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Investigating Untapped Potentials: Velvet Beans as Novel Prebiotic Sources and Their Effects on Gut Microbiota and Short-Chain Fatty Acid Level

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Abstract

Background and Objective: Prebiotics are non-digestible carbohydrates that selectively facilitate growth of beneficial microorganisms in the gut. Legumes naturally contain carbohydrates with potentials as prebiotic sources. However, numerous legume species remain uninvestigated in this context. The aim of this study was to identify such uninvestigated legumes as potential sources of prebiotics.

Material and Methods: Nine legume samples collected from Central Java and East Java, Indonesia, were extracted using maceration method. Digestion with HCl buffer and α-amylase followed by analysis with dinitrosalicylic acid and phenol-sulfuric acid methods assessed quantities of non-digestible carbohydrates. Three legumes with the highest non-digestible carbohydrates levels were assessed *in vitro* to investigate their abilities to promote the probiotics growth. Then, the most promising extract was assessed on mice to assess its effects on short-chain fatty acid levels using GC-2010 Plus and gut microbiota composition using metagenomic 16S rRNA markers.

Results and Conclusion: From the nine legumes assessed, bambara groundnut (23,51 $mg.g^{-1}$), velvet beans (22.36 $mg.g^{-1}$) and chickpeas (12.1 $mg.g^{-1}$) included the highest non-digestible carbohydrates levels. Velvet beans showed a greater ability to stimulate growth of *Lactobacillus plantarum* and *Bifidobacterium bifidum*, compared to bambara groundnut and chickpeas. Administration of velvet beans to mice increased short-chain fatty acid levels in forms of acetate (12.6 mM) and propionate (3.28 mM). Significantly, velvet beans could modify composition of the gut bacteria by increasing diversity, decreasing dominance levels, increasing abundance of *Bacteroides*, *Helicobacter*, *Mucispirillum*, *Bifidobacterium* and *Lawsonia* genera and decreasing abundance of *Lachnospiraceae* NK4A136 group, *Blautia* and *Lachnoclostridium* species.

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1. Introduction

Prebiotics consist of non-digestible carbohydrates (NDCs) and are resistant to stomach acid and digestive enzymes; therefore, they selectively promote growth of beneficial bacteria in the large intestine [1,2]This fermentation process by gut bacteria produces short-chain fatty acids (SCFAs) such as acetate, propionate and butyrate, which can lower pH of the gastrointestinal tract (GIT) and affect gut microbiota compositions [3]. Decreases in pH can decrease number of pathogenic microorganisms and increase growth of beneficial microorganisms, which is linked to the tolerance of the latter microorganisms to acidic conditions. Previous studies have shown that specific prebiotics such as inulin can promote growth of beneficial gut bacteria such as *Lactobacillus* and *Bifidobacterium*. [4,5]. Similar effects were observed with fructo-oligosaccharides, increasing *Bifidobacterium* while decreasing harmful bacteria [6].

Legumes are renowned for their nutritional values, particularly as protein sources. However, they contain NDCs in the form of oligosaccharides and polysaccharides, which include potentials as prebiotics [7]. These components pass through the small intestine undigested and reach the large intestine, where they can promote growth of beneficial gut bacteria [8]. Studies have demonstrated prebiotic effects of common legumes such as cowpeas and black beans,

including decreased pH levels, increased growth of *Bifidobacterium* and *Lactobacillus* and increased SCFA levels [9,10] however, a vast majority of legume species are grouped under the minor category, remaining virtually uninvestigated. Much legumes are consumed only by local communities in Java, Indonesia. Additionally, food industries ignore legumes due to a limited knowledge of their compositions and potential benefits. Investigating prebiotic potentials of the minor legumes presents a compelling opportunity to enhance their economic values.

No reports have been published on the prebiotic potentials of specific minor legume varieties used in the current study. While most studies on prebiotics have focused on familiar major legumes, the current study investigated the lesser-known varieties. Significantly, one minor legume, *Mucuna pruriens* (velvet bean), has shown promises to combat obesity in mice, but its prebiotic potentials must be investigated [11]. In the current study, minor legume samples were selected based on their NDCs content and ability to promote growth of common gut bacteria of *Bifidobacterium bifidum* and *Lactobacillus plantarum* [12,13]. Legumes with the most promising prebiotic potentials were further assessed in mice to assess their effects on SCFA levels and gut microbiota compositions. This study targeted caecum, the major fermentation site in mice (pH 4.4-4.6), and used metagenomic analysis of 16S rRNA gene sequences to characterize the microbial communities. The aim of this study was to investigate novel potential prebiotic sources from several assessed legume candidates.

2. Materials and Methods

2.1. Selection of Legumes

A total of nine types of legumes that were not investigated as prebiotics were species grown and consumed by local people in Java, Indonesia. The minor legumes, including velvet beans (*M. pruriens*), bambara groundnut (*Vigna subterranea*), chickpea (*Phaseolus vulgaris*), calopo beans (*Calopogonium mucunoides*), snow pea (*Pisum sativum* var. *saccharatum*), winged beans (*Psophocarpus tetragonolobus*), sword beans (*Canavalia ensiformis*), red beans or senerek beans (*P. vulgaris*) and turi beans (*Sesbania grandiflora*).

2.2 Extraction of Carbohydrates

The prebiotic components in the legume samples were extracted using maceration method. Ethanol, a polar solvent known for its attraction to polar carbohydrates, was chosen as the extraction solvent. Then, 70% ethanol (v v^{-1}) was used for maceration with a 1:10 sample:solvent ratio [14,15]. The mixture was set for 4 d and then filtered and the solvent was evaporated at 60 °C using rotary evaporator.

2.3. Assessment of non-digestible carbohydrate contents

Carbohydrate resistance of nine minor legume extracts was assessed using simulated stomach acid and digestive enzymes. Each extract was prepared as a 10% stock solution (w v^{-1}) in distilled water (DW). Resistance was assessed using acidic and enzymatic digestions. Acidic digestion involved incubating 200 µl of 1% extract solution (v v^{-1}) with 200 µl of HCl buffer (pH 1) at 37 °C for 4 h (16). The reaction was stopped with 1 N NaOH. Enzymatic digestion was followed by further incubation of 200 µl of acid-digested solutions with 200 µl of α-amylase enzyme (2 U.ml^{-1}) in sodium phosphate buffer at 37 °C for 6 h. Heating at 80 °C for 10 min stopped the reaction. Each digestion was carried out in triplicate [15].

