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Effect of Bambara groundnut (*Vigna subterranea*) on Firmicutes and Bacteroidetes, *FGF21* gene expression, and liver histopathology in mice with low-protein diet

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ABSTRACT

Introduction: The number of Firmicutes and Bacteroidetes in the intestine is influenced by diet. Gut microbiota and fibroblast growth factor 21 (*FGF21*) form a liver-gut axis that mediates the body's response to protein restriction. Bambara groundnut (*Vigna subterranea*), which contains high levels of amino acids, has the potential to be a source of protein. This study aimed to determine the effect of Bambara groundnut on the number of Firmicutes and Bacteroidetes, *FGF21* gene expression, and liver histopathology in mice fed with a low-protein diet. **Methods:** A total of 25 mice were divided into five groups: normal protein diet (N), low-protein diet (LP), and low-protein diet with supplementation of 100 g (LPLB), 200 g (LPMB), and 300 g (LPHB) of Bambara groundnut, respectively. After 2 months of intervention, mice were sacrificed, the number of Firmicutes and Bacteroidetes in the intestines and faeces, as well as *FGF21* gene expression were analysed, while liver histopathology was visualised. **Results:** Results showed that Bambara groundnut supplementation increased the growth of Firmicutes and Bacteroidetes, decreased *FGF21* gene expression, and reduced liver inflammation caused by a low-protein diet. **Conclusion:** Bambara groundnut supplementation has the potential to increase the amount of Firmicutes and Bacteroidetes, decrease the expression of adaptive stress gene *FGF21*, and improve the degree of liver inflammation in a low-protein diet.

Keywords: Bacteroidetes, bambara groundnut, *FGF21*, Firmicutes, low-protein diet

INTRODUCTION

Dietary protein has been known to influence the composition of microbiota in the digestive tract, including Firmicutes and Bacteroidetes. Studies on mice have provided evidence that dietary protein can affect not only the diversity, but also the overall microbial biomass (Bartlett & Kleiner, 2022). A low-protein diet decreases the number of

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Firmicutes and Bacteroidetes, which is related to homeostasis and pathological pathways. Therefore, many therapeutic strategies are suggested to achieve a proper Firmicutes and Bacteroidetes ratio to maintain homeostasis (Abenavoli *et al.*, 2019). Zhao *et al.* (2020) found that 2% dietary protein withdrawal markedly reduced the ratio of Firmicutes to Bacteroidetes. The response of the microbiota to host food produces metabolites that have significant physiological effects on the host. Thus, gastrointestinal microbiota can be used as a parameter of host nutrition.

In addition, a low-protein diet also affects the expression of fibroblast growth factor 21 (*FGF21*) gene (Zhao *et al.*, 2020), which plays a role in controlling nutritional status by regulating glucose, lipid, and energy metabolisms (Tezze *et al.*, 2019). *FGF21* is expressed in several metabolically active organs, including liver, and is only released during a low-protein diet (Solon-Biet *et al.*, 2016), so consumption of a high-protein diet reduces *FGF21* expression (Alemán *et al.*, 2019). Protein restriction activates the GCN2/pEIF2α/ATF4 pathway involved in upregulating *FGF21* gene expression; high availability of amino acids can suppress the signalling pathway (Laeger *et al.*, 2014).

Bambara groundnuts (*Vigna subterranea*) are a source of vegetable protein, which contain 15–25% protein (Oluwole *et al.*, 2022). Bambara groundnuts have a high content of essential amino acids, including isoleucine, leucine, methionine, phenylalanine, threonine, and valine (Mune Mune *et al.*, 2011). Therefore, this study examined the benefits of Bambara groundnuts on the number of intestinal microbiota, especially Firmicutes and Bacteroidetes, and the expression of the *FGF21* gene, as well as the liver histopathological appearance of mice given a low-protein diet.

MATERIALS AND METHODS

Materials

This research used Bambara groundnuts with black testa. Bambara groundnuts in the form of fine powder were sent for proximate analysis at the Centre of Food and Nutrition Studies. The fine powder was also dissolved in ethanol solvent for phenolic compound analysis using high resolution mass spectrometry (HRMS) at the Integrated Research and Testing Laboratory (Q Exactive™ High Resolution Mass Spectrometer, Thermo Fisher Scientific, Waltham, MA, USA).

