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# Acute toxicity of caprine alpha S2-casein protein on the microstructures and mineral profiles of rat ileum

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ABSTRACT

**Objective:** To determine the microstructures and mineral profiles of ileum villi in rats treated acutely with caprine αS2-casein (CSN1S2) protein isolated from Etawah goat milk.
**Methods:** Fifty (male and female) rats were divided into 5 groups, including a group of rats-untreated as a control (untreated) and rats-treated with caprine CSN1S2 protein at doses of 500, 1 000, 2 000, and 4 000 mg/kg body weight by oral single dosage. The microstructures were analyzed by scanning electron microscope. The mineral profiles of ileum were measured by scanning electron microscope-energy dispersive X-ray software version 1.7. The degree of ileum perforation was calculated by BoneJ software.
**Results:** The ileum microstructure of the control group showed a smooth and compact surface, while the treating groups showed less compact surface and minimal perforation. The levels of sodium, sulphur and phosphorus were statistically significant higher in treating females at all doses compared to the control ($P < 0.05$). In contrast, the selenium levels were significantly lower in treating females at all doses than that of the control group ($P < 0.05$).
**Conclusions:** This research concludes that the caprine CSN1S2 protein is able to stimulate the ileum toxicity up to 2 000 mg/kg body weight dosage and change sodium, phosphorus, sulphur and selenium homeostasis of ileum in female rats.

## 1. Introduction

Caprine casein protein contains mainly in αS1-, αS2-, β- and κ-casein, of which those types of casein have different properties and bioactive functions[1,2]. Bioactive peptides have potential as antibacterial, immunomodulatory, antioxidant, antithrombotic and antiinflammation activities[3-5]. Casein is potential as well to be developed as a source of nutrients for the body. Casein derived a lot of bioactive peptides that have a health benefit to control molecular and/or cellular signaling mechanism. Recently in our study, we had identified that the molecular weight of caprine αS2-casein (CSN1S2) protein is separately electroporated at 36 kDa, in contrast we did not find in bovine protein. The function of 36 kDa CSN1S2 protein had been characterized as proper. This study reported that CSN1S2 (αS2) protein isolated from Etawah goat milk consists of 223 amino acids and 8 bioactive peptides, so that there are many interactive sites for negatively and/or positively charged molecules[6]. Our previous *in vitro* study reported that CSN1S2 affected the proliferation of preosteblast cells[7]. Furthermore, *in vivo* study also supported the inflammatory effect of this casein on complete Freund's adjuvant induced rheumatoid arthritis rats[8].

Ileum, the final part of the small intestine, performs the function of absorbing nutrients which have been processed by the stomach. The ileum consists of 4 layers: mucosa, sub-mucosa, muscularis propria (oriented smooth muscles fibers) and outer serosa layer. Mucosa epithelium folds to form villi that increase surface area and sub-mucosa embodies glands that secrete digestive enzymes[9]. Observation using scanning electron microscope (SEM) showed that mucosal villi are shaped like fingers which protrude into the lumen. The villous surface is almost entirely covered with enterocytes that act as absorptive cells[10]. Through absorption in the ileum, milk proteins including the CSN1S2 proteins are broken down into

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smaller peptides which are suggested to have a biological function of the target cells[3,6]. As far as we know, there is no study concerning the effect of CSN1S2 milk protein to organ toxicity especially on ileum. Until now, most of the research reported the ileum toxicity effect was limited to bovine caprine αS1-casein milk protein.

Casein in milk can bind elements such as calcium, iron, sodium, cobalt, and zinc[11-13]. Phosphorus and sulfur are common elements that are bound to proteins commonly consumed and are not toxic to the body. However, high level of iron can be toxic. Signs of toxicity develop in dogs ingesting 20–60 mg/kg of elemental iron[14]. A study showed that the $LD_{50}$ for zinc is 28 g/day for humans, which can induce vomit, tachycardia as well as hyperglycemia[15]. It is indicated that copper exposure increases the oxidative stress in *Mytilus galloprovincialis*[16]. Calcium can also be toxic if consumed in large quantities. Excessive intake of calcium may cause hypercalcemia, a high level of calcium in the blood. This condition can cause nausea, vomit, and constipation[17]. Therefore, this study was aimed to evaluate the acute toxicity of CSN1S2 protein isolated from Etawah goat milk on microstructure of ileum and mineral profile using SEM-energy dispersive X-ray (EDX).