The NDCs content was assessed using dinitrosalicylic acid (DNS) method for reducing sugars before digestion and the phenol sulfuric acid method for total sugars after acid-enzymatic digestion. Acid and enzyme analyses were carried out respectively to simulate digestion in the stomach and small intestine. The DNS method involved preparation of a reagent by dissolving 10 g of 3,5-dinitrosalicylic acid, 2 g of phenol, 0.5 g of sodium sulfite and 10 g of sodium hydroxide in 1 l of DW. Assay involved adding 100 µl of 1% legume extract solution (v v^{-1}) and 100 µl of DNS reagent, followed by vortexing for 30 s, heating at 95 ℃ for 10 min (until color change), adding 33 µl of 40% sodium potassium tartrate (w v^{-1}) and diluting 10× with DW. Absorbance was measured at 540 nm using ELISA reader [17] For the phenol sulfuric acid method, 50 µl of digested solution were mixed with 50 µl of phenol solution and vortexed for 30 s; followed by mixing with 250 µl of H_2SO_4 (sample:phenol:H_2SO_4 ratio of 1:1:5), vortexing for 30 s, diluting 10× with DW and measuring the absorbance at 490 nm using ELISA reader (18). The NDC content in the extracts was calculated using Eq. 1 (15):

$$\text{NDC (mg. g}^{-1}) = \text{Reducing sugar before digestion} - \text{total sugar after digestion} \quad \text{Eq.1}$$

2.4. Probiotics Growth Stimulation

This study used the probiotic *B. bifidum* and *L. plantarum*. Bacterial growth was estimated using standard growth curve based on optical density at 600 nm and colony counts (CFU.ml^{-1})19. *Bifidobacterium bifidum* was incubated anaerobically at 37 °C, while *L. plantarum* was incubated aerobically at 37 °C using shaker incubators. The two bacteria were first propagated on MRS agar media for 24 h at 37 °C before inoculation into MRS broth media. After 24 h, inoculum was used to count cells and colonies. The OD 600 measurement at each dilution (10^0-10^{-10}) estimated the number of cells. Colony counts for dilutions of 10^{-7}, 10^{-8} and 10^{-9} were carried out using total plate count (TPC) method with triplicate plating.

Bifidobacterium bifidum and *L. plantarum* were propagated in each modified MRS broth growth medium. The

MRS broth was prepared using 10 g of peptone, 4 g of yeast extract, 8 g of meat extract, 20 g of carbon sources (glucose, inulin and three types of legume extracts with the highest NDCs), 1 g of Tween 80, 2 g of potassium phosphate dibasic (K_2HPO_4), 5 g sodium acetate ($CH_3COONa.3H_2O$), 2 g of tri-ammonium citrate ($C_6H_{17}N_3O_7$), 0,2 g magnesium sulfate ($MgSO_4 \cdot 7H_2O$) and 0,04 g manganese (II) sulfate ($MnSO_4 \cdot H_2O$), which were dissolved in DW up to 1 l [20]. The OD 600 was measured at 0, 24 and 48 h using ELISA reader. Then, number of the bacterial colonies was estimated from the standard curve.

2.5. Experiments on Animal Models

Promising prebiotic function legumes, based on their NDC levels and probiotic-stimulating abilities, were assessed in mice to investigate their effects on SCFA levels and gut microbiota compositions. Fifteen mice were equally divided into three groups of a negative control group fed with a standard diet, a positive control group fed with a standard diet supplemented with 5% inulin (w w^{-1}) and a treatment group fed with a standard diet supplemented with 5% of the selected legume extract (w w^{-1}). Each mice was housed individually, fed at 5 g per min rate with *ad libitum* water. Body weight and food intake of each mice were assessed daily for 28 d. After 28 d, mice were euthanized and their cecum were collected for further analysis.

2.6. Assessment of Short-Chain Fatty Acid Levels

Analysis of SCFA levels in caecum contents was carried out using gas chromatography (Shimadzu GC-2010 Plus, Japan). First, 0.15 g of the sample was mixed with 1 ml of DW and centrifuged at 1008 × g for 10 min. Then, supernatant was analyzed using GC-202 Plus, Japan, provided by the Food and Agricultural Product Technology Testing Laboratory, Faculty of Agricultural Technology, UGM, Indonesia [21].

2.7. Metagenomic Analysis

Genomic DNA was extracted from the caecum using ZymoBIOMICS DNA mini kit, following the manufacturer's instructions. The extracted DNA concentration was assessed using BioPhotometer Plus (Eppendorf, Germany). Polymerase chain reaction (PCR) amplified the V3 and V4 regions of the 16S rRNA gene using primers of 341F (CCTACGGGRGGCAGCAG) and 806R (GGACTACC-AGGGTTTCTA) [22]. Then, DNA sequencing was carried out using next-generation sequencing (NGS) method and MGISeq platform. All PCR and NGS studies were carried out at PT. Genetic Sciences Jakarta, Indonesia.

2.8. Data Analysis

Data were analyzed using SPSS software v.24.0 and one-way ANOVA with a significance level of $\alpha = 0.05$. For significant differences, Duncan's multiple range test (DMRT) was used for post-hoc analysis. FASTA-formatted sequence data were analyzed with UPARSE v7.0.1001 to group sequences into OTUs (operational taxonomic units). Sequences with ≥97% similarity were assigned to the same OTU. Taxonomic profiling was carried out using QIIME v.1.7.0 and SILVA database. Multiple sequence alignment was carried out using MUSCLE v.3.8.31. Then, OTUs with abundance less than 0.005% were removed. Normalized OTU abundance was used to assess alpha and beta diversities. Alpha diversity analysis was carried out using QIIME v.1.7.0 and visualized using R v.2.15.3. This captured the within-habitat bacterial diversity, including the number of OTUs, Shannon-Wiener diversity index and Simpson index. Beta diversity, reflecting bacterial diversity between the habitats, was estimated using PCoA-based index calculated using FactoMineR, ggplot2 and R v.2.15.3.

3. Results and Discussion

3.1. Quantity of Non-digestible Carbohydrates in Legumes

To qualify a compound as a prebiotic agent, its carbohydrates must resist digestion in the stomach and small intestine. Table 1 shows that velvet beans and bambara groundnut included significantly higher quantities of NDC, compared to other beans (p<0.05). Chickpeas demonstrated a relatively high quantities of NDC, compared to other legumes. In addition to the high quantity of NDCs, the low hydrolysis proportions of velvet beans (4.57%), bambara groundnut (8%) and chickpea (24%) indicated that most of the carbohydrates in these three beans were resistant to acid and enzyme digestions.