Animals

In this study, the number of animals needed was calculated by the Federer formula, which is $(n - 1)(t - 1) > 15$. The n value determines the number of animals per group, while the t value determines the number of treatment groups. Based on the calculation, a total of 25 mice (*Mus musculus*) of the DDy strain, four weeks old, with a body weight of ±20 grammes (g), were divided into five groups: normal protein diet (N); low-protein diet (LP); low-protein diet with low 100 g (LPLB); moderate 200 g (LPMB); and high 300 g (LPHB) Bambara groundnut supplementation. The mice were obtained from the integrated research and testing laboratory and the study was done after receiving approval from the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada (Ref. No. KE/FK/0879/EC/2021). The diet compositions are presented in Table 1.

All study groups were acclimatised for one week with standard care (dark cycle: light 12:12 hours and room temperature 22-25°C) and fed with the standard diet of modified AIN 93 M formulation. After the acclimatisation period, the mice were given dietary treatment according to their groups for

Table 1. Diet composition per 1 kg

Ingredients	*Treatment groups*				
	N (g)	*LP (g)*	*LPLB (g)*	*LPMB (g)*	*LPHB (g)*
Maizena	621	661	561	461	361
Casein	140	100	100	100	100
Sucrose	100	100	100	100	100
Bambara groundnut	0	0	100	200	300
Corn/Soybean oil	40	40	40	40	40
Alpha cell	50	50	50	50	50
Mineral mix	35	35	35	35	35
Vitamin mix	10	10	10	10	10
Methionine	2	1.8	1.8	1.8	1.8
Choline chloride	3	2.5	2.5	2.5	2.5
Tert-Butylhydroquinone	0.00	0.01	0.01	0.01	0.01
Total	1,000	1,000.3	1,000.3	1,000.3	1,000.31

N: Normal protein (14%), LP: Low-protein (10%), LPLB: Low-protein (10%) with 100g of Bambara groundnut supplement, LPMB: Low-protein (10%) with 200g of Bambara groundnut supplement, LPHB: Low-protein (10%) with 300g of Bambara groundnut supplement

two months. Feed intake was calculated based on the amount of feed given and then deducted by the amount of feed remaining the next day. The amount of protein intake was calculated based on the percentage of protein composition in the feed for each group as follows: N 14% (140 g/kg), LP 10% (100 g/kg), LPLB 11.2% (112.89 g/kg), LPMB 12.5% (125.78 g/kg), and LPHB 13.8% (138.67 g/kg). Around 0.5 g of faeces was collected at the end of the experiment by removing the mice for 5-10 minutes, then waiting for the mice to excrete their faeces. Faeces was collected in a sterile tube containing preservation buffer (PB) and stored at -20°C. The PB contained 20 mM ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate, 25 mM sodium citrate trisodium salt dihydrate, and 5.3 M ammonium sulphate. For each treatment, around 2 ml of PB buffer was used. The duration between collection and analysis was about 2-3 hours. After that, all mice were terminated and anaesthetised with a combination injection of 0.5 ml of Ketamine and 0.25 ml of Xylazine. Mice were euthanised by neck dislocation. Liver tissue and intestines were then collected for further analysis.

Examination of gastrointestinal microbiota

Deoxyribonucleic acid (DNA) was extracted from faeces using the Quick DNA Faecal/Soil Microbe Miniprep Kit (D6010; Zymo Research, Irvine, CA, USA). The relative abundance levels of Firmicutes and Bacteroidetes were determined using the quantitative real-time polymerase chain reaction (qRT-PCR) SensiFAST™ SYBR® No-ROX Kit (BIO-98005; Bioline, London, UK).

