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EFFECTS OF LIMA BEAN (*Phaseolus lunatus*) FLOUR ON COGNITIVE FUNCTION AND GROWTH RECOVERY MALNUTRITION RATS

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ABSTRACT

Incorporating lima bean (*Phaseolus lunatus*) flour can replace and complement the content of proteins, macronutrients, and micronutrients, which may be related to curative effects for malnutrition. Therefore, an in vivo approach was undertaken to demonstrate the effect of lima bean flour on cognitive function and growth recovery in malnourished rats. Four groups of male Wistar rats were included in this study: The control group (N), MAL, MAL+25% LB group, and MAL+50% LB group. Morphometric analysis was used to assess growth recovery. Cognitive tests included Y-Maze, Novel Object Recognition Test (NORT), and Water Maze by Morris (MWM) test, and histopathological examination of the brain sections was conducted on the cerebral cortex and hippocampus by hematoxylin and eosin stains. The biological activity prediction of lima bean active compounds was analyzed by the PASS server using an in-silico study. This study found that lima bean flour increased body weight, length, tail increment, ear length, and leg length in malnourished rats at 50% lima bean concentration, similar to the control group. Lima bean flour showed neuroprotective capabilities by reversing the effects of malnutrition on the cerebral cortex and hippocampus by lowering the percentage of degenerated cells and increasing cognitive and behavioral. Eight bioactive compounds in lima bean extract have antioxidant, anti-inflammatory, and antibacterial properties. Lima bean flour, a source of complex nutrients, protects against malnutrition systemically in various organ systems, including the brain.

Keywords: Protein malnutrition, Lima Bean, Body weight, Behavioral effects, Neuroprotective, Rat

INTRODUCTION

Malnutrition is a condition in which an individual's nutritional intake is unbalanced due to insufficient or excessive consumption of nutrients. In 2020, it was estimated that 149 million children under five would be stunted (too short for their age), 45 million would be wasted (too emaciated for their height), and 38.9 million would be overweight or obese. About 45 % of fatalities among children under five are attributable to malnutrition. These are most prevalent in low- and middle-income nations. In developing nations, malnutrition among children under five remains alarming. Study on the Nutritional Status of Toddlers in Indonesia (SSGI) reported that the prevalence of stunting in Indonesia was still relatively high, at approximately 21.6%, while in West Sumatra, Indonesia, it was still relatively high, at approximately 25.2% **(SSGI, 2022).**

Malnutrition may lead to decreased cognitive abilities, metabolic issues, learning difficulties, mental retardation, and the immune system, increasing the risk of infection and death **(Woldehanna *et al*., 2017; Forgie *et al*., 2020)**. Early malnutrition has the most significant long-term behavioural and cognitive impacts, including reduced fine motor abilities, impoverished IQ scores, and attention difficulties **(Galler & Robert Barrett, 2001)**. Tissue damage, growth retardation, dysregulated differentiation, reduction of synapses and synaptic neurotransmitters, delayed myelination, and overall decreased arborization of dendritic development of the developing brain are all consequences of structural malnutrition. There is a disruption in the temporal sequence of brain maturation, which impairs neural network development **(Udani, 1992)**. Long-term changes in brain function have been linked to cognitive impairment **(Leenstra *et al*., 2004; Tonkiss *et al*., 1994, 1991, 1990)**, interference with neurotransmitter systems **(Alamy *et al*., 2005)**, and changes in protein phosphorylation and oxidative condition **(Bonatto *et al*., 2005)**. Chronic malnutrition affects the growth and maturation of the brain **(Ranade *et al*., 2012).** Cognitive deficits caused by malnutrition manifest in memory difficulties, intellectual slowness, or specific learning disabilities in reading, writing or mathematics. The child may have behavioural problems such as attention deficit hyperactivity disorder, emotional regulation, or socialization difficulties **(El Hioui *et al*., 2017).**

Lima bean (*Phaseolus lunatus*) has enormous potential to be a food product when viewed from a nutritional perspective and growth requirements. Lima Bean has the potential to be a good source of nutrition with a protein source of 14.24 -24.92% **(Jayalaxmi *et al*., 2016; Ibeabuchi *et al*., 2019).** Lima Beans contain complex carbohydrates, especially starch and dietary fibre, vitamins B complex, and minerals (zinc, iron, and calcium) **(Campos-Vega *et al*., 2010).** The most abundant proteins are globulin and albumin proteins **(Agarwal, 2017).** In addition to its good macronutrient composition, Lima Bean is considered beneficial to health because of its low glycemic index due to the presence of slow-release carbohydrates **(Bello-Pérez *et al*., 2007).** Compounds found in Lima Beans are reported to have benefits as antioxidants and anti-cancer (**Campos-Vega *et al*., 2010; Alcázar-Valle *et al*., 2020**). **Tamayo *et al*. (2018)** reported that this bean has broad-spectrum anti-microbial activity, antioxidant, and anti-inflammatory. Lima beans are tropical or subtropical plants that are grown in various parts of the world with appropriate conditions. The Indonesian province of West Sumatra is a major producer of lima beans. The high source of nutrients in Lima Beans has the potential to be a source of nutrition in treating malnutrition and overcoming accompanying diseases. This study aims to identify effect of lima bean on growth restoration and cognitive function improvement in malnourished rats. This study found that a lima bean flour diet could enhance memory, morphometric description, and growth by increasing body weight and reducing chronic malnutrition-induced brain degeneration.

MATERIAL AND METHODS

Lima Beans Flour Production

Lima Beans were cleaned and washed with running water, soaked in distilled water with a ratio of 1: 5 for 12 hours at room temperature, and boiled in distilled water with a ratio of 2:1 (water: grain) for 60 minutes. After initial cooking, the Lima Beans were dried in an oven with air circulation at 60 °C until constant weight. After cooling the lima beans and sieving (20 mesh), the flour was stored in polyethylene bags at 8°C (**Jayalaxmi *et al*., 2016**).

Biological Activity Prediction

The Prediction of Activity Spectra for Substances (PASS) server (http://www.pharmaexpert.ru/passonline/) was used to examine the biological activities of substances. The structure-activity relationship (SAR) technique was used to make predictions. Compounds with a similar structure and active cluster were considered comparable activity. The probability that a chemical was active (Pa) was set at 0.7 for the PASS Server, so Pa > 0.7 indicated a compound with a high probability of activity on experimentation (**Gangwar *et al*., 2014; Marisca *et al*., 2020**). Anti-inflammatory, antioxidant, and antibacterial activity were among the biological evaluated. A cluster analysis of candidate substances was used to make the prediction. A combination of molecules with anti-inflammatory, antioxidant, and antibacterial properties was predicted to treat malnutrition.