Table 1. Quantities of Non-digestible Carbohydrates

Samples	NDCs (mg.g^{-1})
Velvet beans (*Mucuna pruriens*)	22.36 ± 1.69 ^{a}
Bambara groundnut (*Vigna subterranea)*	23.51 ± 4.39 ^{a}
Chickpea (*Phaseolus vulgaris*)	12.10 ± 2.65 $^{a.b}$
Calopo beans (*Calopogonium mucunoides*)	4.55 ± 1.72 ^{b}
Snow pea (*Pisum sativum var. saccharatum*)	11.82 ± 6.82 $^{a.b}$
Winged beans (*Psophocarpus tetragonolobus*)	4.19 ± 4.74 ^{b}
Sword beans (*Canavalia ensiformis*)	8.87 ± 3.33 $^{a.b}$
Red beans or senerek beans (*Phaseolus vulgaris*)	5.55 ± 11.09 ^{b}
Turi beans (*Sesbania grandiflora*)	8.63 ± 5.38 $^{a.b}$

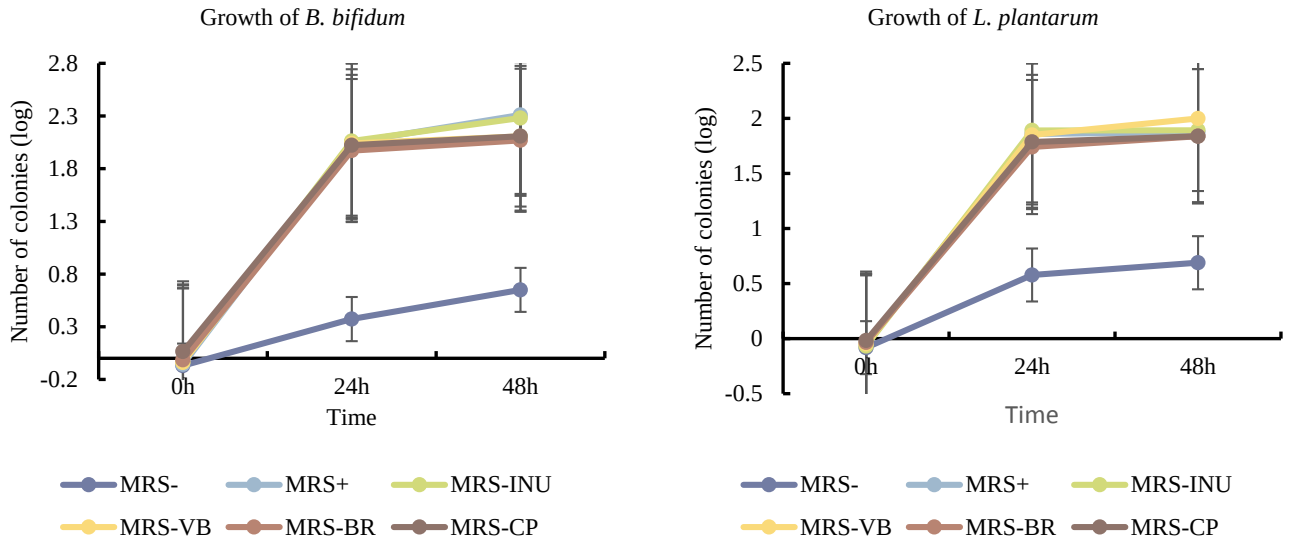
These results suggested that these three beans could reach the large intestine for selective fermentation by the beneficial bacteria. Based on the results, these three beans were further assessed *in vitro* to assess their abilities to stimulate beneficial bacteria.

Velvet beans (*M. pruriens*) are beans with high carbohydrates contents. Previous studies reported that raw velvet bean seeds contained up to 49.22% of carbohydrates [23] Total dietary fiber content of the velvet beans is known to reach 86.6 mg.g^{-1}, which is higher than that of *Canavalia gladiata* and *Vigna unguiculata* [24]. Bambara groundnuts (*V. subterranean*) are known as food sources with high carbohydrate contents, reaching up to 64.4%. Previous studies have shown that most of the carbohydrates contained in bambara groundnut are dominated by oligosaccharides and polysaccharides [25]. Chickpeas (*P. vulgaris*) are known as sources of carbohydrates that consist of starch, fibers and oligosaccharides. Previous studies have reported that the total polysaccharide contents in chickpeas reach to 300-370 mg.g^{-1}, while the indigestible carbohydrate contents in form of oligosaccharides reach to 41.8-85.3 mg.g^{-1} [26].

3.2. Propagation of Probiotics on Various Carbon Sources

The NDCs are qualified as prebiotics if they selectively encourage propagation of beneficial bacteria in the large intestine. This study investigated several prebiotic candidates known for their acid and enzymatic resistances, based on their high quantity of NDCs. The candidates included velvet beans, bambara groundnuts and chickpeas. The study assessed their abilities to stimulate propagation of the representative probiotics of *B. bifidum* and *L. plantarum*, commonly detected in GIT of humans and rodents [27,13]. Bacteria were cultured in MRS broth media with various carbon sources. Each medium contained 2% extracts (w v^{-1}) of velvet beans (MRS-VB), bambara groundnuts (MRS-BR) and chickpeas (MRS-CP). Propagation of *B. bifidum* and *L. plantarum* on these sources was compared with those on cultures using 2% of prebiotic inulin (w v^{-1}) (MRS-INU), 2% of glucose (w.v^{-1}) (MRS^{+}) and no carbon sources (MRS^{-}). Figure 1a,b showed that the growth of *B. bifidum* and *L. plantarum* in MRS^{-} media did not show significant increases after incubation for 48 h. Carbon source in the media is a substrate that is utilized by bacteria to form amino acids and other cell components, making it important for the multiplication of bacterial cells [28].

Figure 1a reveals diverse growth patterns for *B. bifidum* in various MRS media. Significantly, MRS-VB and MRS-CP media stimulated *B. bifidum* propagation after 24 h, compared to MRS-BR. However, the number of *B. bifidum* colonies in MRS-VB and MRS-CP media was lower than that in MRS-INU at 24 h (p<0.05). At 48 h, *B. bifidum* in media with the prebiotic candidate (MRS^{-}VB, MRS-BR and MRS-CP) included a lower number than that it did in MRS^{+} and MRS^{-}INU media (p<0.05). These results indicated that the three prebiotic candidates were not able to stimulate propagation of *B. bifidum* as well as inulin prebiotics. However, *B. bifidum* in MRS^{+} media included a better propagation rate, compared to that in MRS-INU. Despite its positive effects, glucose failed to control harmful bacteria such as *Escherichia coli* and *Salmonella* spp. This suggests that specific media and prebiotics might be needed to support particular beneficial bacteria while limiting harmful ones [29]. .



(a) (b)

Figure 1. Growth of (a) *Bifidobacterium bifidum* and (b) *Lactobacillus. plantarum* in MRS broth media with various carbon sources. Notes: MRS-: Negative control (no carbon source), MRS+: Positive control (2% glucose), MRS-INU: Prebiotic control (2% inulin), MRS-VB: 2% velvet bean extract, MRS-BR: 2% bambara groundnut extract, MRS-CP: 2% chickpea extract.

Figure 1b reveals that *L. plantarum* in MRS-VB, MRS-BR and MRS-CP media included a lower number of colonies at 24 h than that it did MRS-INU (p<0.05). At 48 h, *L. plantarum* in MRS-VB media included the highest number of colonies, compared to that it did in MRS-VB, MRS-CP and MRS-INU media. A carbon source of 2% velvet bean extract could stimulate propagation of *L. plantarum* to reach to 99.83 ±1.45 × 10^7 CFU.ml^{-1}, which was significantly higher than inulin prebiotics and the other two prebiotic candidates (p<0.05). These results indicated that velvet beans included a better ability to stimulate *L. plantarum* during 48 h of incubation, compared to that inulin prebiotics did.

