**Nutritional, Sensory and Hypocholesterolemic Effect of**

**Jack Bean Seed or Sprout Protein Isolates Milk**

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**Abstract**

Plant-based protein (eg. jack bean protein) contains some amino acids that have hypo cholesterol effect on cow's milk cholesterol. Cow’s milk cholesterol is a limiting factor for consumers with obesity and coronary heart disease. The purpose of this study was to determine the effect of germination and concentration of jack bean seed or sprout protein isolates on amino acid profile and reduction in cholesterol level via in vitro digestion. Complete randomized design was used for this study. Seven treatments, that is, cow milk (T0 or control), cow milk plus 3% (T1), 6% (T2) and 9% (T3) jack bean seed protein isolates, and cow milk plus 3% (T4), 6% (T5) and 9% (T6) jack bean sprout protein isolates. Jack bean seed and sprouts were obtained after 0 and 72 hours of germination, respectively. All samples were analyzed for proximate composition and preference level to determine the treatment with the best amino acid profile and cholesterol reduction capacity. The results showed that, cow milk plus 3% jack bean seed protein isolate (T1) and cow milk plus 3% jack bean sprout protein isolate (T4) were the best treatments for amino acid and cholesterol analyses because their level of preference were not significantly different (p>0.05) with cow milk (T0). The protein content and amino acid profile (arginine: lysine ratio) of T4 was significantly higher (p<0.05) but significantly lower (p<0.05) cholesterol level than cow milk (T0). The highest percentage of cholesterol reduction after in vitro digestion was shown by T4 (61.65% cholesterol reduction) compared to other treatments. In conclusion, the additional of 3% jack bean sprout protein isolate to cow milk (T4) showed potential hypo cholesterol effect due to its amino acid profile especially the high arginine: lysine ratio and cholesterol reduction observed via in vitro digestion.

**Keywords:** cow’s milk, protein isolate, jack bean, sprout, amino acid, cholesterol.

**Introduction**

Cardiovascular disease is one of the increasing degenerative diseases. Globally the death rate due to CVD (cardiovascular disease) in 2017 was estimated to be 17.8 million, equivalent to a ratio of 233.1 deaths per 100,000 people (Jagannathan *et al.*, 2019). The death rate due to CVD was reduced in high-income countries from 1990 (271.8 per 100,000 people) to 2017 (128.5 per 100,000 people), while in middle to low-income countries it decreased from 368.2 per 100,000 people in 1990 to 316.9 per 100,000 people in 2017 (Jagannathan *et al.*, 2019). High mortality rates due to cardiovascular diseases are related to total cholesterol levels in the blood (Kwon *et al.*, 2019). Total cholesterol plasma levels between 180-200 mg/dL represent a low mortality rate due to CVD (Kwon *et al.*, 2019). High consumption of refined grains, sugars, salt, unhealthy fats (cholesterol and saturated fatty acids) and animal foods, and low consumption of whole grains, fruits, vegetables, fish and nuts increased the risk of CVD (Anand *et al.*, 2015).

Cow milk is known to be a high animal protein source with essential amino acid content with one limitation of cow milk is its saturated fatty acid content which plays a role in increasing the level of low-density lipoprotein (LDL) cholesterol presenting risk for cardiovascular diseases (Artaud-Wild *et al.*, 1993). Several studies have attempted to eliminate this limitation, one of which was conducted by Ohlsson (2010) who replaced high-fat dairy products with low-fat dairy products reduced LDL cholesterol and the ratio of total cholesterol/HDL in blood plasma. Skeaff *et al.* (2004) also made efforts to reduce cholesterol in cow milk by replacing cow milk fat with goat milk fat. Their results showed a slight decrease in total cholesterol in blood plasma, but did not affect LDL and HDL. Koury *et al.* (2014) stated that, the consumption of animal and vegetable protein have varied effects on blood cholesterol levels which sometimes depend on the amino acid composition of the protein.

The use of protein sources derived from plant foods that have potential hypo cholesterol effect in reducing the risk of cardiovascular diseases have also been widely studied. Chalvon-Demersay *et al.* (2017) showed that, soy isoflavones play a role in reducing the risk of cardiovascular diseases such as hypercholesterolemia and hypertension. Research conducted by Slamet and Kanetro (2017) showed a decrease in triglyceride, total cholesterol and LDL cholesterol levels in hypercholesterolemic rats fed yogurt made from winged bean protein isolate. Vallabha *et al.* (2016) also examined the cholesterol-lowering effect of a soy protein diet based on its amino acid profile, especially the Arginine: Lysine (Arg: Lys) ratio in hypertension, atherosclerosis and hypercholesterolemia patients. They found the highest hypercholesterolemic prevention at a ratio of 8.7: 1 for Arg: Lys. In the form of seeds, several studies on nut germination also had a positive effect on the reduction of blood cholesterol levels, one of which was a study conducted by Asrullah *et al.* (2019) which showed that, total cholesterol and plasma MDA levels in mice fed a high-fat diet plus green bean sprouts and vitamin E were lower than mice fed normal high fat diet.

