TPM Vol. 32, No. S7, 2025
ISSN: 1972-6325
https://www.tpmap.org/

NUTRITIONAL AND BIOSAFETY ASSESSMENT OF A NOVEL SOY-WHEY HYBRID PROTEIN CROSSLINKED BY MICROBIAL TRANSGLUTAMINASE IN SPRAGUE DAWLEY RATS

MUHAMMAD ABDULLAH BUTT
EMAIL: muhammadabdullahbuttfst@gmail.com

MUHAMMAD UMAIR ARSHAD
EMAIL: umair.arshad@gcuf.edu.pk

ALI IMRAN
EMAIL: dr.aliimran@gcuf.edu.pk

MUHAMMAD AFZAAL
DEPARTMENT OF FOOD SCIENCE, GOVERNMENT COLLEGE UNIVERSITY, FAISALABAD, PAKISTAN, EMAIL: muhammadafzaal@gcuf.edu.pk

Abstract
This study evaluated the preclinical efficacy and biosafety of novel hybrid proteins produced via microbial transglutaminase (MTG)-mediated crosslinking of soy and whey protein isolates. Despite their superior functional properties, limited data exist on their nutritional profile and biosafety. Sprague Dawley rats were divided into five dietary groups: a control group receiving cereal protein, and four treatment groups consuming crosslinked soy-whey hybrids with varying whey ratios (100% soy, 95:5, 90:10, and 85:15 soy-whey blends). Nutritional assessment included growth parameters, anthropometrics, feed and water intake, nitrogen balance, protein digestibility, and biosafety via hematological and organ health evaluations. Rats fed hybrids with higher whey content showed marked improvements in body weight, lean muscle mass, and protein utilization—attributed to superior bioavailability and feed efficiency. The 85:15 soy-whey formulation exhibited the most favorable outcomes in body composition metrics including BMI and Lee index, alongside enhanced protein digestion. No adverse effects were observed across groups; organ weights, liver and kidney functions, and hematological profiles remained within safe physiological ranges. These findings highlight the nutritional potential and biosafety of soy-whey hybrid proteins, particularly those with increased whey content, suggesting promising applications in human nutrition and dietetics.
Keywords: Microbial transglutaminase, Protein utilization, superior bioavailability, feed efficiency, hematological profiles

1. INTRODUCTION

Protein crosslinking is the process that involves formulation of very strong covalent bonds between the polypeptide chains of the proteins that result in the formulation of distinct protein networks and thus the resultant functional properties of this newer protein are also distinct in their nature (Heck *et al.,* 2013). The natural in vivo (in any biological system) process of protein crosslinking dose exists, but it can also be performed in vitro via several methods such as chemical crosslinking and enzymatic crosslinking. Another technique that can be applied is chemoenzymatic crosslinking. This approach makes the usage of both of the previously mentioned methodologies (Gupta *et al.,* 2020; Uy and Wold, 1977). The current study makes the usage of enzymatic crosslinking of whey and soy protein isolates. Among the common enzymes that are used for the preparation of newer hybrids are hydrolases, transferases and oxidoreductases (Heck *et al.,* 2013). An important type of transferase enzyme used for crosslinking is transglutaminase enzyme, which is usually calcium dependent on its action. It catalyzes acyl transfer reactions mainly by utilizing those residues of glutamine amino acid that are protein bound (Keillor *et al.,*2014). The type of transglutaminase used for the current study is microbial transglutaminase enzyme (MTG) which is calcium independent for its mode oof action and because of its lower molecular weight it is very suitable for industrial applications (Marapana *et al.,* 2010). Overall, crosslinking is an interesting technique that has multiple applications in the domains of biology, chemistry and biotechnologies. Some of the applications include formulation of the aggregate of enzymes, bioconjugates of

enzymes and reusable enzymes (Gupta *et al.,* 2020). In the field of food science and technology this process can be utilized for the creation of contemporary and novel textures, maintenance of required tangibility and the required mouthfeel of the products. Besides, it is also used to develop nondairy alternatives and meat analogues (Sulaiman *et al.,*2022).

In the recent study, soy and whey protein isolates were crosslinked to create a hybrid protein that would have superior functional properties. As Cui *et al.* (2020) showed that the crosslinked soy and whey proteins improve gel properties including the water holding capacity and hardness. Besides, improving the functional role, the act of crosslinking can also improve nutritional properties as the MTG has shown to increase the true digestibility of soya bean protein to that extent at which it became compatible with the meat-based protein sources (Volken de Souza *et al.,* 2009). Moreover, both soy and whey protein, though being nutritionally very excellent still, are not perfect proteins. For example, whey contains higher amounts of certain amino acids, namely threonine, methionine, lysine, valine, and isoleucine etc. as compared to soy protein. While soy contains higher quantities of certain other amino acids as compared to whey proteins, namely phenylalanine, histidine, arginine and glycine (Gorissen *et al.,* 2018). Hence, the act of developing a newer hybrid protein through soy and whey proteins is apparently nutritionally and functionally very beneficial. But the biosafety assessment of any edible product before commercialization is very necessary to identify and eliminate any potential hazard or safety risk associated with that product.

However, before commercialization of this novel protein for human consumption the assurance of its biosafety is very necessary. Though, in the past successful attempts have been made to develop hybrid crosslinked proteins. But one of the possible reasons for failure of its mass production would be lack of biosafety research of these sorts of products. The current study focuses on efficacy on animal models (rats) for assessing preclinical effect of hybrid protein on health. Studies on animal models, especially rodents (rats and rabbits) do have a very important role in assessing the health implications and possible allergens present in the new food products. These types of models are vital for evaluation of the effects of multiple factors on the health and physiology of humans where the actual human efficacy is either not possible or not easily feasible (Nematizadeh *et al.,* 2020). Another benefit of animal-based efficacy is their shorter lifespan, the provision of controlled environment and in case of rats and mice the genetic similarity with humans is another plus point (Mitchell *et al.,* 2015; Vanhooren and Libert *et al.,* 2013). The objective of this 28-day study was to comprehensively assess the physiological and health impacts of a novel hybrid protein, formed through the microbial transglutaminase (MTG) enzymatic crosslinking of soy and whey protein isolates, in rats. Furthermore, this research sought to generate essential preclinical data regarding its digestibility, nutritional advantages, and overall biosafety, thereby laying the groundwork for its potential commercialization as a food ingredient for human consumption.

2. MATERIALS AND METHODS

This research was conducted in the Department of Food Science of the Faculty of Life Sciences of the Government College University, Faisalabad and Al-Khidmat Laboratory Gulberg, Faisalabad

2.1. Procurement of chemicals, equipment and rats

90 % pure whey proteins were procured from Protein factory company. 90 % pure soy protein isolates were ordered from Nutrena company, and microbial transglutaminase enzyme (MTG) was purchased from Sunson Industry of China. Sprague Dawley rats were procured from the local market, rest of the reagents and gadgets were also purchased from Faisalabad city.