Primer sequences for Firmicutes were:

Sense : 5'-GTCAGCTCGTGTCGTGA-3'

Antisense : 5'-CCATTGTAACGTGTGT-3'

Primer sequences for Bacteroidetes were:

Sense : 5'-AGCAGCCGCGGTAAT-3'

Antisense : 5'-CTAGCATTTCACCGCTA-3'

Examination of *FGF21* gene expression

Total RNA was extracted from liver cells using the Direct-zol RNA Miniprep Plus (R2071; Zymo Research, Irvine, CA, USA) according to manufacturer's protocol. It was then reverse transcribed to complementary DNA using the SensiFAST cDNA Synthesis Kit (Bio-65054; Meridian Bioscience, Cincinnati, OH, USA) according to manufacturer's protocol. The expression levels of *FGF21* were determined using the quantitative real-time polymerase chain reaction (qRT-PCR) SensiFAST™ SYBR® No-ROX Kit (BIO-98005; Bioline, London, UK). Relative expression was calculated by the 2–ΔΔCt method.

Primer sequences for *Mus musculus FGF21* were:

Sense :
5'-GAGGATGGAACAGTGGTAGG-3'

Antisense :
5'-CAAAGTGAGGCGATCCATAGAG-3'

Primer sequences for Mus musculus Beta Actin were:

Sense :
5'-AAGATCAAGATCATTGCTCCTCC-3'

Antisense :
5'-TAACAGTTCCGCCTAGAAGCA-3'

Statistical analysis

The data collected were analysed using IBM SPSS Statistics for Windows version 20.0 (IBM Corp., Armonk, New York, NY, USA). Data with normal distribution were analysed using the one-way analysis of variance (ANOVA) with Tukey's Honest Significant Difference (HSD) post-hoc test. Data with skewed distribution were analysed using the Kruskal-Wallis test. The *p*-value was considered significant if it was lower than or equal to 0.05 ($p \leq 0.05$).

Results of the gene expression of intestinal and faecal Bacteroidetes and Firmicutes showed normally distributed data (indicated by $p > 0.05$). Because the data were normally distributed, a homogeneity test was carried out ($p > 0.05$ indicated that the data were homogeneous). Next, a follow-up test was carried out with Tukey's HSD test ($p < 0.05$). Meanwhile, the results of *FGF21* gene expression showed abnormally distributed data, therefore analysis was carried out using a non-parametric test (Kruskal-Wallis test) ($p < 0.05$). To determine significant notation, the Mann-Whitney test was carried out ($p < 0.05$).

Histopathology

Hepatic tissues were fixed in a neutral buffered formalin solution (10%) and embedded in paraffin. Then, 4-µm thick sections were placed on adhesive slides and stained with haematoxylin-eosin. Samples were visualised using an Olympus microscope (Olympus CX21) at 100x-400x magnification using standard procedure. All imaging was performed with the group identity blinded. Images were then quantified using imaging software (Optilab; Miconos, Yogyakarta, Indonesia). Histologic examination of the hepatic cells was reviewed by a single expert pathologist who was blinded to all other features of the sample's characteristics.

All samples were evaluated for the presence or absence of the following liver damage features: sinusoidal dilatation, cloudy swelling, and inflammation. Centrilobular sinusoidal dilatation and cloudy swelling were graded according to the altered hepatic lobule zone. Liver inflammation was graded as mild if it only extended to the periportal area, mild if it extended to the periportal and intraparenchymal areas, and severe if it had reached the periportal and intraparenchymal areas with bridging necrosis (Trefts, Gannon & Wasserman, 2017).

RESULTS

Analysis of Bambara groundnut

Proximate analysis showed that Bambara groundnuts contained 10.61% water, 3.15% ash, 12.89% protein, 7.24% fat, and 66.11% carbohydrate (by difference). In proximate analysis, the Soxhlet method with Weibull modification was used for fat content analysis, the Kjedahl method for protein content analysis, the gravimetric method for water and ash analyses, and for analysis of carbohydrate content, it was a deduction of all proximate components. Amino acid analysis showed that L-lysine was the highest amount of essential amino acid in Bambara groundnuts (8,021.00 mg/kg), followed by L-histidine (4,109.70 mg/kg), L-phenylalanine (3,255.80 mg/kg), L-leucine (2,716 mg/kg), L-isoleucine (2,452.20 mg/kg), L-valine (549.10 mg/kg), L-methionine (222.60 mg/kg), L-tryptophan (93.30 mg/kg), and L-threonine (78.10 mg/kg). High Resolution Mass Spectrometry (HRMS) analysis also detected some phenolic