Jack bean (*Canavalia ensiformis*) is a plant that has a good protein content and potentials to grow in Indonesia. It contains the same protein content and amino acid profile as soybeans although it has higher lysine, histidine and phenylalanine (Solomon *et al.*, 2018) and lower arginine contents compared to soybeans (Leon *et al.*, 1989). Thus, Jack bean has potential hypo cholesterol effect and the ability to prevent the risk of cardiovascular diseases. According to Rahmawati *et al.* (2018) white jack bean (*Canavalia ensiformis*) flour could improve the blood lipid profile of hypercholesterolemic rats. Another study by Naufaliana *et al.* (2018) demonstrated the linearity of improving serum lipid profiles of hypercholesterolemic Sprague Dawley rats with increasing doses of jack bean extract. The potential of jack bean (*Canavalia ensiformis*) to prevent hypercholesterolemia underlies the need to study it role and ability with cow milk in reducing the risk of cardiovascular diseases when consumed. Therefore, this study determined the effect of adding cow milk to different levels of jack bean seed protein isolates and jack bean sprout protein isolates on their proximate composition and sensory preference. It also determined the amino acid and cholesterol reduction ability of the most preferred cow milk plus jack bean seed protein isolates and jack bean sprout protein isolates.

**Materials and methods**

**Cow’s milk, jack bean seed and reagents**

The cow milk used in this research was a commercial pasteurized cow milk, Greenfield brand. The Jack bean seed (*Canavalia ensiformis*) used in this study was white in color with a diameter of ± 13-14 mm. It was obtained from farmers in Kulonprogo, Yogyakarta, Indonesia.

The chemicals/reagents used in this study were HCl, NaOH/KOH, CuSO4, Na2SO4, H2SO4, KCl, KH2PO4, NaHCO3, NaCl, MgCl2(H2O)6, (NH4)2CO3, CaCl2(H2O)2 and distilled water obtained from Agricultural Product Processing Laboratory (PHP) Mercu Buana University Yogyakarta. Pancreatin and pepsin enzymes used for in vitro digestion tests were purchased from Xi'an Sheerherb Biological Technology Co., Ltd. Shanxi, China.

**Preparation of jack bean (*Canavalia ensiformis*) seed and sprout protein isolates**

The preparation of jack bean seed protein isolate and jack bean sprout protein isolate (referred to as jack bean seed or sprout protein isolates) followed the modification method of Kanetro and Setyowati (2013). Jack bean seeds (*Canavalia ensiformis*) were sorted, washed with running water until clean and soaked for 24 hours in water at a ratio of 1:3. After soaking, the water was drained and sow (germinated) for 0 (for seeds) and 72 hours (for sprouts) at room temperature and 100% relative humidity. The seeds and sprouts produced were dried using a cabinet dryer at 50 - 55oC for 12 hours. After drying, they were ground into flour and later dissolved in distilled water at a ratio of 1 (flour): 10 (distilled water). The mixture was then heated using a magnetic stirrer to 40oC and pH adjusted to 4.0 using 10% (w/v) potassium hydroxide and 20% citric acid solutions. After which, the mixture was filtered through a tofu filter cloth and the precipitate obtained was dried to obtain jack bean seed or sprout protein isolate powder.

The powdered protein isolate from the jack bean seeds and sprouts were then used at 3%, 6%, and 9% addition to cow milk (that is, 3% (T1), 6% (T2) and 9% (T3) jack bean seed protein isolates plus cow milk; and 3% (T4), 6% (T5) and 9% (T6) jack bean sprout protein isolates plus cow milk). Mixing and dissolving were done using Blender (Philips HR2116) at a speed of 1500 rpm for 5 minutes. Cow milk (T0) without the addition of jack bean seed or protein isolates served as the control. Cow milk and the mixed samples were then subjected to organoleptic and proximate tests. The most preferred sample from the organoleptic test was then analysed for amino acids and cholesterol. Cholesterol determination was done before and after in vitro digestion process.

**Sensory Evaluation**

The sensory evaluation adapted a modified method from the Indonesian National Standard No. 01-2346 (Badan Standardisasi Nasional, 2006). Sensory evaluation of cow milk, and cow milk plus jack bean seed protein isolate and jack bean sprouts protein isolate (T0-T6) was carried out to find out the most preferred because one limitation of adding plant protein to cow milk is aroma and taste acceptance. The sensory evaluation was carried out using the hedonic test from a scale of 1 - 9 according to the Indonesian National Standard guidelines for sensory testing (Badan Standardisasi Nasional, 2006). The test parameters were suitability for color, aroma, taste, and overall preference, while the number of panellists used was 17 trained panellists.

**Proximate test**

Proximate analyses which included moisture, protein, fat and ash contents were done according to the Indonesian National Standard (Badan Standardisasi Nasional, 2011). The samples (T0-T6) were subjected to proximate analyses at the laboratory of the University of Mercu Buana Yogyakarta. Carbohydrate was calculated using the following equation: Carbohydrate = 100 – moisture content – protein content – fat content – ash content.