2.2. Enzymatic Crosslinking

The entire methodology of Cui *et al.* (2020) which included novel ultrasonication technique for better occurrence of crosslinking among the glutamine and lysine amino acids was followed (Cui *et al.,* 2020; Sun and Arntfield, 2012). For ultrasonication the VCX 750 probe type ultrasonicator manufactured by Sonics and materials, inc manufactured by U.S.A was used. The little modification that was made in the methodology was that for the deactivation of enzyme heating was performed at 80^{o}C for 10 minutes. Later, freeze drying of the protein was done through VaCo 5 laboratory freeze dryer following the methodology of Simoni *et al.* (2017). The above-mentioned methodologies were used to create four different types of crosslinked proteins, namely T_0 (100 % crosslinked soy protein), T1 (95 % Soy crosslinked with 5 % Whey protein), T2 (90 % soy and 10 % whey), and T3 (85 % Soy and 15 % Whey).

2.3. Animal study design:

After the review and approval of bioethical board of Government College University, Faisalabad (No.17/12/2024) male Sprague Dawley rats (8 weeks old) weighing 130 to 150 grams were taken and divided into 5 groups, namely R_0, R_1, R_2, R_3 and R_4. Each group contained a total of 8 rats. They were housed in the cages that were present in an air-conditioned room having 12 hours dark and light cycle. The lights were turned on at 7:30 am and were turned off at 7:30 pm. The rats were initially fed on a basal diet for 7 days as pretreatment of efficacy. The purpose was to neutralize the impact of any potential variation in the previous feed. Later during the efficacy all 5 groups were

subjected to variations of crosslinked proteins (No protein to R_0, T_0 to R_1, T_1 to R_2, T_2 to R_3 and T_3 to R_4). For water, bottles connected to tubes were provided for 24/7 water supply.

2.4. Dosage determination and delivery method

The dosage for rats was calculated based on protein requirement of humans which is around 1 g (1000 mg)/kg of body weight. For the calculation of Animal equivalent dose (AED) the formula given below was used (Jacob *et al.,* 2022; Nair and Jacob *et al.,* 2016)

AED (mg / kg) = Human does (mg / kg) × K_m ratio

K_m ratio = Animal Km/Human km

Huaman Km = 37

Rat km = 6

Human dose (mg/kg) = 1000 mg/kg

Calculations:

K_m ratio = 6/37 = 0.162

AED for rats = 1000 mg/kg x 0.162 = 162 mg/kg

The dosage was given via the oral gavage method.

2.5. Growth and anthropometrical performance parameters

The initial and final body weight gains of the animals were determined following the methodology of Novelli *et al.* (2007). The difference in these two weights was utilized to find out the weight gain in grams and in percentage (Brower *et al.,* (2015). To understand the compositional changes in the body in terms of weight, the specific rate of body mass gain was measured following the methodology of Novelli *et al.* (2007). The dimensions of quadricep (height, width, length) and other calculations based on these findings were obtained following the methodology of Suwankanit *et al.* (2022). However, in the current study a newer calculation that was obtained was the dimensions of upper arm, for this the exact methodology of Suwankanit *et al.* (2022) defined for taking measurements of quadriceps was applied on the upper arms as well. The organ to body ratios of vital organs (heart, liver and kidney) that are influenced by the consumption of protein-based diet was determined by following the methodology of Li *et al.* (2024). In order to observe potential obesity, amount of fat in body and amount of muscle gain in the rats, the BMI and Lee index of rats were measured. And the difference between the initial and final values was also measured to observe the amount of fat decreased and muscle mass gained. For finding out these values the nose anus length (NAL) was also calculated **(**Novelli *et al.,* 2007).

2.6. Nutritional parameters

For the nutritional study of rats, initial and final feed and water intake and other relevant data was observed (Laaksonen *et al.,* 2013; Morsy *et al.,* 2024). Furthermore, Feed efficiency was also determined (Naim *et al.,* 1980). Besides, protein efficiency ratio was also determined following the methodology of Gil *et al.* (2015). For finding out the initial and final nitrogen balance and apparent protein digestibility, fecal and urinary nitrogen was calculated at both stages (Salles *et al.,* 2021).

2.7. Biosafety parameters

In order to assess the biosafety of the novel hybrid protein in rats and observe any potential damage to the kidneys and liver, as well as the immune response and inflammation, multiple analyses were performed. Such as the complete blood count (CBC) was examined by following the methodology of Ali *et al.* (2021). Liver function test (LFT) and Renal function test (RFT) were performed following the methodology of Muthukumaran and Begum. (2020). C-Reactive Protein (CRP) as the marker to evaluate the systematic inflammation was determined following the methodology of Oliynyk *et al.* (2022).

2.8. Statistical analysis

One-way analysis of variance (ANOVA) under a completely randomized design (CRD) was used in an IBM SPSS Statistics 25 software. The methodology described by Montgomery. (2019) was followed. Means were interpreted using Tukey's HSD test.

3. RESULTS

3.1. Growth and anthropometrical performance parameters

The results depicted in Table-1 show that final body weight gains were highest in R_4 (45.16±4.95 g), followed by R_3 (41.06±2.98 g). This weight gain trend shows increased protein quality, with maximum specific growth rate in R_4 group (22.77±4.31 g/kg). Similarly, muscle width, height, and length gains were explicitly more in R_3 and R_4, with upper arm muscle width increase rate of 19.28±0.73% in R_4, compared to 6.25±0.37% in R_0. Similarly, the quadricep dimensions, specifically width and height, improved in those groups that had higher amount of whey protein crosslinked along with the soy protein isolates. In Table-1 it can be witnessed that the organ weights, including heart, liver, spleen, and kidneys, varied less between groups, indicating that these organs were less influenced by diet type.