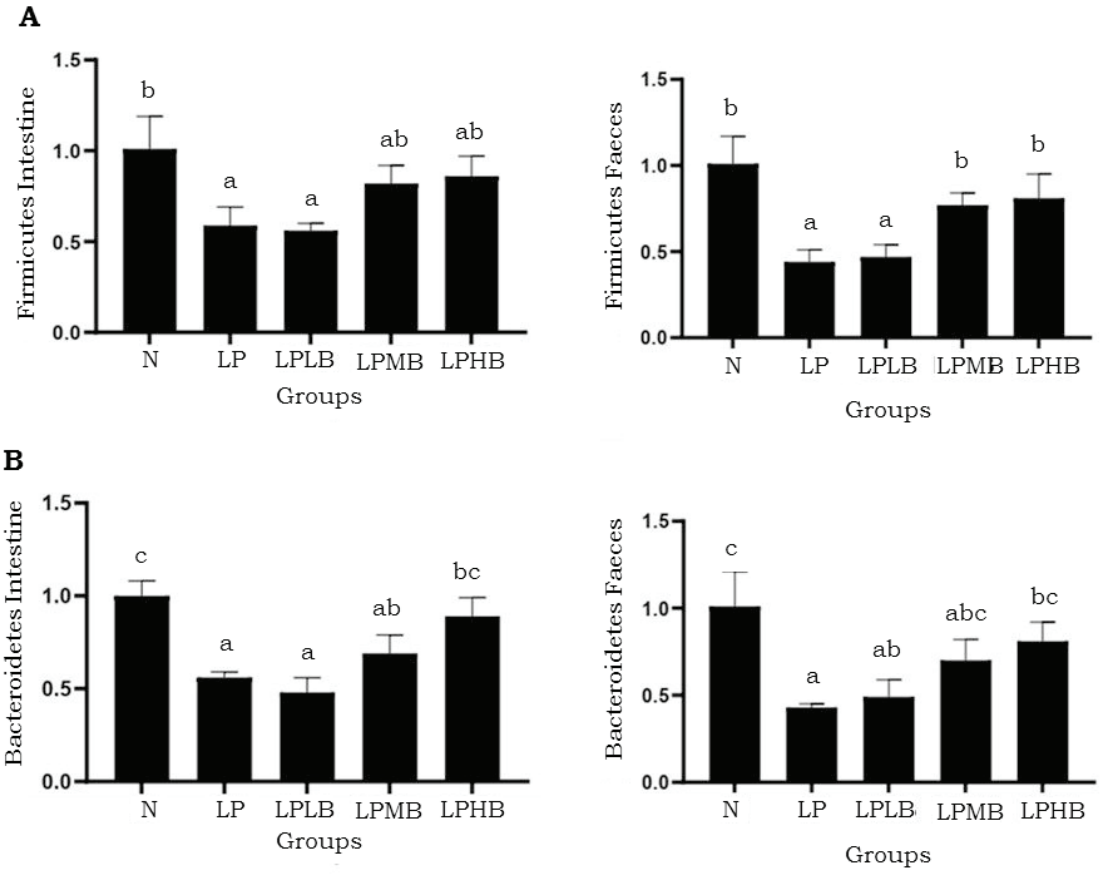

Figure 1. The effect of Bambara groundnut supplementation on the gene expression of Firmicutes (A) and Bacteroidetes (B) in low-protein diet mice

Note: (N) Normal protein diet; (LP) Low-protein diet; (LPLB) Low-protein diet + 100g of Bambara groundnut; (LPMB) Low-protein diet + 200g of Bambara groundnut; (LPHB) Low-protein diet + 300g of Bambara groundnut. Data are shown as means±standard deviation. Different letters of a, b, and c indicate significant differences between groups ($^{*}p<0.05$).