**Amino acid test**

The amino acid profile test was done using the Waters method (Waters, 2012). The most preferred cow milk plus jack bean seed/sprout protein isolate was analysed for amino acid profile using the UPLC method. First, a standard concentration points of amino acids to serve as an internal standard was made. After obtaining this point, the sample weighing 0.1 - 1 g was put in a 20 mL headspace vial and hydrolyzed using 12N HCl solution. After that, the hydrolyzed sample was transferred into a 50 mL measuring flask and distilled water was added until the mark and homogenized. The homogenized solution was filtered using a 0.2 µm syringe filter. The resulting filtrate was then added to the standard solution for derivatization. The derivatized solution was injected into the UPLC system (UPLC-ESI-QTOF Waters Xevo G2-S QTOF) with a C18 column at a temperature of 49oC.

**Cholesterol analysis**

Cholesterol analysis was done for T0 (cow milk), T1 (cow milk plus 3% jack bean seed protein isolate) and T4 (cow milk plus 3% jack bean sprout protein isolate) using the Gas Chromatography method based on the ASEAN Manual of Nutrient Analysis (Puwastien *et al.*, 2011). Samples weighing 0.5 g were put in a 50 mL falcon tube. After which, 50% KOH solution and 4 mL of ethanol were added. It was then reflux in a water bath at 85 - 120oC for ± 60 minutes and cooled. 2.5 mL of distilled water and 5 mL of n-hexane was added to the cooled samples and vortex for 15 seconds until the solution separates. The organic layer was taken and transferred into a 30 ml test tube. The extraction was repeated 3 times with hexane. The solvent was then steam to dry with N2 gas at 40oC. The dry sample obtained was reconstituted using 3 mL dimethylformamide (DMF) solution. 0.2 mL of HMDZ and 0.1 mL of TMCS were added to 1 mL of the reconstituted solution, vortexed for 1 minute. After that, 1 mL of the standard internal solution (5α-cholestane 100 mg/L) was added and vortexed for 15 seconds. 10mL of distilled water was added and shook vigorously for 1 minute. The heptane layer formed was injected into a Gas Chromatography (GC ECD Perkin Elmer Clarus 680) with the capillary column type HP-5MS UI using nitrogen carrier gas at a constant flow rate of 2 mL/minute. The injection was performed using a 10: 1 split injection mode, with an injection volume of 1 µL at a temperature of 305oC. The oven temperature was set at a gradient of 40oC to 315oC. The detector used was a Parkin Elmer FID detector with a temperature of 305oC, a hydrogen flow rate of 45 mL/minute and air of 450 mL/minute.

**In vitro digestion testing**

The in vitro digestion test was adapted from Minekus et al., (2014) which consists of 3 distinct stages namely; oral, gastric, and intestinal. The oral stage is optional for liquid products so it was not done in this study. For the stomach stage, 10 ml of each samples (T0, T1 and T4) was mixed with 7.5 ml of SGF (Simulated Gastric Fluid) stock electrolyte solution (a solution of 6.9 mL KCl 0.5 mmol/L + 0.9 mL KH2PO4 0.5 mmol/L+ 12.5 mL NaHCO3 1 mmol/L + 11.8 mL NaCl 2 mmol/L+ 0.4 mL MgCl2(H2O)6 0.15 mmol/L + 0.5 mL (NH4)2CO3 0.5 mmol/L). After which water was added to achieve a final ratio of 50: 50 (v/v) for liquid sample and SGF. Following this, 1.6 mL of pepsin stock enzyme solution (25000 U/m) was made by mixing pepsin (3200 - 4500 U/mg) with SGF electrolyte solution. 5 µL of 0.3M CaCl2 and 0.2 mL of 1 M HCl were added to achieve a pH of 3.0 to mimic the pH of the digestive system. Finally, 0.695 µL of water was added and incubated for 2 hours at 37oC in a shaker bath. For the the intestinal stage, 20 ml of the gastric digestion mixture was mixed with 11 ml of the SIF (Simulated Intestinal Fluid) electrolyte stock solution. This was followed by the addition of 5 mL of 800 U/mL pancreatin solution which was made by mixing SIF electrolyte solution (a solution of 6.8 mL KCl 0.5 mmol/L + 0.8 mL KH2PO4 0.5 mmol/L+ 42.5 mL NaHCO3 1 mmol/L + 9.6 mL NaCl 2 mmol/L+ 1.1 mL MgCl2(H2O)6 0.15 mmol/L), and pancreatic enzyme based on trypsin activity. 2.5 mL of fresh bile (160 mM in fresh bile), 40 µL of 0.3M CaCl2, and 0.15 mL of 1M NaOH were added to attain a pH of 7.0. Thereafter, 1.31 mL of distilled water was added and incubated for 2 hours at 37 °C. The final sample from the digestion stage was then analyzed for cholesterol and fatty acid profiles.

**Statistic test**

Statistical analysis was done using SPSS v. 22.0 (SPSS Inc., Chicago, Illinois, USA) and significance was tested using the Duncan Multiple Range Test (DMRT) variant analysis method at the 95% confidence level (P ≤ 0.05).