Table 1 Growth and physiological parameters of rats fed different protein diets

Parameters	R_0	R_1	R_2	R_3	R_4
Initial body weight (g)	140.00 ± 7.25^{a}	153.33 ± 2.86^{b}	141.00 ± 6.48^{a}	139.33 ± 8.73^{a}	141.67 ± 5.55^{a}
Final body weight (g)	167.17 ± 4.12^{a}	188.76 ± 7.85^{b}	177.64 ± 7.59^{ab}	180.39 ± 5.25^{ab}	186.83 ± 3.51^{b}
Mean weight (day 0-14) (g)	159.83 ± 10.86^{a}	186.04 ± 18.97^{a}	178.01 ± 22.18^{a}	178.59 ± 23.03^{a}	176.65 ± 19.29^{a}
Net weight gain (g)	27.17 ± 2.3^{a}	35.43 ± 4.9^{b}	36.64 ± 4.0^{b}	41.06 ± 2.98^{c}	45.16 ± 4.95^{d}
Net weight gain (%)	19.41 ± 1.11^{a}	23.11 ± 1.55^{b}	25.99 ± 1.19^{c}	29.47 ± 2.24^{d}	31.88 ± 2.12^{e}
Specific rate of body mass gain (g/kg)	13.86 ± 1.55^{a}	16.50 ± 3.13^{b}	18.56 ± 3.01^{b}	21.04 ± 2.24^{c}	22.77 ± 4.31^{c}
Initial upper arm width (cm)	0.45 ± 0.01^{a}	0.47 ± 0.02^{a}	0.46 ± 0.02^{a}	0.45 ± 0.03^{a}	0.46 ± 0.01^{a}
Final upper arm width (cm)	0.48 ± 0.03^{a}	0.52 ± 0.02^{a}	0.51 ± 0.04^{a}	0.53 ± 0.03^{a}	0.57 ± 0.05^{a}
Increase in upper arm muscle width (%)	6.25 ± 0.37^{a}	9.61 ± 0.62^{b}	9.80 ± 0.26^{b}	15.09 ± 0.57^{c}	19.28 ± 0.73^{d}
Initial upper arm length (cm)	2.50 ± 0.04^{a}	2.50 ± 0.06^{a}	2.50 ± 0.11^{a}	2.50 ± 0.03^{a}	2.50 ± 0.05^{a}
Final upper arm length (cm)	2.60 ± 0.07^{a}	2.65 ± 0.08^{a}	2.67 ± 0.09^{a}	2.73 ± 0.08^{b}	2.80 ± 0.10^{c}
Increase in upper arm muscle length (%)	4.00 ± 0.12^{a}	5.66 ± 0.26^{b}	6.36 ± 0.67^{c}	8.42 ± 0.73^{d}	10.71 ± 0.37^{e}
Initial upper arm height (cm)	1.50 ± 0.05^{b}	1.60 ± 0.06^{a}	1.55 ± 0.05^{ab}	1.50 ± 0.07^{b}	1.55 ± 0.06^{ab}
Final upper arm height (cm)	1.60 ± 0.07^{d}	1.80 ± 0.08^{c}	1.90 ± 0.07^{b}	2.00 ± 0.08^{ab}	2.10 ± 0.09^{a}
Increase in upper arm muscle height (%)	6.67 ± 0.24^{e}	12.50 ± 0.11^{d}	22.58 ± 0.28^{c}	33.33 ± 0.97^{b}	35.48 ± 1.27^{a}
Final upper arm muscle weight (g)	0.8 ± 0.1^{e}	1.2 ± 0.2^{d}	1.4 ± 0.2^{c}	1.8 ± 0.3^{b}	2.2 ± 0.3^{a}
Upper arm weight (g)/body weight (Kg)	5.44 ± 0.75^{e}	7.12 ± 0.82^{d}	8.87 ± 1.02^{c}	11.24 ± 1.12^{b}	13.18 ± 1.27^{a}
Initial quadricep width (cm)	0.70 ± 0.02^{b}	0.72 ± 0.03^{a}	0.71 ± 0.02^{ab}	0.71 ± 0.03^{ab}	0.73 ± 0.03^{a}
Final quadricep width (cm)	0.75 ± 0.03^{d}	0.78 ± 0.03^{c}	0.79 ± 0.03^{c}	0.82 ± 0.03^{b}	0.85 ± 0.04^{a}
Increase in quadricep width (%)	7.14 ± 0.22^{e}	8.33 ± 0.53^{d}	11.27 ± 1.97^{c}	15.49 ± 2.36^{b}	16.44 ± 2.28^{a}
Initial quadricep length (cm)	3.5 ± 0.07^{b}	3.6 ± 0.09^{a}	3.5 ± 0.08^{b}	3.5 ± 0.10^{b}	3.6 ± 0.07^{a}
Final quadricep length (cm)	3.7 ± 0.08^{e}	3.9 ± 0.09^{d}	4.0 ± 0.09^{c}	4.1 ± 0.10^{b}	4.3 ± 0.10^{a}
Increase in quadricep length (%)	5.71 ± 0.47^{a}	8.33 ± 0.28^{b}	14.29 ± 1.37^{c}	17.14 ± 1.85^{d}	19.44 ± 1.44^{e}
Initial quadricep height (cm)	1.90 ± 0.05^{a}	2.00 ± 0.06^{b}	1.95 ± 0.05^{ab}	1.90 ± 0.07^{a}	1.95 ± 0.06^{ab}
Final quadricep height (cm)	2.00 ± 0.07^{a}	2.20 ± 0.08^{b}	2.30 ± 0.08^{c}	2.50 ± 0.09^{d}	2.60 ± 0.10^{e}
Increase in upper arm muscle height (%)	5.26 ± 0.26^{a}	10.00 ± 1.76^{b}	17.95 ± 1.95^{c}	31.58 ± 1.58^{d}	33.33 ± 1.44^{d}
Final quadricep muscle weight (g)	4.2±0.3^{a}	4.5±0.4^{ab}	4.8±0.5^{b}	5.2±0.5^{c}	5.6±0.6^{c}

Quadricep muscle weight (g)/body weight (Kg)	3.00±0.4[a]	2.94±0.5[a]	3.40±0.5[a]	3.25±0.6[a]	3.34±0.7[a]
Heart weight (g)	0.77±0.09[a]	0.86±0.11[a]	0.79±0.10[a]	0.81±0.11[a]	0.83±0.10[a]
Heart to body weight ratio (%)	0.46±0.05[a]	0.45±0.06[a]	0.44 ± 0.04[a]	0.44±0.05[a]	0.44±0.06[a]
Liver weight (g)	7.05±0.69[a]	7.95±0.81[a]	7.35±0.74[a]	7.50±0.77[a]	7.65±0.77[a]
Liver to body weight ratio (%)	4.21± 0.3[a]	4.21±0.35[a]	4.13±0.28[a]	4.15±0.31[a]	4.09±0.32[a]
Spleen (pancreases) weight	0.94±0.09[a]	1.09±0.12[a]	0.99±0.10[a]	1.02±0.10[a]	1.05±0.11[a]
Spleen to body weight ratio (%)	0.56± 0.05[a]	0.57± 0.06[a]	0.55± 0.04[a]	0.56± 0.05[a]	0.56± 0.06[a]
Kidney weight (mg)	0.67±0.06[a]	0.73±0.08[a]	0.69±0.06[a]	0.70±0.07[a]	0.72±0.07[a]
kidney to body weight ratio (%)	0.40± 0.04[a]	0.38± 0.05[a]	0.38± 0.03[a]	0.38± 0.04[a]	0.39± 0.05[a]
Initial nose to anus ratio (NAL) (cm)	16± 0.24[a]	17± 1.11[a]	16.5± 1.05[a]	16.8±1.21[a]	17.5 ± 2.52[a]
Final NAL (cm)	17.2±1.17[a]	18.2±2.02[a]	17.7±2.52[a]	18.1±2.22[a]	18.9±3.18[a]
Increase in NAL (%)	7.5 ± 1.25[a]	7.06 ± 1.15[a]	7.27 ± 1.2[a]	7.74 ± 1.3[a]	8.0 ± 1.2[a]
Initial lee index (g/cm)	0.51 ± 0.02[a]	0.48 ± 0.02[a]	0.50 ± 0.03[a]	0.51 ± 0.03[a]	0.50 ± 0.03[a]
Final lee index (g/cm)	0.32±0.02[a]	0.31±0.02[a]	0.31±0.03[a]	0.31±0.02[a]	0.30±0.03[a]
Decrease in Lee index (%)	37.25± 2.5[a]	35.41± 2.8[a]	38.00± 3.0[a]	39.21± 2.7[a]	40.00± 2.6[a]
Initial body mass index (BMI) (g/cm)	0.35±0.04[a]	0.32±0.03[a]	0.34±0.03[a]	0.35±0.03[a]	0.34±0.03[a]
Final BMI (g/cm)	0.56±0.03[a]	0.56±0.03[a]	0.56±0.02[a]	0.55±0.02[a]	0.52±0.02[a]
Decrease in BMI (%)	60 ± 2.5[b]	75 ± 3[a]	64.70 ± 2.8[b]	57.14 ± 2.3[c]	52.94 ± 2.6[c]

