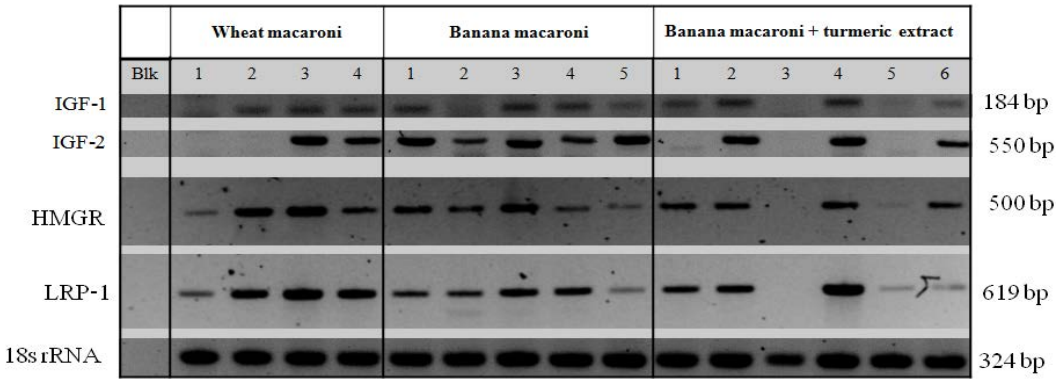

Figure 3. Agarose gel electrophoresis showing expression levels of genes involved in cholesterol metabolism (HMGR and LRP-1) and metabolic syndrome (IGF-1 and IGF-2) assessed by semi-quantitative RT-PCR