## 2. Materials and methods

### 2.1. Animal

Fifty Wistar rats (half male and half female), 8 weeks old, were obtained from the Laboratory of Experimental Animal, Technical Implementation Units, Integrated Research and Testing Laboratory, Gadjah Mada University, Yogyakarta. All rats were acclimatized for 1 week in Biosains Laboratory, Brawijaya University, Malang (conditions prior to experimental manipulation). Rats were exposed to a 12 h light and 12 h dark cycles at room temperature (24 ℃) and had free access to standard laboratory diet and drinking water *ad libitum*. Rats then were randomly divided into 5 groups (each group consisted of 5 male and female rats) as follows: control (untreated) rats at dose of 0 mg/kg body weight ($AMC_0$/$AFC_0$; AMC is acute male casein; AFC is acute female casein), and rats-treated with CSN1S2 protein at doses of 500 mg/kg body weight ($AMC_{500}$/$AFC_{500}$), 1 000 mg/kg body weight ($AMC_{1000}$/$AFC_{1000}$), 2 000 mg/kg body weight ($AMC_{2000}$/$AFC_{2000}$), and 4 000 mg/kg body weight ($AMC_{4000}$/$AFC_{4000}$), respectively. All animal conditions and handling were approved by the Institutional Ethics Committee of Brawijaya University (Ethical Clearance No. 205-KEP-UB).

### 2.2. Preparation and administration of CSN1S2 protein of goat milk

Casein was prepared according previous methods[7]. The rats were fasted overnight before treatment. Single oral dose of CSN1S2 (500, 1 000, 2 000, and 4 000 mg/kg body weight) was dissolved in distilled water according to the previous study with dose modification[18]. The treated group was administered by oral gavage. Animals were monitored for behavioral changes and mortality once a day for 14 days. The rats were then weighed, euthanized, and necropsied after 14 days. The ileum was taken and subsequently stored at -80 ℃ prior to SEM analysis.

### 2.3. Analysis of ileum microstructure and mineral profile

The preparation of rat ileum for microstructure and mineral profile was using standard protocol with some modifications[10]. Ileum was coated with gold prior to SEM analysis. The ileum microstructures of rats were analyzed using SEM (Hitachi TM-3000 table top). Mineral profile was measured using EDX spectroscopy (SEM-EDX) software version 1.7. The size of ileum perforation was evaluated by the BoneJ software.

### 2.4. Statistical analysis

Data were presented as mean ± SD and differences among groups were analyzed using ANOVA test with SPSS 16.0 software. $P$-value < 0.01 was considered statistically significant for perforation and $P$ < 0.05 for mineral profile.

## 3. Results

### 3.1. Ileum microstructure

The microstructure of ileum mucosa in control and CSN1S2-treated group had different structure characterization (Figure 1). There was a compact and smooth surface of the ileum in the both gender control groups. The first dose of CSN1S2 protein ($AMC_{500}$/$AFC_{500}$) of goat milk induced minimal changes of ileum microstructure which is characterized by a compact texture with small perforations. The second dose of CSN1S2 protein ($AMC_{1000}$/$AFC_{1000}$) were reduced the cell integrity. The ileum structures with massive perforation and less compact surface were found in the two highest doses of CSN1S2 protein, $AMC_{2000}$/$AFC_{2000}$ and $AMC_{4000}$/$AFC_{4000}$ rat groups. There was similar pattern of ileum microstructure between females and males.

### 3.2. Perforation

Interestingly, the size of ileum perforation was significantly higher in the highest dose of CSN1S2 protein of goat milk compared to the control group ($P$ < 0.01) as presented in Figure 2. This was found in both sexes.