Proliferative Index was reported from log cell number at 48 h minus log cell number at 0 h. Table 2 shows proliferative index of the probiotic bacteria during 48 h of incubation in media containing various prebiotic carbon sources. Significantly, *B. bifidum* in MRS-VB media included a higher proliferative index and was significantly different, compared to that it did in MRS-BR and MRS-CP (p<0.05). However, the proliferative index of *B. bifidum* in MRS-VB media included a lower value, compared to that it did in MRS-INU and MRS^+ (p<0.05). These results indicated that *B. bifidum* utilized inulin and glucose better than that the prebiotics in velvet beans did. These results were similar to previous results, which stated that propagation of *B. bifidum* could only be stimulated by inulin [30]. This was because *B. bifidum* could not utilize oligosaccharides such as fructooligosaccharides (FOS) and galactooligosaccharides (GOS) in legumes. Previous studies have reported that FOS-type prebiotics could stimulate propagation of all *Bifidobacterium* spp., except *B. bifidum* [30]. This was because *B. bifidum* did not include genes encoding β-fructofuranosidase enzyme, functioning to hydrolyze FOS [31].

The proliferative index of *L. plantarum* (Table 2) in MRS-VB media showed the highest value, compared to that the other groups did (p<0.05). These results indicated that *L. plantarum* could better utilize carbohydrates in velvet beans better, compared to that inulin prebiotics and the other two prebiotic candidates did. Velvet beans (*M. pruriens*) are known to contain FOS prebiotics, which consist of glucose and fructose units linked by β(2-1) glycosidic bonds [32,33]. Previous studies have shown that *L. plantarum* can utilize FOS as a selective carbon source because it includes β-fructofuranosidase enzyme, which plays roles in FOS degradation [34,33]. Nucleotide sequence of the *L. plantarum* genome has demonstrated presence of the *sac*A gene, which expresses an enzyme that can hydrolyze FOS internally. Unlike extracellular enzymes in *L. pentosus* and *L. paracasei*, intracellular enzymes in *L. plantarum* can maximally utilize FOS prebiotics because there are no hydrolysis products that are consumed by other bacterial species in the large intestine. This reveals that *L. plantarum* utilizes FOS to compete with other microorganisms in the large intestine. Fermentation of FOS by *L. plantarum* produces secondary metabolites that inhibit propagation of pathogenic bacteria such as *E. coli* that produce β-glucuronidase enzyme [34].

3.3. Assessment of the prebiotic effects with prebiotic index

A prebiotic index value greater than 1 (>1) shows that a carbohydrate includes positive effects on propagation of the probiotic bacteria. Figure 2 shows that the prebiotic index of velvet beans and inulin in *L. plantarum* culture includes values greater than 1 (>1). Velvet beans included the highest value, compared to that inulin and other prebiotic candidates did (p<0.05). In *B. bifidum* culture, the three legume candidates included a lower proliferative index, compared to that inulin prebiotics did (p<0.05). These results demonstrated that prebiotics in velvet beans included good abilities to stimulate *L. plantarum*, compared to that the inulin did. Moreover, propagation of *B. bifidum* could only be stimulated by the inulin prebiotics.

3.4. Experiments on Animal Models: Changes in Body Weight and Food Consumption

Identified as promising prebiotic candidates, velvet beans were assessed *in vivo* using mice models. Mice in all three groups included similar body weights at baseline (Day 0) (Table 3). On Day 7, all groups gained weight with the treatment group (13.3%) showed the highest increase (not statistically significant), compared to the negative (12,3%) and positive controls (12,67%). By Days 21 and 28, all groups gained weight steadily with no significant differences between them (Table 4). However, all groups consumed more than 78% of the provided foods, demonstrating that prebiotics reached digestive system of all mice. Although significantly indifferent, treatment and positive control groups included lower body weights than that the negative control group did. A relatively high feed consumption in the treatment group did not cause significant increases in body weight of the mice. Previous studies have reported that velvet beans include anti-obesity effects by decreasing body weight in obese groups [11].

3.5. Short-chain fatty acid levels in animal models

Anaerobic bacteria primarily produce SCFA such as acetic, propionic and butyric acids via fermenting NDCs [35]. Significantly, positive control and treatment groups demonstrated higher levels of these SCFAs, compared to the negative control (Table 5). Levels of acetic and propionic acid in the treatment group significantly higher than those in negative control group (p<0.05), but significantly lower than those in positive control group (p<0.05). Levels of butyric acid in treatment and negative control groups were significantly lower than those in positive control group (p<0.05). This suggests that velvet beans supported propagation of the bacteria that produced these SCFAs, potentially leading to shifts in gut microbiome metabolism and SCFA production with benefits to gut health.

Table 2. Proliferative Index of *Bifidobacterium bifidum* and *Lactobacillus plantarum* for 48 Hours

Groups	*Bifidobacterium bifidoum*	*Lactobacillus plantarum*
MRS-	0.71±0.03 [a]	0.77±0.07 [a]
MRS+	2.36±0.02 [d]	1.92±0.02 [b.c]
MRS-INU	2.31±0.05 [d]	1.96±0.04 [c]
MRS-VB	2.14±0.03 [c]	2.04±0.02 [d]
MRS-BR	2.08±0.02 [b]	1.87±0.01 [b]
MRS-CP	2.04±0.01 [b]	1.85±0.04 [b]

Note: ab: notation for comparing between treatment groups; Different notations indicate significant differences at the 5% significance level (p<0.05). Notes: MRS-: Negative control (no carbon source), MRS+: Positive control (2% glucose), MRS-INU: Prebiotic control (2% inulin), MRS-VB: 2% velvet bean extract, MRS-BR: 2% bambara groundnut extract, MRS-CP: 2% chickpea extract.

Table 3. Changes in Body Weight of the Animal Models

Groups	Weight (g)				
	D0	D7	D14	D21	D28
Negative Control	25.26±1.7[a]	28.86±3.1[a]	31.2±2.4[a]	32.26±3.8[a]	33.13±5.1[a]
Positive Control	24.3±2.0[a]	27.76±4.2[a]	26.4±3.7[a]	29.16±4.7[a]	30.7±4.9[a]
Treatment	24.7±0.9[a]	28.5±1.3[a]	30.46±3.0[a]	31.43±3.9[a]	33±4.3[a]

Note: ab: notation for comparing between treatment groups; Different notations indicate significant differences at the 5% significance level ($p < 0.05$). Note= negative control: standard diet, positive control: standard diet +5% inulin, treatment: standard diet + 5% velvet bean extract.

