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Pre-Clinical Study of the High Protein Food Based on Denaturized Whey Protein

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ABSTRACT

One source of high protein food origin of animal protein is whey protein as a cheese processing waste. The addition of whey protein causes the product become tougher by using heat. Whey protein modification due to denaturation process can act as a structure enhancer by changes in solubility to result the brittle product. Whey protein used to increase biological value, sensory properties and the muscle mass also develop physical properties of food and to reduce the obesity, unfortunately the pre-clinical data remain unclear. This experiment performed the pre-clinical study of denaturized whey protein concentrate (WPC) by using male (*Sparague-Dawley*) for 21 concecutive days of denaturized WPC administration. The rats divided into five diet treatments (Standard Food (SF) + Semi Solid High Protein Food (SSHPF) with denaturized WPC 6.59%, 10%, 15%, 15% satellite and control. There was an increase in body weight after SSHPF treatment, and showed that body weight gain elevate significantly ($p<0.05$) among treatment groups. Final hematological parameters showed an increase in Hb (Hemoglobin), RBC (Red Blood Cell) and Hct (Hematocrit) levels, meanwhile tended to decrease in MCH (Mean corpuscular hemoglobin) and WBC (White blood cell) level but still within normal ranges. Hb and MCH levels were significantly different in the treatment group ($p <0.05$). But RBC, Hct, WBC levels showed no significant difference between the treatment groups ($p>0.05$). This result indicates that addition of denaturized WPC consist in SSHPF increase body weight, Hb, RBC and Hct and not induce the adverse effect after administration for 21 consecutive days.

Keywords: Denaturized whey protein; high protein food; hematology; rat; weight gain.

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INTRODUCTION

High protein food has been popular strategies in trained athlete to increase muscle mass and beneficial effects on Body Mass Index (BMI) in nutritional problem [1,2]. High protein food is a type of food with a high enough amino acid (AA) contents (both that naturally synthesized by the body or not). The criteria for high protein biological value generally determined by digestibility and composition of amino acids contained in a product [3]. Protein is categorized as the main nutrients required for growth and development, especially in children and the elderly [4]. Protein food intended for children contains standard protein 7,5% of total energy, while high protein food contains up to 15% of total energy [5,6].

Whey protein is a substance high protein that is rich in amino acids and contained in milk about 20%. Whey protein contains various types of amino acids such as isoleucine, leucine, arginine, valine, lysine and cysteine. In addition, whey is very easy to digest so it can increase the concentration of amino acids in plasma and also accelerate protein synthesis by body tissues and this good quality protein can trigger the synthesis of hemoglobin [4,7–9]. Whey protein also used to increase biological value, develop physical properties, texture or function of food, improve sensory properties, as a formulation of high-protein or low-lactose food products and the formation of structures in food products including emergency food [10,11].

The addition of whey protein commercialized (native) causes the product to become harder by a thermal process, where the higher protein content allow to the harder the resulting texture. However, whey protein that is engineered by the denaturation process can act as a structure enhancer because it causes solubility changes to be lower or not soluble in water so that the resulting product is more brittle [12]. Protein denaturized itself is a modification of the secondary, tertiary, and quaternary structures of protein molecules without breakdown of covalent bonds. Denaturized whey protein preparation follows Khairunnisa *et al.,* [12] and Siddiq [13] method by separated the sweet, whey protein fat, then conditioned to pH 4,6 the isoelectric point, heated by pasteurization at 90°C for 30 min, then separated by centrifugation at 5000 rpm, 15 min and finally dryed with spray dry into denaturized whey protein concentrate (WPC) powder.

Various forms of whey protein development as a food product has carried out, such as bakery, snack, sport drinks and even as an additional component in emergency food for children [12]. Intermediate Moisture Food (IMF) is a product that highly recommended as high protein food because it has a soft texture and moisture content 10-40% with a_w value around 0.60-0.85, makes microbes unable to thrive [14].

Denaturized WPC in high protein food product's formulation was used to obtain a products which which

easy to distribute, easy to consume, safe for consumption, has an acceptable taste, and has a complete nutritional content by utilized denaturized WPC as a protein source in 6,9%, 10% and 15% which is the optimal amount of high protein product's structure formulation in children [12,13] and maximum limit protein in high protein food products to avoids kidney damage [15], sweet potato flour as a source of carbohydrates and vitamins and minerals to complement the product's micronutrients to achieve growth rate in rats.

Several studies has been conducted to examine the effect of protein type using rats and human trial. Wróblewska *et al.,* [16] examined the effect of commercial whey protein and soy based proteins designed for athletes. The results of the study reported that rats fed casein (10.21% protein) and whey protein (10.84% protein) had 2 times more lean body mass than soy protein (10.45% protein). A study by Masarwi *et al.,* [17] shows that rats fed back with casein and whey had a higher epiphysal growth plate (EGP) than the normal diet. Whey was reported to produce slower bone growth, resulting in lower body weight gain than casein. WPC administration by Bahwere *et al.,* [18] and Stobaugh *et al.,* [19] showed WPC diets increase body weight by 2-3 g/day in malnourished children. Even, hematology hasn't show significant difference in infant and infant rats, it's demonstrated that whey diet maintain clinical sign of body [20,21]. Many studies have reported that using commercial whey protein but still a few that use modified whey protein.

Therefore, it's interesting and vital to investigate the quality of semi-solid high protein food products using denaturized WPC as nutritional supplements. This research was conducted on rats (*Sparague Dawley)* to know the product's physiological and to investigate the safety efficacy before its applied to human consumption through food intervention program.

MATERIAL AND METHODS

Material, whey protein denatured, and semi-solid high protein food production

Sweet whey protein purchased at local dairy industry taken from KPBS Pangalengan, West Java, Indonesia. Sweet whey then separated with a cream separator, conditioned to isoelectric pH (pH 4.6), pasteurization at 90°C for 30 m, then separated by centrifugation at 5000 rpm, 15 m and dried on spray dying with T inlet 180°C and T outlet 80°C to a fine WPC powder. High protein food production by mixing denaturized WPC powder, sweet potato flour, mineral mix, B-Complex vitamin, sugar, vegetable oil, and water mixed and then followed by 12D (12 cycle) thermal process packaged with metalized [12,13]. The denaturized WPC was then analyzed for amino acid determination and the result is shown in this following table.

Table 1: Amino Acid Profil of Denaturated Whey Protein Concentrate

Amino Acid	Component Level (mg/kg)
L-Metionine	7322.50
L-Histidine	7728.03
L-Threonine	25232.11
L-Proline	17422.75
L-Tyrosine	11482.50
L-Leucine	38503.26
L-Aspartate Acid	42338.80
L-Lysin	42407.97
Glysine	9123.68
L-Arginin	11347.52
L-Alanine	20520.21
L-Valine	23434.69
L-Isoleusine	22032.48
L-Phenylalanine	14604.84
L-Glutamate Acid	65940.46
L-Serine	20620.28

Animal trial and ethics statement

4-week-old health male *Sprague Dawley* rats were randomly distributed into five experimental groups (125-128 g, five rats each group) housed in animal cages Pharmacology and Therapy Laboratory, Faculty of Medicine, Universitas Padjadjaran, Indonesia for 21-d long study. Rats were acclimatized for a week with free access to water and food (*ad libtum*) and kept under controlled conditions (12 h-12h light-dark cycle; temperature: 22 ±3°C), bed cleaned 2-times a week [22]. In the end, 21-d study, all animals were fasted overnight and sacrificed under anesthesia with ketamine-xylazine [23]. The organs such as liver, kidney, lymph, intestine, and stomach were quickly removed, weighed, and preserved used Buffer Neutral Formalin (BNF) 10% for further analysis [22]. Before sacrificed, diet rats in the satellite group without treatment (SSHPF) for a week to see the effect. All animal and procedure experiments were submitted to the Research Ethics Commission at Universitas Padjadjaran Faculty of Medicine (approval number Reg: 0819091293, October 2019, which can be accessed at http://kepk.fk.unpad.ac.id) Bandung, West Java, Indonesia.