3.2. Nutritional parameters

In the Table-2 the initial and final feed and water intake continuously increased across groups, with R_4 exhibiting the highest increase in both feed (34.48±2.24%) and water intake (48.80±3.45%). Fecal nitrogen levels decreased, particularly in R_4, where final fecal nitrogen was the lowest (0.006± 0.0001mg), showing the best protein absorption. Apparent protein digestibility also improved across groups, with the highest value in R_4 (87.63± 1.15 %), highlighting that crosslinked proteins with increased ratio of whey protein are better absorbed and utilized.

Table 2 Nutritional parameters of rats fed different protein diets

Parameters	R_0	R_1	R_2	R_3	R_4
Initial feed intake (g)	14.0±1.2[a]	15.0±1.3[a]	14.5±1.2[a]	14.3±1.1[a]	14.5±1.1[a]
Final feed intake (g)	16.0±1.5[a]	17.5±1.6[a]	17.0±1.5[a]	18.0±1.5[a]	19.5±1.5[a]
Mean feed intake of 14 days (g)	15.1 ± 0.4[c]	16.3 ± 0.5[a]	15.7 ± 0.4[b]	15.9 ± 0.5[b]	16.6 ± 0.6[a]
Total feed consumed (g) during 14 days	211.4 ± 1.5[c]	228.2 ± 1.9[a]	219.8 ± 1.5[b]	222.6 ± 1.9[b]	232.4 ± 2.2[a]
Increase in feed intake (%)	12.5±1.5[a]	16.67±2.58[b]	17.24±3.22[c]	25.87±1.13[d]	34.48±2.24[e]
Initial water intake (ml)	8.0±0.7[a]	8.5±0.8[a]	8.3±0.7[a]	8.2±0.7[a]	8.4±0.7[a]
Final water intake (ml)	9.0±0.8[a]	10.5±0.9[a]	10.0±0.9[a]	11.0±0.9[a]	12.5±1.0[a]

Increase in water intake (%)	12.50±0.41^{e}	23.52±1.84^{c}	20.48±2.18^{d}	34.14±2.97^{b}	48.80±3.45^{a}
Feed efficiency	0.129±0.011^{e}	0.155±0.022^{c}	0.167±0.019^{d}	0.184±0.014^{b}	0.194±0.022^{a}
Net protein consumed (g)	0.3878± 0.0001^{c}	0.3878± 0.0001^{c}	0.3626± 0.0001^{b}	0.3626± 0.0001^{a}	0.3724± 0.0001^{a}
Protein efficiency ratio	70.1 ± 1.2^{e}	91.5 ± 1.7^{d}	101.1 ± 1.8^{c}	113.2 ± 1.9^{b}	121.5 ± 2.1^{a}
Biological value	68.70±2.34^{a}	74.73±2.61^{b}	76.69±2.73^{bc}	79.83±2.99^{c}	82.86±3.11^{d}
Initial fecal nitrogen (mg)	0.014 ± 0.0001^{a}	0.013±0.0001^{ab}	0.013±0.0001^{ab}	0.013±0.0002^{ab}	0.013±0.0001^{b}
Final fecal nitrogen (mg)	0.013± 0.0001^{a}	0.012± 0.0001^{ab}	0.010± 0.0001^{b}	0.011± 0.0001^{b}	0.006± 0.0001^{c}
Initial urinary nitrogen (mg)	0.021± 0.0002^{a}	0.018±0.0001^{b}	0.017±0.0001^{b}	0.018±0.0001^{b}	0.018±0.0001^{b}
Final urinary nitrogen (mg)	0.024± 0.0001^{a}	0.023± 0.0001^{ab}	0.024± 0.0002^{a}	0.018± 0.0001^{c}	0.014± 0.0001^{d}
Initial nitrogen balance (mg)	0.0013 ± 0.0001^{a}	0.0087 ± 0.0002^{b}	0.0065 ± 0.0001^{bc}	0.0051 ± 0.0001^{c}	0.0057 ± 0.0002^{c}
Final nitrogen balance (mg)	0.0093 ± 0.0001^{a}	0.0189 ± 0.0002^{b}	0.0189 ± 0.0001^{b}	0.0178 ± 0.0001^{b}	0.0244 ± 0.0002^{c}
Initial apparent protein digestibility (%)	67.67 ± 1.23^{a}	73.42 ± 0.89^{b}	71.68 ± 1.02^{b}	72.22 ± 0.95^{b}	73.14 ± 1.10^{b}
Final apparent protein digestibility (%)	69.98 ± 1.12^{a}	75.43 ± 0.97^{b}	78.26 ± 1.03^{c}	76.47 ± 0.89^{b}	87.63 ± 1.15^{d}

3.3. Biosafety parameters

The hematological data presented in Table 3 highlights the biosafety and physiological impacts of consuming crosslinked soy and whey protein isolates in comparison to inferior quality cereal-based protein (R_0 group) over a 28-day study period.