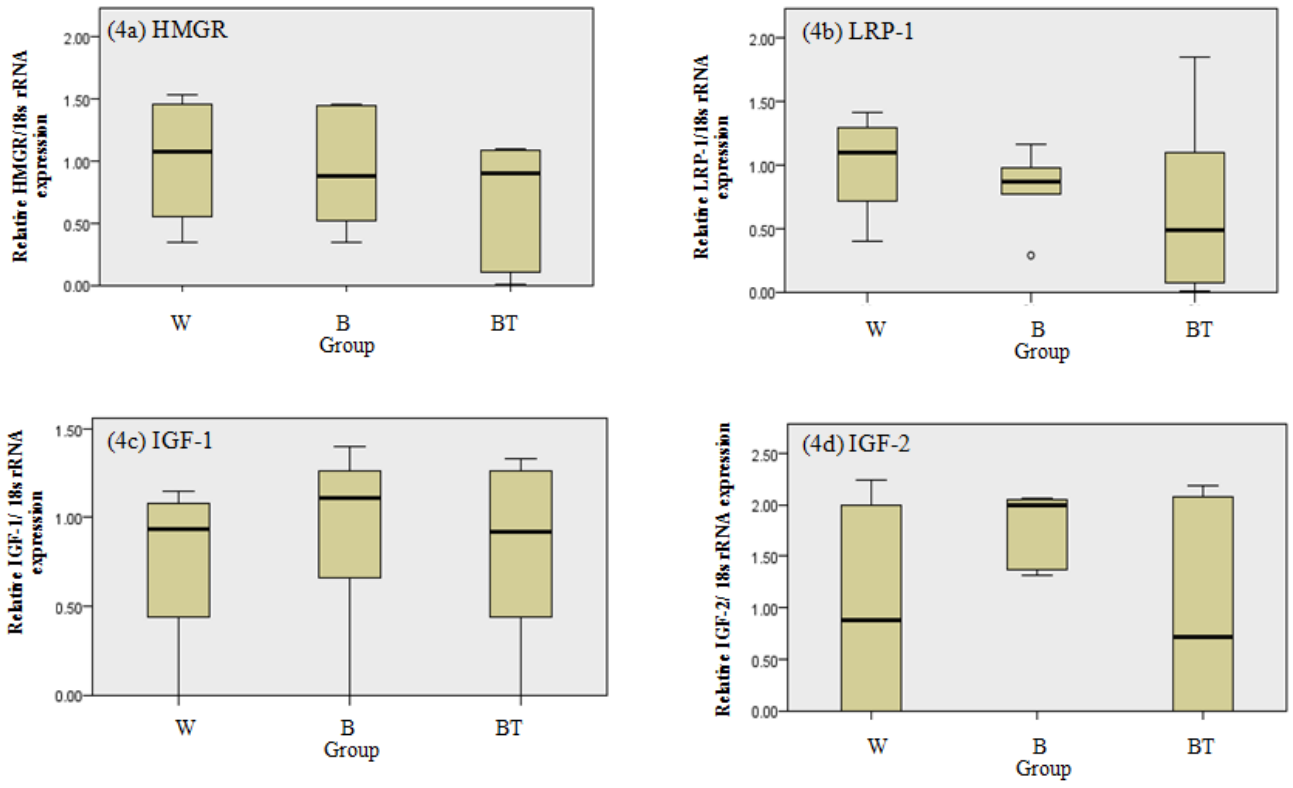

Figure 4 Boxplots showing relative expression levels of HMGR (4a), LRP-1 (4b), IGF-1 (4c) and IGF-2 (4d) in relation to 18S rRNA in liver tissues of rats fed with wheat macaroni (W) with banana RS4 macaroni (B) or with banana RS4 supplemented with turmeric extract macaroni (BT)

The results also showed that expression levels of IGF-1 and IGF-2 (Figure 3, Figure 4) were not significantly different between each groups. Nevertheless, IGF-2 expression in rats received banana RS4 macaroni were interestingly increased to the detectable and similar levels in every rats, while the expression in other groups were varied with about half of all the rats expressed undetectable levels of IGF-2 (Figure 3, Figure 4d).

3.5. Influence of Banana RS4 and Turmeric Extract on Gut Microbiome Profiling

The V3-V4 region of the 16S rRNA gene was amplified and sequenced to determine the compositional changes of the microbiota upon intervention with wheat macaroni, banana RS4 macaroni and banana RS4 macaroni supplemented with turmeric extract.