Animals and Research Design

Twenty-four male Wistar rats (Rattus norvegicus) aged 6 weeks, obtained from the animal house of Muaropalam (West Sumatra, Indonesia. Animals were maintained in the laboratory room for at least one week before experiment under controlled environmental conditions: a constant temperature of 25±2°C, humidity of 60±10%, and 12-hour light/dark cycles. A standard pellet diet and water were allowed ad libitum. The care and use of animals, as well as the experimental procedures, were conducted following the Declaration of Helsinki and the standard guideline for the care and use of animals for experimental purposes (No.985/UN. 16.2/KEP-FK/2022) established by Research Ethics Commission of the Faculty of Medicine, Andalas University. Six male Wistar rats were given a standard diet containing 20% protein (N group). Meanwhile, 18 Wistar rats were treated using pellet feed with 11% protein (nutrition deficit group) for six weeks. In the seventh week, eighteen malnourished rats were randomly distributed into 3 groups: 6 rats were fed with low protein (11%) (MAL group); 6 rats were fed with 25% lima bean flour-substituted low protein feed (11%) (MAL+25% LB group); and 6 rats were fed with 50% lima bean flour-substituted low protein feed (11%) (MAL+50% LB group) for 6 weeks. Food and drink for rats were given ad libitum.

Morphometric Analysis

Once a week, body weight, body length, tail length, right ear length, and right hind leg length were measured using a lab animal scale and a measuring tape with a minor scale in cm (**Islam *et al*., 2021**).

Histopathological Analysis

After the sacrifice of all animals, the dissected rat brains were fixed in a 10% formalin solution and enclosed in paraffin. Sections of 5 µm thickness were obtained at the level of the hippocampus and cerebellum, stained with hematoxylin and eosin, and examined by a light microscope for histological examination (**Bancroft *et al.*, 2001**). Neuron density was counted using Image J manual cell counter and in 5 high-power field areas on each histological slide. Each brain tissue was sectioned into three serial sections. The average cell number was reported as the mean cell density per unit area.

Cognitive Test

The Y-Maze, Novel object recognition test (NORT), and Water Maze by Morris (MWM) testing order was randomized before behaviour tests. Go-PRO (HERO) was used to record mouse movements. Blinded analysis with ANY-Maze software or a blinded observer was used to track and score videos.

Water Maze by Morris (MWM)

The Morris water maze (MWM) tested rats' spatial learning and memory. Rat was tested in a pool with a 116 cm diameter and a 55 cm height (the water was between 21 and 23 ºC). The pool is divided into the N, S, E, and W quadrants. After testing, the rats were gently warmed and dried before being placed back in their cage. A platform was submerged in the water for the test, and the time it took the rats to get there was recorded (**Shi *et al*., 2021**).

The Y-Maze Test

Spatial memory is also tested using the Y-maze test. Three arms with length x width x height (40 cm x 8 cm x 15 cm) and an angle of 1200 from the center are employed in this Y-maze. Each arm will be identified, and access to it will be restricted. Two arms will be exposed during the test, with one arm serving as the beginning point. Mice will have 5 minutes to explore two open arms. The mice were re-evaluated after 2 hours of rest. Rats with high spatial memory explore new arms more frequently (**Kraeuter *et al*., 2019**).

Novel Object Recognition Test (NORT)

Two identical objects were set opposite sides of the OFT box to evaluate exploratory behaviors. The test consists of two phases, acclimatization on the first day, training on the second and third days, and testing two hours after training. A blinded observer documented interaction times. We defined rat interaction for this test as sniffing and/or positioning the nose on the object (**Miedel *ß*., 2017**).

Compounds from the Lima Bean (*Phaseolus lunatus*) Extract

Active compounds were identified from datamining the Knapsack herbal database (http://www.knapsackfamily.com/knapsack_core/top.php). The structure of each compound with further analyzed through the simplified molecular-input line-entry system (SMILES) in PubChem (http://pubchem.ncbi.nlm.nih.gov).

Statistical Analysis

Statistical comparisons were performed by one-way analysis of variance (ANOVA) using GraphPad PRISM 9 version. If ANOVA analysis indicated significant differences, Tukey's post hoc test was performed to compare mean values between treatment groups and control. ****p.0001, ***p.001, **p.01, *p.05, and ns = no significant correlation were used to denote statistical significance. Unless otherwise specified, analyses are expressed as the mean with SEM.

RESULTS AND DISCUSSION

Biological Activity of Lima Bean (*Phaseolus lunatus*) Extract

The Knapsack database and SMILE analysis from PubChem was used to identify roughly 36 chemicals in lima bean rind extract. Genistein, luteone, 2,3-Dehydrokievitone, kaempferol 3-O-isorhamninoside, 7,8,2',4'-Tetrahydroxyisoflavone, 5,7,8,2',4'-Pentahydroxyisoflavone and lunatone have a high probability (Pa>0.700) for antioxidant activity, five among them have a high probability (Pa>0.700) of anti-inflammatory activity, and kaempferol 3-isorhamninoside is only the one compound has antioxidant, anti-inflammatory, and antibacterial activity (Figure 1).

Genistein, luteone, 2,3-Dehydrokievitone, kaempferol 3-O-isorhamninoside, 7,8,2',4'-Tetrahydroxyisoflavone, 5,7,8,2',4'-Pentahydroxyisoflavone and lunatone belongs to the class of organic compounds polyphenols and flavonoids (**Wang *et al*., 2020; Yu *et al*., 2016; Yu *et al*., 2022 and Wissem *et al*., 2012**). Isoflavone has strong anti-inflammatory characteristics through improved antioxidant activity, NF-B regulation, and lower pro-inflammatory enzyme and cytokine levels, suggesting its use in a variety of inflammatory diseases (**Yu *et al*., 2016**). The aglycone component of kaempferol 3-isorhamninoside, has received substantial research attention for its possible health advantages. It has anti-inflammatory, antioxidant, anticancer, and cardioprotective effects. Its ability to scavenge free radicals, alter signaling pathways, and interact with diverse cellular targets is attributable to these functions (**Dastidar *et al*., 2004; Orhan *et al*., 2010**), and antiaging (**Gupta *et al*., 2014**). **Doria *et al*, (2012)** found 14.1 µg quercetin per gram of flour in an Italian lima bean cultivar. Phenolic substances like quercetin and condensed tannins include hydroxyl functional groups. Therefore plant extracts with more significant phenolic content have more –OH and hydrogen atoms and antioxidant activity (**Mukhtar *et al*., 2022**). Soybeans contain isoflavones like genistein and daidzein, which have been studied for their neuroprotective effects, improving memory and cognitive function by promoting neuronal survival and plasticity while reducing oxidative stress (**Lu *et al*., 2018**). Protein deficiency impacted oxidative stress, free radical concentration, thiobarbituric acid-reactive compounds, and lipid peroxidation in the cerebellum and cerebral cortex. Catalase activity and total antioxidant reactivity in protein-malnourished rats' cerebellum and cerebral cortex decreased considerably (**Feoli *et al*., 2006**). Lima bean bioactive components have antioxidant effects, which protect cells from oxidative stress and neurological disorders. Organic compounds like polyphenols and flavonoids have the potential to reduce inflammation, which is beneficial for overall health, including brain function. Furthermore, they affect the development and breakdown of neurotransmitters, which are molecules that communicate information across nerve terminals. Bioactive compound on lima bean required for proper development and can promote general health by preventing malnutrition, decreasing the impact of malnutrition, and protecting against chronic diseases.