Table 3 Hematological examination of rats fed different protein diets as the depiction of biosafety perspectives of consumed protein

Examination Type	**Test**	**Results**					**Normal reference values for Male Sprague Dawley rats (Delwatta**
		R_0	**R_1**	**R_2**	**R_3**	**R_4**	

							et al., 2018)
Erythrocytes (Red blood cells)	**Hemoglobin (g/dl)**	11.2 ± 0.5[a]	15.0 ± 0.4[b]	15.2 ± 0.3[b]	15.3 ± 0.3[bc]	15.4 ± 0.3[c]	10.4-16.5
	Packed cell volume (hematocrit) %	25.9 ± 1[a]	44.3 ± 1.1[b]	45 ± 1[bc]	46 ± 1.[c]	47 ± 1.3[d]	18-48
	Total RBC (× 10^6/µL)	4.1 ± 0.2[a]	6.02 ± 0.3[b]	6.15 ± 0.2[bc]	6.16 ± 0.2[bc]	6.22 ± 0.2[c]	3.8-6.68
	M.C.V (fL)	53.1 ± 2[a]	54.2± 2[a]	54.6 ± 2[a]	55.1 ± 2[a]	57.6 ± 2[a]	29.41-123.07
	M.C.H (Pg)	18.6 ± 0.2[a]	19.6± 0.2[b]	19.9± 0.5[bc]	20.4± 0.4c	21.9± 0.1[d]	18.37-36.98
	M.C.H.C (g/dl)	30.8 ± 1[a]	36.7 ± 0.5[b]	37.6 ± 0.5[c]	38.2 ± 0.4[c]	38.5 ± 0.5[d]	25.41-80.55
Leucocytes (White blood cells)	**Total WBC count (per mm^3) or (K/uL)**	3.3 ± 0.2[a]	4.5 ± 0.3[b]	4.6 ± 0.3[b]	4.8 ± 0.25[bc]	5.4 ± 0.3[c]	4400-14 800 or 4.4-14.8
	Neutrophils (%)	25.6 ± 2[a]	28.4 ± 1.5[b]	29.5 ± 1.4[b]	30.13 ± 1.1[b]	31.5 ± 1.5[c]	13-36
	Lymphocytes (%)	70 ± 3[a]	64 ± 2[b]	63.5 ± 2[b]	63 ± 2[b]	62.5 ± 2[b]	61-86
	Monocytes (%)	0.11 ± 0.01[a]	0.16 ± 0.01[b]	0.18 ± 0.1[b]	1 ± 0.01[c]	1 ± 0.04[c]	0-1
	Basophils (%)	0.24 ± 0.05[a]	0.22 ± 0.05[a]	0.25 ± 0.01[a]	0.25± 0.01[a]	0.25± 0.01[a]	0-2
	Eosinophils (%)	2 ± 0.2[a]	2.03 ± 0.2[a]	2.07 ± 0.7[a]	2.11 ± 0.2[a]	2.14 ± 0.5[a]	0-6
Thrombocytes	**Platelet count (× 105 /µL)**	3.64± 0.2[a]	3.78± 0.1[a]	3.84 ± 0.6[a]	4.17± 0.1[a]	4.47± 0.8[a]	1.7-5.57
	E.S.R by Westergren Method (mm 1st Hr)	Negative	Negative	Negative	Negative	Negative	-

In the Table-4 the biochemical examination of five groups of male rats (R_0 to R_4) provides the details into the biosafety profile of these crosslinked protein treatments.

Table 4 Biochemical examination of rats fed different protein diets as the depiction of biosafety perspectives of consumed protein

Examination Type	**Test**	**Results**					**Normal reference values for Male Sprague Dawley rats (Delwatta et al., 2018; Kurtz et al., 2017)**
		R_0	**R_1**	**R_2**	**R_3**	**R_4**	
Liver function test (LFT)	**Total bilirubin (mg/dl)**	0.7 ± 0.03[a]	0.68 ± 0.03[a]	0.6 ± 0.02[b]	0.6 ± 0.02[b]	0.5 ± 0.02[c]	0.06-0.8
	Conjugated bilirubin (mg/dl)	0.25 ± 0.01[a]	0.23± 0.03[a]	0.2± 0.02[b]	0.2± 0.01[b]	0.2± 0.03[b]	0.05 to 0.3

	Unconjugated bilirubin (mg/dl)	0.45 ± 0.02[a]	0.45± 0.01[a]	0.4± 0.02[b]	0.4± 0.01[b]	0.3± 0.01[c]	0.1 - 0.5
	Alkaline phosphatase (ALP) (U/I)	360 ± 10[a]	330 ± 10[b]	320 ± 10[b]	310 ± 10[c]	305 ± 10[c]	21-367
	Aspartate aminotransferase (AST) (U/L)	360 ± 10[a]	330 ± 10[b]	320 ± 10[b]	310 ± 10[c]	305 ± 10[c]	0.2-838.3
	Alanine aminotransferase (U/I)	95.15 ± 5[a]	84.17± 2[b]	78.26± 5[b]	70.83± 3[c]	61.65± 3[c]	6-114
Renal (Kidney) function test	**Blood urea (mg/dl)**	28 ± 1[a]	26.3 ± 1[b]	22 ± 1[b]	24 ± 1[c]	18 ± 1[c]	13-29
	Serum Creatinine (mg/dl)	0.8± 0.01[a]	0.7± 0.03[b]	0.6± 0.01[b]	0.6± 0.02[c]	0.6± 0.01[c]	0.45-1.5
	Serum albumin (gm/dl)	3.1 ± 0.1[a]	3.8 ± 0.1[b]	3.9 ± 0.1[b]	4.0 ± 0.1[c]	4.1 ± 0.1[c]	2.9-4.8
Immune response and Inflammation	**C. Reactive protein (CRP)**	Negative	Negative	Negative	Negative	Negative	-

4. DISCUSSION

The trend of growth measures discussed in the results section indicated enhanced muscle development in those rat groups that consumed crosslinked proteins having higher content of whey protein, as seen in R_4 with a 33.33±1.44% increase in quadricep height. This muscle gain could be because of the antioxidant properties of whey proteins that are very good against oxidative stress (Teixeira *et al.,* 2016). Another reason could be the high leucine amino acid content of whey protein that through the mTOR pathway results in the synthesis of proteins within the body (Phillips *et al.,* 2016).

The muscle-specific weight, such as quadriceps and upper arm muscles relative to body weight, showed explicit increase, with R4 again displaying the highest values, suggesting improved muscle-to-body weight ratio with higher whey content. A study related to humans showed that the whey protein supplementation not only increased muscle strength but also increased muscle mass (Kim *et al.,* 2023). Another study, which was done on the rats showed that the consumption of whey protein led to the changes at the molecular level and changed the gene expression of muscle protein synthesis (mTOR) and degradation (MAFbx and MuRF-1). This change resulted in the development of bigger bodies having higher muscle mass (Haraguchi *et al.,* 2014). NAL values showed minimal differences across groups, with a slight increase in R_4.

The Lee index and BMI revealed decrease in Lee index, depicting muscle mass gain and increase in BMI in groups with higher whey content, resulting in buffed body composition. Specifically, the highest BMI increase was in R_1 at 75±3%, whereas R_4 showed a lower but still significant and relatively healthiest BMI increase at 52.94± 2.6%, suggesting different patterns in body mass distribution among the protein diets. Overall, the findings indicate that increasing the extent of whey in the crosslinked protein positively impacts body weight, muscle development, and composition, with R_4 demonstrating the greatest improvements across most parameters. Literature also shows that the consumption of whey protein contributes to the increase in muscle mass of the body and decrease in the total bodily fat (Kim *et al.,* 2023; Sepandi *et al.,* 2022).