Animal diet treatment

The animal diet conducted in this study was equivalent with children diet, age 5-9 y.o were 200 grams, which diet

20-30% of calories a day [24] following the recommendation of Institute of Medicine [15,25] and nutritional requirement value [26]. Dose conversion for rats was 0.018, then treatment diet consumed by rats is 200 grams x 0.018 = 3.6 grams. After adaption period the rats were randomized into five groups control (A1); SFC + SSHPF with WPC 6.59% (A2); SFC + SSHPF with WPC 10% (A3); SFC + SSHPF with WPC 15% (A4) and SFC + SSHPF with 15% WPC (satellite) (A5) based on previous research [12,13].

Body weight measurement

Body weight (BW) was measured every 24 hours since the day before treatment until 21 days of treatment. Weight gain was calculated based on the final BW minus the weight of the previous weighing and then divided by the number of days. BW graphics expressed in average in weekly changes.

Blood sampling and blood analysis

Blood samples were collected before treatment, and the end of the study used microhematocrit after one-night fasting. A 0,5 mL blood was collected in EDTA (Ethylenediaminetetraacetic acid) tube for hematology analysis (Hb, RBC, Hct, MCH and WBC). Blood samples tested with Abaxis (VETSCAN® HM5 Hematology Analyzer) in Chemical Laboratory of Educational Animal Hospital Universitas Padjadjaran, Indonesia. A 1,5 ml blood collected in microtube for serum. Data calculation and hematology graphic expressed in final value result with baseline value information in a table.

Statistical analysis

Results were expressed as average ± Standard Error Mean (SEM) and tested for significance by One-Way-Anova at p< 0,05, and further test carried out used Tukey test.

RESULT

The comparison of BW increasing before and after treatment with standard food consumption in this study showed in Table 2.

Table 2: Growth and food consumption of rats

Variable	A1	A2	A3	A4	A5
Baseline BW (g)	128,2 ± 2,48	126,3 ± 2,25	126,8 ± 3,47	126,5 ± 2,18	127,5 ± 2,10
Final BW (g)	197,0 ± 4,54	213,3 ± 2,25	214,5 ± 6,86	239,8 ± 8,33	223,3 ± 11,39
SFC (g)	21 ± 0,70	18 ± 1,22	18 ± 0,99	19 ± 0,61	22 ± 0,60

Values are Average ± SEM, n=5. BW, body weight; SFC, standard food consumption

Table 2. shows the initial BW 126,3 -128,2 g to 197.0 - 239.7 g. Standard food consumption for 21-d study resulted a change significantly between treatment groups. Body weight of all rats elevated every days, mainly on treatment groups (A2, A3, A4 and A5) (Figure 1). In Figure 3. 21 d total weight gain of rats in the A1 test group; A2; A3; A4 and A5 are respectively; 68,8; 87; 87,75, 113,25 and 95,75 g.

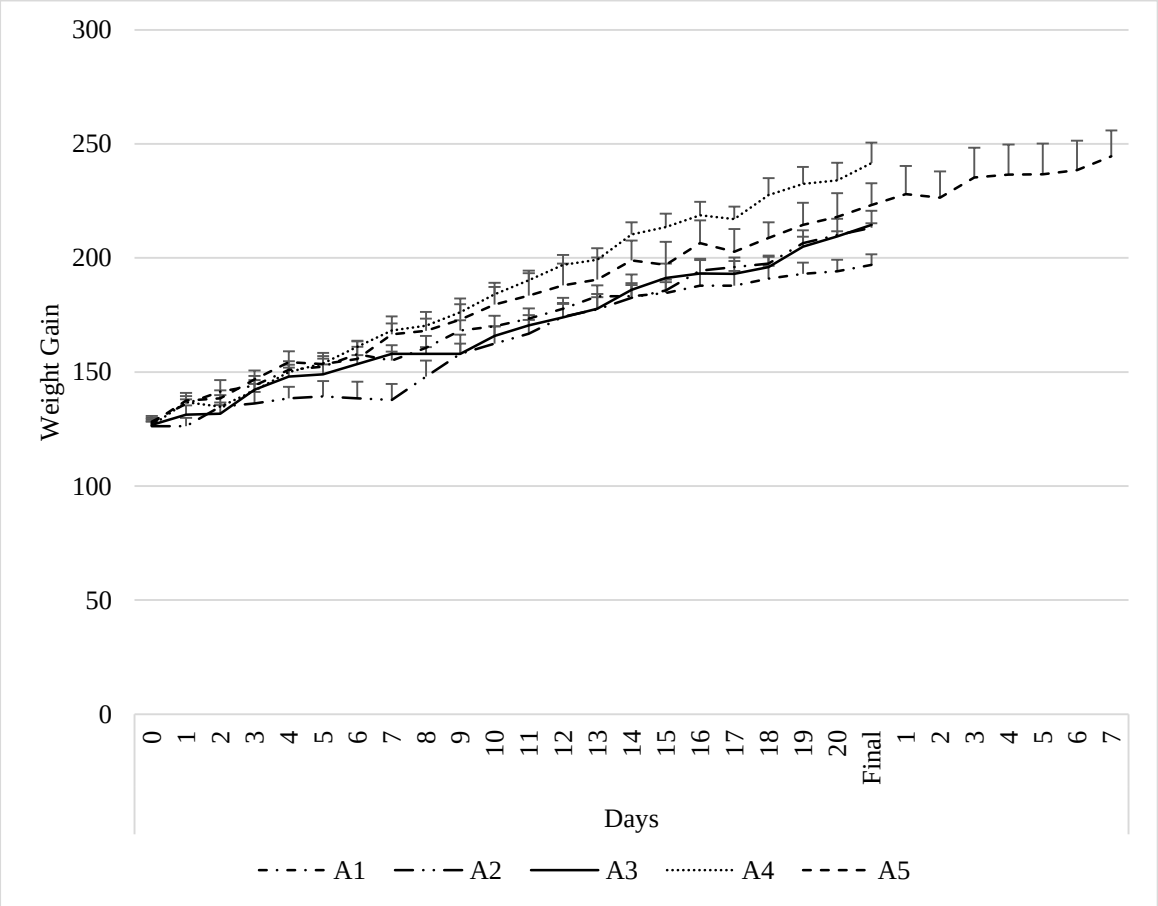

Figure 1: Average of body weight during treatment A1 (control); A2 (SFC+SSHPF with WPC 6,59 %); A3 (SFC+SSHPF with WPC 10 %); A4 (SFC+SSHPF WPC 15% %); and A5 (satellite with WPC 15%). Data are expressed as average ± SEM

The results of ANOVA analysis of body weight showed that significant difference ($p < 0.05$) between treatments group in first and third week observations, but not significant different ($p > 0.05$) compared to the second week of observation (Figure 2.).

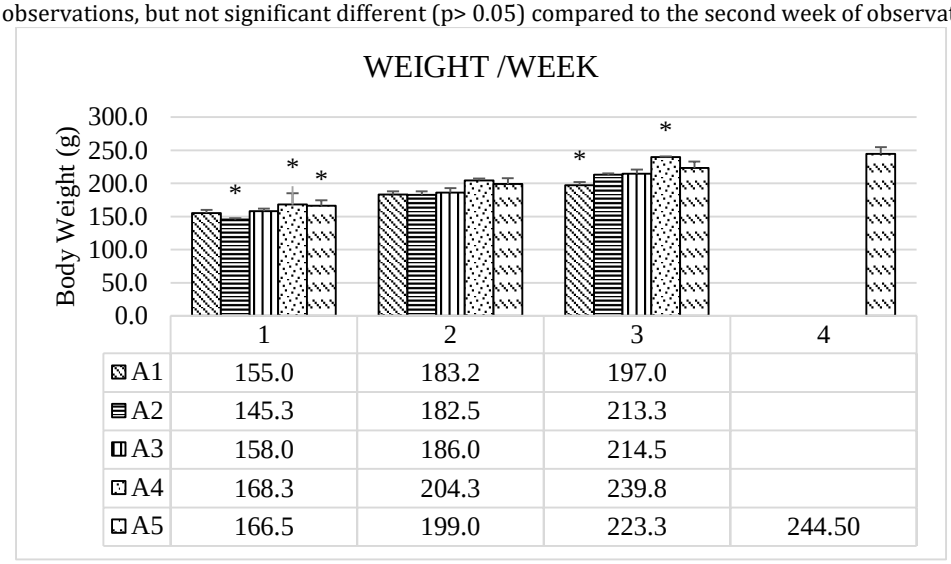

Figure 2: Body weight gain of treatment A2 (SFC+SSHPF with WPC 6,59 %); A3 (SFC+SSHPF with WPC 10 %); A4 (SFC+SSHPF WPC 15% %); A5 (satellite with WPC 15%) and A1 (control) in weeks. Data are expressed as average ± SEM. Means with a * denote significant

At the first week observation, the BW of A2 treatment (6,59% protein concentration) was significantly different from A4 and A5 (15% protein concentration and satellite treatment, respectively), but not significant compared with A3 (10% protein concentration) and A1 (control). At the third week observation, A4 treatment group was significant increase compared to A1, but not significant against A2, A3 and A5 treatment groups (Figure 2.).