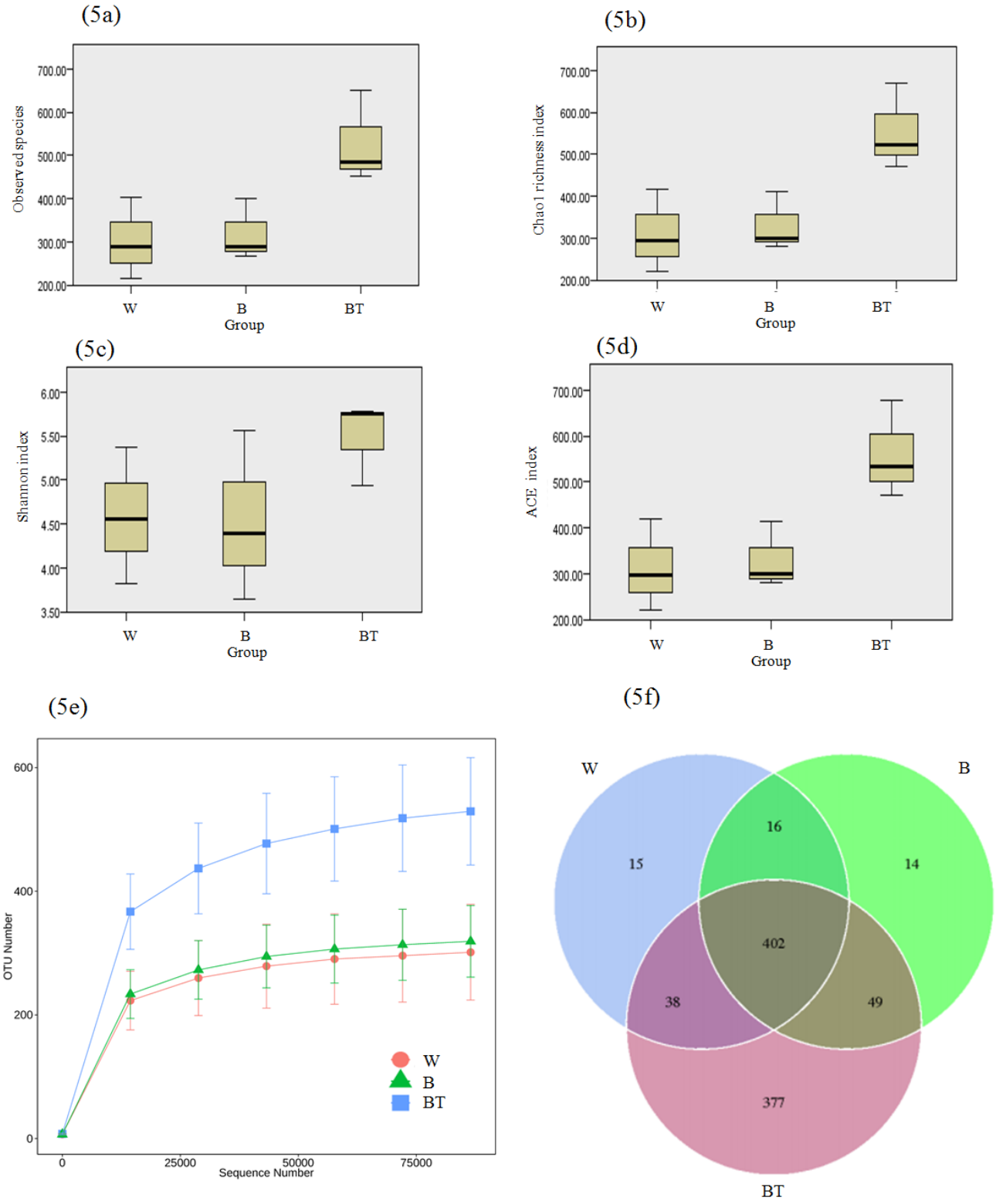

Figure 5. Analysis of alpha diversity in rats fed with banana RS4 macaroni with or without turmeric extract compared with wheat macaroni control. Observed species (5a), Chao1 richness index (5b), Shannon index (5c), ACE index (5d), species accumulation curves (5e), Venn and Flower diagram (5f). Rats were fed with wheat macaroni (W) or with banana RS4 macaroni (B) or with banana RS4 supplemented with turmeric extract macaroni (BT)

Table 4. Effect of banana RS4 macaroni and turmeric extract on insulin sensitivity and serum lipid profile

Type of macaroni given / Weight	Wheat (control)	Banana RS4	Banana RS4 + Turmeric
Glucose homeostasis			
Fasting Blood Glucose (mg/dL)	165.8 ± 22.0	150.6 ± 15.8	162.0 ± 13.6
Fasting insulin μU/mL)	9.2 ± 1.2	9.5 ± 0.31	9.9 ± 1.15
HOMA-IR index	3.8 ± 0.66	3.5 ± 0.44	3.9 ± 0.48
QUICKI index	0.315 ± 0.010	0.318 ± 0.004	0.315 ± 0.005
Lipid profile			
Triglyceride	115.3 ± 85.3	**54.4 ± 5.7***	**62.8 ± 22.2***
Total cholesterol	96.8 ± 13.0	91.4 ± 17.7	81.2 ± 9.3
HDL-cholesterol	38.8 ± 13.0	30.6 ± 4.1	30.0 ± 3.5
LDL-cholesterol	32.5 ± 6.5	30.0 ± 7.9	28.3 ± 6.1

Note: * significant different from control group at $p<0.05$ by Kruskal-Wallis rank-sum test.

3.5.1. Richness and Diversity Analysis

The alpha diversity indices, including observed species, Chao1, Shannon index, and abundance-based coverage estimator (ACE), were calculated for each data set. Our results showed that the fecal microbiota of rats fed with banana RS4 supplement with turmeric macaroni (BT) had overall higher alpha diversity than those of the control rats fed with wheat macaroni (W) or rats fed with banana RS4 macaroni (B), although no significant difference was observed by Kruskal-Wallis test (Figure 5a-d). Venn and Flower diagram shows the analysis of both the common and unique information for different groups. The result shows that rats in BT group has adopted more unique information than those in the other two groups (Figure 5f). Beta diversity, which refers to the diversity between samples was evaluated with weighted-UniFrac analysis. Boxplot generated shows that rats fed with RS4 banana macaroni or RS4 banana supplemented with turmeric extract macaroni has adopted higher level of diversity between samples (Figure 6), although it was not statistically significant.