Similarly, the feed efficiency, protein efficiency and the mean feed intake improved in those groups which had higher levels of whey protein, with R_4 showing the highest values, indicating better utilization of the nutrients. Though according to Zhou *et al.* (2011) dietary whey protein resulted in decreased feed consumption in rats, in current study increased hunger and feed intake in rats having starch-based diet coupled with soy protein crosslinked with whey protein as the source of protein, had occurred because of the increase in metabolic rate, and digestibility. Wróblewska *et al.* (2018) also showed that those rats that consumed whey proteins had better digestion and skeletal anabolism as compared to those which had consumed just soy proteins. Nitrogen metabolism results show an improvement in protein retention, especially in the R_4 group.

By the end of the study, urinary nitrogen decreased in those groups that consumed higher amounts of whey protein. The R4 group again showed the lowest value, indicating reduced nitrogen loss and better protein utilization. The nitrogen balance, which reflects net nitrogen retained, was highest in R_4, depicting enhanced growth and protein

digestion compared to other groups. The study of Poullain *et al.* (1989) showed that in contrast to rat groups consuming other types of proteins and amino acids, whey protein consuming groups had better retention of nitrogen, which resulted in enhanced growth.

The trend of the results of the apparent protein digestibility suggests that while pure crosslinked soy protein is better than simple cereal based protein, the combination of soy and whey protein enhances digestibility and the nutritional quality of the diet, ultimately supporting higher protein efficiency and better overall growth outcomes in rats. Literature also shows that while the soy protein in the crosslinked form can improve digestibility growth (Volken de Souza et al., 2009), whey protein outperforms soy protein in promoting the anabolism of skeletal muscles and overall growth (Bar-Maisels *et al.,* 2021; Wróblewska *et al.,* 2018). Besides, the whey protein containing diets result in enhanced weight gain, mineral density of the bones, and cortical thickness of soy protein diets (Bar-Maisels *et al.,* 2021).

It can be seen about the erythrocyte-related parameters, that there is a clear upward trend in hemoglobin (Hb), packed cell volume (PCV), total RBC count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) across groups R_1 to R_4, indicating improved erythropoiesis and oxygen-carrying capacity with increasing levels of whey protein isolate in the diet. The R_0 group had the lowest levels in all erythrocyte parameters, suggesting a lesser impact on red blood cell production and lower quality protein intake. Notably, all groups' values fell within normal reference ranges, underscoring the safety and suitability of the protein hybrids. Leukocyte profiles also showed positive responses with higher white blood cell (WBC) counts in groups consuming soy-whey protein hybrids (R_1 to R_4) than in R_0. These increases were still within the normal range, indicating a potentially better immune response without overstimulation. Similarity, the distribution of neutrophils, lymphocytes, monocytes, basophils, and eosinophils remained within safe limits and reflected a balanced immune cell profile across all groups. Thrombocyte or platelet counts also demonstrated an increase in groups R_1 through R_4, with the highest count in R_4, further reflecting enhanced hematopoietic activity from the crosslinked soy-whey protein. Notably, all groups had negative erythrocyte sedimentation rates (ESR), indicating no inflammation. Overall, the data supports the biosafety and beneficial effects of crosslinked soy and whey protein consumption on hematological parameters, with soy-whey hybrids, and among them, those having higher concentration of whey protein did promoted better erythrocyte, leukocyte, and platelet production compared to cereal-based proteins. These findings prove that crosslinked soy and whey protein isolates do enhance blood health and immune status without causing any adverse hematological effects. Several other studies also support the current trend as in as study, a mixture of soy and whey protein resulted in enlargement of hematopoietic stem cells in mice, moreover their white blood cell recovery was also improved. Furthermore, the thymus and spleen also showed healthy results among the transplanted mice (Wu *et al.,* 2022). In the study of Mehal *et al.* (2019) it was witnessed that the consumption of soy-based beverage was not only safe but also did not cause any significant changes in hematological parameters. A study on athletes showed that the supplementation of whey protein resulted in an increase in white blood cell counts and phagocytic activity of neutrophils (Abbas and Fathi, 2018). In another study it was found that whey protein caused better enhancement in intersystem, particularly in the response of plaque-forming cells as compared to other protein sources.

The LFT results reveal that all groups had bilirubin levels within normal ranges, showing no stress on the liver. Interestingly, the total bilirubin levels were slightly lower in groups with increasing whey protein content (R_2 to R_4), indicating a positive effect on liver health. Alanine aminotransferase (ALT) levels, an indicator of liver function, were highest in R_0 and decreased across the groups, with R_4 showing the lowest ALT, showing improved liver health in those groups that consumed hybrid proteins having higher concentration of whey protein. AST and alkaline phosphatase (ALP) levels, both liver enzymes, also showed a gradual decline from R_0 to R_4, remaining within normal ranges. This decrease aligns with the trend in ALT levels, implying a dose-dependent improvement in liver function with higher whey content in the hybrid protein diet. In the renal function test, blood urea and serum creatinine levels were well within normal ranges, with the lowest values observed in R_4. The gradual reduction in blood urea from R_0 to R_4 shows enhanced kidney efficiency with the increase of whey protein. Serum albumin levels increased progressively from R_0 to R_4, showing improved protein utilization and metabolic health, as albumin is crucial for maintaining osmotic balance and nutrient transport. The immune response and inflammation marker, C-reactive protein (CRP), remained negative across all groups, indicating no inflammatory response or adverse immune reactions from any of the protein diets. Overall, the biochemical profiles of all the rats depict that increasing the proportion of whey protein crosslinked with soy protein dose enhances liver and kidney function and improves metabolic health, affirming the biosafety of the crosslinked protein blends across all groups. Interestingly, different types of claims are available in literature, some of which support the current study and some are not so supportive. Nunes *et al.* (2013) showed that excessive supplementation by whey protein alone may lead to increased dysfunction of liver and kidney. But Vieira *et al.* (2021), showed that the combination of whey protein supplementation along with resistance training gave better results without impairing renal functions. Barbosa *et al.* (2021) showed that the higher doses of whey protein (2 to 6 g/kg/day) not only do not damage liver but also have hepatoprotective effects. However, Aparicio *et al.* (2014) showed that in comparison to soy protein, whey protein diet result in more acidic pH of the urine, higher

calcium and low level of citrate in the urine, this potentially increases the risk of nephrolithiasis. Interestingly, among rats the whey protein supplementation results in regulation of feed intake, but higher consumption may lead to reduction in feed intake (Barbosa *et al.,* 2021). In the current study the delivery of optimum concentration of protein was opted for the rats. That's why not only feed intake but also the healthy weight and size was also achieved without having any negative impact on the liver, kidney and immune system. From the findings of the current study and the data available in the literature it can be concluded that when in the rats having a sedentary lifestyle, the consumption of this soy and whey hybrid can have these good results, what would be the level of betterment among the humans having active lifestyle. Hence cross-linked, soy and whey are extremely safe and healthy for those people who hit the gym or are involved in athletics.