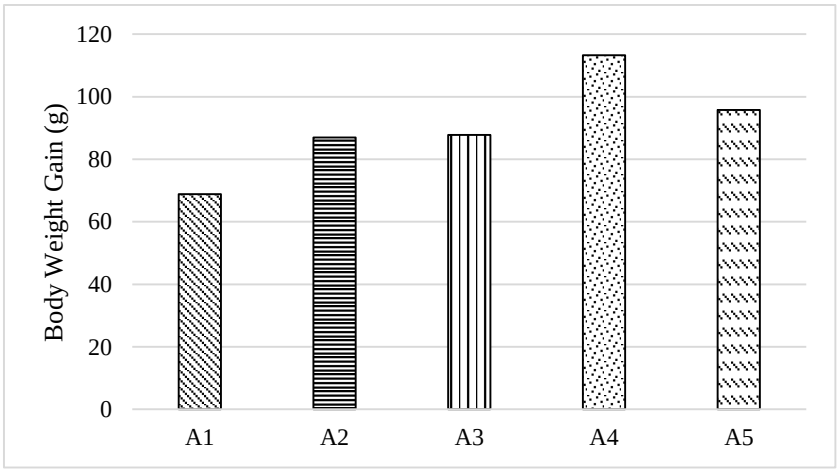

Figure 3: Rats weight gain for 21 (A1,A2,A3 and A4) and 28 (A5) concecutive days of denaturized WPC administration

Blood sample were collected and analyzed to investigate hematology data after treatment in this study and reported in Table 3. and Figure 4.

Table 3: Average Hematology Value of Rats

Group	Variabel				
	Hb (g/dL)	Hct (%)	MCH (pg)	RBC (10^{12}/L)	WBC (fl)
Baseline					
A1	14.5 ± 0.11	45.81 ± 0.79	19.47 ± 0.16	7.15 ± 0.19	10.89 ± 0.37
A2	14.4 ± 0.11	46.81 ± 0.60	19.77 ± 0.07	7.09 ± 0.14	14.01 ± 1.41
A3	15.0 ± 0.40	46.41 ± 1.96	19.00 ± 0.09	7.99 ± 0.26	8.73 ± 0.42
A4	14.9 ± 0.17	45.73 ± 0.34	19.50 ± 0.18	7.84 ± 0.09	11.09 ± 0.40
A5	15.2 ± 0.27	47.15 ± 0.16	19.57 ± 0.21	7.36 ± 0.12	8.75 ± 0.53
Final					
A1	14.7 ± 0.07	48.64 ± 0.38	18.70 ± 0.12	8.5 ± 0.23	9.10 ± 0.98
A2	14.8 ± 0.11	48.13 ± 0.72	18.90 ± 0.12	8.0 ± 0.09	10.44 ± 0.68
A3	15.1 ± 0.07	51.12 ± 0.38	18.10 ± 0.16	8.2 ± 0.15	7.87 ± 0.78
A4	16.1 ± 0.23	49.74 ± 0.87	17.93 ± 0.11	8.5 ± 0.17	10.78 ± 0.86
A5	15.6 ± 0.19	49.89 ± 0.51	18.73 ± 0.07	8.8 ± 0.16	8.13 ± 0.23

A1 (control); A2 (SFC+SSHPF with WPC 6,59 %); A3 (SFC+SSHPF with WPC 10 %); A4 (SFC+SSHPF WPC 15% %); A5 (satellite with WPC 15%). Values are Average ± SEM. Hb, hemoglobin; Hct, hematocrit; MCH, mean cospucular hemoglobin; RBC, red blood cell; WBC, white blood cell.

This study found that Hb levels of rat tended to increase as compared to pre-treatment (baseline) (Tabel 3.). Although Hb levels relatively increase, the results were still within normal limits (Table 3.), in range 13.6-17.7 g/dL [27]. The highest increase of Hb in A4 treatment group (Figure 4a.).

The result of ANOVA analysis showed that there were a differences between treatment groups ($p < 0.05$). Hb levels was significantly changed in the protein treatment group 10% (A4) to control group, protein treatment group 6.56%, and 10% (A1, A2, and A3) ($p < 0.05$) (Figure 4a.).

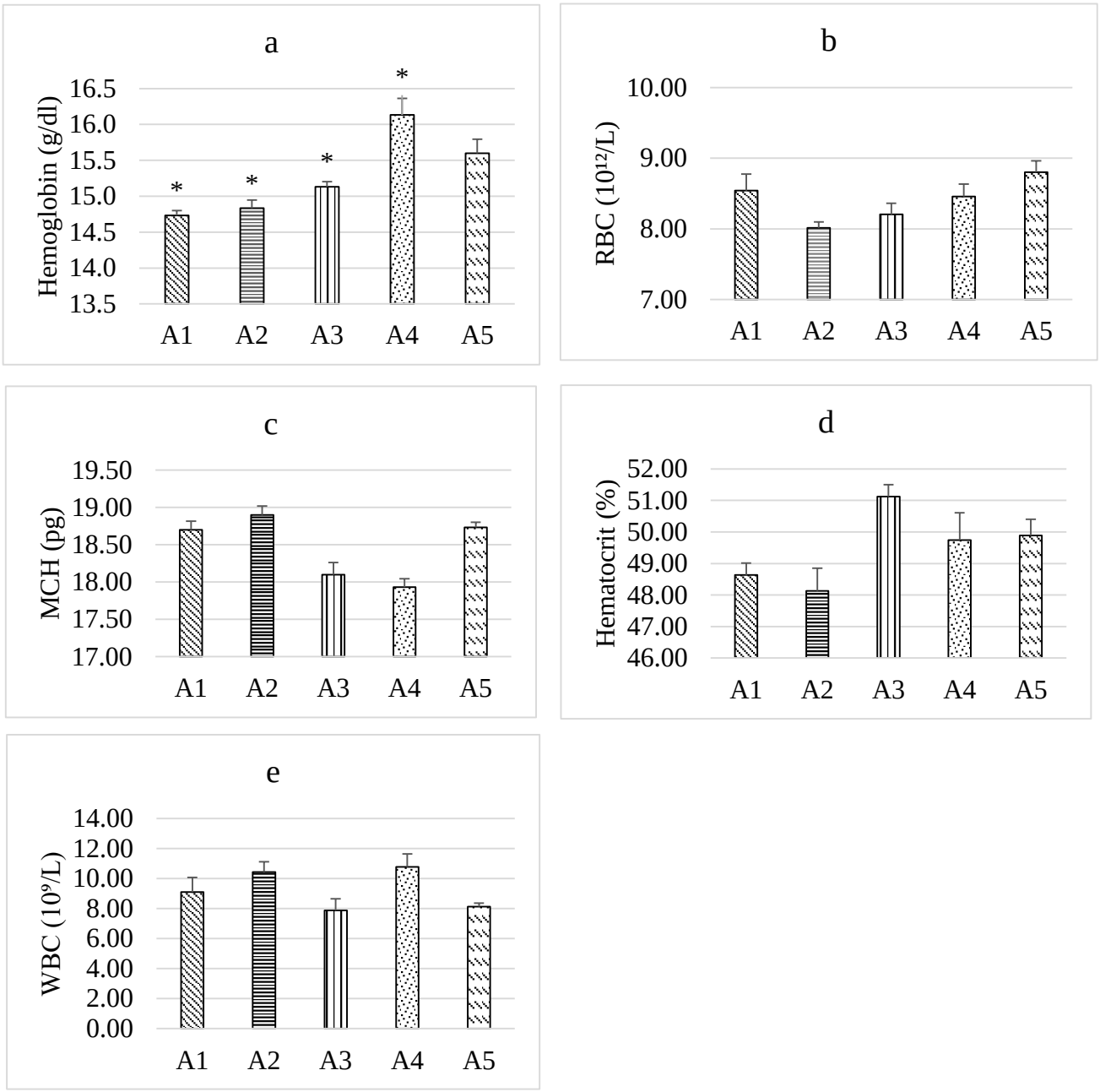

Figure 4: The effect of treatment group A2 (SSHPF with WPC 6,59 %); A3 (SSHPF with WPC 10 %); A4 (SSHPF WPC 15% %); A5 (satellite with WPC 15%) and A1 (control) on (A) Hemoglobin (B) RBC (C) MCH (D) Hct (E) WBC. Data are expressed as final value average ± SEM. Means with a * denote significant

RBC levels were found that A5 (satellite) had the highest level of RBC. Meanwhile, RBC levels of A2, A3, and A4 treatment groups were lower than the control (A1) (Figure 4b.), although lower, RBC levels of A2, A3, and A4 protein treatment group (Table 3.) are still in normal limits with a range around 6.7-9.0 10^{12}/L [27]. However, RBC levels of the analysis tended to increase compared to before treated (baseline) (Table 3.). The ANOVA analysis showed no significant difference between the treatment groups ($p > 0.05$).