Table 4. Food Consumption by the Animal Models within 28 Days

Groups	Food intake (g)
Negative Control	4,41±0,61 [a]
Positive Control	3,90±0,59 [a]

Table 5. Sort-Chain Fatty Acid Levels in Mice

Groups	Acetic Acid (m Molar)	Propionic Acid (m Molar)	Butyric Acid (m Molar)
Negative Control	8.08±0.16[a]	2.25±0.27[a]	1.94±0.16[a]
Positive Control	14.71±0.07[c]	4.12±0.16[c]	2.18±0.05[b]
Treatment	12.60±0.19[b]	3.28±0.07[b]	1.98±0.09[a]

Note: ab: notation for comparing between treatment groups; Different notations indicate significant differences at the 5% significance level ($p < 0.05$). Note= negative control: standard diet, positive control: standard diet +5% inulin, treatment: standard diet + 5% velvet bean extract.

Previous studies have reported that feeding germinated velvet beans can increase SCFA production in the cecum of broilers [36].

3.6. Diversity of the Gut Microbiota in Mice

Structure of the microbiota community in each group was assessed using various alpha diversity indices (Table 5). Figure 3 shows a flattened rarefaction curve indicating that all OTUs in the three groups were detected.

Based on Table 5, Shannon index in the treatment group with velvet bean supplementation showed a lower value compared to the negative control (standard diet), but higher compared to the positive control (inulin supplementation). The Shannon index (H') is positively correlated with the diversity and evenness of bacterial species in a bacterial community [37]. These results revealed that species diversity and evenness in the treatment group were higher than the positive control but lower than the negative control. This was similar to the higher number of OTUs (species richness) in the treatment group compared to the positive control, but lower compared to the negative control. In addition to Shannon index, Simpson index was used as an indicator to estimate the diversity of gut microbiota in mice. A high Simpson index value demonstrates that the diversity of bacteria in a community is low. Furthermore, a higher Simpson index value indicates greater dominance of a particular bacterial species in the community [38]. Based on Table 5, it was detected that the Simpson index in the treatment group included a lower value, compared to the positive and negative control groups. These results showed that species diversity in the treatment group was higher than that in the controls. Moreover, a low Simpson index in the treatment group demonstrated a low dominance level, hence, it could be assumed that no community balances existed in the mouse gut bacteria. Bacterial community balance can create a positive ecosystem that includes good resistances to pathogens and improves digestive track health of the host [38].

Table 6. The Alpha Diversity Indices

Groups	OTU	Shannon	Simpson
Negative Control	524	5.175	0.987
Positive Control	359	4.629	0.982
Treatment	457	4.697	0.969

Note= negative control: standard diet, positive control: standard diet +5% inulin, treatment: standard diet + 5% velvet bean extract.

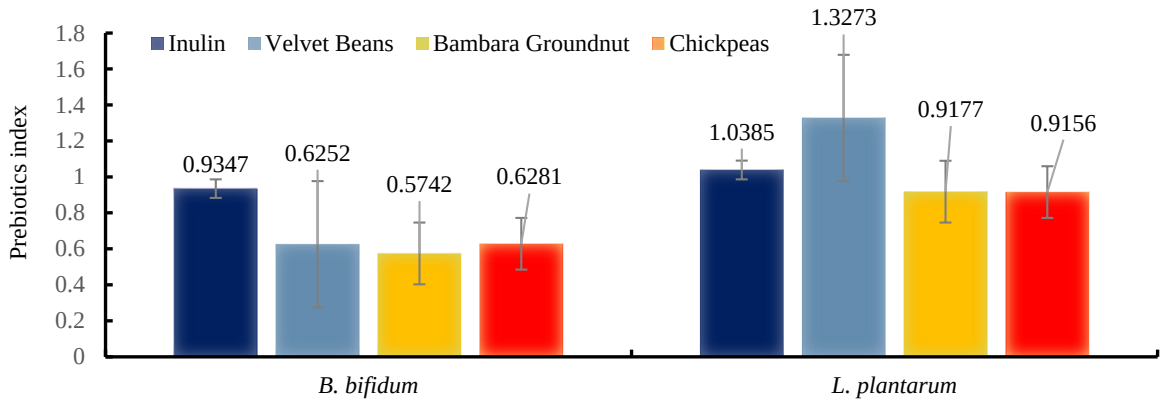


Figure 2. Prebiotic Index Value. abc, notation for comparing between the groups. Different notations indicate significant differences at 5% significance levels (p<0.05).

Diversity between the groups (beta diversity) was analyzed using principal coordinates analysis (PCoA). Figure 4 shows the PCoA analysis, revealing distinct variations in the bacterial communities within the groups. Distances between the points that represent positive control and treatment groups on the PCoA plot show a closer proximity, indicating that the bacterial composition of these two groups included a greater similarity, compared to the negative control group. This verified that the bacterial communities in positive control and treatment groups were more similar to each other than the negative control.

3.7. Abundance and Composition of the Gut Microbiota in Mice

Metagenomic analysis of the bacterial communities in mice cecal samples (Figures 5A) revealed that the Firmicutes and Bacteroidetes phyla included the highest relative abundance in all three groups. This finding is similar to that of previous studies, which showed that Firmicutes and Bacteroidetes were the dominant phyla in the gut of healthy individuals [41]. Abundance of Firmicutes in the treatment group (45%) was relatively lower than that in the positive (64%) and negative (62%) control groups. However, abundance of Bacteroidetes did not show significant differences between the three groups (20,23 and 21%). The Firmicutes/Bacteroidetes (F/B) ratio indicates homeostasis of the GIT. Increases or decreases in the F/B ratio reveal dysbiosis, which is associated to metabolic and inflammatory disorders. Increased F/B ratios are associated to obesity, which is characterized by increased abundances of the Firmicutes phylum [42]. Previous studies have reported that obese individuals received high-fiber diets for 1 y had decreased Firmicutes abundances [43]. Similar studies have reported that African children who consumed high-fiber diets had lower Firmicutes abundances than that children who consumed fast food [44] The current study revealed that the F/B ratio in the treatment group was lower than that in positive and negative control groups. This suggests that a 5% supplementation of velvet bean extract includes anti-obesity characteristics in mice. In addition to the dominant phyla, treatment group demonstrated enrichments in Proteobacteria (2%) and Actinobacteria (0.8%), compared to the control groups (respectively 0.8-4 and 0.5-1%).

Significantly, Desulfobacterota, Deferribacterota and Campylobacerota showed significant enrichments in the treatment group (14, 12, 8%), compared to controls (respectively 4-7, 3, 2-5%).These findings highlight potential shifts in gut microbial composition following the interventions.

Figure 5B reveals that Lachnospiraceae dominated gut microbiota in all groups. However, the treatment group (21%) showed significantly lower abundance of Lachnospiraceae, compared to positive control (39%) and negative control (40%). While genera within this family produce beneficial metabolites, the high abundance was linked to glucose metabolism disorders and inflammatory bowel disease (IBS) [45,46].