**Results and discussion**

**Proximate composition**

The proximate composition of cow milk, cow milk plus various levels of jack bean seed protein isolate and jack bean sprout protein isolate (cow milk plus jack bean seed or sprout protein isolates) is shown in Table 1. The moisture content of the cow milk plus jack bean seed or sprout protein isolates showed a decreasing trend as the amount of jack bean seed or sprout protein isolates increased. The moisture content of the cow milk, cow milk plus various levels of jack bean seed or sprout protein isolates was lower than the moisture content of cow milk (89.2%) reported by the Indonesian National Standard (Badan Standardisasi Nasional, 2011). There was no significant difference (p >0.05) among the cow milk plus various levels of jack bean seed or sprout protein isolates. This could be due to the small differences (3, 6 and 9%) in the amount of jack bean seed protein isolates or jack bean sprout protein isolates used in this study.

The fat content of cow milk plus various levels of jack bean seed or sprout protein isolates showed an increasing trend with increasing amount of jack bean seed or sprout protein isolates. Generally, the fat content of cow milk plus jack bean sprout protein isolate were lower that of cow milk plus jack bean seed protein isolate. This could be due to the differences in the fat content of the raw materials (jack bean seed protein isolate versus jack bean sprout protein isolate) used. The fat content of jack bean sprout protein isolate is presumably lower than the jack bean seed protein isolates. According to Kanetro (2017) during germination there is lipid mobilization in the cotyledons as an energy source for growth. The lipid in the cotyledons is transported to the embryo where it is used as a source of energy for germination. For jack bean sprout protein isolate plus cow milk, there was no significant difference (p >0.05) in fat content between concentrations 3% and 6%, but a significant difference (p <0.05) occurred between the 3% and 9% concentrations. This condition suggests that a difference of 6% (addition of 6%) jack bean sprout protein isolate is needed to bring about a significant difference. Due to the reduction in the fat content of the 3% jack bean seed or sprout protein isolates plus cow milk as compared to the 6% and 9% jack bean seed or sprout protein isolates plus cow milk, they showed a potential hypo cholesterol effect in cow milk.

The protein content of cow milk plus various levels of jack bean seed or sprout protein isolates also showed an increasing trend with increasing amount of jack bean seed or sprout protein isolates. The protein content was highest (p<0.05) in the 9% jack bean sprout protein isolate plus cow milk (47.43%) and lowest (p<0.05) in the 3% jack bean seed protein isolate plus cow milk (35.49%). This is because the protein content of the jack bean sprout protein isolate is higher than that of jack bean seed protein isolate. Devi *et al.* (2015) reported a higher protein content for pea seed germinated for 24 hours at 25oC. This was attributed to the loss of weight as carbohydrate was being used as a source of energy during germination (Kanetro, 2017). In this study, the protein content of cow milk plus various levels of jack bean seed or sprout protein isolates was higher that the protein content of cow milk reported by the Indonesian National Standard (Badan Standardisasi Nasional, 2011). This was due to the high protein content in jack bean seed or sprout protein isolates used in this study.

Similarly, to the protein content, the ash content of the cow milk plus various levels of jack bean seed or sprout protein isolates showed an increasing trend with increasing amount of jack bean seed or sprout protein isolates. The ash content was highest (p<0.05) in the 9% jack bean sprout protein isolate plus cow milk (6.19%) and lowest (p<0.05) in the cow milk (4.57%). The ash content of the jack bean sprout protein isolate plus cow milk were generally higher than the jack bean seed protein isolate plus cow milk. According to Lorenz and D’Appolonia (1980) minerals increase during germination due to the discharge of complex compounds as energy source for respiration. The mineral contents of the cow milk plus various levels of jack bean seed or sprout protein isolates were significantly higher (p<0.05) that of the cow milk. This is due to the high mineral content in jack bean seed or sprout protein isolates as compared to cow milk.

The carbohydrate content of the cow milk plus various levels of jack bean seed or sprout protein isolates also showed an increasing trend with increasing amount of jack bean seed or sprout protein isolates. The carbohydrate content was highest (p<0.05) in 3% jack bean seed protein isolate plus cow milk (30.30%) and lowest (p<0.05) in 9% jack bean sprout protein isolate plus cow milk (17.30%). Generally, the carbohydrate content of cow milk was higher than cow milk plus various levels of jack bean seed or sprout protein isolates. This is due to the low carbohydrate content of jack bean seed or sprout protein isolates as compared to cow milk. Kanetro (2017) reported that carbohydrates are the first and major energy source for germination process.

**Sensory evaluation**

Sensory evaluation in this study used the hedonic test method which included the level of color preference, aroma, taste and overall preference based on a scale of 1 - 9 (higher numbers indicate the most liked). This hedonic test used 17 trained panelists (Badan Standardisasi Nasional, 2006). The color, aroma, taste and overall preference test scores for the cow milk, and cow milk plus various levels of jack bean seed or sprout protein isolates are presented in Table 2.