Figure 4c. showed that the highest MCH level is in A2 group (protein 6.59%). MCH levels in A3 and A4 groups showed lower values than A1 (control). In this study, treatment of rats by SSHPF tended to showed a decrease in MCH levels compared to before treated and group A4 was the group with the highest decreased trend compared to the other groups (Table 3.). Based on these results, ANOVA analysis showed a significant difference between treatment groups ($p < 0.05$). Tukey's test for further test showed that MCH levels of A4 treatment group was significantly different ($p < 0.05$) from A1, A2, A3, and A5.

Hct levels showed all groups in normal levels, although group A2 was lower. According to ANOVA analysis, Hct levels did not showed significant difference in the

treatment group ($p < 0.05$) (Figure 4d.). Hct level in A1-A5 group tended to elevated compared to before treated, although A2 lower in the final result but all value within normal range 38-52% (Table 3.) [27].

Based on Figure 4e. it was found that A4 treatment group had the highest levels, then A3 and A5 groups had lower levels than A1 (control). ANOVA analysis results showed that there were no differences between groups ($p > 0.05$) in WBC levels (Figure 4e.). WBC levels tended to decrease compared to pre-treatment levels, but this reduction was still around normal range (baseline) (Table 3.).

DISCUSSION

Effect of SSHPF on body weight

The use of various concentrations protein in SSHPF products is intended to investigate the effect of protein intake on enhancing rats weight. Protein influences the growth process by increasing the body weight of rats. Following by Harianti [24] and Frisilia, Adi & Ismawati [28] that amount of food intake and nutritional content in foods such as protein greatly affect the growth process in individual. In this study, all test groups experienced an increase of body weight, especially in protein treatment group where the body weight greater as compared to control group (A1) (Table 1.). All groups of rats received same type of feed and amount of weight food, but there were differences in protein concentration to each group of rats. Average consumption of standard feed increased every day with more feed consumed in the control (A1) and satellite (A5) groups (Table 1.) and after treatment ended, standard feed consumption in the satellite group did not show any decrease. The increase in feed consumption is possible because the older rats, the needs for food intake will also increase [29].

Lower standard feed consumption in A2, A3 and A4 groups than the control group indicates that there is an effect of treatment in amount of standard feed eaten. Arrivals of SSHPF products in the stomach and digested in the gut, small peptides and AA (phenylalanine, histidine, alanine, serine, proline, glutamic acid, arginine and lysine) from denaturized WPC act as signaling molecules in enteroendocrine cells, secreted satiety hormones such as glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), hormones and Peptide tyrosine tyrosine (PYY). This hormone reduces food intake by increase post-meal satiety and inhibit gastric emptying [۲۰,۲۱]. Previous study demonstrated that whey protein consumption suppressed hunger initiates at weeks 1-3 [32] and increased plasma concentration of PYY and reduced food intake compared to control group [33].

Most of rats in this study has a weight gain with various number of increments, the higher weight gain occurs on treatment groups (A2, A3, A4 and A5) in 4 to 6 days (Figure.1). According to Kong *et al.* [34] The IECs (layering cell in the luminal surface of intestinal epithelium), which is function as nutrients absorption zone, regenerates every 4-5 days. Therefore, it can be assumed that during regeneration the new IECs layer, absorption of nutrition is higher than on the IECs layer aged 3-5 days after regeneration. Regeneration of IECs were carried out by stem cells in crypts on base of the intestinal gland, furthermore the mature of IECs layer will undergo apoptosis and discharged into intestinal lumen 34.

Our data show that all of treatment group experienced an increase in body weight, where the higher concentration of denaturized WPC, the greater body weight gain (15%) (Figure 3.). Meanwhile, satellite group (A5) supposed showed highest increase in body weight same with A4 group (SSHPF WPC 15%) (Figure. 3). This result was occurred because of age factor of rats in A5 group in fourth week of treatment (8 weeks age) has entered sexual of adult phase so that the growth rate begins to decrease and accumulated increase in body weight becomes smaller. Rats that are still in their growth phase can carry out rapid biosynthesis, so they produce a lot of energy. Energy has an important role in the body's metabolic processes, it's meant more energy produced, induce the easier metabolic process. Good metabolism greatly affects the body weight of an individual during its growth stage. So it can be concluded that the A5 group of rats has entered the adult phase and affect increase in body weight which is not too high [28,35]. Although the body weight gain of A5 group was not higher as compared to A4 group, overall there were body weight increased in each week and quite high.

Adequate dietary intake from SSHPF product consumption with standard feed gave effect of rat body weight increased and did not affect decreased of appetite after treatment. The results of body weight increased in treatment group of this study was supported by Teixeira *et al.,* [36] showed that weight gain of rats in casein control group was 3.47 ± 0.24 g/day, 2.75 ± 0.17 g/day and whey group was WS 3.81 ± 0.36 g/day and WE 3.57 ± 0.4 g/day. Another study showed that giving AIN-93M diet contain whey protein to Fischer male rats increased the body weight of whey rat group greater (WS 313 ± 15 g, WE 308 ± 20 g) compared to control group (CS 302 ± 13 g, CE 264 ± 10 g) [37].

The study, which was conducted by Teixeira, showed greater increasing body weight than our study. It explained that the form of whey protein used by Teixeira was whey protein isolate (WPI) powder, where WPI had a higher protein content than our study. Generally, WPI was made from 90% protein with less or no lactose and fat, whereas maximum protein content on WPC powder in our study was 15% with modest amount of fats, lactose, and minerals [38,39]. The high concentration of this protein is in line with the increasement in AA absorption in the body and even higher in body weight. In (Figure 1.) also showed a higher body weight increasement in line with increasing whey concentration were used. Hayes and Cribb showed the concentration of whey combined in supplements is an important factor in the formation of muscle protein. Whey protein, according to Hayes and Cribb, is categorized based on the concentration of whey contained in the product. Whey protein isolate (WPI) with a concentration of 90% protein and whey protein concentrate (WPC) with a concentration of 80% protein [40]**.** This indicated that consumption of whey protein (concentrate or isolate) can improve body composition (proportion of fat and non fat mass in the body).

Protein plays role as main structural component in muscle building and tissue [41]. Protein content in SSHPF product which is denaturized WPC has an amino acid profile that is very similar to muscle protein Branced-chain amino acid (BCAA) (Table 1.), to increase protein synthesis and stimulate muscle protein synthesis. Protein in whey that we consumed was digested by digesting enzymes into polypeptides and broken down into shorter peptides (tripeptides, dipeptides and some free amino acids) in intestine by pancreatic enzymes and mainly absorbed by Intestinal epithelial cells (IECs) in the small intestinal mucosa [34]. This absorbed amino acid then enters portal vein blood and carried to liver, and some of it carried to tissue cells for metabolism and protein syntesis [42]. Amino acid leucine in denaturized WPC acted directly as stimulator for protein synthesis by activated mTOR signaling pathway and activated several intracellular

signals that activated S6K1 and 4E-BP1 (Eukaryotic translation initiation factor 4E-binding protein 1) ribosomal proteins to trigger protein synthesis translation, so that muscle protein synthesis will increase and increase lean body mass (muscle) [43–47]. Other amino acids such as alanine, proline, glutamine, arginine and lysine work to stimulate growth hormone, which is an anabolic hormone to promote growth [9,48]. By the role of amino acid that work together in the body, growth in rats occurs.