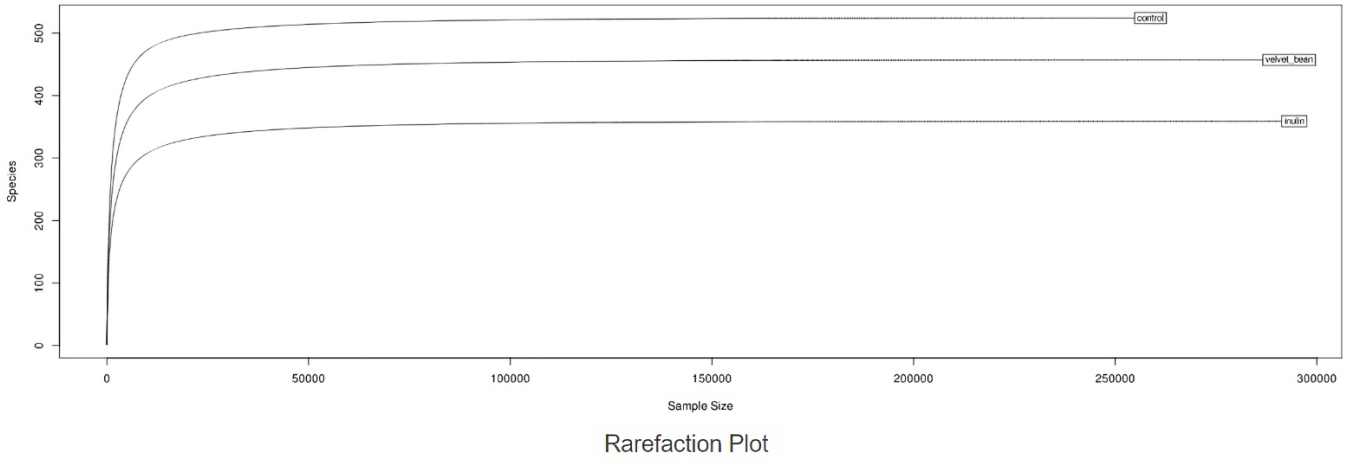


Figure 3. Rarefaction curve. The flat rarefaction curve in all three groups indicate that all OTUs were identified.

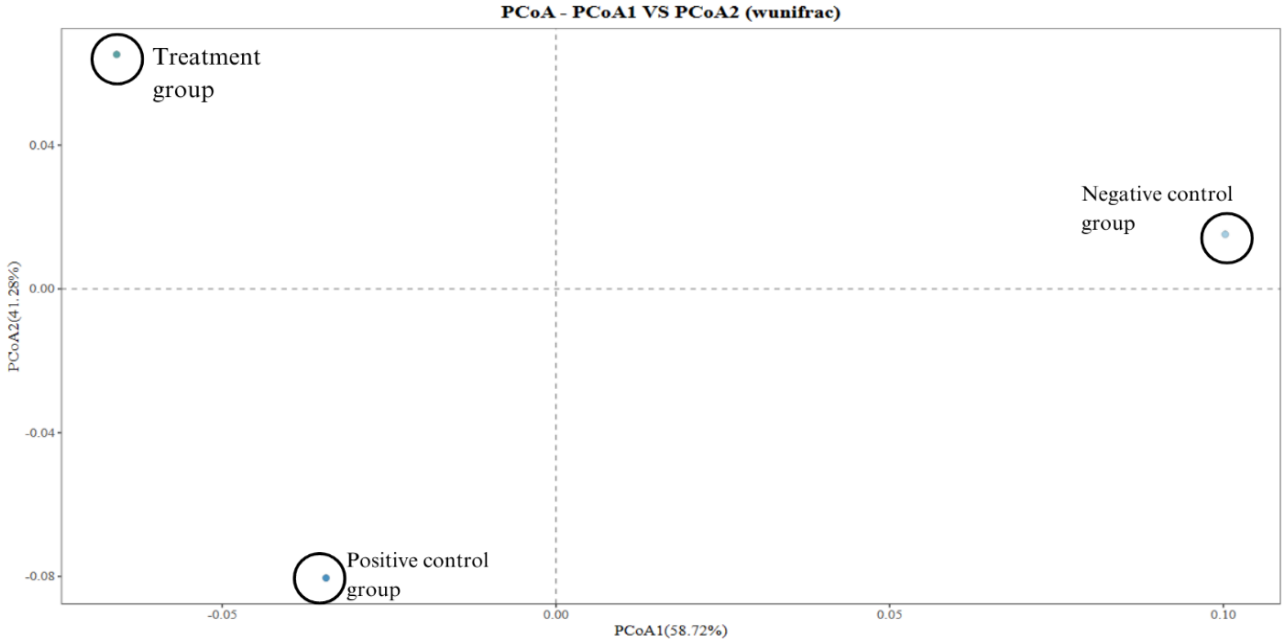


Figure 4. The PCoA Plot. The PCoA analysis based on unweighted UniFrac distance was generated from the abundance of bacterial taxa detected in the gut (39). Closely spaced points on the plot indicate community similarities between the groups (40).