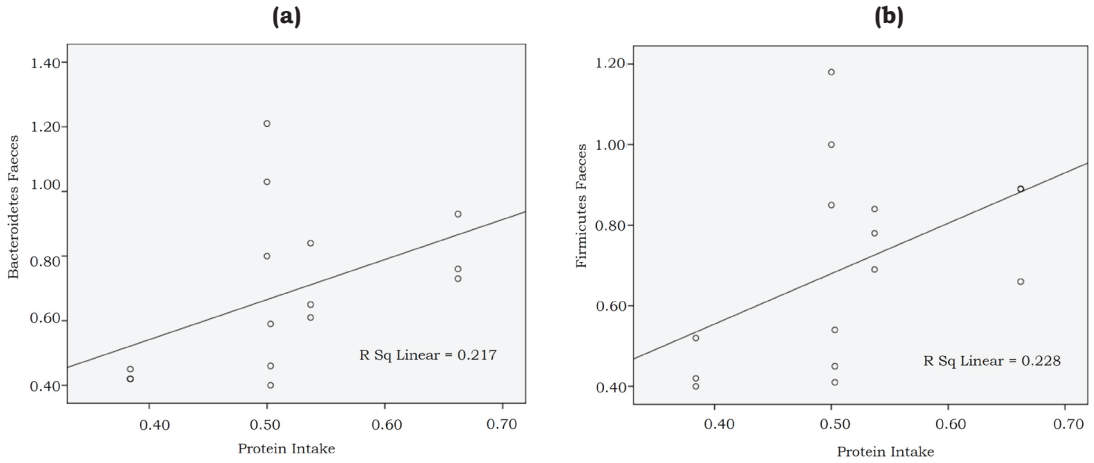

Figure 2. Correlation between protein intake and the number of (a) Bacteroidetes and (b) Firmicutes in faeces.
Correlation coefficient: 0.400*, Sig. (2-tailed): 0.048

compounds in Bambara groundnuts, such as genistin (432.10 g/mol), daidzin (416.11 g/mol), myricetin (318.03 g/mol), genistein (270.05 g/mol), and daidzein (254.06 g/mol).

Effect of Bambara groundnuts on the number of Firmicutes and Bacteroidetes in low-protein diet mice

This research revealed that a low-protein diet caused the number of Firmicutes and Bacteroidetes to greatly decrease. However, the supplementation of Bambara groundnuts did not cause significant changes to the Firmicutes and Bacteroidetes ratio in the intestine and faeces of mice (Bartlett & Kleiner, 2022). Figure 1 shows a significant increase in the number of Firmicutes and Bacteroidetes in the intestine and faeces after Bambara groundnut supplementation. The supplementation of Bambara groundnuts as much as 200 g (LPMB) and 300 g (LPHB), respectively, was proven to give better results compared to supplementing Bambara groundnuts at only 100 g. As shown in Figure 2, it appears that the higher the protein intake, the higher the number of Firmicutes and Bacteroidetes.

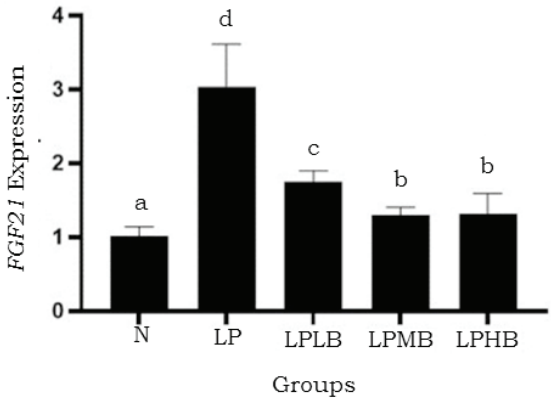

Figure 3. The effect of Bambara groundnut supplementation on the gene expression of *FGF21* in low-protein diet mice
Note: (N) Normal protein diet; (LP) Low-protein diet; (LPLB) Low-protein diet + 100g of Bambara groundnut; (LPMB) Low-protein diet + 200g of Bambara groundnut; (LPHB) Low-protein diet + 300g of Bambara groundnut. Data are shown as means±standard deviation. Different letters of a, b, c and d indicate significant differences between groups ($^{*}p<0.05$).