In the A5 treatment group (satellite), further observation was carried out with only standard feed intake (without treatment) in the fourth week, to find out whether SSHPF products have an addictive effect which, if the intake is stopped, and caused significant weight loss. We found that A5 (satellite) group still has an increase in body weight in the fourth week compared to third week. The increment showed from three to fourth week was 21.2 g, where the increase was not higher than when given SSHPF products. Lower increase body weight may affect because nutritional content of SSHPF products is better than standard feed, so the effect on increasing body weight is not as good as when given SSHPF products.

This showed that SSHPF denaturized WPC product does not have an uncontrolled additive effect. It explained by AA Cysteine, as food addictive amino acid in whey protein has rearrangement tertiary structure through denaturation process [49], combining heat temperature (90°C) and low pH (4,6), as results the free thiol group in cysteine becomes accessible and allowing to react with disulfide bonds that present in β-laktoglobulin (β-Lg) or α-laktalbumin (α-La) formed disulfide bond reaction (SH-SS), which this SH-SS reaction also prevents protein binding with other components that can cause addictive reactions, and shaped an whey protein aggregates that can be used as texturizer [50–52]. Although the heat and low pH mechanisms are not completely 100% denaturing but in this process, the SH-SS bonding process has occurred to minimize the occurrence of this process during the product-making process and even lower when consumed by humans. So when denaturized WPC product consumed, the uncontrolled additive effect has been minimized or even none. In other words, SSHPF denaturized WPC product didn't showed uncontrolled effect on growth in rats even after not being treated.

Hematological effect of SSHPF treatment

Consumption of animal protein such as whey protein stimulates increase of blood cells formation, namely erythrocytes formation (RBC) where 95% of RBC contains Hb which acts as oxygen carrier to tissues [53]. This explains that hemoglobin and RBC levels are closely related to each other or in other words, RBC levels can be determined by measuring Hb in serum or blood plasma through visual inspection, hemolytic index or microfluid analysis [54,55]. Therefore, Hb is a conjugated protein in RBC which is synthesized through globin formation (protein) and synthesis of heme (iron where globin is a functional protein in the form of polypeptide chain that forms Hb) [56]. We found that RBCs and Hb levels increased, and greater in SSHPF 15% denaturized WPC (A4 and A5). Denaturized WPC in SSHPF contains high branched-chain amino acid protein -BCAA and other AA which can trigger good amino acids come in the body so that it can increase globin synthesis rate. The import of good amino acids in the body can trigger an increase in Hb and RBC levels. AA Arginine and Leucine were best activity type of AA compared to other types in regulated mTORC1 signaling in Hb and RBC formation. The formation process was knowned as erythropoiesis. Erythropoiesis process began with mTORC1 signaling in metabolism, which is assisted by amino acids that enter the body, then new Hb and RBC are formed from the results of this process. SSHPF whey protein can increase Hb and RBC levels in rats [57,58].

Therefore, we can assumed that SSHPF denaturized WPC beneficial to human body, because is less likely to cause anemia. Low of RBCs number and lack of Hb levels in the body can triggers anemia [59–61]. Anemia is a condition when RBC low cause formation of Hb reduced [62]. Lack of Hb levels in the blood cause symptoms of fatigue (blood difficult in binding oxygen as Hb functions an an oxygen carrier low), reduces endurance (immunity mechanism affected by RBCs production within bone marrow reduced) result in infection, affected cognitive development and these are associated with increased of morbidity [63–65]. Actually anemia is a sign of a disease process rather that disease itself, and it was sign of malnutrition clinically [65]. So RBCs and Hb values it's really important to maintain our body health.

However, we also found that RBC levels had slight decrease in A2, A3, and A4 groups compared to control, this may occur due to regulatory mechanisms controlling marker pathways, iron utilization and complicated red blood cell production, which involves hormones such as erythropoietin (EPO), which is main regulatory glycoprotein hormone that regulated proliferation, differentiation, and cell maturation of red blood cells in individuals [66,67]. Another consideration is that parameters of subject before treatment were in normal range and effects of dietary supplementation [68]. Results of this study were same as Mehta *et al.,* [69] and Naclerio *et al.,* [68] which also showed a slight decrease in RBC levels after whey protein treatment (Pre 5 10^6/mm^3, post 4.9 10^6/mm^3) with RBC levels in still normal range.

Percentage of Hct obtained by measuring the volume of the RBC compared to the total volume of blood (red blood cells and plasma) [70]. Hct measurement can also be used to indicate blood disorders, where low hematocrit levels indicate anemia [71]. Based on the results of this study, Hct level has increased along with the increase in RBC, and the increase in value is still categorized as normal. A high Hct value can indicate dehydration and polycythemia (the state of too many red blood cells in the blood) [70,72]. This result is different from the research conducted by Mehta *et al.,* [69] who showed a reduction in hematocrit in whey protein supplement group.

SSHPF diet in rats, as previously described, was able to have an effect on increasing Hb and RBC levels. MCH value obtained from average hemoglobin level per number of red blood cells, so that protein intake automatically affects the MHC value [73]. Meanwhile our study found that MCH levels tended to decrease, even though they decreased, MCH levels of the test results was still in normal value range, 17.9-21.20 pg [27,74]. MCH level showed amounts of total hemoglobin weight in red blood, therefore the decrease in MCH value after treatment can also be due to the smaller size of erythrocytes in the body after treatment [75]. Due to the influence of hemoglobin values, red blood cell counts, and MCV, MCH and MCHC values also become abnormal because these indices are calculated and not directly measured [73]. Although in normal value, a slight decrease in MCH levels also occurred in the control and satellite groups, overall SSHPF product treatment has not a negative effect.

WBC in the body plays a role as the immune system, or it said protect body against infection [48]. Medically an increase WBC count within normal indicates as

Leukocytosis, an infection or inflammation respond in our body [76,77]. WBC levels in this study showed a decreased trend. Denaturized WPC as protein source in SSHPF did not cause an increase in WBC levels, so we assume that no infection or inflammation occurs along this study (Table 3, Figure. 4e). Lower value in WBC may affected by AA contain in denaturized WPC such as Lysine excessed than needed which can affect intracellular Arginin depletion and caused the number of leukocytes (WBC) decreased [78]. Thus, fasted condition before blood sampling can affect arginine plasma concentration decreased, overexpressed of arginase in small intestine and stress hormone [48,79]. Some studies have shown same result in no effect of whey and amino acid supplementation on WBC [78,80]. The effects of whey protein are quite beneficial for the blood system's health of animals and humans. Several previous studies have successfully demonstrated the effects of various form of whey protein (isolate or other whey protein powder) on improving the function of blood system and reducing the risk of hypertension, lipid profile, and metabolic syndrome [8,38,39]. Although the protein content in WPI is higher when compared to WPC, this study has managed to show an equal good effect on body health when it consumed [38].

Overall, greater body weight gain and the conditions of the blood value in treatment rats showed a good health and did not show any signs of anemia (Hb, RBC and Hct value increase) or lean body weight, which is an indicator of malnutrition. Besides that, the immune system showed by WBC value hasn't increased. So that denaturized WPC may be promising candidate to improves and maintains the nutritional status in children ages of 5-9 y.o.

CONCLUSION

The addition of whey protein to SSHPF enhance the growth phase, it appears that SSHPF treatment can increase the body weight of rats compared to controls, where protein intake (whey protein) with a higher concentration (15%) A4 and A5 (satellite) has the highest body weight increased that other treatment. Hematological results of Hb, Hct, and RBC showed normal values and even tended to increase during treatment, where these conditions illustrate that the body's transport system can function properly through metabolism of whey protein. Although the levels of MCH and WBC tended to decreased, they were still within normal ranges. Satellite group (A5) also did not showed irregular result, so that providing SSHPF products does not have an uncontrolled effect on nutritional status by maintaining and reduce risk of decreased nutritional status. And this study gives the new evidence regarding preclinical data, the whey protein in SSHPF is safe and useful to gain the body weigh in growth phase and no adverse effect induce by denatured WPC consumption for 21 days.