3.5.2. Community Differences Analysis

According to the taxonomic annotation results, top 10 phyla of each group were selected to form the distribution histogram of relative abundance of phyla. The relative abundance of taxa in the phylum is illustrated in Figure 7a. Although it was not statistically significant, fecal samples from rats in BT group showed higher relative abundance of *Firmicutes* and lower *Bacteroidetes* thus have adopted an increase ratio of *Firmicutes* to *Bacteroidetes* (F/B). (Figure 7b-7d). However, information from the taxonomic abundance cluster heatmap, which was plotted according to the top 35 genus presented in all samples showed that the relative abundance of bacteria in *Firmicutes* phyla was decreased in fecal samples from rats fed with banana RS4 macaroni (B group) compared to controls (W group). The addition of turmeric extract into banana RS4 macaroni did cause an increase of relative abundance of bacteria in *Firmicutes* Phyla, but different strains of bacteria within the *Firmicutes* Phyla was increase (Figure 7e).

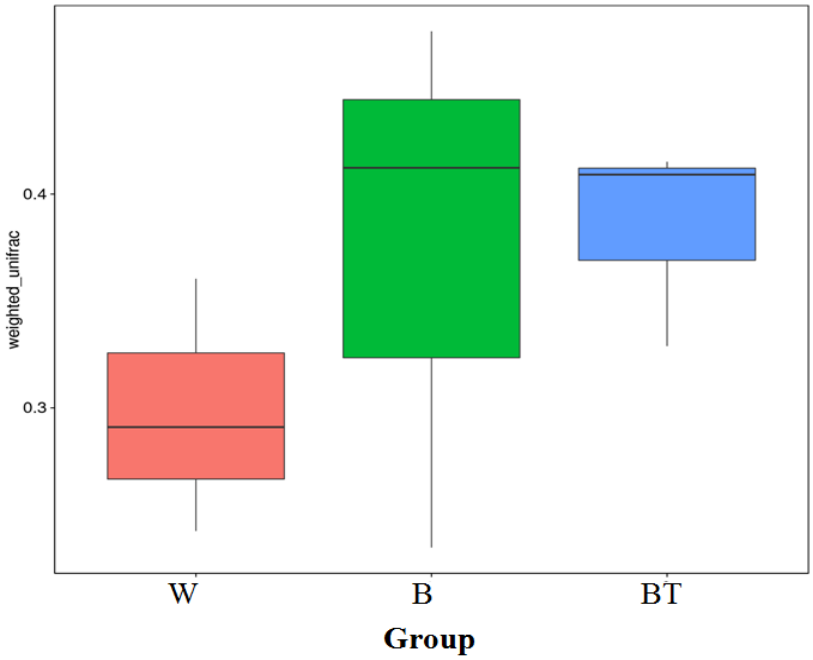

Figure 6. Analysis of beta diversity in rats fed with banana RS4 macaroni with (BT) and without (B) turmeric extract compared with wheat macaroni control (W)