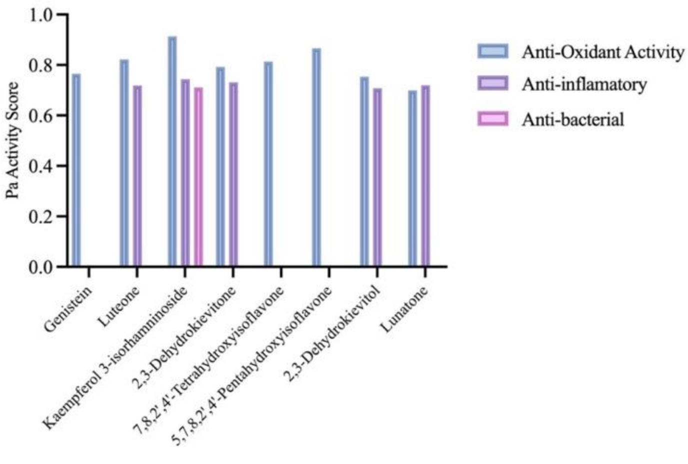

Figure 1 Eight compounds with the most active prediction as antioxidant, antiinflamatory, and antibacterial activity in lima beans

Morphometric Analysis

This study aimed to examine the effects of lima bean flour on early cognitive development in malnourished Wistar rats. At the six weeks period, decrease in body weight of malnutrition group animals were observed, with clear physical changes, when compared to the control group (N). Body weight reduction can be used as a basic indicator for malnutrition condition (**Estrela *et al*., 2014**). Moreover, at the end of experimental period, the average weight gain of the rats increased in MAL+ 25% and 50% LB group more than control group (N). In contrast, the MAL group experienced weight loss than initial weight after 12 weeks of low protein feeding treatment. Body length growth, tail increment, ear length growth, and leg length gain data in Table 1 indicate that the MAL + 50% group had the fastest growth recovery compared to the MAL + 25% group, with a significant difference from the MAL group. This result suggests that lima bean flour treatment in malnourished rats fed a low-protein diet for six weeks may trigger improvement and growth like the control group. Table 1 displays the results.

Table 1 Morphometrics data results of malnourished rats treated with lima bean flour

Groups	N	MAL	MAL+25% LB	MAL+50% LB
Initial Body Weight (g)	$118 \pm 18,70$	$113 \pm 15,72$	$113 \pm 4,04$	$121 \pm 8,98$
Body Weight (g) after 6 weeks treatment low protein (11 %)	$200 \pm 13,98$*	$100 \pm 9,528$	$109 \pm 4,256$	$112 \pm 8,29$
Final Body Weight (g)	$243 \pm 18,71$*	$96 \pm 9,713$	$164,7 \pm 8,81$	$179,3 \pm 7,31$
Weight Gain (g) for 6 weeks	43	- 4	55, 7	67,3
Body length growth (cm)	$1,567 \pm 0,21^{a}$	$0,233 \pm 0,08^{b}$	$0,900 \pm 0,05^{c}$	$1,500 \pm 0,11^{a}$
Tail increment (cm)	$2,833 \pm 0,16^{a}$	$0,200 \pm 0,05^{b}$	$0,966 \pm 0,14^{c}$	$2,600 \pm 0,30^{a}$
Ear length growth (cm)	$0,5333 \pm 0,06^{a}$	$0,000 \pm 0,00^{b}$	$0,300 \pm 0,05^{c}$	$0,600 \pm 0,05^{a}$
Leg length gain (cm)	$0, 3750 \pm 0,02^{a}$	$0,050 \pm 0,02^{b}$	$0,300 \pm 0,04^{a}$	$0,4750 \pm 0,04^{a}$

All data are presented in the form of Means ± SEM, where means having a different letter are significantly different at $P \leq 0,005$, and symbols *: Final body weight after treatment with a normal diet (18–20% protein)

The growth and development of bone tissues are influenced by nutritional and hormonal functions that affect the growth rate, bone formation, and bone size. Widening of the bone is affected by several hormones but the most conspicuous by the pituitary growth hormone and IGF-1 (**Florencio *et al*., 2015). Sari *et al*. (2021)** stated that nutritional deficiencies or malnutrition, such as protein deficiency, will cause low Insulin-like Growth Factor (IGF-1) and Growth Hormone (GH). Lima beans are known for their high protein, carbohydrate, fat, and micronutrient sources needed by the body and contain a variety of essential amino acids that plays an important role in the growth process (**Ishaya *et al*., 2019; Van Vught *et al*., 2013**). Lima beans contain the amino acids leucine, isoleucine, lysine, arginine, and glutamine, which play a role in growth and development (**Adebo, 2023**). Arginine is an amino acid involved in growth hormone production, which regulates growth and metabolism (**Kanaley, 2008**). The importance of leucine and isoleucine in improving muscle protein synthesis and promoting muscular growth is well recognized (**Norton *et al*., 2006**). Isoleucine supplementation can promote muscle protein synthesis and recovery after exercise (**Shimomura *et al*., 2004**). Lysine is required for collagen production, which is necessary for connective tissue growth and repair. It also helps with calcium absorption and bone health (**Zdzieblik *et al*., 2017**). Glutamine is required for growth and is important in cellular proliferation, tissue healing, and immunological function (**Castell *et al*., 1998**).

Lima beans contain amino acids and fatty acids that play an important role in growth and development. Lima legumes contain saturated, monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) (**Adewole, 2021**). Fatty acids, as is well known, contribute to the structure and fluidity of cell membranes and play an essential role in brain development and function (**Lauritzen *et al*., 2016;** Bradbury et al., 2011), hormone production (**Khorram *et al*., 2014**), nutrient assimilation (**Xu *et al*., 2021**), inflammation, and immune function (**Calder *et al*., 2017**). Compared to carbohydrates or proteins, fatty acids provide more than twice as much energy per gram as fatty acids (**Rolls *et al*., 2009**). This study found that the protein, carbohydrate, fat, and micronutrients in lima bean flour were proven to increase the body weight of rats after weight loss in the protein energy malnutrition state.

Histopathological Analysis

As shown in Figure 2, photomicrographs of the cerebral cortex and hippocampus sections stained with hematoxylin and eosin from the normal control group revealed a normal histopathological appearance of the cerebral cortex (Figure 2 A, E) and hippocampus (Figure 2 I, M). Malnutrition groups with severe malnutrition showed histological changes in the cerebral cortex (Figure 2 B, F) and hippocampus (Figure 2 J, N) areas in the form of degenerated neuron cells. Some neuronal cells have pyknotic nuclei, hyperchromatic cells, and shriveled cells.