No significant differences (p >0.05) were observed in color among some of the samples examined. The 3%, 6% and 9% jack bean seed protein isolates plus cow milk did not differ significantly (p >0.05) from each other, but the 3% and 9% jack bean sprout protein isolates plus cow milk differed significantly (p <0.05). The 3% jack bean sprout protein isolate plus cow milk was significantly higher (p <0.05) than the 9% jack bean sprout protein isolate plus cow milk. There was a decreasing trend in color liking with increasing inclusion level of jack bean seed or sprout protein isolates. Color liking was highest (p <0.05) in the 3% jack bean sprout protein isolate plus cow milk (7.50) and lowest (p <0.05) in 9% jack bean sprout protein isolate plus cow milk (6.31). The white color of jack bean and the effect of peeling the skin after soaking contributed to the differences in color of the cow milk plus jack bean seed or sprout protein isolates. According to Gilang et al. (2013) soaking will remove the pigment in the jack bean skin.

Significant differences (p <0.05) were observed in aroma among some of the samples examined. The 3%, 6% and 9% jack bean seed protein isolates plus cow milk did not differ significantly (p >0.05) from each other, but the 3% and 9% jack bean sprout protein isolates plus cow milk differed significantly (p <0.05). The 3% jack bean sprout protein isolate plus cow milk was significantly higher (p <0.05) than the 9% jack bean sprout protein isolate plus cow milk. There was a decreasing trend in aroma liking with increasing inclusion level of jack bean seed or sprout protein isolates. Aroma liking was highest (p <0.05) in the 3% jack bean seed protein isolate plus cow milk (6.94) and lowest (p <0.05) in 9% jack bean sprout protein isolate plus cow milk (5.63). The unpleasant or beany smell of jack bean seed or sprout protein isolate affected the preference for aroma since aroma decreased with increasing level of jack bean seed or sprout protein isolates. Kanetro (2018) reported that, the unpleasant smell of seeds or sprouts is due to lipoxygenase activity in the bean.

Significant differences (p <0.05) were observed in taste among some of the samples examined. The 9% jack bean seed protein isolate plus cow milk, the 6% jack bean sprout protein isolate plus cow milk, and the 9% jack bean sprout protein isolate plus cow milk were significantly lower (p<0.05) than the rest of the treatments. Taste liking was highest (p <0.05) in the 3% jack bean seed protein isolate plus cow milk (6.94) and lowest (p <0.05) in 9% jack bean sprout protein isolate plus cow milk (4.50). There was a decreasing trend in taste liking with increasing inclusion level of jack bean seed or sprout protein isolates except 6% jack bean sprout protein isolate plus cow milk (4.44) which was numerically lower than the 3% jack bean sprout protein isolate plus cow milk (4.50). The differences in taste observed among some of the samples is due to the sandiness nature of starch which is thought to come from complex carbohydrates. Rahmawati *et al.* (2018) stated that, the extraction process involved in making protein isolates leads to the production of complex carbohydrates such as dietary fiber, starch and resistant starch.

The overall liking preference was based on the trained panelists' preference for all the preference attributes (taste, texture, appearance/color, and aroma). Similarly, to taste, texture, color and aroma, significant differences (p <0.05) were observed in overall liking among some of the samples examined. The 3% jack bean seed protein isolate plus cow milk (6.38), the 6% jack bean seed protein isolate plus cow milk (6.38), the 3% jack bean sprout protein isolate plus cow milk (7.06) and cow milk (7.00) were significantly higher (p<0.05) than the rest of the treatments. Overall liking was highest (p <0.05) in the 3% jack bean sprout protein isolate plus cow milk (7.06) and lowest (p <0.05) in 6% jack bean sprout protein isolate plus cow milk (5.00). The trend observed for overall all liking was similar to that of taste, therefore, the panelists’ preference for taste attribute is in line with their preference for overall liking. Based on the current results, the 3% jack bean seed protein isolate plus cow milk and 3% jack bean sprout protein isolate plus cow milk had better ratings for aroma, color, taste and overall liking, and worth considering for further analysis.

**Amino acid profile**

The amino acid profile of cow milk, 3% jack bean seed protein isolate plus cow milk and jack bean sprout protein isolate plus cow milk is shown in Table 3. The 3% jack bean seed protein isolate plus cow milk and 3% jack bean sprout protein isolate plus cow milk were chosen because they were most preferred in terms of aroma, color, taste and overall liking. Generally, the beany taste and sandy texture associated with plant protein were not noticeable in these samples.