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REFERENCES

1. Acheson K. Higher-protein diets for health ? Eur J Clin Nutr [Internet]. 2012;66:763–4. Available from: https://www.nature.com/articles/ejcn201246
2. Antonio J, Ellerbroek A, Silver T, Vargas L, Tamayo A, Buehn R, et al. A High Protein Diet Has No Harmful Effects : A One-Year Crossover Study in Resistance-Trained Males. J Nutr Metab [Internet]. 2016;1–5. Available from: https://www.hindawi.com/journals/jnme/2016/9104792/
3. Weindl I, Ost M, Wiedmer P, Schreiner M, Neugart S, Klopsch R, et al. Sustainable food protein supply reconciling human and ecosystem health: A Leibniz Position. Glob Food Sec [Internet]. 2020 Jun;25(March):100367. Available from: https://linkinghub.elsevier.com/retrieve/pii/S2211912420300201
4. Camargo L da R, Doneda D, Oliveira VR. Whey protein ingestion in elderly diet and the association with physical, performance and clinical outcomes. Exp Gerontol [Internet]. 2020 Aug;137(March):110936. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0531556520301571
5. Kabir I, Rahman MM, Haider R, Mazumder RN, Khaled MA, Mahalanabis D. Increased Height Gain of Children Fed a High-Protein Diet during Convalescence from Shigellosis: A Six-Month Follow-Up Study. J Nutr [Internet]. 1998 Oct 1;128(10):1688–91. Available from: https://academic.oup.com/jn/article/128/10/1688/4723068
6. Koletzko B, Demmelmair H, Grote V, Prell C, Weber M. High protein intake in young children and increased weight gain and obesity risk. Am J Clin Nutr [Internet]. 2016 Feb 1;103(2):303–4. Available from: https://doi.org/10.3945/ajcn.115.128009
7. Dinika I, Verma DK, Balia R, Utama GL, Patel AR. Potential of cheese whey bioactive proteins and peptides in the development of antimicrobial edible film composite: A review of recent trends. Trends Food Sci Technol [Internet]. 2020 Sep;103:57–67. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0924224420305185
8. Ronghui S. The Reasearch on the Anti-Fatigue Effect of Whey Protein Powder in Basketball Training. Open Biomed Eng J [Internet]. 2015;9:330–4. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4787274/
9. Hoppe C, Andersen GS, Jacobsen S, Mølgaard C, Friis H, Sangild PT, et al. The use of whey or skimmed milk powder in fortified blended foods for vulnerable groups. J Nutr [Internet]. 2008;138(1):145S-161S. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18156417
10. Alimoradi F, Hojaji E, Jooyandeh H, Moghadam SAHZ, Moludi J. Whey Protein : Health Benefit and Food Applications Whey Protein. J Int Res Med Pharm Sci [Internet]. 2016;9(2):63–73. Available from: https://www.researchgate.net/publication/299286785_WHEY_PROTEINS_HEALTH_BENEFITS_AND_FOOD_APPLICATIONS
11. Andoyo R, Dianti Lestari V, Mardawati E, Nurhadi B. Fractal Dimension Analysis of Texture Formation of Whey Protein-Based Foods. Int J Food Sci [Internet]. 2018;2018. Available from: https://www.hindawi.com/journals/ijfs/2018/7673259/
12. Khairunnisa S, Andoyo R, Marta H, Saripudin GLU. Process optimization of emergency food originated

from denatured whey protein concentrate and dried sweet potato puree. {IOP} Conf Ser Earth Environ Sci [Internet]. 2018 May;157:12032. Available from: https://doi.org/10.1088%2F17551315%2F157%2F1%2F012032
13. Siddiq N. Optimasi Formula dan Kondisi Proses Sterilisasi Pangan Darurat Semi Padat Berbasis Whey Protein Concentrate Terdenaturasi dan Tepung Ubi Jalar. Padjajaran University; 2018.
14. Prabhakar K. Intermediate Moisture Foods. In: Batt CA, Tortorello MLBT-E of FM (Second E, editors. Encyclopedia of Food Microbiology [Internet]. 2nd ed. Oxford: Academic Press; 2014. p. 372–6. Available from: http://www.sciencedirect.com/science/article/pii/B9780123847300001701
15. Institute Of Medicine. High-Energy, Nutrient-Dense Emergency Relief Food Product [Internet]. High-Energy, Nutrient-Dense Emergency Relief Food Product. Washington DC: National Academy Press; 2002. 143 p. Available from: https://pubmed.ncbi.nlm.nih.gov/25057600/
16. Wróblewska B, Juśkiewicz J, Kroplewski B, Jurgoński A, Wasilewska E, Złotkowska D, et al. The effects of whey and soy proteins on growth performance, gastrointestinal digestion, and selected physiological responses in rats. Food Funct [Internet]. 2018;9(3):1500–9. Available from: http://xlink.rsc.org/?DOI=C7FO01204G
17. Masarwi M, Gabet Y, Dolkart O, Brosh T, Shamir R, Phillip M, et al. Skeletal effect of casein and whey protein intake during catch-up growth in young male Sprague–Dawley rats. Br J Nutr [Internet]. 2016 Jul 14;116(1):59–69. Available from: https://www.cambridge.org/core/product/identifier/S0007114516001781/type/journal_article
18. Bahwere P, Banda T, Sadler K, Nyirenda G, Owino V, Shaba B, et al. Effectiveness of milk whey protein-based ready-to-use therapeutic food in treatment of severe acute malnutrition in Malawian under-5 children: A randomised, double-blind, controlled non-inferiority clinical trial. Matern Child Nutr [Internet]. 2014;10(3):436–51. Available from: https://onlinelibrary.wiley.com/doi/full/10.1111/mcn.12112
19. Stobaugh HC, Ryan KN, Kennedy JA, Grise JB, Crocker AH, Thakwalakwa C, et al. Including whey protein and whey permeate in ready-to-use supplementary food improves recovery rates in children with moderate acute malnutrition: a randomized, double-blind clinical trial. Am J Clin Nutr [Internet]. 2016;103(3):926–33. Available from: https://pubmed.ncbi.nlm.nih.gov/26864368/
20. Hernell O, Lönnerdal B. Nutritional evaluation of protein hydrolysate formulas in healthy term infants: plasma amino acids, hematology, and trace elements. Am J Clin Nutr [Internet]. 2003 Aug 1;78(2):296–301. Available from: https://academic.oup.com/ajcn/article/78/2/296/4689939 DOI: 10.1093/ajcn/78.2.296
21. Dyer AR, Burdock GA, Carabin IG, Haas MC, Boyce J, Alsaker R, et al. In vitro and in vivo safety studies of a proprietary whey extract. Food Chem Toxicol [Internet]. 2008 May;46(5):1659–65. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0278691508000136
22. Berrazaga I, Mession J., Laleg K, Salles J, Guillet C, Patrac V, et al. Formulation , process conditions , and biological evaluation of dairy mixed gels containing fava bean and milk proteins : Effect on protein retention in growing young rats. J Dairy Sci [Internet]. 2019;102(2):1–17. Available from: http://dx.doi.org/10.3168/jds.2018-14610
23. Abril-Gil M, Garcia-Just A, Pérez-Cano FJ, Franch À, Castell M. Development and Characterization of an Effective Food Allergy Model in Brown Norway Rats. Chatenoud L, editor. PLoS One [Internet]. 2015 Apr 29;10(4):e0125314. Available from: https://dx.plos.org/10.1371/journal.pone.0125314
24. Harianti R. Pengaruh Pemberian Biskuit Tinggi Protein Berisi Krim Probiotik Fungsional Terhadap Profil Mikrobiota Fekal dan Berat badan Tikus [Internet]. IPB University; 2009. Available from: https://repository.ipb.ac.id/handle/123456789/43801
25. Institute Of Medicine. Estimated Mean Per Capita Energy Requirements for Planning Emergency Food Rations [Internet]. Washington DC: National Academy Press; 1995. 44 p. Available from: https://www.nap.edu/catalog/9095/estimated-mean-per-capita-energy-requirements-for-planning-emergency-food-aid-rations
26. Kementrian Kesehatan RI. Angka Kecukupan Gizi yang Dianjurkan Untuk Masyarakat Indonesia [Internet]. 28 Indonesia; 2019. Available from: https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&cad=rja&uact=8&ved=2ahUKEwj76qecgp3sAhWKH7cAHYRxB58QFjAAegQIARAC&url=http%3A%2F%2Fhukor.kemkes.go.id%2Fuploads%2Fproduk_hukum%2FPMK_No__28_Th_2019_ttg_Angka_Kecukupan_Gizi_Yang_Dianjurkan_Untuk_Masyarakat_Indonesia.pdf&usg=AOvVaw1d45UKKyBFYivhTfUuKCTJ
27. Zhong-ze H, Hong-de X, Kwang-ho K, Tae-hwan A, Jin-sook B, Ji-young L, et al. Reference Data of the Main Physiological Parameters in Control Sprague-Dawley Rats from Pre-clinical Toxicity Studies 재료 및 방법. Laboratiry Anim Res [Internet]. 2010;26(2):153–64. Available from: https://agris.fao.org/agris-search/search.do?recordID=KR2011004514
28. Frisilia J, Adi AC, Ismawati R. Efficacy of Local Food-Based Tabaro dange Against Weight of Gain and Levels of Hemoglobin (Hb) White Rats (Rattus Norvegicus Strain Wistar). Indian J Public Heal Res dan Dev [Internet]. 2020;11(04):701–6. Available from: https://medicopublication.com/index.php/ijphrd/article/view/7202
29. National Research Council. Nutrient Requirements of Laboratory Animals [Internet]. 4th ed. Washington DC: National Academy Press; 1995. 192 p. Available from: https://www.nap.edu/catalog/4758/nutrient-requirements-of-laboratory-animals-fourth-revised-edition-1995
30. Santos-Hernández M, Miralles B, Amigo L, Recio I. Intestinal Signaling of Proteins and Digestion-Derived Products Relevant to Satiety. J Agric Food Chem [Internet]. 2018 Oct 3;66(39):10123–31. Available from: https://pubs.acs.org/doi/10.1021/acs.jafc.8b02355
31. Kondrashina A, Brodkorb A, Giblin L. Dairy-derived peptides for satiety. J Funct Foods [Internet]. 2020;66(September 2019):103801. Available from: https://doi.org/10.1016/j.jff.2020.103801
32. Ridge A, Devine A, Lyons-wall P, Conlon J, Lo J. The impact of whey protein supplementation in older