In the nutshell, it can be concluded that the crosslinked soy-whey protein hybrids showed improved health, nutritional and biosafety related results in a rat model, particularly the hybrid with 85% soy and 15% whey protein, which led to the greatest improvements in growth, muscle mass, and protein retention. Improvement in digestibility and nitrogen retention in the groups having higher extent of whey shows the potential of this novel hybrid protein to improve nutritional intake and support muscle development, while having no negative impact on the immune system, kidney and liver. These preclinical findings show the hybrid protein's potential for commercialization as a high-quality, safe, cheap and sustainable protein that could meet consumer demands for nutritious and functional food products. Further studies are needed to confirm these effects on human trials.

Authors' Contribution

Muhammad Abdullah Butt conceived the study, designed the experimental protocol, conducted the efficacy trial, and prepared the initial draft of the manuscript. Muhammad Umair Arshad supervised the study, guided the experimental design and analysis, and contributed to manuscript revision. Ali Imran provided technical input on protein crosslinking and reviewed the scientific content critically. Muhammad Afzaal assisted in laboratory work, particularly in biochemical assays and data interpretation. All authors reviewed, revised, and approved the final version of the manuscript.

REFERENCES

1. Abbas, A.K. and Q.M. Fathi (2017). Effect of whey protein supplement on physiological parameters in building body athletes. Iraqi J. Sci. 58(4C): 2332-2337.
2. Ali, M.M., N.R. Azad, M.I. Haque, K.M. Sujan, K.R. Islam and M.K. Islam (2020). Performance of complete blood count (CBC) upon use of different anticoagulants in rats. Asian J. Med. Biol. Res. 6(1): 16-21.
3. Aparicio, V.A., E. Nebot, M. Tassi, D. Camiletti-Moirón, C. Sanchez-Gonzalez, J.M. Porres and P. Aranda (2014). Whey versus soy protein diets and renal status in rats. J. Med. Food 17(9): 1011-1016.
4. Bar-Maisels, M., C. Menahem, Y. Gabet, S. Hiram-Bab, M. Phillip and G. Gat-Yablonski (2021). Different effects of soy and whey on linear bone growth and growth pattern in young male Sprague-Dawley rats. Front. Nutr. 8: 739607.
5. Bounous, G., P.A. Kongshavn and P. Gold (1988). The immunoenhancing property of dietary whey protein concentrate. Clin. Invest. Med. 11(4): 271-278.
6. Brower, M., M. Grace, C.M. Kotz and V. Koya (2015). Comparative analysis of growth characteristics of Sprague Dawley rats obtained from different sources. Lab. Anim. Res. 31: 166-173.
7. Cui, Q., G. Wang, D. Gao, L. Wang, A. Zhang, X. Wang and L. Jiang (2020). Improving the gel properties of transgenic microbial transglutaminase cross-linked soybean-whey mixed protein by ultrasonic pretreatment. Process Biochem. 91: 104-112.
8. da Silva Barbosa, A.K., A.C. Navarro, B.A.S. Pinto and F. Navarro (2021). Efeitos sobre biomarcadores hepáticos e o consumo alimentar de ratos Wistar suplementados com Whey Proteins. Res. Soc. Dev. 10(12): e282101220563.
9. Delwatta, S.L., M. Gunatilake, V. Baumans, M.D. Seneviratne, M.L. Dissanayaka, S.S. Batagoda and P.B. Walpola (2018). Reference values for selected hematological, biochemical and physiological parameters of Sprague-Dawley rats at the Animal House, Faculty of Medicine, University of Colombo, Sri Lanka. Anim. Models Exp. Med. 1(4): 250-254.
10. Gorissen, S.H., J.J. Crombag, J.M. Senden, W.H. Waterval, J. Bierau, L.B. Verdijk and L.J. van Loon (2018). Protein content and amino acid composition of commercially available plant-based protein isolates. Amino Acids 50: 1685-1695.
11. Gupta, M.N., M. Perwez and M. Sardar (2020). Protein crosslinking: uses in chemistry, biology and biotechnology. Biocatal. Biotransform. 38(3): 178-201.
12. Haraguchi, F.K., C.L. de Brito Magalhães, L.X. Neves, R.C. dos Santos, M.L. Pedrosa and M.E. Silva (2014). Whey protein modifies gene expression related to protein metabolism affecting muscle weight in resistance-exercised rats. Nutr. 30(7–8): 876-881.