From Table 3, the Glycine, L-Alanine and L-Serine contents of the 3% jack bean seed protein isolate plus cow milk and 3% jack bean sprout protein isolate plus cow milk were significantly higher (p <0.05) than the cow milk. The 3% jack bean sprout protein isolate plus cow milk were also significantly higher (p <0.05) than the 3% jack bean seed protein isolate plus cow milk in terms of their Glycine, L-Alanine and L-Serine contents. The values for Glycine were 0.56% (cow milk), 0.64% (3% jack bean seed protein isolate plus cow milk and 0.67% (3% jack bean sprout protein isolate plus cow milk). The L-Alanine values were 0.83% (cow milk), 0.87% (3% jack bean seed protein isolate plus cow milk) and 0.92% (3% jack bean sprout protein isolate plus cow milk). The L-Serine values were 1.73% (cow milk), 1.78% (3% jack bean seed protein isolate plus cow milk) and 1.84% (3% jack bean sprout protein isolate plus cow milk). Glycine is known to have a lowering effect on the risk of metabolic disorders that leads to conditions such as cardiovascular diseases, inflammatory diseases, cancers, diabetes and obesity (Le Douce *et al.*, 2020). Alanine and Serine play a major role in the prevention of neurological diseases such as Alzheimers, insomnia and Parkisons’ in humans (Chernoff *et al.*, 2017; Le Douce *et al.*, 2020). In general, the amino acid profile of 3% jack bean sprout protein isolate plus cow milk were higher than the 3% jack bean seed protein isolates plus cow milk. This is because the amino acid composition of jack bean sprout protein isolate is higher than that of jack bean seed protein isolate. Lorenz and D’Appolonia (1980) reported that germination causes an increase in enzyme activity resulting into protein modification and subsequently changes in amino acid composition and accretion.

The levels of L-Tyrosine and L-glutamic were significantly higher (p <0.05) in cow milk than the 3% jack bean seed protein isolate plus cow milk and 3% jack bean sprout protein isolate plus cow milk. The L-Tyrosine and L-glutamic levels of the 3% jack bean sprout protein isolate plus cow milk were also significantly higher (p <0.05) than the 3% jack bean seed protein isolates plus cow milk. Germination causes a high protease activity to change proteins become free amino acids (Kanetro, 2017).

Based on Table 3, cow milk with the addition of 3% jack bean sprouts protein isolate (0.40) had the highest Arginine and Lysine ratio compared to the Arginine and Lysine ratio of cow milk (0.38) and 3% jack bean seed protein isolate (0.38). The highest ratio of Arginine and Lysine levels in 3% jack bean sprouts protein isolate is expected to reduce cardiovascular disease risk. According to Bahadoran *et al.*, (2016), L-arginine from vegetable protein has the potential to reduce the risk of coronary heart disease by regulating blood pressure.

**Cholesterol levels before and after in vitro digestion**

The cholesterol level of cow milk, 3% jack bean seed protein isolate plus cow milk and 3% jack bean sprout protein isolate plus cow milk is shown in Table 4. Based on Table 4, the 3% jack bean seed protein isolate plus cow milk and 3% jack bean sprout protein isolate plus cow milk had significantly lower (p <0.05) cholesterol levels compared to the cow milk. Furthermore, the cholesterol level of the 3% jack bean sprout protein isolate plus cow milk was significantly lower (p <0.05) than that of the 3% jack bean sprout protein isolate plus cow milk.

This results show the effectiveness of reduction in cholesterol levels in animal protein due to isoflavone antioxidant compounds contained in jack bean seeds or sprouts. According to Yildiz (2006), genistein and isoflavones showed the highest anti-oxidative activity as antioxidants and had hypo cholesterolemia effects. Total cholesterol levels reduction by genistein and isoflavones from soybean seeds has also been studied by Carolyn *et al.* (2019) with result cholesterol reduction was influenced by protein, monounsaturated fatty acids, anthocyanins and isoflavones.

The highest cholesterol levels reduction (61.65%) after in vitro digestion was shown in cow milk with 3% addition jack bean sprouts protein isolate. This observation is due to the higher content of isoflavone aglycone compounds in jack bean sprouts compared to jack bean seeds. Sikder *et al.* (2014), found in their study that, aglycone and glycone (two kind of flavonoids) effectively reduced cholesterol in diets thereby effectively preventing epatotoxicity and inflammation. According to Yoshiaraa *et al.* (2018), the highest aglycone compounds was found in soybean germinated for 144 hours. The aglycone compounds were 2.24 mg/100g and 149.61 mg/100gr for soybean germinated for 0 and 144 hours, respectively. However, the levels of aglycone compounds decreased after 144 hours of germination.

The highest cholesterol reduction in the milk added with the jack bean sprouts protein isolate compared to the other two samples (cow milk and cow milk with 3% jack bean seed protein isolate) was also related to the high level of leucine in the sample, which was 2.80%. Leucine levels in the other two samples were 2.66% for cow milk and 2.56% for cow milk with 3% jack bean seed protein isolate. Coker *et al.* (2015) found that giving humans aged above 50 years supplementary diets high in leucine and phytosterol led to significant reduction in total cholesterol and triglycerides in blood plasma.