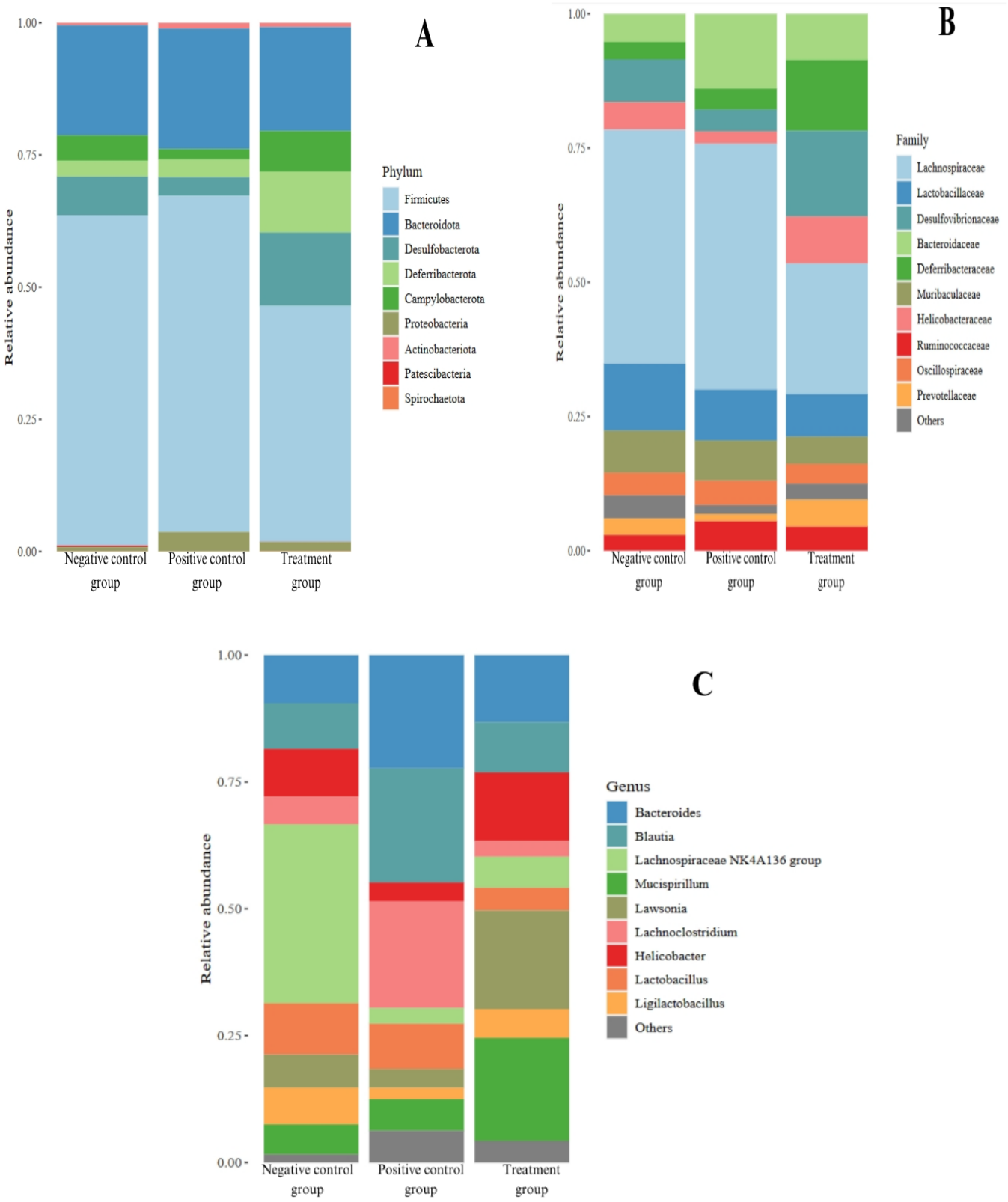


Figure 6. Relative abundance of the gut microbiota in the caecum of mice across groups, identified at various taxonomic levels of **(A)** Phylum, **(B)** Family and **(C)** genus levels.

Interestingly, Helicobacteraceae (8%), Deferribacteraceae (12%) and Desulfovibrionaceae (14%) were significantly enriched in the treatment group, compared to positive and negative control groups (2-4 and 3-7%, respectively). Significantly, Lactobacillaceae (7–11%) was abundant within all groups (Figure 5C).

Figure 6C shows genera that belonged to the top 10 highest abundances in all three groups. The *Lachnospiraceae* NK4A136 group, *Blautia* and *Lachnoclostridium* were the most identified genera from the Lachnospiraceae family. The *Lachnospiraceae* NK4A136 group included a lower relative abundance in the treatment (3%) and positive control (2%) groups, compared to the negative control group (18%). The *Lachnospiraceae* NK4A136 group include anaerobic bacteria that could produce SCFAs through the fermentation of polysaccharides, making them beneficial bacteria. However, previous studies reported that the negative control group included higher abundance of *Lachnospiraceae* NK4A136 group, compared to the group fed prebiotic tape ketan diets. The study showed that the *Lachnospiraceae* NK4A136 group was more abundant in stressed mice and its abundance decreased after feeding with prebiotic tape ketan diets [47].

Blautia genus, belonging to the Lachnospiraceae family, exhibited a diminished relative prevalence in the treatment (6%) and negative control (5%) groups in contrast to the positive control group (12%). *Blautia* is addressed for its involvement in synthesis of SCFAs, particularly propionate and butyrate. Similarly, *Lachnoclostridium* genus demonstrated a comparatively decreased prevalence in the treatment (2%) and negative control (3%) groups, compared to the positive control group (11%). *Lachnoclostridium* is highlighted for its anti-inflammatory characteristics and production of butyrate (48, 49). The higher propionate and butyrate levels in the positive control group (Table 5) support the current findings, as these levels were significantly different from that of the treatment and negative control groups.

Based on Figure 6C, *Helicobacter* genus from Helicobacteraceae included a higher relative abundance in the treatment group (8%), compared to the positive (2%) and negative (5%) control groups. Several *Helicobacter* spp. were detected in the treatment group, including *H. typhlonius, H. bilis, H. japonicus, H. apodemus, H. rodentium* and *H. ganmani.* Although most *Helicobacter* spp. in the GIT are potential pathogens, studies have investigated interactionS of *Helicobacter* spp. with other microbiota members that create positive effects. *Helicobacter* in the mouse GIT can enhance resistance to infectious diseases caused by *Citrobacter rodentium* and prevent infection without adaptive immunity [50]. Previous studies reported that *H. bilis* colonization in the cecum could induce specific immune responses in form of immunoglobulin G (IgG) activation, which could protect mucosal layers from *Mucispirillum schaedleri* infections [51].

Mucispirillum, part of the Deferribacteraceae family, showed higher abundance in treatment group (12%), highlighting potential interactions with the velvet bean interventions, compared to the control groups (3%). Identified species from this genus included *M. schaedleri*, which is known to invade the cecal mucosal layers and cause intestinal inflammations [52]. In addition to its negative effects, *Mucispirillum* is known to interact with the mucus and create a positive environment for the bacterial propagation in GIT. Previous studies detected that *M. schaedleri*, which colonized the mucosal layers played positive roles in protecting the host's GIT by forming a mucus barrier against the pathogen infections [53]. The studies showed that mice infected with *Salmonella typhimurium* that were previously colonized with *M. schaedleri* experienced significant decreases in intestinal inflammation, compared to the controls. Generally, *M. schaedleri* is antagonistic to *S. typhimurium* and can therefore inhibit virulence factors, tissue invasion and inflammation [52].

Bacteroides spp. in the treatment (8%) and negative control (12%) groups included higher abundances, compared to the positive control group (5%) (Figure 6C). *Bacteroides* spp. produce SCFAs that can be absorbed by the intestine and include positive effects on the hosts [53]. Studies have shown that *Bacteroides* spp. are low in abundance in people with diarrhea, indicating that high abundances of *Bacteroides* spp. are positively correlated with digestive health [54]. Identified species from the genus *Bacteroides* included *B. uniformis, B. acidifaciens* and *B. vulgatus*. Techniqually, *B. uniformis* has been identified as a potential therapeutic agent for obesity due to its demonstrated capacity to restrict weight gain and increase butyrate concentrations within the intestinal tract [55]. Detected broadly in high-fiber diets, commensal gut bacteria of *B. acidifaciens* offer multiple health benefits. These bacteria help prevent obesity and improve insulin sensitivity, potentially decreasing risks of type 2 diabetes mellitus [56]. Known for its enzyme production, *B. vulgatus* degrades complex polysaccharides into SCFAs, offering gut health benefits [57]. This versatile bacterium possesses the ability to fight against pathogenic infections associated with inflammatory bowel disease (IBD) [58]. . Additionally, *B. vulgatus* serves as an anti-obesity agent and helps hyperlipidemia treatment, expanding its health effects [59].