Effect of Bambara groundnuts on *FGF21* gene expression in low-protein diet mice

A low-protein diet could significantly increase *FGF21* gene expression compared to control group (Figure 3). All low-protein diet groups supplemented with Bambara groundnuts showed a decrease in *FGF21* gene expression. Both Bambara groundnut supplements at 200 g and 300 g, respectively, were found to give better results than Bambara groundnuts supplement at 100 g.

Effect of Bambara groundnuts on liver histopathology

Figure 4 shows that Bambara groundnut supplementation reduced inflammation features in liver histopathologic findings. Mice on a low-protein diet with Bambara groundnut supplementation had lower scores of sinusoidal congestion, cloudy swelling, and inflammation compared to low-protein diet mice.

DISCUSSION

Bacteria in the intestine are represented by more than 1000 species that belong to six dominant phyla: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia. Bacteria from the phyla Firmicutes and Bacteroidetes are the most common, representing 90% of intestinal microbiota (Rinninella *et al.*, 2019). The ratio between these two phyla has been associated with maintaining homeostasis and changes in this ratio can lead to various pathologies and disorders (Lopetuso *et al.*, 2013; Statovci *et al.*, 2017; Park & Kim, 2020). Balancing the intestinal ecosystem is an important aspect of maintaining normal human body function (Abenavoli *et al.*, 2019). In addition to being a regulator of digestive processes, the intestinal microbiota also plays a role in metabolic processes and systemic immune responses (Statovci *et al.*, 2017). Several studies suggest that changes in the composition of intestinal microbiota (increasing F/B ratio) can be influenced by dietary factors such as nutrients and bioactive compounds from foods, contributing to direct and indirect effects on the intestinal microbiota (Zou *et al.*, 2020). This is in accordance with this study, where a low-protein diet significantly decreased the number of Firmicutes and Bacteroidetes compared to the control mice group (adequate protein diet) and supplementation of Bambara groundnuts in low-protein diet mice increased the number of Firmicutes and Bacteroidetes. Several studies regarding low-protein diets stated that protein deficiency will affect the intestinal microbiota (Hsu *et al.,* 2021; Masuoka *et al.*, 2020) and research on mice showed that protein levels in the diet not only change the diversity, but also the overall biomass of microbes in the intestine (Bartlett & Kleiner, 2022).

To understand the interactions between dietary protein and the microbiota, one must first consider the process of protein digestion. Protein digestion and absorption influence how much protein reaches the colon, where most of the microbiota resides. This undigested dietary protein serves as a substrate for microbial metabolism. Undigested dietary protein that reaches the large intestine is hydrolysed into peptides and amino acids, which may be used by the microbiota as a source of carbon, nitrogen, and energy through some diverse metabolic pathways, thus influencing the abundance of the microbiota (Moreno-Pérez *et al.*, 2018; Oliphant & Allen-Vercoe, 2019). Furthermore, a study from Masuoka *et al.* (2020) showed that dietary protein quantity was influential on microbiota, particularly through providing nitrogen, a limiting nutrient for the intestinal microbiota. Similar evidence was found