The histopathological showed a positive effect on the histological of the cerebral cortex (Figure 2 C, G MAL+25% LB, Figure 2 D, H MAL+50% LB) and hippocampus (Figure 2 D, H MAL+25% LB, Figure 2 L, P MAL+50% LB) sections malnourished rat fed with lima bean flour. In the MAL Group, neuron density was lower than in the control group. The administration of lima bean supplementation showed cerebral cortex tissue with higher neuronal density than malnourished controls and fewer degenerated neuronal cells, especially in the treatment with 50% LB (MAL+50% LB group), which was mainly in the interlobular and perivascular septa. There was a mild increase in the distribution of lymphocytes in the tissue and an increase in mast cells. Likewise, in the hippocampus tissue, the feeding of five beans showed a higher density of neurons than the MAL group and fewer degenerated neuron cells, and the MAL+50% LB treatment was closer to the control group (N), especially in the interlobular and perivascular septa. A slight increase in the distribution of lymphocytes in the tissue and an increase in mast cells. The lima bean feeding described lower degenerative changes in brain tissue with a decreased proportion of degenerated cells. The histological data at 50% LB (MAL+50% LB group) showed histological morphology closer to normal rats.

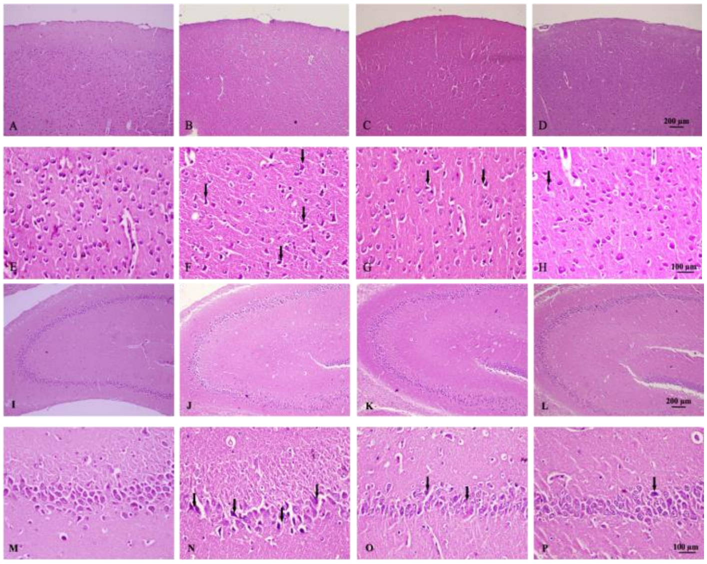

Figure 2 Histopathology of the Cerebral Cortex (A-H) and Hippocampus (I-P) sections of malnourished rat with H&E x40 (A-D, I-L), x100 (E-H, M-P). Normal control group (A, E, I, M), MAL Group (B, F, J, N), MAL + 25% LB (C, G, K, O), and MAL + 50% LB (D, H, L, P). Histopathology of the cerebral cortex and hippocampus tissues of malnourished rats showed some neurons have degenerated with neuronal pyknotic, hyperchromatic (↓), lower neuron density compared to the normal control group, and malnutrition treated with lima bean flour

Malnutrition can have significant histopathological effects on the cortex and hippocampus of rats, especially in neuron cell density and degeneration of neuron cells. Figure 3 shows the results of neuron cells density measurements in the cerebral cortex (Figure 3 A; N 119±3,51; MAL 86,67±1,45; MAL+25% LB 98,67±0,33; MAL+50% LB 111,3±3,48 cells/unit area) and hippocampus (Figure 3 B ; (N 82,33±0,88; MAL 55,33±2,02; MAL+25%LB 67,33±0,88; MAL+50%LB 76,67±0,88 cells/unit area). Cerebral cortex and hippocampus neuron cell density shown in the MAL+50% LB group was similar in the normal control group but significantly different from the MAL group and the MAL+25% LB group.

The percentage of the cerebral cortex and hippocampus neuron cell degeneration shown in Figure 3 C and D (N 1,34±0,09; MAL 8,5±0,67; MAL+25% LB 5,77±0,38; MAL+50% LB 2,5±0,14 % and N 1,30±0,03; MAL 11,29±0,04; MAL+25% LB 9,04±0,25; MAL+50% LB 2,87±0,19 %, respectively). In the MAL+50% LB group, there was a reduction in cell degeneration in the cerebral cortex, similar to the situation in the control group and significantly different from the MAL and MAL+25% LB groups. In contrast, cell degeneration in the hippocampus of the MAL+50% group was comparable to the control group but was not significantly different from the MAL and MAL+25% LB groups.

Histopathological effects of malnutrition on the cortex and hippocampus may vary depending on the duration and severity of malnutrition, as well as the specific nutritional deficiencies involved (e.g., protein, vitamins, minerals). Severe malnutrition can result in the loss of neurons cells in the cortex, leading to decreased cell density and disrupted cortical architecture (**Granados *et al*., 2002)**. Thickness of cerebral cortex in figure 3 E (N 1094±0,88; MAL 870,1±12,32; MAL+25%LB 993,5±6,21; MAL+50%LB 1051±15,90 µm) shown that MAL group significantly different with control group after 12 weeks fed with low protein. In contrast, the thickness of cerebral cortex in MAL+50% LB group similar with control group.

Several studies have shown that a low protein diet during brain development has a negative impact on hippocampus structure and function, including neuronal and synaptic loss and behavioral changes (**Galler *et al*., 1994; Garc'ia *et al*., 1993; Granados *et al*., 2002**). Significant changes were seen in the activities of the enzyme SOD, lipoperoxidation, and protein oxidation in the hippocampus of 21 and 75-day-old rats fed 25% protein with methionine. The diet's protein composition and the necessary amino acid methionine may affect the brain's antioxidant system and redox status (**Bonatto *et al*., 2005**). The improvement of the histological data shows the potential of lima bean flour containing protein with complete amino acids, fat, and other micronutrients in overcoming tissue damage due to severe malnutrition, including in the brain.