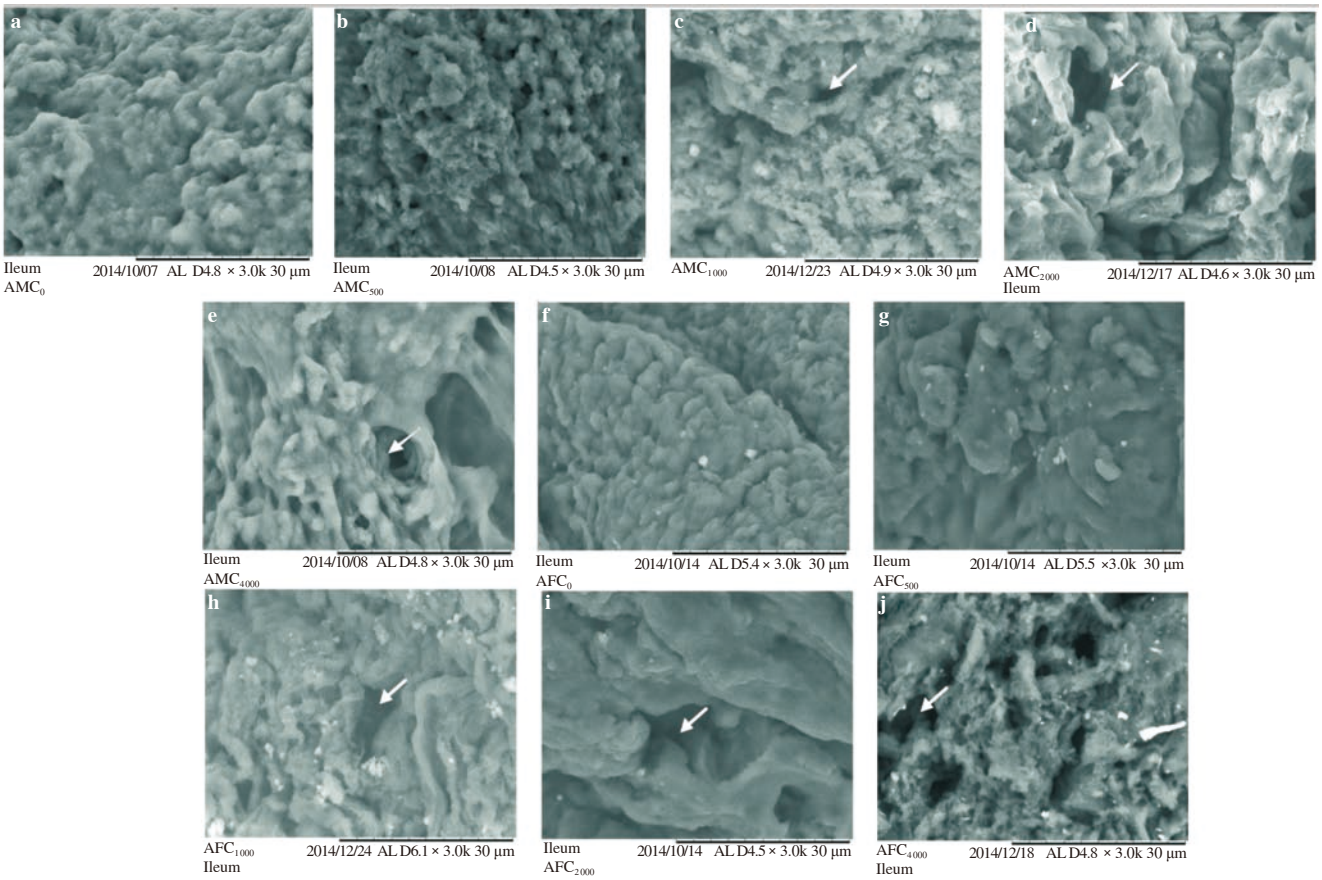


**Figure 1.** The microstructure of ileum mucosa in untreated and CSN1S2-treated groups (SEM, magnification × 3 000).
(a) $AMC_0$: Control male rats; (b) $AMC_{500}$: Male rats administered with CSN1S2 at dose of 500 mg/kg body weight; (c) $AMC_{1000}$: Male rats administered with CSN1S2 at dose of 1 000 mg/kg body weight; (d) $AMC_{2000}$: Male rats administered with CSN1S2 at dose of 2 000 mg/kg body weight; (e) $AMC_{4000}$: Male rats administered with CSN1S2 at dose of 4 000 mg/kg body weight; (f) $AFC_0$: Control female rats; (g) $AFC_{500}$: Female rats administered with CSN1S2 at dose of 500 mg/kg body weight; (h) $AFC_{1000}$: Female rats administered with CSN1S2 at dose of 1 000 mg/kg body weight; (i). $AFC_{2000}$: Female rats administered with CSN1S2 at dose of 2 000 mg/kg body weight; (j) $AFC_{4000}$: Female rats administered with CSN1S2 at dose of 4 000 mg/kg body weight. White arrow is perforation.