The presence of higher arginine in cow milk plus 3% jack bean sprout protein isolate (0.92%) compared to cow milk (0.85%) and cow milk plus 3% jack bean seed protein isolate (0.83%) is thought to contribute to the highest cholesterol reduction after in vitro digestion. This finding is in line with the research conducted by Børsheim et al. (2012) with result the provision diets containing extra 11g of essential amino acid and arginine twice a day to glucose tolerant elders aged 60-70 years resulted in a reduction of total cholesterol and very low-density cholesterol (VLDL) in blood plasma. Kanetro *et al.* (2019) showed that, addition of mung bean sprout (germinated for 12 hours) flour to composite flour increased the arginine level (2 times). They stated that germination can increase the levels of arginine in foods. In addition to L-Leucine and L-Arginine, other amino acids such as L-Glycine, L-Isoleucine and Lysine also played a role in reducing total cholesterol levels in cow milk plus 3% jack bean sprout protein isolate. The L-Glycine, L-Isoleucine and L-Lysine in cow milk plus 3% jack bean sprout protein isolate were 0.67%, 1.46% and 2.30%, respectively. These three amino acids were higher in cow milk plus 3% jack bean sprout protein isolate than cow milk and cow milk plus 3% jack bean seed protein isolate. Park *et al.* (1999) found that high-cholesterol rats fed taurine and glycine diets experienced 40% reduction in total cholesterol for rats on taurine diet and 27% reduction in total cholesterol for rats on glycine diet. Mice fed high-fat diet had significant amount of total cholesterol, but not on plasma lipid metabolites. O’rielly *et al.* (2020) studied mice that were fed a high-fat diet, and reported a significant effect on total cholesterol although it did not affect plasma lipid metabolites.

Based on the composition of amino acids in cow milk plus 3% jack bean sprout protein isolate and its capacity to reduce cholesterol levels, it can be said to be a potential milk-based functional drink that can reduce the risk of cardiovascular disease. Ma and Shieh (2006) stated that cholesterol and saturated fatty acids play an important role in human heart health, and high cholesterol levels can cause cardiovascular diseases such as heart attacks and strokes. Kanetro et al. (2019) who investigated the cholesterol and blood glucose profiles of volunteers aged 15-50 years who were given a composite flour diet mixed with mung bean sprouts flour, and reported a reduction in LDL cholesterol levels.

**Conclusions**

The most preferred diets were cow milk plus 3% jack bean seed protein isolate and cow milk plus 3% jack bean seed protein isolate. The addition of jack bean seed protein isolate and jack bean sprout protein isolate to cow milk caused an increase in amino acids such as Glycine, L-alanine and L-Serine; which can play a positive role in controlling the risk of human degenerative diseases such as cardiovascular and neurological diseases. Arginine and Lysine ratio was highest in cow milk plus 3% jack bean sprout protein isolate, followed by cow milk plus 3% jack bean seed protein isolate. The cholesterol levels of cow milk plus 3% jack bean sprout protein isolate were lower than the cow milk and cow milk plus 3% jack bean seed protein isolate, and this sample showed the highest reduction in cholesterol after in vitro digestion.

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**Table 1** Proximate composition of cow milk, cow milk plus jack bean seed or sprout protein isolates

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Percentage jack bean seed or sprout protein isolates on cow’s milk** | | **Moisture**  **(%)** | **Fat**  **(%)** | **Protein**  **(%)** | **Carbohydrate**  **(%)** | **Ash**  **(%)** |
| Concentration jack bean seed protein isolates | 3% | 87.90 ± 0.11 ab | 28.30 ± 0.53 bc | 35.49 ± 0.60 a | 30.30 ± 0.33 d | 5.91 ± 0.06 b |
| 6% | 87.85 ± 0.06 ab | 28.55 ± 0.00 bc | 41.96 ± 2.04 b | 23.48 ± 1.18 bc | 6.01 ± 0.12 bc |
| 9% | 87.80 ± 0.05 ab | 28.80 ± 0.64 c | 42.50 ± 1.66 b | 22.51 ± 2.10 b | 6.19 ± 0.06 d |
| Concentration jack bean sprout protein isolates | 3% | 87.89 ± 0.08 ab | 27.41 ± 0.70 a | 40.24 ± 2.24 b | 26.25 ± 1.69 c | 6.11 ± 0.00 cd |
| 6% | 87.83 ± 0.09 ab | 27.93 ± 0.12 ab | 41.22 ± 0.57 b | 24.41 ± 0.82 bc | 6.45 ± 0.06 e |
| 9% | 87.75 ± 0.23 a | 28.66 ± 0.69 c | 47.43 ± 2.64 c | 17.30 ± 2.15 a | 6.61 ± 0.12 f |
| Cow milk (0% jack bean seed or sprout protein isolates) | | 88.00 ± 0.03 b | 28.81 ± 0.34 c | 36.70 ± 2.47 a | 29.90 ± 2.97 d | 4.57 ± 0.09 a |

Note: numbers followed by the same letters in the same column are not significantly different at 95% confidence level and vice versa.