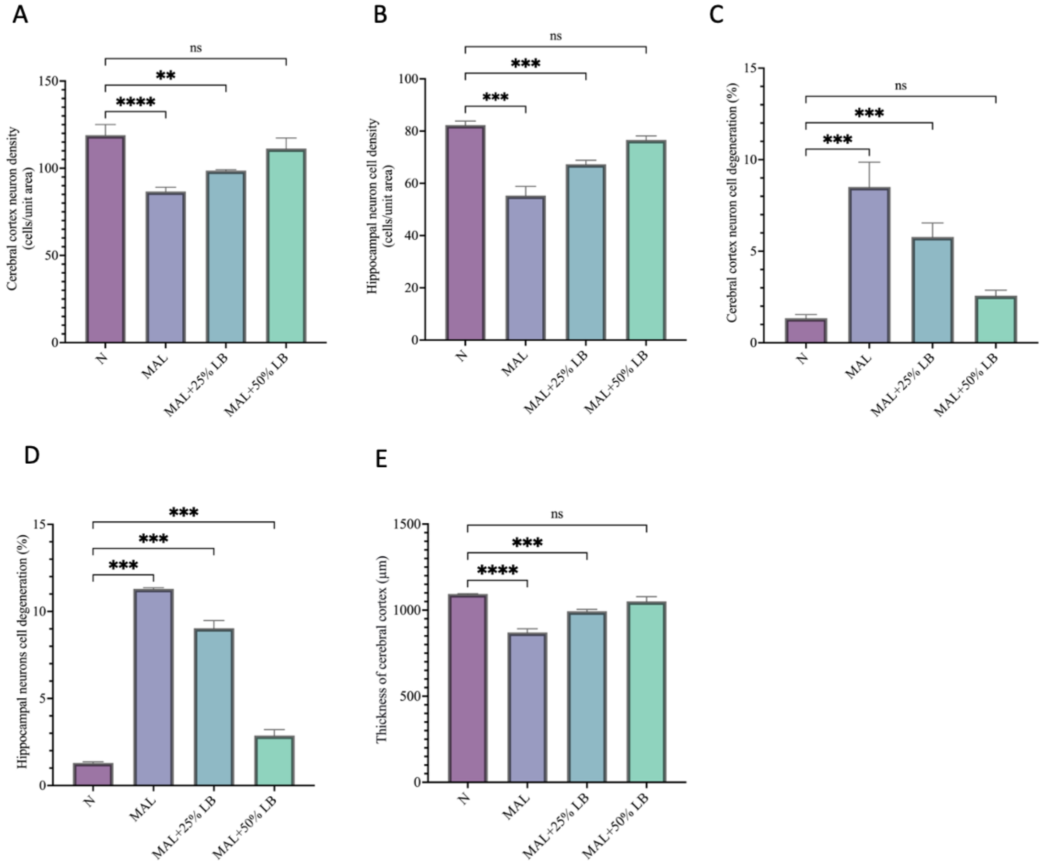

Figure 3 (A) Cerebral cortex neuron density (cells/unit area), (B) Hippocampal neuron cell density (cells/unit area), (C) Cerebral cortex neuron cell degeneration, (D) Hippocampal neurons cell degeneration, and (E) Thickness of cerebral cortex (µm) data of Cerebral Cortex and Hippocampus sections of malnourished rat with control group (N), MAL Group, MAL + 25% LB, and MAL + 50% LB. Data represent means ± SEM (n = 4). ****p.0001, ***p.001, **p.01, *p.05, and ns = no significant vs. control in each group

Cognitive Analysis

Latency, Spontaneous Alternation (%), and Novel Object Recognition (%) tests were performed in the final week of treatment to determine the cognitive performance of malnourished rats fed lima beans. Table 2 shows the cognitive test results. The latency test data of the Morris Water Maze test show that the MAL group takes a long time to find the platform compared to the other treatment groups. In contrast, the control group takes less time. Compared to the MAL group, the MAL +25% or 50% LB group had the same platform finding time as the control group (N) and was significant. The long-time taken by the MAL group to locate the platform suggests that malnutrition can impair spatial and semantic memory. The rapid time to locate the platform in the lima bean-fed group suggests a propensity for memory improvement in the MAL+25% or 50% group, as individuals can recall the platform's location. Data from the Y Maze test revealed that the control group and MAL+25% or 50% had significantly different spontaneous alternation than the MAL treatment group. The Y-maze can be used to test rats' short-term memory. A rat with a good working memory will remember which arms of the maze it has visited and enter fewer often visited arms, and this requires interactions in several different brain regions, such as the hippocampus and prefrontal cortex (**Kraeuter *et al*., 2019**). Data from the Novel Object Recognition Test show that the MAL group has the lowest percentage of novel object time compared to the other groups. In contrast, the control group (N) has the highest percentage of novel object time. The MAL+25% or 50% group had nearly the same item similarity percentage as the control group (P<0.05). The percentage of novel objects is used to assess episodic, recognition, and semantic memory. The low percentage of object novelty time in this test implies a deterioration in memory function in rats. This result demonstrates that treating lima bean flour improves cognitive performance in rats. According to **Wahl *et al*. (2017)**, amino acids act as precursors of serotonin, a neurotransmitter that governs learning and memory. Malnutrition-induced cognitive deficiencies appear as memory issues, intellectual slowness, or specific learning disabilities in reading, writing, or math. The degree of cognitive impairment is proportional to the severity of malnutrition.

Table 2 Latency, Spontaneous Alternation (%), and Novel Object Recognition (%) experiments yield experimental data results

Group	MWM (s)	Y Maze (%)	Novel Object Recognition (%)
N	2.77±0.73^{a}	41.11±8.39^{a}	100.0±0.0^{a}
MAL	8.39±2.13^{b}	18.1±3.29^{b}	0.00±0.00^{b}
MAL+25% LB	3.46±0.42^{a}	42.5±6.61^{a}	73.33±46.1^{a}
MAL+50% LB	3.07±44^{a}	43±1.35^{a}	69.0±29.61^{a}

Lima beans contain 0.32-2% fat, 0.9% total oil, 28.7% saturated fatty acid, 10.5% monounsaturated fatty acid, and 69.8% polyunsaturated fatty acid (**USDA, 2018; Adebo, 2023; Bonita G *et al*., 2020; Adewole *et al*., 2021**). Linoleic acid and palmitic acid are the two most important components. Linoleic acid is essential to human nutrition since it is a precursor to EPA and DHA (**Rossignoli *et al*., 2018**). Linolenic acid in lima beans may help the body synthesize DHA and EPA (**Goyens *et al*., 2006**). DHA in neuronal membrane glycerophospholipids regulates neurogenesis, synaptogenesis, and neurite outgrowth. DHA affects neurotransmitter release, gene expression, membrane-bound enzyme and ion channel activity, and synaptic plasticity (**Horrocks *et al*., 2004**). Polyunsaturated fatty acids (PUFAs) are important in preserving brain processes such as learning and memory, which decrease with age (**De Souza *et al*., 2011**). Numerous studies have shown that omega-3 fatty acids can improve learning and memory. omega-3 fatty acids have been suggested to be protective in moderate cognitive impairment, dementia, and Alzheimer's disease (**Waitzberg *et al*., 2014**). Long-chain ω-3 PUFA accumulates in brain areas linked with learning and memory, such as the cerebral cortex and hippocampus (**Chung *et al*., 2008**) Long-chain -3 PUFAs are important for learning and memory throughout life (**Joffre *et al*., 2014; Luchtman *et al*., 2013**). **Bach *et al*. (2014)** found that n-3 fatty acids affect how long rats remember things by ensuring they have enough DHA and brain-derived neurotrophic factors and affecting the activity of NR2B and Fyn when memories are formed. **Heng *et al*, (2014)** found that adding PUFA to the food of diabetic rats is neuroprotective through an antiapoptotic pathway and improves their ability to learn and remember. **Pérez *et al*, (2013)** found that giving ω-3 to stressed rats helped them in two ways: it made them less nervous and helped them learn better. In vivo dietary with lima bean flour increased spatial memory, according to our findings. We hypothesize that lima bean flour influences learning and memory.