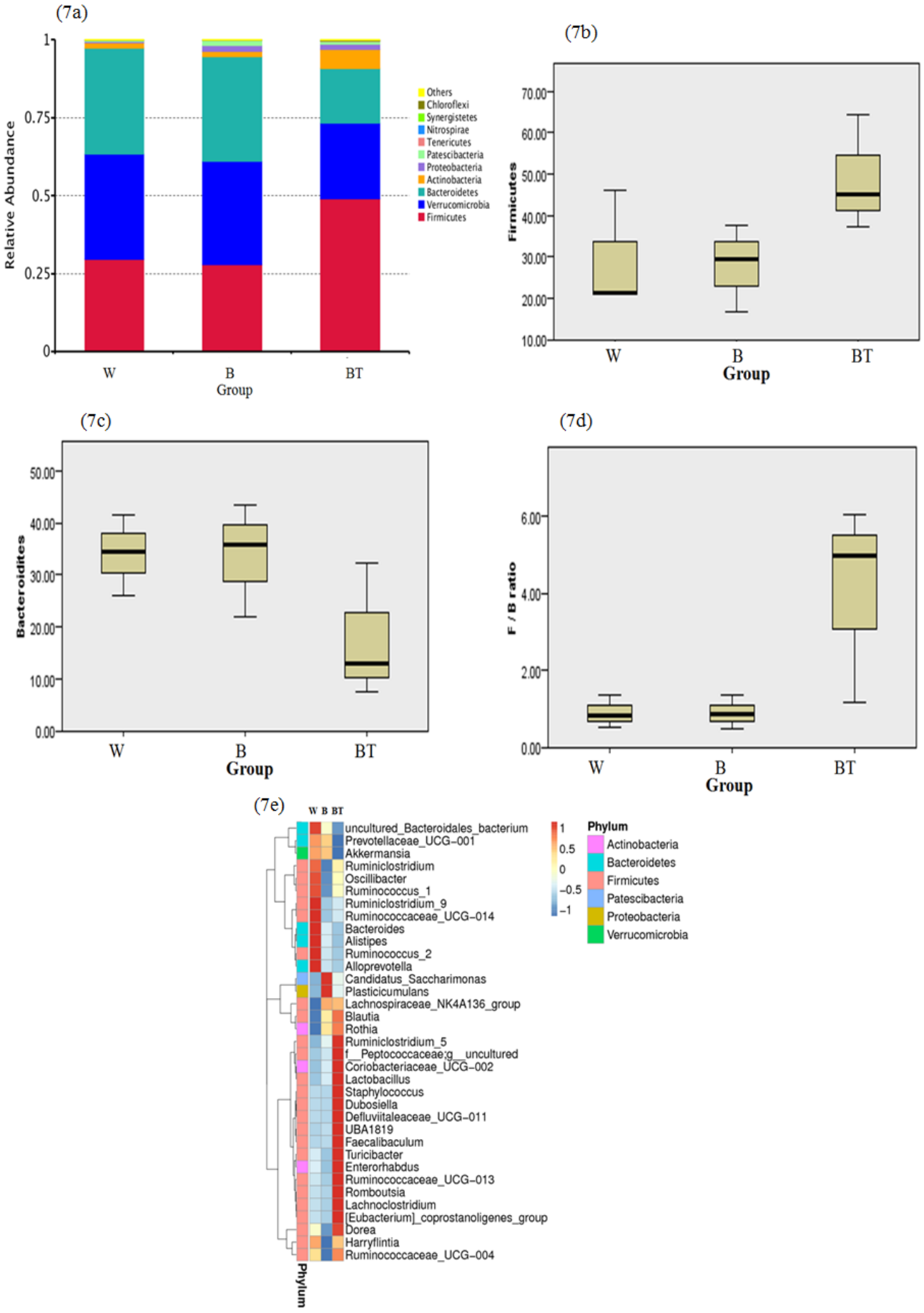

Figure 7. Composition of gut microbiota in rats fed with banana RS4 macaroni with (BT) and without (B) turmeric extract compared with wheat macaroni control (W). The relative abundance of 10 most common phyla (7a) Relative abundance of *Firmicutes* phylum (7b), *Bacteroidetes* phylum (7c), *Firmicutes / Bacteroidetes* (F/B) ratios (7d) and taxonomic abundance cluster heatmap (7e)