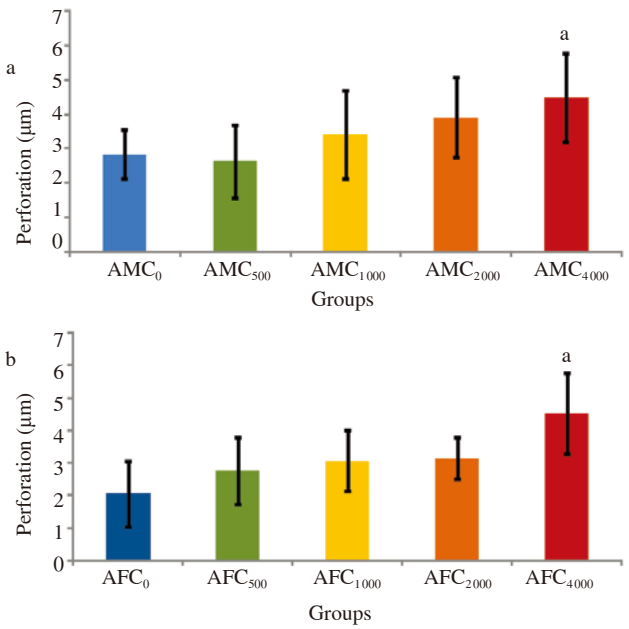


**Figure 2.** The size of ileum perforation was analyzed by BoneJ software. Values were presented as mean ± SD. [a]: $P < 0.01$ was considered statistically significant compared with the control group.

### *3.3. Mineral profiles*

In the ileum of male rats, the levels of carbon, oxygen, sodium, magnesium, aluminum, phosphorus, sulfur, calcium, vanadium, iron, cobalt, nickel, copper, arsenic, selenium, cadmium, and ytterbium were unfortunately not significant ($P > 0.05$) different among groups, as presented in Table 1.

The mineral profiles in ileum of female rats were presented in Table 2. The levels of sodium were significantly ($P < 0.05$) greater in the first (1.360 ± 0.494)%, second (1.206 ± 1.251)%, and fourth (0.940 ± 0.506)% doses compared to the control group (0.013 ± 0.026)%. The phosphorus levels were significantly ($P < 0.05$) higher in the first (1.107 ± 0.500)% and third (1.112 ± 0.937)% doses than that of the control group (0.073 ± 0.046)%. The levels of sulfur were significantly ($P < 0.05$) higher in all doses of treatment compared to the control group.

Among all doses of CSN1S2 protein of goat milk, only the lowest (0.048 ± 0.077)% and the highest (0.058 ± 0.081)% doses significantly ($P < 0.05$) decreased the selenium levels that was lower than the control group (0.265 ± 0.193)%.

**Table 1**
Mineral profiles of ileum male rats treated with acutely CSN1S2 protein of goat milk.

| Mineral (%) | $AMC_0$ | $AMC_{500}$ | $AMC_{1000}$ | $AMC_{2000}$ | $AMC_{4000}$ |
|---|---|---|---|---|---|
| Carbon | 72.710 ± 11.353 | 75.000 ± 10.131 | 76.405 ± 9.127 | 76.737 ± 9.597 | 82.981 ± 11.167 |
| Oxygen | 23.760 ± 11.271 | 19.993 ± 11.913 | 18.842 ± 10.050 | 18.799 ± 9.647 | 13.108 ± 12.110 |
| Sodium | 1.141 ± 0.704 | 0.736 ± 0.417 | 1.300 ± 0.444 | 0.999 ± 0.518 | 0.729 ± 0.489 |
| Magnesium | 0.021 ± 0.032 | 0.101 ± 0.073 | 0.087 ± 0.069 | 0.069 ± 0.089 | 0.058 ± 0.051 |
| Aluminum | 0.038 ± 0.040 | 0.478 ± 0.880 | 0.075 ± 0.109 | 0.057 ± 0.064 | 0.076 ± 0.033 |
| Phosphorus | 0.970 ± 0.602 | 1.107 ± 0.494 | 1.154 ± 0.265 | 0.802 ± 0.401 | 0.982 ± 0.491 |
| Sulphur | 0.527 ± 0.152 | 0.897 ± 0.359 | 0.700 ± 0.223 | 0.618 ± 0.243 | 0.679 ± 0.218 |
| Calcium | 0.199 ± 0.132 | 0.458 ± 0.118 | 0.091 ± 0.095 | 0.249 ± 0.155 | 0.377 ± 0.577 |
| Vanadium | 0.036 ± 0.035 | 0.058 ± 0.101 | 0.139 ± 0.168 | 0.129 ± 0.162 | 0.162 ± 0.048 |
| Iron | 0.162 ± 0.275 | 0.192 ± 0.343 | 0.227 ± 0.207 | 0.505 ± 0.520 | 0.152 ± 0.186 |
| Cobalt | 0.065 ± 0.116 | 0.026 ± 0.064 | 0.059 ± 0.096 | 0.027 ± 0.054 | 0.067 ± 0.105 |
| Nickel | 0.013 ± 0.033 | 0.566 ± 1.106 | 0.406 ± 0.705 | 0.307 ± 0.400 | 0.139 ± 0.023 |
| Copper | 0.148 ± 0.201 | 0.068 ± 0.081 | 0.185 ± 0.307 | 0.233 ± 0.325 | 0.101 ± 0.129 |
| Arsenic | 0.034 ± 0.068 | 0.069 ± 0.084 | 0.034 ± 0.054 | 0.042 ± 0.045 | 0.102 ± 0.126 |
| Selenium | 0.050 ± 0.072 | 0.114 ± 0.105 | 0.155 ± 0.202 | 0.092 ± 0.093 | 0.068 ± 0.052 |
| Cadmium | 0.067 ± 0.085 | 0.104 ± 0.142 | 0.063 ± 0.085 | 0.120 ± 0.124 | 0.084 ± 0.071 |
| Ytterbium | 0.047 ± 0.120 | 0.149 ± 0.159 | 0.189 ± 0.313 | 0.352 ± 0.362 | 0.171 ± 0.108 |