CONCLUSION

This study has demonstrated that providing feed with lima bean flour could increase growth, mainly through increasing body weight and preventing brain degeneration due to chronic malnutrition, in line with improving cognitive function and morphometric images. As a source of complex nutrients, Lima bean flour protects against malnutrition systemically in various organ systems, including the brain.

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REFERENCES

Agarwal, A. (2017). Proteins in Pulses. *Journal of Nutritional Disorders & Therapy*, 07(01). http://dx.doi.org/10.4172/2161-0509.1000e129.

Adebo, J.A. (1996). A Review on the Potential Food Application of Lima Beans (*Phaseolus lunatus* L.), an Underutilized Crop. *Appl. Sci*, 2023, 13. https://doi.org/10.3390/app13031996

Adewole, E., Ojo, A., Oludoro, O., Osasona, I. (2021). Fatty acid profiles of *Phaseolus* species. *Food Research*, 5 (5) , 131 – 135. http://dx.doi.org/10.26656/fr.2017.5(5).439

Alamy, M. and Bengelloun, W.A. (2012). Malnutrition and brain development: An analysis of the effects of inadequate diet during different stages of life in rat, *Neuroscience & Biobehavioral Reviews*, 36(6), 1463–1480. https://doi.org/10.1016/j.neubiorev.2012.03.009

Alcázar-Valle, M. (2020). Bioactive Compounds, Antioxidant Activity, and Antinutritional Content of Legumes: A Comparison between Four Phaseolus Species. *Molecules 2020*, 25(15), 3528. https://doi.org/10.3390/molecules25153528

Bach, S.A., De, S.L.V., Müller, A.P., Oses, J.P., Quatrim, A., Emanuelli, T., Moreira, J.D. (2014). Dietary omega-3 deficiency reduces BDNF content and activation NMDA receptor and Fyn in dorsal hippo- campus: implications on persistence of long-term memory in rats. *Nutritional Neuroscience*, 17, 186–189. https://doi.org/10.1179/1476830513y.0000000087

Bancroft, J.D., Gamble, M. (2001). Theory and Practice of Histological Techniques (5th ed.). Edinburgh, Scotland: Churchill Livingstone. 175–331.

Bello-Pérez, L.A. (2007). Proximal composition and in vitro digestibility of starch in lima bean (*Phaseolus lunatus*) varieties. *Journal of the Science of Food and Agriculture*, 87(14), 2570–2575. http://dx.doi.org/10.1002/jsfa.3005

Bonatto, F., Polydoro, M., Andrades, M.E., da Frota Júnior, M.L., Dal-Pizzol, F., Rotta, L.N., Souza, D.O., Perry, M.L., Moreira, J.C. (2005). Effect of protein malnutrition on redox state of the hippocampus of rat. *Brain Res*, 25, 1042(1), 17-22. https://doi.org/10.1016/j.brainres.2005.02.002

Calder, P.C. (2017). Omega-3 fatty acids and inflammatory processes: from molecules to man. *Biochem Soc Trans*. 15, 45(5), 1105-1115. https://doi.org/10.1042/bst20160474

Campos-Vega, R. (2010). Bean (*Phaseolus vulgaris* L.) polysaccharides modulate gene expression in human colon cancer cells (HT-29). *Food Research International*, 43(4), 1057–1064. https://doi.org/10.1016/j.foodres.2010.01.017

Castell, L.M., Newsholme, E.A. (1998). Glutamine and the effects of exhaustive exercise upon the immune response. *Can J Physiol Pharmacol*, 76(5), 524-32. https://doi.org/10.1139/cjpp-76-5-524

Chung, W.L., Chen, J.J., Su, H.M. (2008). Fish oil supplementation of control and (n-3) fatty acid-deficient male rats enhances refer- ence and working memory performance and increases brain regional docosahexaenoic acid levels. *Journal of Nutrition*, 138, 1165–1171. https://doi.org/10.1093/jn/138.6.1165

Dastidar, S.G., Manna, A., Kumar, K.A., Mazumdar, K., Dutta, N.K., Chakrabarty, A.N., Motohashi, N., Shirataki, Y. (2004). Studies on the antibacterial potentiality of isoflavones. *Int. J. Antimicrob. Agents*, 23, 99–102. https://doi.org/10.1016/j.ijantimicag.2003.06.003

De Souza, A.S., Fernandes, F.S., Do Carmo, M.D. (2011). Effects of maternal malnutrition and postnatal nutritional rehabilitation on brain fatty acids, learning, and memory. *Nutrition Reviews*, 69, 132–144. https://doi.org/10.1111/j.1753-4887.2011.00374.x

Doria, E., Campion, B., Sparvoli, F., Tava, A., Nielsen, E. (2012). Anti-nutrient components and metabolites with health implications in seeds of 10 common bean (*Phaseolus vulgaris* L. and *Phaseolus lunatus* L.) landraces cultivated in southern Italy. *J. Food Compos. Anal*, 26, 72–80. https://doi.org/10.1016/j.jfca.2012.03.005

El Hioui, M. (2017). The Relationship Between Nutritional Status and Educational Achievements in the Rural Schooil Children of Morocco. Jurnal of Neurology and Neurological Disorders, 3(1). http://dx.doi.org/10.15744/2454-4981.3.101

Estrela, A.D., Lemes, . C.G., Guimarães, A.T., Malafaia, G. (2014). Effects of short-term malnutrition in rats. *Sci Plena*, 10, 1–13. https://www.scientiaplena.org.br/sp

Feoli, A.M., Siqueira, I.R., Almeida, L., Tramontina, A.C., Vanzella, C., Sbaraini, S., Schweigert, I.D., Netto, C.A., Perry, M.L., Gonçalves, C.A. (2006). Effects of protein malnutrition on oxidative status in rat brain. *Nutrition*. 22(2):160-5. doi:10.1016/j.nut.2005.06.007.