The relative abundance of *Lactobacillus* was lower in the treatment group (3%), compared to the positive and negative control groups (5% each). *Lactobacillus* is a genus known as probiotic bacteria widely used in the production of functional foods. However, abundance of *Lactobacillus* spp. is relatively low in the GIT [60]. Although *Lactobacillus* spp. include positive roles in host health as studies have reported that *Lactobacillus* spp. with high abundance are detected in groups with digestive infection diseases, IBD, diarrhea and IBS [61]. A majority of species detected in the treatment and

negative control groups were *L. intestinalis*, while the species detected in the positive control group were *L. intestinalis* and *L. hominis*. The *L. intestinalis*, recently recognized as a probiotic agent, offers protective and homeostatic benefits for GIT. Studies have shown that the microorganism modulates the immune system to combat colitis infections in mice [62]. In addition to *Lactobacillus*, *Ligilactobacillus* genus was detected to include a higher relative abundance in the treatment group (3%) than the positive control (1%). Species of the *Ligilactobacillus* genus that have been investigated as probiotics include *L. salivarius* [63]. However, this study was unable to identify *Ligilactobacillus* to the species level.

The relative abundance of *Bifidobacterium* spp. in the treatment (0.2%) and positive control (0,3%) groups was higher than that in the negative control group (0.04%). Similar to the established prebiotic inulin, supplementing mice with velvet beans significantly increased the presence of *Bifidobacterium* spp. in their caecum. This genus largely includes probiotic species. *Bifidobacterium* genus detected in the positive control group was dominated by *B. animalis*, while the treatment group was dominated by *B. pseudolongum.* The *B. animalis*, a probiotic species, modulates gut microbiota composition for anti-obesity effects and protects GIT by enhancing intestinal barrier functions [64]. Studies have shown that the microorganism improves insulin sensitivity and restores blood glucose homeostasis in diabetic mice [65]. Furthermore, *B. animalis* produces beneficial organic acids such as acetic acid, linoleic acid and DHA [66]. Well-known for its anti-tumor characteristics, *B. pseudolongum* as a probiotic that combats non-alcoholic fatty liver disease (NAFLD) boosts intestinal barrier functions and restores healthy gut microbiota compositions [67,68].

4. Conclusion

This study assessed several legumes with potentials of development as prebiotic sources or functional foods. Bambara groundnuts, velvet beans and chickpeas passed the initial selection due to their high quantities of NDCs as a prebiotic characteristic. Velvet beans showed a better ability to stimulate *B. bifidum* and *L. plantarum* probiotics that were assessed *in vitro*, compared to bambara groundnuts and chickpeas. Thus, these passed the second selection. The abundance of *Lactobacillus* spp. in the velvet bean group (3%) was lower than that in the two controls (5%). Presence of prebiotic characteristics in velvet beans was validated by the increased levels of SCFAs in the cecum of mice as a result of NDC fermentation. Supplementation of velvet beans in mice can alter gut microbiota compositions. This alteration involves balancing the bacterial community, which is positively correlated with enhanced host defense systems.

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6. Conflict of Interest

This study was supported financially by Sebelas Maret University.

8. Authors Contributions

Conceptualization and design of the experiments, A.P and S.L.A.S; methodology, A.P and A.E.P; carry out of the experiments, A.E.P; analysis of data, A.E.P; contribution to reagents/materials/analysis tools, A.P; original draft preparation, A.E.P; review and edition, A.P. and S.L.

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بررسی پتانسیل‌های استفاده نشده: باقلاهای مخملی به‌عنوان منابع جدید کمک زیست‌یار و اثرات آنها بر میکروبیوتای روده و میزان اسیدهای چرب کوتاه زنجیر

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- اینولین
- *لاکتوباسیلوس پلانتاروم*
- کربوهیدرات غیرقابل هضم
- آنالیز متاژنومیک
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چکیده

سابقه و هدف: کمک زیست‌یارها[1] کربوهیدرات‌های غیر قابل هضمی هستند که به طور انتخابی رشد میکروارگانیسم‌های مفید موجود در روده را تسهیل می کنند. حبوبات به‌طور طبیعی حاوی کربوهیدرات هایی با پتانسیل به‌عنوان منابع کمک زیست‌یار هستند. با این حال، گونه‌های حبوبات متعددی وجود دارند که در این خصوص بررسی نشده‌اند. هدف از این مطالعه شناسایی این گونه حبوبات بررسی نشده به‌عنوان منابع بالقوه کمک زیست‌یار بود.

مواد و روش ها: 9 نمونه حبوبات از جاوه مرکزی و جاوه شرقی اندونزی جمع آوری و با استفاده از روش خیساندن عصاره‌گیری شدند. هضم با بافر HCl و α-آمیلاز به دنبال آن با آنالیز توسط دی‌نیتروسالیسیلیک اسید و فنل-سولفوریک اسید، مقادیر کربوهیدرات‌های غیرقابل هضم مورد بررسی قرار گرفت. سه حبوبات با بیشترین میزان کربوهیدرات‌های غیرقابل هضم در شرایط آزمایشگاهی مورد بررسی قرار گرفتند تا توانایی آنها برای افزایش رشد زیست‌یارها[2] بررسی شود. سپس، امیدواربخش‌ترین عصاره روی موش‌ها به‌منظور بررسی اثرات آن بر سطوح اسیدهای چرب کوتاه زنجیر با استفاده از GC-2010 Plus و ترکیب میکروبیوتای روده با استفاده از نشانگرهای متاژنومی S rRNA16 مورد ارزیابی قرار گرفت.

یافته‌ها و نتیجه‌گیری: از 9 حبوبات مورد بررسی، بادام زمینی بامبارا ($23/51 mg.g^{-1}$)، باقلا مخملی ($mg.g^{-1}$ 22/36) و نخود ($12/1 mg.g^{-1}$) دارای بیشترین میزان کربوهیدرات غیرقابل هضم بودند. باقلا مخملی در مقایسه با بادام زمینی بامبارا و نخود، توانایی بیشتری برای تحریک رشد *لاکتوباسیلوس پلانتاروم* و *بیفیدوباکتریوم بیفیدوم* نشان دادند. خوراندن باقلا مخملی به موش موجب افزایش سطح اسیدهای چرب کوتاه زنجیره به صورت استات (12/6 mM) و پروپیونات (3/28 mM) شد. به طور معنی‌داری، باقلای مخملی می‌تواند با افزایش تنوع، کاهش میزان برتری، افزایش فراوانی *باکتری‌ها*، *هلیکوباکتر*، *موسیسپیریلوم*، *بیفیدوباکتریوم* و جنس *Lawsonia* و کاهش فراوانی *Lachnospiraceae* NK4A136 و گروه *Lachnospiraceae* NK4A136، ترکیب باکتری‌های روده را تغییر دهد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[1] Prebiotics غذاهای غیرقابل‌هضمی که باعث تحریک رشد ریزاندامگان‌های کولون می‌شوند

[2] probiotics غذاها یا مکمل‌هایی که به دلیل داشتن ریزاندامگان‌ها قادر به بازسازی یا تغییر گیاگان میکربی روده هستند و در حفظ سلامت انسان مؤثرند