Values were presented as mean ± SD; $AMC_0$: Male control group; $AMC_{500}$: Male dosage 500 mg/kg body weight; $AMC_{1000}$: Male dosage 1000 mg/kg body weight; $AMC_{2000}$: Male dosage 2000 mg/kg body weight; $AMC_{4000}$: Male dosage 4000 mg/kg body weight.

**Table 2**
Mineral profiles of ileum female rats treated with acutely CSN1S2 protein of goat milk.

| Mineral (%) | $AFC_0$ | $AFC_{500}$ | $AFC_{1000}$ | $AFC_{2000}$ | $AFC_{4000}$ |
|---|---|---|---|---|---|
| Carbon | 97.441 ± 1.020 | 65.191 ± 4.768[a] | 77.233 ± 18.152[a] | 78.431 ± 16.608 | 75.907 ± 11.247[a] |
| Oxygen | 0.000 ± 0.000 | 30.553 ± 5.569[a] | 18.701 ± 17.410[a] | 16.542 ± 15.605 | 19.984 ± 10.250[a] |
| Sodium | 0.013 ± 0.026 | 1.360 ± 0.494[a] | 0.812 ± 0.622 | 1.206 ± 1.251[a] | 0.940 ± 0.506[a] |
| Magnesium | 0.060 ± 0.053 | 0.020 ± 0.023 | 0.080 ± 0.115 | 0.042 ± 0.042 | 0.007 ± 0.012 |
| Aluminum | 0.136 ± 0.098 | 0.026 ± 0.025 | 0.089 ± 0.072 | 0.070 ± 0.087 | 0.039 ± 0.046 |
| Phosphorus | 0.073 ± 0.046 | 1.107 ± 0.500[a] | 0.727 ± 0.453 | 1.112 ± 0.937[a] | 0.784 ± 0.544 |
| Sulphur | 0.094 ± 0.103 | 0.574 ± 0.247[a] | 0.635 ± 0.324[a] | 0.489 ± 0.272[a] | 0.519 ± 0.246[a] |
| Calcium | 0.270 ± 0.298 | 0.369 ± 0.271 | 0.636 ± 0.337 | 0.405 ± 0.298 | 0.332 ± 0.180 |
| Vanadium | 0.128 ± 0.166 | 0.057 ± 0.098 | 0.086 ± 0.109 | 0.040 ± 0.099 | 0.030 ± 0.066 |
| Iron | 0.192 ± 0.266 | 0.030 ± 0.061 | 0.017 ± 0.029 | 0.280 ± 0.478 | 0.188 ± 0.196 |
| Cobalt | 0.171 ± 0.302 | 0.080 ± 0.080 | 0.197 ± 0.235 | 0.129 ± 0.155 | 0.080 ± 0.166 |
| Nickel | 0.729 ± 0.858 | 0.082 ± 0.141 | 0.249 ± 0.375 | 0.535 ± 0.836 | 0.487 ± 1.103 |
| Copper | 0.000 ± 0.000 | 0.302 ± 0.238 | 0.131 ± 0.301 | 0.065 ± 0.174 | 0.376 ± 0.485 |
| Arsenic | 0.103 ± 0.088 | 0.038 ± 0.062 | 0.091 ± 0.060 | 0.144 ± 0.120 | 0.090 ± 0.114 |
| Selenium | 0.265 ± 0.193 | 0.048 ± 0.077[a] | 0.123 ± 0.107 | 0.171 ± 0.115 | 0.058 ± 0.081[a] |
| Cadmium | 0.059 ± 0.135 | 0.044 ± 0.066 | 0.111 ± 0.188 | 0.080 ± 0.147 | 0.063 ± 0.080 |
| Ytterbium | 0.259 ± 0.243 | 0.111 ± 0.166 | 0.073 ± 0.085 | 0.254 ± 0.233 | 0.120 ± 0.130 |