Florencio-Silva, R., Sasso, G.R.D.S., Sasso-Cerri, E., Simoes, M.J., Cerri, P.S. (2015). Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells. *Biomed Res Int*, 2015, 421746. https://doi.org/10.1155/2015/421746

Forgie, A.J. (2020). The impact of maternal and early life malnutrition on health: A diet-microbe perspective. *BMC Medicine*, 18(1). https://doi.org/10.1186/s12916-020-01584-z

Galler, J.R., Tonkiss, J., Maldonado-Irizarry, C.S. (1994). Prenatal protein malnutrition and home orientation in the rat, *Physiol. Behav*. 55 (1994) 993–996. https://doi.org/10.1016/0031-9384(94)90379-4

Galler, J.R., Robert Barrett, L. (2001). Children and famine: long-term impact on development. *Ambulatory Child Health*, 7(2), 85–95. http://dx.doi.org/10.1046/j.1467-0658.2001.00109.x

Gangwar, M.K., Gautam, A.K., Sharma, Y.B., Tripathi, R.K.,. Goel, G.N. (2014). Antioxidant Capacity and Radical Scavenging Effect of Polyphenol Rich *Mallotus philippenensis* Fruit Extract on Human Erythrocytes: An *in vitro* Study. *The Scientific World Journal*, 279451. https://doi.org/10.1155/2014/279451

Garc´ıa-Ruiz, M., Da´z-Cintra, S., Cintra, L., Cordiki, G. (1993). Effect of protein malnutrition on CA3 hippocampal pyramidal cells in rats of three ages, *Brain Res*, 625 (1993), 203 – 212. https://doi.org/10.1016/0006-8993(93)91060-6

Goyens, P.L., Spilker, M.E., Zock, P.L., Katan, M.B., Mensink, R.P. (2006). Conversion of alpha-linolenic acid in humans is influenced by the absolute amounts of alpha-linolenic acid and linoleic acid in the diet and not by their ratio. *American Journal of Clinical Nutrition*, 84, 44–53. https://doi.org/10.1093/ajcn/84.1.44

Granados-Rojas, L., Larriva-Sahd, J., Cinta, L., Gutierrez-Ospina, G., Ronda A., Cintra, S. D. (2002). Prenatal protein malnutrition decreases mossy fibers-CA3 thorny excrescences asymmetrical synapses in adult rats, *Brain Res*. 933 (2002), 164–171. https://doi.org/10.1016/s0006-8993(02)02314-4

Gupta, C., Prakash, D. (2014). Phytonutrients as therapeutic agents. *J. Complement. Integr. Med*. 11, 151–169. https://doi.org/10.1515/jcim-2013-0021

Horrocks, L.A., Farooqui, A.A. (2004). Docosahexaenoic acid in the diet: Its importance in maintenance and restoration of neural membrane function. Prostaglandins. *Leukotrienes & Essential Fatty Acids*, 70, 361–372. https://doi.org/10.1016/j.plefa.2003.12.011

Ibeabuchi, J.C. (2019). Effect of Dehulling on Proximate Composition and Functional Properties of Lima Bean (*Phaseolus lunatus*) Grown in Enugu State. *Journal of Food Research*, 8(2). http://dx.doi.org/10.5539/jfr.v8n2p116

Ishaya, F.A. Aletor, O. (2019). Nutritive Potential and Functional Attributes of Lima Bean (*Phaseolus lunatus*) and Pigeon Pea (*Cajan cajanus*) Protein Isolates. *Journal of Integrative Food Sciences & Nutrition*, 3, 1-6. https://scientonline.org/

Islam, M.M. (2021). Morphometric Study of *Mus musculus*, *Rattus norvegicus*, and *Rattus rattus* in Qatar. *Animals*, 11(8), 2162. https://doi.org/10.3390/ani11082162

Jayalaxmi, B. (2016). Effect of different processing methods on proximate, mineral and antinutrient content of lima bean (*Phaseolus lunatus*) seeds. *Legume Research*, 39(4), 543–549. http://dx.doi.org/10.18805/lr.v0iOF.7108

Heng, L.J., Jia, D., Yang, R.H., Gao, G.D. (2014). Fish oil improves learning impairments of diabetic rats by blocking PI3K/AKT/nuclear factor-κB-mediated inflammatory pathways. *Neuroscience*, 258, 228– 237. https://doi.org/10.1016/j.neuroscience.2013.11.016

Joffre, C., Nadjar, A., Lebbadi, M., Calon, F., Laye, S. (2014). n-3 LCPUFA improves cognition: The young, the old and the sick. *Prostaglandins Leukotrienes and Essential Fatty Acids*, 91, 1–20. https://doi.org/10.1016/j.plefa.2014.05.001

Kanaley, J.A. (2008). Growth hormone, arginine and exercise. *Current Opinion in Clinical Nutrition and Metabolic Care*, 11(1), 50-54. https://doi.org/10.1097/mco.0b013e3282f2b0ad

Khorram, O., Keen-Rinehart, E., Chuang, T.D., Ross, M.G., Desai, M. (2015). Maternal undernutrition induces premature reproductive senescence in adult female rat offspring. *Fertil Steril*, 103(1), 291-8.e2. https://doi.org/10.1016/j.fertnstert.2014.09.026

Kraeuter, A.K., Guest, P.C., Sarnyai, Z. (2019). The Y-Maze for Assessment of Spatial Working and Reference Memory in Mice. *Methods in Molecular Biology*. 1916, 105–111. https://doi.org/10.1007/978-1-4939-8994-2_10/COVER

Lauritzen, L., Brambilla, P., Mazzocchi, A., Harsløf, L.B., Ciappolino, V., Agostoni, C. (2016). DHA Effects in Brain Development and Function. Nutrients, 4;8(1):6. https://doi.org/10.3390/nu8010006

Leenstra, T. (2004). Prevalence and severity of malnutrition and age at menarche; cross-sectional studies in adolescent schoolgirls in western Kenya, *European Journal of Clinical Nutrition 2005*, 59:1, 59(1), 41–48. https://doi.org/10.1038/sj.ejcn.1602031

Luchtman, D.W., Song, C. (2013). Cognitive enhancement by ome- ga-3 fatty acids from child-hood to old age: Findings from animal and clinical studies. *Neuropharmacology*, 64, 550–565. https://doi.org/10.1016/j.neuropharm.2012.07.019

Lu, C., Wang, Y., Wang, D., Zhang, L., Lv, J., Jiang, N., Fan, B., Liu, X., Wang, F. (2018). Neuroprotective Effects of Soy Isoflavones on Scopolamine-Induced Amnesia in Mice. *Nutrients*. 30;10(7):853. doi: 10.3390/nu10070853.