**Table 2** Preference test for cow milk, cow milk plus jack bean seed or sprout protein isolates

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Percentage jack bean seed or sprout protein isolates on cow’s milk** | | **Preference score \*** | | | |
| **Color** | **Aroma** | **Taste** | **Overall Liking** |
| Concentration jack bean seed protein isolates | 3% | 7.13 ± 1.45 ab | 6.94 ± 1.39 c | 6.94 ± 1.44 b | 6.38 ± 1.59 b |
| 6% | 6.69 ± 1.49 ab | 6.75 ± 1.65 bc | 6.19 ± 1.72 b | 6.38 ± 1.36 b |
| 9% | 6.44 ± 1.46 a | 6.13 ± 1.71 abc | 4.81 ± 1.38 a | 5.06 ± 1.24 a |
| Concentration jack bean sprout protein isolates | 3% | 7.50 ± 1.03 b | 6.75 ± 1.98 bc | 6.69 ± 1.49 b | 7.06 ± 1.34 b |
| 6% | 6.69 ± 1.74 ab | 5.81 ± 1.91 ab | 4.44 ± 1.86 a | 5.00 ± 1.79 a |
| 9% | 6.31 ± 1.89 a | 5.63 ± 1.93 a | 4.50 ± 1.90 a | 5.13 ± 1.96 a |
| Cow milk (0% jack bean seed or sprout protein isolates) | | 7.31 ± 0.95 b | 6.81 ± 0.75 c | 6.69 ± 1.14 b | 7.00 ± 1.21 b |

Note: numbers followed by the same letters in the same column are not significantly different at 95% confidence level and vice versa. \* Score scale 1: Most dislike, 2: Very dislike, 3: Dislike, 4: Somewhat dislike, 5: Neutral, 6: Somewhat like, 7: Like, 8: Very like and 9: Most like

**Table 3** Amino acid profile of cow milk, cow milk plus 3% jack bean seed and sprout protein isolates

|  |  |  |  |
| --- | --- | --- | --- |
| **Amino acids type** | **Amino Acid Content (%)** | | |
| **Cow milk** | **Cow milk plus 3% jack bean seed protein isolate** | **Cow milk plus 3% jack bean sprout protein isolate** |
| **Non-essential amino acid** | | | |
| L-Alanine | 0.83 ± 0.0009 a | 0.87± 0.0030 b | 0.92 ± 0.0012 c |
| L-Arginine \*\* | 0.85 ± 0.0006 b | 0.83 ± 0.0011 a | 0.92 ± 0.0033 c |
| L- Aspartic acid | 1.81 ± 0.0084 a | 1.82 ± 0.0067 a | 1.91 ± 0.0042 b |
| L-Glutamic acid | 5.32 ± 0.0072 c | 4.82 ± 0.0179 a | 5.15 ± 0.0136 b |
| Glycine \*\* | 0.56 ± 0.0003 a | 0.64 ± 0.0001 b | 0.67 ± 0.0007 c |
| L-Proline | 2.41 ± 0.0035 b | 2.21 ± 0.0081 a | 2.41 ± 0.0102 b |
| L-Serine | 1.73 ± 0.0007 a | 1.78 ± 0.0024 b | 1.84 ± 0.0061 c |
| L-Tyrosine | 1.13 ± 0.0007 c | 1.00 ± 0.0031 a | 1.10 ± 0.0024 b |
| **Essential amino acid** | | | |
| L-Histidine | 0.77 ± 0.0005 b | 0.73 ± 0.0002 a | 0.80 ± 0.0008 c |
| L-Isoleucine \* | 1.39 ± 0.0021 b | 1.35 ± 0.0072 a | 1.46 ± 0.0007 c |
| L-Leucine \* | 2.67 ± 0.0003 b | 2.56 ± 0.0057 a | 2.80 ± 0.0077 c |
| L-Lysine \* | 2.24 ± 0.0009 b | 2.17 ± 0.0070 a | 2.30 ± 0.0064 c |
| L-Phenylalanine | 1.36 ± 0.0047 b | 1.27 ± 0.0017 a | 1.39 ± 0.0051 c |
| L-Threonine | 1.42 ± 0.0008 a | 1.41 ± 0.0048 a | 1.51 ± 0.0059 b |
| L-Valine | 1.67 ± 0.0037 b | 1.61 ± 0.0042 a | 1.74 ± 0.0033 c |
| Ratio Arginine: Lysine | 0.38 | 0.38 | 0.40 |
| Protein (%) | 35.28 ± 0.14 a | 35.49 ± 0.60 a | 40.24 ± 2.24 b |

Note: numbers followed by the same letters in the same row are not significantly different at 95% confidence level and vice versa.

\* Amino acids associated with high circulating cholesterol levels

\*\* Amino acids associated with low cholesterol levels in the blood circulation

**Table 4** Cholesterol levels of cow milk, cow milk plus 3% jack bean seed and sprout protein isolates before and after in-vitro digestion

|  |  |  |  |
| --- | --- | --- | --- |
| **Cholesterol content**  **(mg/100mL)** | **Cow milk** | **Cow milk plus 3% jack bean seed protein isolate** | **Cow milk plus 3% jack bean sprout protein isolate** |
| Cholesterol before in vitro digestion | 118.71 ± 0.41 c | 102.09 ± 1.46 b | 94.14 ± 0.68 a |
| Cholesterol after in vitro digestion | 59.04 ± 0.41 c  (49.74%) | 52.13 ± 0.54 b  (51.06%) | 36.09 ± 0.26 a  (38.35%) |
| % reduction during in vitro digestion | 50.26% | 48.94% | 61.65% |

Note: numbers followed by the same letters in the same row are not significantly different at 95% confidence level and vice versa.

Values in the brackets was percentage of cholesterol after to before in vitro digestion