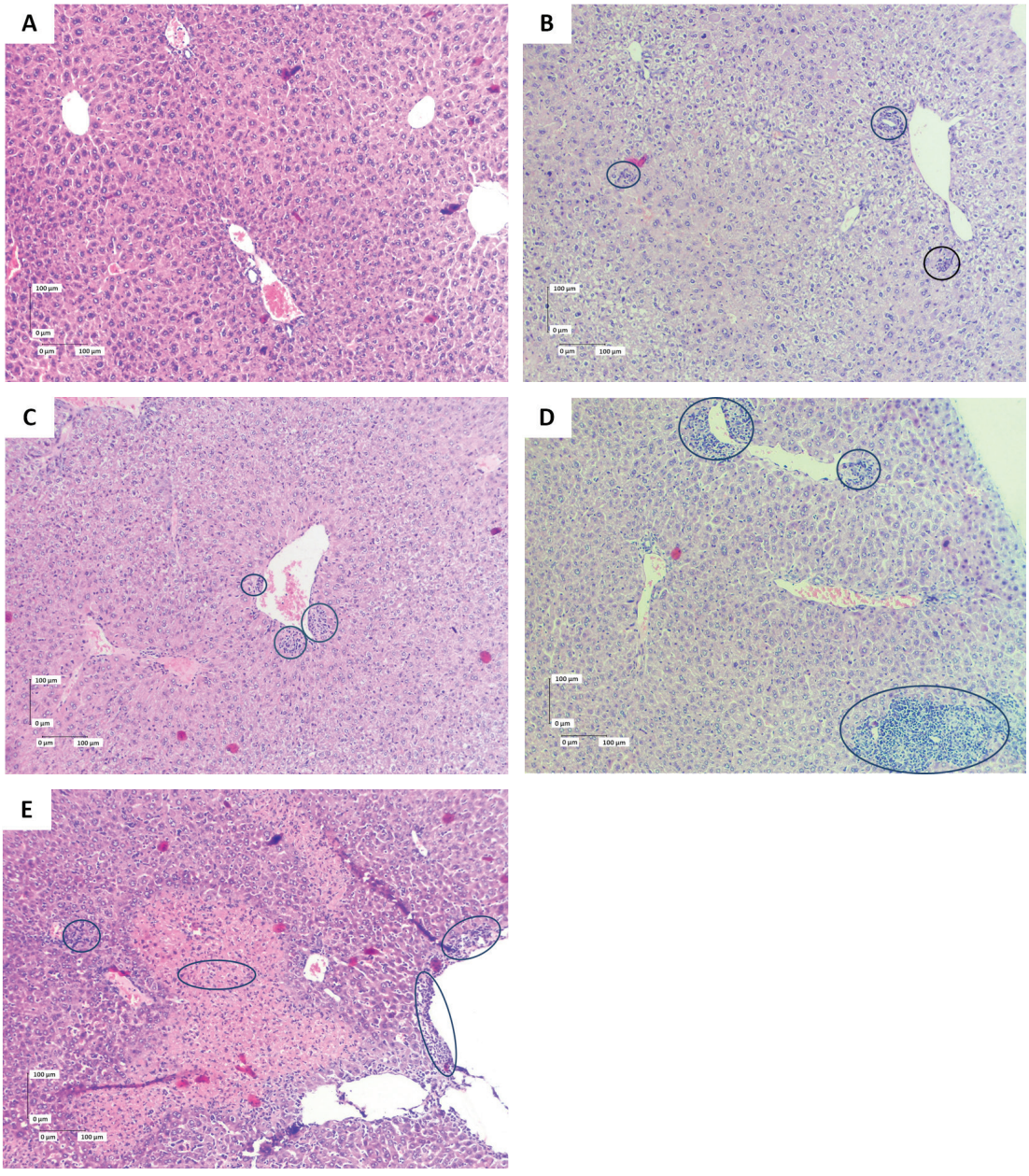

Figure 4. Liver histopathology under light microscope with 100x magnification (scale bar 100 µm): normal (A), mild inflammation in LPMB (B), moderate inflammation in LPHB (C), severe inflammation in LPLB (D), and severe inflammation with necrosis in LP (E). The inflammatory area is marked with a black circle. Swollen liver cells are surrounded by lymphocyte cells. In the area of necrosis, many liver cells appear damaged and shrunken, and lymphocyte cells are clustered in this area. Note: (N) Normal protein diet; (LP) Low-protein diet; (LPLB) Low-protein diet + 100g of Bambara groundnut; (LPMB) Low-protein diet + 200g of Bambara groundnut; (LPHB) Low-protein diet + 300g of Bambara groundnut.

in this study, where a very low number of Firmicutes and Bacteroidetes were detected in the low-protein mice group compared to the control group with a normal protein diet. Meanwhile, the low-protein diet mice groups supplemented with Bambara groundnuts at 200 g per kg feed (LPMB) and 300 g per kg feed (LPHB), respectively, showed a higher amount of Firmicutes and Bacteroidetes compared to the control group.