adults on nutrient intakes and satiety over an 11-week exercise intervention. Food Qual Prefer [Internet]. 2018;68:72–9. Available from: https://doi.org/10.1016/j.foodqual.2018.01.013
33. Zapata RC, Singh A, Chelikani PK. Peptide YY mediates the satiety effects of diets enriched with whey protein fractions in male rats. FASEB J [Internet]. 2018 Feb;32(2):850–61. Available from: https://pubmed.ncbi.nlm.nih.gov/29042449/
34. Kong S, Zhang YH, Zhang W. Regulation of intestinal epithelial cells properties and functions by amino acids. Biomed Res Int [Internet]. 2018;2018:10. Available from: https://www.hindawi.com/journals/bmri/2018/2819154/
35. Sengupta P. The Laboratory Rat: Relating Its Age With Human's. Int J Prev Med [Internet]. 2013 Jun;4(6):624–30. Available from: https://pubmed.ncbi.nlm.nih.gov/23930179
36. Teixeira KR, Silva ME, Neves LX, Santos RC dos, Pedrosa ML, Haraguchi FK. Whey protein improves HDL/non-HDL ratio and body weight gain in rats subjected to the resistance exercise. Brazilian Arch Biol Technol [Internet]. 2012 Dec;55(6):943–50. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1516-89132012000600019&lng=en&tlng=en
37. Teixeira KR, Silva ME, de Lima WG, Pedrosa ML, Haraguchi FK. Whey protein increases muscle weight gain through inhibition of oxidative effects induced by resistance exercise in rats. Nutr Res [Internet]. 2016 Oct;36(10):1081–9. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0271531716303232
38. Pal S, Jane M, McKay J, Ho S. Whey Protein and the Metabolic Syndrome [Internet]. 2nd ed. Bioactive Food as Dietary Interventions for Diabetes. Elsevier Inc.; 2019. 103–120 p. Available from: http://dx.doi.org/10.1016/B978-0-12-813822-9.00007-2
39. Patel S. Functional food relevance of whey protein: A review of recent findings and scopes ahead. J Funct Foods [Internet]. 2015;19:308–19. Available from: http://dx.doi.org/10.1016/j.jff.2015.09.040
40. Hayes A, Cribb PJ. Effect of whey protein isolate on strength, body composition and muscle hypertrophy during resistance training. Curr Opin Clin Nutr Metab Care. 2008;11(1):40–4.
41. Hoffman JR, Falvo MJ. Protein - Which is Best? J Sports Sci Med [Internet]. 2004 Sep;3(3):118–30. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3905294/
42. Monirujjaman M, Ferdouse A. Metabolic and Physiological Roles of Branched-Chain Amino Acids. Adv Mol Biol [Internet]. 2014;2014:1–6. Available from: https://www.hindawi.com/journals/amb/2014/364976/
43. Brody T. Nutritional Biochemistry [Internet]. 2nd ed. California: Academic Press; 1999. 1006 p. Available from: https://www.sciencedirect.com/book/9780121348366/nutritional-biochemistry
44. Muza SF, Peres AP, Degáspari CH. Development of Yogurt Enriched With Whey Protein. Cad da Esc Saúde [Internet]. 2014;11:79–89. Available from: https://scholar.google.com/scholar_lookup?hl=en-US&volume=1&publication_year=2014&pages=79-89&author=S.+F.+Muza&author=A.+P.+Peres&author=C.+H.+Degáspari&title=Desenvolvimento+de+iogurte+enriquecido+com+proteína+do+soro+do+leite
45. Nie C, He T, Zhang W, Zhang G, Ma X. Branched Chain Amino Acids: Beyond Nutrition Metabolism. Int J Mol Sci [Internet]. 2018 Mar 23;19(4):954. Available from: http://www.mdpi.com/1422-0067/19/4/954
46. Rieu I, Sornet C, Giraudet C, Pujos E, Grizard J, Dardevet D. Leucine supplementation improves muscle protein synthesis in elderly men independently of hyperaminoacidaemia. J Physiological [Internet]. 2006;1(575):305–15. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1819434/#__ffn_sectitle
47. Walzem RL, Dillard CJ, German JB. Whey Components: Millennia of Evolution Create Functionalities for Mammalian Nutrition: What We Know and What We May Be Overlooking. Crit Rev Food Sci Nutr [Internet]. 2002 Jul 1;42(4):353–75. Available from: https://doi.org/10.1080/10408690290825574
48. Li P, Yin Y, Li D, Kim SW, Wu G. Amino acids and immune function. Br J Nutr [Internet]. 2007;98(2):237–52. Available from: https://pubmed.ncbi.nlm.nih.gov/17403271/
49. Food and Drug Administration. PART 172 -- Food Addictives Permitted for Direct Addition to Food and Human Consumption [Internet]. CFR - Code of Federal Regulations Title 21. Washington DC; 2019 [cited 2020 Sep 16]. Available from: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?fr=172.320
50. Floris R, Bodnár I, Weinbreck F, Alting AC. Dynamic rearrangement of disulfide bridges influences solubility of whey protein coatings. Int Dairy J [Internet]. 2008 May;18(5):566–73. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0958694607002427
51. Trivedi M, Laurence J, Siahaan T. The Role of Thiols and Disulfides on Protein Stability. Curr Protein Pept Sci [Internet]. 2009 Dec 1;10(6):614–25. Available from: http://www.eurekaselect.com/openurl/content.php?genre=article&issn=1389-2037&volume=10&issue=6&spage=614
52. Nguyen NHA, Streicher C, Anema SG. The effect of thiol reagents on the denaturation of the whey protein in milk and whey protein concentrate solutions. Int Dairy J [Internet]. 2018 Oct;85:285–93. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0958694618301584
53. Lin P, Chang CC, Yuan KC, Yeh HJ, Fang SU, Cheng T, et al. Red blood cell aggregation-associated dietary pattern predicts hyperlipidemia and metabolic syndrome. Nutrients [Internet]. 2018;10(8):1–13. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6115951/
54. Jung SM, Kim Y-J, Ryoo SM, Kim WY. Relationship Between Low Hemoglobin Levels And Mortality In Patients With Septic Shock. Acute Crit Care [Internet]. 2019;34(2):141–7. Available from: https://pubmed.ncbi.nlm.nih.gov/31723919/
55. Tran AK, Sapkota A, Wen J, Li J, Takei M. Linear relationship between cytoplasm resistance and hemoglobin in red blood cell hemolysis by electrical impedance spectroscopy & eight-parameter equivalent circuit. Biosens Bioelectron [Internet]. 2018 Nov;119(August):103–9. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0956565