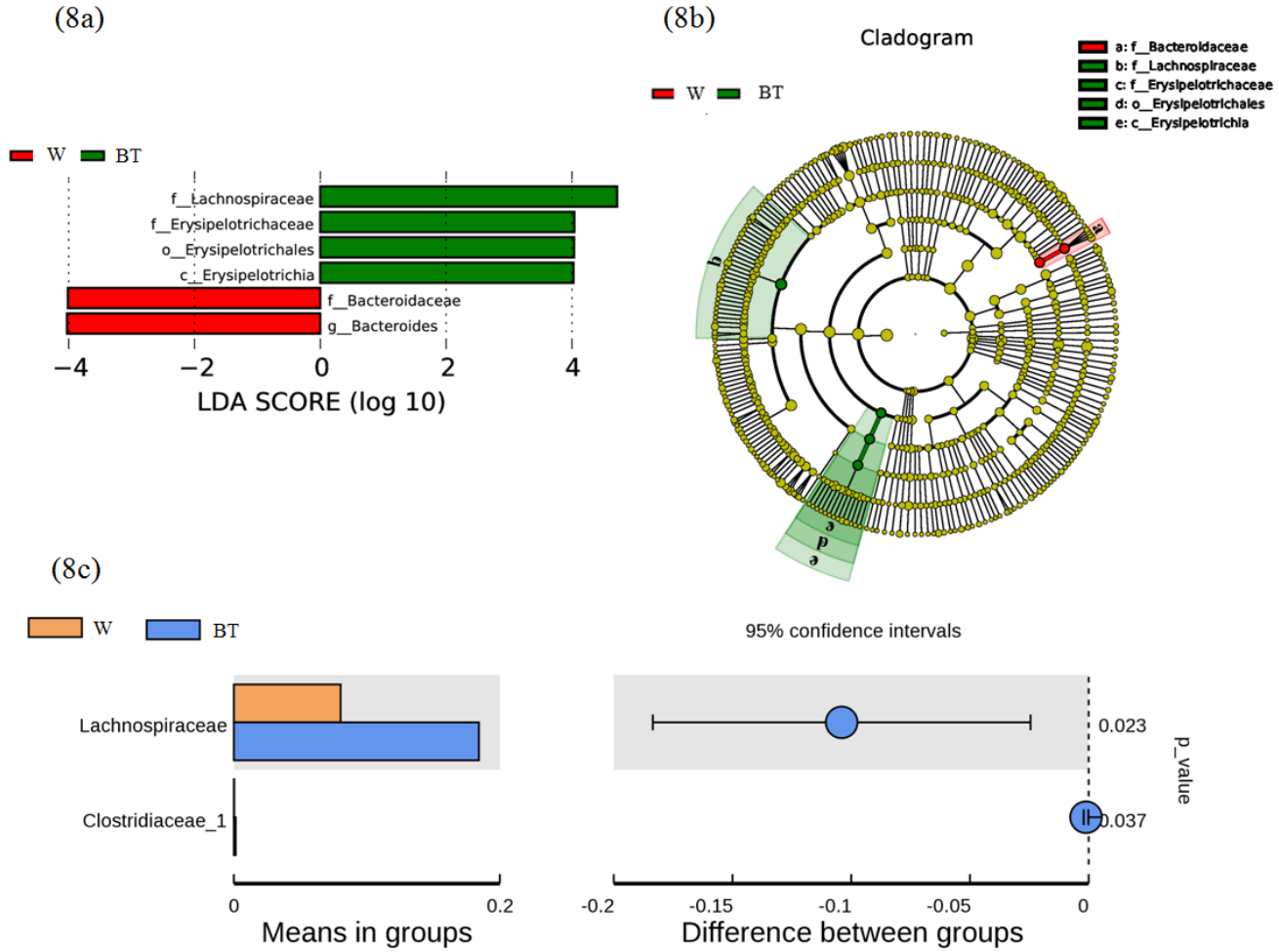

Figure 8. Taxonomic differences of gut microbiota were detected between rats fed with banana RS4 macaroni supplemented with turmeric extract (BT) and those fed with wheat macaroni control (W). Linear discriminative analysis (LDA) effect size (LEfSe) analysis (8a). Cladogram showing differentially abundant taxonomic clades (8b). The relative abundance of *Lachnospiraceae* (P=0.023) and *Clostridiaceae* (P=0.037) was significantly higher in BT group compared with W group (8c)