Low-protein diet leads to reduced delivery of amino acids to the liver, activating the kinase general control nonderepressible 2 (GCN2), leading to increased eukaryotic initiation factor 2α (eIF2α) phosphorylation and activation of activating transcription factor 4 or 5 (ATF4/ATF5). ATF4/5 binds amino acid response elements (AAREs) within the *FGF21* promoter, leading to increased liver *FGF21* production and increased circulating *FGF21* (Laeger *et al.*, 2014). *FGF21* is a liver-derived circulating hormone (hepatokine) belonging to the FGF family and it has been shown to respond to different nutritional signals. *FGF21* is expressed in several metabolically active organs and interacts with different tissues. It regulates nutritional status through the control of glucose, lipids, and energy metabolisms (Tezze *et al.*, 2019). Increased *FGF21* is related to liver inflammation and the lower the protein content in the diet, the more severe the degree of inflammation in the histopathological appearance of the liver. Previous studies reported that *FGF21* is elevated when dietary protein is low (Solon-Biet *et al.*, 2016; Li *et al.*, 2019). This is consistent with our findings, where a low-protein diet significantly increased *FGF21* gene expression compared to the control group.

During long-term dietary protein restriction, the intestinal microbiota undergoes metabolic adaptations that stimulate hepatic *FGF21* adaptive metabolic pathways (Wu *et al.*, 2022). In this study, the LPMB mice group that consumed feed supplemented with 200 g of Bambara groundnuts per kg of feed had much lower *FGF21* expression than mice fed a low-protein (LP) diet. Bambara groundnuts, as a source of protein, have a high content of essential amino acids, including isoleucine, leucine, methionine, phenylalanine, threonine, and valine (Tan *et al.*, 2020). This research showed that consumption of foods with sufficient or high protein levels reduces *FGF21* expression. This result is in line with the findings of Alemán *et al.* (2019). Chalvon-Demersay *et al.* (2016) also reported that a high-protein diet decreased the expression and circulating levels of *FGF21*.

Based on the HRMS examination, Bambara groundnuts contained isoflavones. The current study affirms the finding from Okafor *et al.* (2022), who reported that Bambara groundnuts are rich in polyphenolic compounds, including flavonoid subgroups such as isoflavones. Isoflavones, the most well-known subgroup of phytoestrogens, play protective roles against chemically induced liver injuries through several molecular mechanisms. The hepatoprotective effects of isoflavones are partly associated with their antioxidant, anti-inflammatory, immunomodulatory, and anti-fibrotic properties. Moreover, isoflavones can reduce gut-derived endotoxins, accelerate alcohol metabolism, stimulate detoxification of hepatotoxic chemicals, suppress the bioactivation of these chemicals, inhibit hepatocyte apoptosis, and restore autophagy activity during chemically induced liver diseases (Sarhan *et al.,* 2012; Yao *et al.*, 2021). This is a possible mechanism by which in this study, supplementation of Bambara groundnuts in mice with a low-protein diet had the effect of reducing the severity of sinusoidal congestion, swelling,

and cloudy inflammation. This finding is in line with Folayan *et al.* (2022), who showed that liver damage can be improved by supplementing feed with antioxidant effects. Sarhan *et al.* (2012) also reported that soy supplementation rich in isoflavones showed some protective effects against liver damage in rats due to its antioxidant activity.

CONCLUSION

Overall, this study revealed that Bambara groundnut supplementation in low-protein diet mice has the potential to increase the number of Firmicutes and Bacteroidetes, decrease the expression of adaptive stress gene *FGF21*, and improve the inflammation degree of liver histopathologic features.

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Authors' contribution

Gunanegara RF, conceptualisation, formal analysis, original draft preparation; Dewanto A, project administration; Sunarti, writing, reviewing, and editing of manuscript.

Conflict of interest

Authors declare that there is no conflict of interest in this study.

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