66318306006
56. Augustyn A, Bauer P, Duignan B, Eldridge A, Gregersen E, McKenna A, et al. Hemoglobin [Internet]. Encyclopædia Britannica. Encyclopædia Britannica, inc.; 2018 [cited 2020 Apr 26]. Available from: https://www.britannica.com/science/hemoglobin
57. Chung J, Bauer DE, Ghamari A, Nizzi CP, Deck KM, Kingsley PD, et al. The mTORC1/4E-BP pathway coordinates hemoglobin production with L-leucine availability. Sci Signal [Internet]. 2015 Apr 14;8(372):ra34–ra34. Available from: https://stke.sciencemag.org/lookup/doi/10.1126/scisignal.aaa5903
58. Wolfson RL, Chantranupong L, Saxton RA, Shen K, Scaria SM, Cantor JR, et al. Sestrin2 is a leucine sensor for the mTORC1 pathway. Science (80-) [Internet]. 2016 Jan 1;351(6268):43–8. Available from: https://www.sciencemag.org/lookup/doi/10.1126/science.aab2674
59. Gupta A. Hemoglobin. In: Comprehensive Biochemistry for Dentistry [Internet]. 1st ed. Singapore: Springer Singapore; 2018. p. 77–100. Available from: https://www.springer.com/gp/book/9789811310348
60. Fentie K, Wakayo T, Gizaw G. Prevalence of Anemia and Associated Factors among Secondary School Adolescent Girls in Jimma Town , Oromia Regional State , Southwest Ethiopia. Anemia [Internet]. 2020;2020. Available from: https://www.hindawi.com/journals/anemia/2020/5043646/
61. Honda T, Pun VC, Manjourides J, Suh H. Anemia prevalence and hemoglobin levels are associated with long-term exposure to air pollution in an older population. Environ Int [Internet]. 2017 Apr;101(2):125–32. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0160412016303737
62. Yuniarti E, Hasanah L, Advinda L, Indika PM. Effect of wheat grass juice (triticum aestivum l.) against the erythrocytes and hemoglobin in male mice (mus musculus l.) anemia induced by sodium nitrite. J Phys Conf Ser [Internet]. 2019 Oct;1317:012069. Available from: https://iopscience.iop.org/article/10.1088/1742-6596/1317/1/012069
63. Provan D, Baglin T, Dokal I, Vos J de. Oxford Handbook of Clinical Haematology [Internet]. Oxford, UK: Oxford University Press; 2015. Available from: https://oxfordmedicine.com/view/10.1093/med/9780199683307.001.0001/med-9780199683307
64. Beard JL, Connor JR. Iron status and neural functioning. Annu Rev Nutr [Internet]. 2003 Jul;23(1):41–58. Available from: http://www.annualreviews.org/doi/10.1146/annurev.nutr.23.020102.075739
65. Abbaspour N, Hurrell R, Kelishadi R. Review on iron and its importance for human health. J Res Med Sci [Internet]. 2014 Feb;19(2):164–74. Available from: https://pubmed.ncbi.nlm.nih.gov/24778671
66. Adamsom J. The relationship of erythropoietin and iron metabolism to red blood cell production in humans. In: Semin Oncol [Internet]. New York; 1994. p. 9–15. Available from: https://www.ncbi.nlm.nih.gov/pubmed/8202725
67. Singh VK, Saini A, Chandra R. Role of Erythropoietin and Other Growth Factors in *Ex Vivo* Erythropoiesis. Ichim T, editor. Adv Regen Med [Internet]. 2014;2014:426520. Available from: https://doi.org/10.1155/2014/426520
68. Naclerio F, Seijo M, Larumbe-Zabala E, Ashrafi N, Christides T, Karsten B, et al. Effects of Supplementation with Beef or Whey Protein Versus Carbohydrate in Master Triathletes. J Am Coll Nutr [Internet]. 2017 Nov 17;36(8):593–601. Available from: https://doi.org/10.1080/07315724.2017.1335248
69. Mehta KJ, Seijo M, Larumbe-Zabala E, Ashrafi N, Christides T, Karsten B, et al. Case studies: Effects of beef, whey and carbohydrate supplementation in female master triathletes. J Hum Sport Exerc [Internet]. 2019;14(1):170–84. Available from: http://hdl.handle.net/10045/82110
70. Billett HH. Hemoglobin and Hematocrit. In: Clinical Methods: The History, Physical, and Laboratory Examinations [Internet]. 3rd ed. Boston: Butterworths; 1990. Available from: https://www.ncbi.nlm.nih.gov/books/NBK259/
71. Carneiro IA, Drakeley CJ, Owusu-Agyei S, Mmbando B, Chandramohan D. Haemoglobin and haematocrit: is the threefold conversion valid for assessing anaemia in malaria-endemic settings? Malar J [Internet]. 2007 Dec 22;6(1):67. Available from: https://malariajournal.biomedcentral.com/articles/10.1186/1475-2875-6-67
72. Medline Plus. Hematocrit Test [Internet]. Medline Plus. 2020 [cited 2020 Apr 26]. Available from: https://medlineplus.gov/lab-tests/hematocrit-test/
73. Sarma PR. Red Cell Indices. In: Walker H, Hall W, Hurst J, editors. Clinical Methods: The History, Physical, and Laboratory Examinations [Internet]. 3rd ed. Boston: Butterworths; 1990. Available from: https://www.ncbi.nlm.nih.gov/books/NBK260/
74. He Q, Su G, Liu K, Zhang F, Jiang Y, Gao J, et al. Sex-specific reference intervals of hematologic and biochemical analytes in Sprague-Dawley rats using the nonparametric rank percentile method. Johnson C, editor. PLoS One [Internet]. 2017 Dec 20;12(12):e0189837. Available from: https://doi.org/10.1371/journal.pone.0189837
75. Hidayat M, Prahastuti S, Dewi E, Safitri D, Farah S, A.Soemardji A. Subchronic Toxicity Test of Combination of Soybean and Jati Belanda Extract towards Wistar Rat Hematology. J Ilmu Kefarmasian Indones [Internet]. 2017;15(1):114–9. Available from: https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&cad=rja&uact=8&ved=2ahUKEwjf28X5_5zsAhVh7HMBHSVcCFUQFjAAegQIAhAC&url=http%3A%2F%2Fjifi.farmasi.univpancasila.ac.id%2Findex.php%2Fjifi%2Farticle%2Fdownload%2F495%2F314&usg=AOvVaw2xJc3vvYE4i36gQx2zA3YT
76. George E, Panos A. Does a high WBC count signal infection? Nursing2020 [Internet]. 2005;35(1). Available from: https://journals.lww.com/nursing/Fulltext/2005/01000/Does_a_high_WBC_count_signal_infection_.14.aspx
77. Riley LK, Rupert J, Air E, Base F, Medicine F, Air E, et al. Evaluation of Patients with Leukocytosis. Am Fam Physician [Internet]. 2015;92(11):1004–11. Available from: https://www.aafp.org/afp/2015/1201/p1004.html
78. Xiao CW, Wood C, Bertinato J, Amylase AMY. Dietary supplementation with l -lysine affects body weight and blood hematological and biochemical parameters in rats. Mol Biol Rep [Internet]. 2018;0(0):0. Available

from: http://dx.doi.org/10.1007/s11033-018-4492-1
79. Bassit RA, Sawada A, Bacurau RFP, Navarro F, Martins E, Santos RVT, et al. Branched-Chain Amino Acid Supplementation and the Immune Response of Long-Distance Athletes. Nutrition [Internet]. 2002;18(5):376–9. Available from: https://pubmed.ncbi.nlm.nih.gov/11985939/
80. Abbas AK, Fathi QM. Effect of Whey Protein Supplement on Physiological Parameters in Building Body Athletes. IRAQI J Sci [Internet]. 2017 Dec 31;58(4C):2332–7. Available from: http://scbaghdad.edu.iq/eijs/index.php/eijs/article/view/102/13