Values were presented as mean ± SD; [a]: $P < 0.05$ in comparison with control group; $AFC_0$: Female control group; $AFC_{500}$: Female dosage 500 mg/kg body weight; $AFC_{1000}$: Female dosage 1000 mg/kg body weight; $AFC_{2000}$: Female dosage 2000 mg/kg body weight; $AFC_{4000}$: Female dosage 4000 mg/kg body weight.

## 4. Discussion

Nutrient-derived proteins and peptides may play important roles in the intestinal tract before being hydrolyzed into amino acid residues and absorbed into the bloodstream. The proteins and peptides induce the regulation of digestive enzymes and modulation of nutrient absorption in the intestinal tract[3]. The ileum is the final section of small intestine. As a part of the gastrointestinal tract, ileum has a function as a nutrients absorber. This role is supported by the epithelium lining the ileum which provides protection and selective barrier between nutrients and body tissues[19].

In this study, we found that the ileum of male and female rats gave the similar response to the CSN1S2 protein of goat milk. The ileum in male control rats showed a compact and a smooth surface. On the contrary, the ileum in the two highest doses of CSN1S2 protein in both sexes showed massive perforation and less compact surface. Moreover, there was a positive correlation between the surface structure as well as the perforation size of ileum and the dose of CSN1S2 protein of goat milk. Our finding indicated that the CSN1S2 protein of goat milk induces the changes in ileum morphology, including the surface structure and formation of perforation. The size of ileum perforation was significantly higher in the highest dose of CSN1S2 protein of goat milk than that of the control group ($P < 0.01$). This finding indicated that the CSN1S2 protein of goat milk at dose of not more than 2000 mg/kg body weight was able to repair the ileum perforation. Meanwhile, the ileum repairing mechanisms fail at higher dose. This result is not accordance with the result of previous study that the administration of caprine αS1-casein to the rats at dose of 2000 mg/kg body weight had no effect on intestinal histopathology[18].

Sodium is absorbed in jejunum, ileum, and also colon[20,21]. Sodium can be absorbed through the enterocyte by cotransport with other mineral and one of them is phosphorus[22]. The levels of sodium were significantly ($P < 0.05$) greater in female rats given the first,

second, and fourth doses of CSN1S2 protein compared to the control group. Moreover, phosphorus levels were significantly ($P < 0.05$) higher in female rats given the first and third doses of CSN1S2 protein than that of the control group. Our finding indicated that at the lower dose of the CSN1S2 protein, the phosphorus acts as cotransport for sodium. However, in the second, third, and fourth doses of CSN1S2 protein of goat milk, we hypothesized there is substitution mechanism for sodium and phosphorus.

Otherwise, the CSN1S2-treated group at doses of 500 and 4 000 mg/kg body weight showed a significant ($P < 0.05$) reducing in selenium comparing with the control. On the contrary, the levels of sulfur were significantly ($P < 0.05$) elevating in all doses of treatment except in the control group. Selenium and sulfur compete each other to enter the enterocyte[23]. The CSN1S2 protein of goat milk contains methionine amino acid residue, in which selenium can be replaced for sulfur[7]. Moreover, goat milk casein can also act as chelating agent that is capable of binding to the metal ions[4,24]. We hypothesized that the presence of goat milk CSN1S2 protein may induce the replacement of sulfur with selenium in methionine residue in the ileum.

In conclusion, based on the analysis of ileum microstructures and mineral profile, we concluded that the maximum recommended safety dosage of CSN1S2 protein isolated from Ethawah breed goat milk is 2 000 mg/kg body weight.

## Conflict of interest statement

We declare that we have no conflict of interest.

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