Marisca, E.G., Sutiman, B.M., Kusworini, H., Bambang, P., Wahyu, W.D.H.U. (2020). A Computational Study to Predict Wound Healing Agents from the Peel of the Mangosteen (Garcinia mangostana L.) Extract. Int J Bioautomation, 24 (3), 265-276. http://dx.doi.org/10.7546/ijba.2020.24.3.000607

Miedel, C.J. (2017). Assesment of Spontaneous Atmlternation, Novel Object Recognition and Limb Clasping in Transgenic Mouse Models of Amuloid and Tau Neuropathology. Journal of Vemisualized Experiments, 123. https://doi.org/10.3791/55523

Mukhtar, H., Ahmed, D.V., Arez, H., Judit, M. (2022). The impact of functional food in prevention of malnutrition, *PharmaNutrition*, 19, , 100288, ISSN 2213-4344. https://doi.org/10.1016/j.phanu.2022.100288

Norton, L.E., Layman, D.K. (2006). Leucine regulates translation initiation of protein synthesis in skeletal muscle after exercise. *J Nutr*, 136(2), 533S-537S. https://doi.org/10.1093/jn/136.2.533s

Orhan, D.D., Ozcelik, B., Ozgen, S., Ergun, F. (2010). Antibacterial, antifungal, and antiviral activities of some flavonoids. Microbiol. *Res. 2010*, 165, 496–504. https://doi.org/10.1016/j.micres.2009.09.002

Pérez, M.Á., Terreros, G., Dagnino-Subiabre, A. (2013). Long-term ω-3 fatty acid supplementation induces anti-stress effects and improves learning in rats. *Behavioral and Brain Functions*, 9, 25. https://doi.org/10.1186%2F1744-9081-9-25

Ranade, S.C., Sarfaraz, N.M., Kumar, R.P., Rose, A.J., Gressens P. (2012). Early protein malnutrition disrupts cerebellar development and impairs motor coordination. *Br J Nutr*, 107, 1167-1175. https://doi.org/10.1017/s0007114511004119

Rolls, B.J. (2009). The relationship between dietary energy density and energy intake. *Physiol Behav*, 14, 97(5), 609-15. https://doi.org/10.1016/j.physbeh.2009.03.011

Rossignoli, C.P., Dechandt, C.R.P., Souza, A.O., Sampaio, I.H., Vicentini, T.M., Teodoro, B.G., Alberici, L.C. (2018). Effects of intermit- tent dietary supplementation with conjugated linoleic acid and fish. *J Nutr Biochem*, 60, 16-23. https://doi.org/10.1016/j.jnutbio.2018.07.001

Sari, Y.O., Aminuddin., Hamid, F. (2021). Malnutrition in children Associated with Low Growth Hormone (Gh) Levels. *Gaceta Sanitaria*, 35, S327-S329. https://doi.org/10.1016/j.gaceta.2021.10.046

Shi, X. (2021). Behavioral Assessment of Sensory, Motor, Emotion, and Cognition in Rodent Models of Intracerebral Hemorrhage. *Frontiers in Neurology*, 12. https://doi.org/10.3389/fneur.2021.667511

Shimomura, Y., Murakami, T., Nakai, N., Nagasaki, M., Harris, R.A. (2004). Exercise promotes BCAA catabolism: effects of BCAA supplementation on skeletal muscle during exercise. *J Nutr*, 134(6 Suppl), 1583S-1587S. https://doi.org/10.1093/jn/134.6.1583s.

Study on the Nutritional Status of Toddlers in Indonesia. (2022). SSGI Hasil Survei Status Gizi Indonesia. Kementrian Kesehatan RI.

Tamayo, J. (2018). Antimicrobial, Antioxidant and Anti-Inflammatory Activities of Proteins of *Phaseoulus lunatus* (Fabaceae) Baby Lima Beans Produced in Ecuado. *bioRxiv*, 401323. https://doi.org/10.1101/401323

Tonkiss, J., Galler, J.R. (1990). Prenatal protein malnutrition and working memory performance in adult rats. *Elsevier*. https://www.sciencedirect.com/science/article/pii/016643289090002V

Tonkiss, J., Foster, G., Galler, J. (1991). Prenatal protein malnutrition and hippocampal function: partial reinforcement extinction effect. *Elsevier*. https://doi.org/10.1016/0361-9230(91)90213-4

Tonkiss, J. (1994). An analysis of spatial navigation in prenatally protein malnourished rats. *Elsevier*. https://www.sciencedirect.com/science/article/pii/0031938494901260

USDA. (2018). National Nutrient Database for Standard Reference 1, Legacy Release. U.S. Department of Agriculture, Agricultural Research Service. https://www.ars.usda.gov/nutrientdata

Van Vught, A.J.A.H. (2013). Dietary arginine and linear growth: the Copenhagen School Child Intervention Study. *British Journal of Nutrition*, 109(6), 1031–1039. https://doi.org/10.1017/s0007114512002942

Wahl D, Sean CP Coogan, Samantha M Solon-Biet, Rafael de Cabo, James B Haran, David Raubenheimer, Victoria C Cogger, Mark P Mattson, Stephen J Simpson & David G Le Couteur (2017). Cognitive and behavioral evaluation of nutritional interventions in rodent models of brain aging and dementia, *Clinical Interventions in Aging*, 12:, 1419-1428, DOI: 10.2147/CIA.S145247

Waitzberg, D.L., Garla, P. (2014). Contribution of omega-3 fatty acids for memory and cognitive function. *Nutricion Hospitalaria*, 30, 467– 477. https://doi.org/10.3305/nh.2014.30.3.7632

Wang, J.F., Liu, S.S., Song, Z.Q., Xu, T.C., Liu, C.S., Hou, Y.G., Huang, R., Wu, S.H. (2020). Naturally Occurring Flavonoids and Isoflavonoids and Their Microbial Transformation: A Review. *Molecules 2020*, 25, 5112. https://doi.org/10.3390/molecules25215112.

Wissem, B., Jihed, B., Soumaya, K., Kamel, G., Leila, C.G. (2012). Flavonoids from Rhamnus alaternus L. (Rhamnaceae): Kaempferol 3-O-β-isorhamninoside and rhamnocitrin 3-O-β-isorhamninoside protect against DNA damage in human lymphoblastoid cell and enhance antioxidant activity. *South African Journal of Botany*, 80, 57-62. https://doi.org/10.1016/j.sajb.2012.02.005

Woldehanna, T. (2017). The effect of early childhood stunting on children's cognitive achievements: Evidence from young lives Ethiopia. 31(2), 75-84. https://www.researchgate.net/publication/321332864_The_effect_of_early_childhood_stunting_on_children's_cognitive_achievements_Evidence_from_young_lives_Ethiopia.

Xu, E., Chen, C., Fu, J., Zhu, L., Shu, J., Jin, M., Wang, Y., Zong, X. (2021). Dietary fatty acids in gut health: Absorption, metabolism and function. *Anim Nutr*. 7(4), 1337-1344. https://doi.org/10.1016/j.aninu.2021.09.010

Yu, H., Liu, J., Wang, L., Guan, S., Jin, Y., Zheng, J., Xiang, H., Wang, D., Liu, D. (2022). 2,3-Dehydrokievitone combats methicillin-resistant *Staphylococcus aureus* infection by reducing alpha-hemolysin expression. *Front Microbiol*. 23(13), 969215. https://doi.org/10.3389/fmicb.2022.969215

Yu, J., Bi, X., Yu, B., Chen, D. (2016). Isoflavones: Anti-Inflammatory Benefit and Possible Caveats. *Nutrients*, 10, 8(6):361. https://doi.org/10.3390%2Fnu8060361

Zdzieblik, D., Oesser, S., Gollhofer, A., König, D. (2017). Improvement of activity-related knee joint discomfort following supplementation of specific collagen peptides. *Appl Physiol Nutr Metab*, 42(6), 588-595. https://doi.org/10.1139/apnm-2016-0390