We further compared taxa in the wheat control group versus B or BT groups by discriminant analysis effect size (LEfSe). LEfSe analysis revealed that *Lachnospiraceae, Erysipelotrichaceae, Erysipelotrichales* and *Erysipelotrichia, (Firmicutes* phylum) were all significantly more abundant in fecal samples from the rats fed with RS4 banana supplemented with turmeric extract, and conversely significantly less abundant of *Bacteroidaceae* and *Bacteroides* (*Bacteroidetes* phylum) compare to wheat control group (Figure 8a). A cladogram shown in Figure 8b represented the connection between the significantly different taxa at different taxonomic levels showing that *Erysipelotrichaceae* (family) is under *Erysipelotrichales* (order) which is under *Erysipelotrichia* (class). Microbial compositions showed that relative abundance of *Lachnospiraceae* (p = 0.023) and *Clostridiaceae* (p= 0.037), which are classed in *Firmicutes* phylum, in BT rats was statistically significantly higher than those in rats fed with wheat control macaroni (Figure 8c).

4. Discussion

Resistant starch (RS) is now recognized to have beneficial effects against diabetes, obesity and cardiovascular diseases through its mechanisms as a prebiotic dietary fiber subjected to fermentation by the gut microbiota in the intestine [4,32]. Intakes of RS of 15-20 grams per day are recommended for supporting human bowel health [33]. However, the intake of RS per person is reported to be generally low with average of 3–10 g/d [34] worldwide, which are too low to have beneficial effects [35]. Although unripe banana is considered the RS-richest non-processed food, it is not usually consumed directly due to its hardness and high astringency. However, when the native resistant starch (RS2) of unripe banana is cooked, its resistant starch become digestible. The banana RS4 used in this study was able to maintain its indigestibility after being cooked, thus replaced digestible starch in formulating into macaroni pasta. The experimental animals were subjected to consume macaroni pasta at concentration of 4 g/kg bodyweight of rat/ day, which equals the amount of 15-20 grams of RS per day in human. Our results show that ingestion of theses amount of banana RS4 macaroni did not show any harmful effect, but conferred health beneficial effects to healthy rats consuming regular diet and low dose of turmeric extract supplementation significantly altered gut microbiota.

The results also showed that although the total body weight of experimental animal was not significantly different from each other, rats fed with banana RS4 macaroni significantly have lower weight of an overnight

fasted stomach and total GI tract compared to wheat macaroni control group. Digestion of RS is believed to occur over a 5–7 hours period, in contrast to digestible starch that occur immediately, thus reduce postprandial glycemia and insulinemia and increase the period of satiety. RS4 starch have been previously reported to lower glucose responses due to its distal absorption and the ability to delay gastric emptying [36]. Stomach functions as a primary source of satiety. As RS has been previously reported to reduce food intake [37,38], therefore, it is possible that the smaller size of stomach and overall GI tract in rats fed with banana RS4 may due to the lower amount of food regularly consumed by the animals.

Wistar rats receiving banana RS4 macaroni showed significantly lower level of triglyceride ($p<0.05$, Kruskal Wallis test) and a trend of lower fasting blood glucose and increased level of IGF-2. With a number of previous studies frequently reported the decrease of IGFs expression levels in metabolic syndrome [39,40], the induction of IGF-2 in rats consuming banana RS4 macaroni indicate its potential health benefits for inducing and balancing liver IGF-2 expression levels. Although it was not significantly different, rats given banana RS4 macaroni supplemented with turmeric extract showed the tendency to have decreased levels of HMGR an LRP1 expression, which was consistent with the reduction of serum cholesterol and LDL-C in this group of rats. The inhibitory effect of turmeric extract on HMGR expression has been previously reported [41], but to our knowledge the inhibition of LRP1 expression has not been previously described. Several studies have demonstrated the protective effects of curcumin, an active component of turmeric, against many chronic diseases [42]. Consumption of 2.1 g/day turmeric extract has been shown to reduce HbA1c, FPG, triglyceride and LDL-C in hyperlipidemic type 2 diabetes patients [43]. Due to its bitter taste, the allowable maximum proportion of turmeric extract in banana RS4 macaroni was only 0.15% (w/w). Rats receiving 4g/kg bodyweight of banana RS4 macaroni supplemented with 0.15% (w/w) is equaled about 300 mg per day for human only. Addition of turmeric extract in banana RS4 macaroni provided additional effect on the suppression of serum cholesterol, although it was not statistically significant. Nevertheless, turmeric extract supplementation statistically affected gut microbiota. This may partly due to its poor absorptivity as it has been reported that an oral dose of 1,000 mg/kg of curcumin administered to rats resulted in as much as 75% of the dose being excreted in feces [44].