13. Heck, T., G. Faccio, M. Richter and L. Thöny-Meyer (2013). Enzyme-catalyzed protein crosslinking. Appl. Microbiol. Biotechnol. 97: 461-475.
14. Jacob, S., A.B. Nair and M.A. Morsy (2022). Dose conversion between animals and humans: A practical solution. Indian J. Pharm. Educ. Res. 56: 600-607.
15. Keillor, J.W., C.M. Clouthier, K.Y. Apperley, A. Akbar and A. Mulani (2014). Acyl transfer mechanisms of tissue transglutaminase. Bioorg. Chem. 57: 186-197.
16. Kim, C.B., J.H. Park, H.S. Park, H.J. Kim and J.J. Park (2023). Effects of whey protein supplement on 4-week resistance exercise-induced improvements in muscle mass and isokinetic muscular function under dietary control. Nutrients 15(4): 1003.
17. Kurtz, D.M. and G.S. Travlos (Eds.) (2017). The clinical chemistry of laboratory animals. 3rd Ed. CRC Press; Boca Raton (USA). 882 p.
18. Laaksonen, K.S., T.O. Nevalainen, K. Haasio, I.H.E. Kasanen, P.A. Nieminen and H.M. Voipio (2013). Food and water intake, growth, and adiposity of Sprague-Dawley rats with diet board for 24 months. Lab. Anim. 47(4): 245-256.
19. Li, T., M. Liang, J. Luo and X. Peng (2024). Metabolites of Clostridium leptum fermenting flaxseed polysaccharide alleviate obesity in rats. Int. J. Biol. Macromol. 264: 129907.
20. Marapana, R.A.U.J. and B. Jiang (2010). Protein cross-linking in food by microbial transglutaminase (MTGase) and its application & usefulness in food industry – A review. Trop. Agric. Res. Ext. 7.
21. Mehal, W., Suyavaran, A., Dioletis, E., Paiva, R., Secor, E., Weiss, T., ... & Ali, A. (2019). Hematology Safety Data for the Fermented Soy Beverage Q-CAN® Plus (P12-033-19). Current Developments in Nutrition, 3, nzz035-P12. doi: https://doi.org/10.1093/cdn/nzz035.P12-033-19
22. Mitchell, S. J., Scheibye-Knudsen, M., Longo, D. L., & de Cabo, R. (2015). Animal models of aging research: implications for human aging and age-related diseases. Annu. Rev. Anim. Biosci., 3(1), 283-303.
23. Montgomery, D.C. (2019). Introduction to statistical quality control. 8th Ed. John Wiley & Sons; Hoboken, NJ (USA). 768 p.
24. Morsy, M.B., M. Abd ElHameed and I.A. Fathy (2024). The possible modulatory effect of vitamin E administration on submandibular salivary gland of albino rats receiving fat rich diet: A histological and ultrastructural study. Egypt. J. Histol. 47(2): 678-780.
25. Muthukumaran, P. and V.H. Begum (2020). Effect of Poorna Chandrodayam Chendooram (PCM-metallic drug) on lipid profile, liver function and kidney function parameters of rats. Asian J. Pharm. Anal. 10(1): 27-31.
26. Naim, M., J.G. Brand, M.R. Kare, N.A. Kaufmann and C.M. Kratz (1980). Effects of unpalatable diets and food restriction on feed efficiency in growing rats. Physiol. Behav. 25(5): 609-614.
27. Nair, A.B. and S. Jacob (2016). A simple practice guide for dose conversion between animals and human. J. Basic Clin. Pharm. 7(2): 27.
28. Nematizadeh, M., M. Payab, M. Gholami, B. Arjmand, B. Larijani and A. Tayanloo-Beik (2020). Preclinical studies for development of biomedical products. In: Biomedical Product Development: Bench to Bedside. pp. 49-60.
29. Novelli, E.L.B., Y.S. Diniz, C.M. Galhardi, G.M.X. Ebaid, H.G. Rodrigues, F. Mani and J.L.V.B. Novelli Filho (2007). Anthropometrical parameters and markers of obesity in rats. Lab. Anim. 41(1): 111-119.
30. Nunes, R., P. Silva, J. Alves, G. Stefani, M. Petry, C. Rhoden and C.D. Schneider (2013). Effects of resistance training associated with whey protein supplementation on liver and kidney biomarkers in rats. Appl. Physiol. Nutr. Metab. 38(11): 1166-1169.
31. Oliynyk, Z., M. Rudyk, V. Svyatetska, T. Dovbynchuk, G. Tolstanova and L. Skivka (2022). Systemic inflammation biomarkers in 6-OHDA- and LPS-induced Parkinson's disease in rats. Ukr. Biochem. J. 94: 33-43.
32. Phillips, S.M. (2016). The impact of protein quality on the promotion of resistance exercise-induced changes in muscle mass. Nutr. Metab. 13: 1-9.
33. Poullain, M.G., J.P. Cezard, L. Roger and F. Mendy (1989). Effect of whey proteins, their oligopeptide hydrolysates and free amino acid mixtures on growth and nitrogen retention in fed and starved rats. J. Parenter. Enteral Nutr. 13(4): 382-386.
34. Salles, J., C. Guillet, O. Le Bacquer, C. Malnero-Fernandez, C. Giraudet, V. Patrac and S. Walrand (2021). Pea proteins have anabolic effects comparable to milk proteins on whole body protein retention and muscle protein metabolism in old rats. Nutrients 13(12): 4234.
35. Sepandi, M., M. Samadi, H. Shirvani, Y. Alimohamadi, M. Taghdir, F. Goudarzi and I. Akbarzadeh (2022). Effect of whey protein supplementation on weight and body composition indicators: A meta-analysis of randomized clinical trials. Clin. Nutr. ESPEN 50: 74-83.
36. Simoni, R.C., G.F. Lemes, S. Fialho, O.H. Gonçalves, A.M. Gozzo, V. Chiaradia and F.V. Leimann (2017). Effect of drying method on mechanical, thermal and water absorption properties of enzymatically crosslinked gelatin hydrogels. An. Acad. Bras. Ciênc. 89: 745-755.

37. Sulaiman, N.S., M.D. Sintang, H.M. Zaini, E. Munsu, P. Matanjun and W. Pindi (2022). Applications of protein crosslinking in food products. Int. Food Res. J. 29(4): 723-739.
38. Sun, X.D. and S.D. Arntfield (2012). Gelation properties of myofibrillar/pea protein mixtures induced by transglutaminase crosslinking. Food Hydrocoll. 27(2): 394-400.
39. Suwankanit, K. and M. Shimizu (2022). Rat model of quadriceps contracture by joint immobilization. Biology 11(12): 1781.
40. Teixeira, K.R., M.E. Silva, W.G. de Lima, M.L. Pedrosa and F.K. Haraguchi (2016). Whey protein increases muscle weight gain through inhibition of oxidative effects induced by resistance exercise in rats. Nutr. Res. 36(10): 1081-1089.
41. Uy, R. and F. Wold (1977). Introduction of artificial crosslinks into proteins. In: Protein Crosslinking: Biochemical and Molecular Aspects. pp. 169-186.
42. Vanhooren, V. and C. Libert (2013). The mouse as a model organism in aging research: Usefulness, pitfalls and possibilities. Ageing Res. Rev. 12(1): 8-21.
43. Vieira, E.P., A.C. Navarro, A.J.S. Silva and F. Navarro (2021). Efeitos do treinamento resistido e da suplementação com whey proteins sobre os marcadores creatinina e ureia de ratos. Rev. Bras. Fisiol. Exerc. 20(2): 130-140.
44. Volken de Souza, C.F., J. Guimarães Venzke, S. Hickmann Flôres and M.A. Zachia Ayub (2009). Nutritional effects of mechanically deboned chicken meat and soybean proteins cross-linking by microbial transglutaminase. Food Sci. Technol. Int. 15(4): 337-344.
45. von Krziwanek, S., E. Grassmann and M. Kirchgessner (1978). Effect of graduated protein supply (0–50%) on hematologic values as well as protein and urea concentrations in the plasma of rats. Arch. Tierernähr. 28(2): 99-109.
46. Wróblewska, B., J. Juśkiewicz, B. Kroplewski, A. Jurgoński, E. Wasilewska, D. Złotkowska and L. Markiewicz (2018). The effects of whey and soy proteins on growth performance, gastrointestinal digestion, and selected physiological responses in rats. Food Funct. 9(3): 1500-1509.
47. Wu, X., Q. Hou, Z. Zhao, J. Wang, Y. Guo, L. Lu and J. Han (2022). Effects of soy–whey protein nutritional supplementation on hematopoiesis and immune reconstitution in an allogeneic transplanted mice. Nutrients 14(15): 3014.
48. Zhou, J., M.J. Keenan, J.N. Losso, A.M. Raggio, L. Shen, K.L. McCutcheon and R.J. Martin (2011). Dietary whey protein decreases food intake and body fat in rats. Obesity 19(8): 1568-1573.