Consumption of banana RS4 supplemented with turmeric extract increased alpha diversity of fecal microbiome as indicated by Chao1 richness index, Shannon index and ACE index. LEfSe analysis revealed that consumption of banana RS4 supplemented with turmeric extract statistical significantly increased relative abundance of *Lachnospiraceae* and *Erysipelotrichaceae* while reduced the abundant of *Bacteroidaceae* in rat's fecal samples in comparison to those from the control rats fed wheat macaroni. Previous study has also reported that turmeric increased the number of taxa and drove expansions of *Erysipelotrichaceae* and *Lachnospiraceae* [45]. These families of bacteria exhibit the character of fermenting a wide variety of sugar and amino acid which highlights the effect of medicinal herb in restructuring fecal microbiota.

Lachnospiraceae belong to the core of gut microbiota, colonizing the intestinal lumen from birth and increasing species richness and relative abundances during the host's life. *Lachnospiraceae* family are the main producers, among *Firmicutes* phylum, of short-chain fatty acids (SCFAs). However, different taxa of *Lachnospiraceae* are also associated with different intra- and extra-intestinal diseases indicating its controversial role in human health. Martinez *et al*. reported that whole grain consumption increased the microbial diversity (alpha diversity) and abundance of *Firmicutes*, which was primarily derived from an increased abundance of *Blautia* and *Roseburia* (members of *Lachnospiraceae* family) [46]. A recent study also positively correlated *Lachnospira* which is a member in the *Lachnospiraceae* family to the intake of beta-carotene, vitamin E and vegetable fat whereas a negative correlation was found with meat, total proteins, and cholesterol [47]. In contrast, *Lachnospiraceae* has been reported to actively impair glucose metabolism, leading to inflammation and promoting the onset of type 1 diabetes (T1D) [48] and may also associate with type 2 diabetes (T2D) [49].

Reports documenting a potential role of *Erysipelotrichaceae* family, another member of *Firmicutes* phylum, in host physiology and/or disease are on the rise. The specific taxa within *Erysipelotrichaceae* may be correlated to inflammation [50], while others are highly immunogenic [51]. Higher levels of *Erysipelotrichaceae* in obese individuals [52], or mice on high-fat or western diet have been observed. Microbial compositions analysis also showed that relative abundance of *Clostridiaceae* was significantly increase in rats fed with banana RS4 macaroni supplemented with turmeric extract. The *Clostridiaceae* are a family of the bacterial class Clostridia, and contain the genus Clostridium, which has been reported to attenuate inflammation and allergic diseases effectively owing to their distinctive biological activities, although there are some risks like toxins release and some challenges in application [53]. Nevertheless, although the contribution of *Erysipelotrichaceae, Lachnospiraceae* and *Clostridiaceae* to animal's health is not fully understood, the finding that low dose of turmeric extract could significantly increase the relative abundance of these three families of bacteria in healthy animals suggesting that further investigations are needed.

In conclusion, this study has demonstrated that banana RS4 macaroni is not harmful, but confers health benefits towards improving blood chemistry profile associated with metabolic syndrome in healthy rats consuming regular diet and low dose of turmeric extract could significantly altered fecal microbiota. Nevertheless, it remains to be investigated whether the health beneficial effect of turmeric extract is mediated through the induction bacterial families of *Erysipelotrichaceae, Lachnospiraceae* and *Clostridiaceae*.

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