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# Rumen fermentation, methane production, and microbial composition following *in vitro* evaluation of red ginseng byproduct as a protein source

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## Abstract

The main objective of this *in vitro* study was to evaluate red ginseng byproduct (RGP) as a protein resource and its effects on rumen fermentation characteristics, microflora, $CO_2$, and $CH_4$ production in ruminants. Four treatments for *in vitro* fermentation using buffered rumen fluid over a 48 h incubation period were used: 1, RGP; 2, corn gluten feed (CGF); 3, wheat gluten (WG); and 4, corn germ meal. *In vitro* dry matter digestibility (IVDMD), *in vitro* neutral detergent fiber digestibility (IVNDFD), *in vitro* crude protein digestibility (IVCPD), volatile fatty acids, pH, and ammonia nitrogen ($NH_3$-N) were estimated after 48 h incubation. Gas production was investigated after 3, 6, 12, 24, 36 and 48 h. The $CO_2$ and $CH_4$ were evaluated after 12, 24, 36, and 48 h. A significant difference in total gas production and $CO_2$ emissions was observed ($p < 0.01$) at all incubation times. $CH_4$ production in RGP were higher ($p < 0.05$) than that in other treatments but a higher $CH_4$ portion in the total gas production was observed in WG ($p < 0.05$) at 48 h incubation. The IVDMD, IVNDFD, and IVCPD of RGP was lower than those of other conventional ingredients ($p < 0.01$). The RGP had the lowest $NH_3$-N value among the treatments ($p < 0.01$). The RGP also had the lowest total VFA concentration ($p < 0.01$), but presented the highest acetate proportion and acetate to propionate ratio among the treatments (both, $p < 0.01$). The abundance of *Prevotella ruminicola* was higher in RGP than in WG ($p < 0.01$), whereas RGP has lower methanogenic archaea ($p < 0.01$). In conclusion, based on the nutritive value, IVDMD, low $NH_3$-N, and decreased methanogenic archaea, RGP inclusion as a protein source in ruminant diets can be an option in replacing conventional feed sources.

**Keywords:** Red ginseng byproduct, *In vitro* fermentation, Methane production, Microbial community

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The protocols for this study regarding animal use were reviewed and accepted by the Animal Research Ethics Committee of Pusan National University (Pusan, Korea, PNU-2019-2239).

# INTRODUCTION

The broad gap between demand and feed supply in Korea calls for increased nutrient production from present feed supplies or by exploiting new non-conventional feed resources for livestock feed. These non-conventional feed resources include agro-industrial waste, seed residues, and certain by-products from the animal feed industry [1]. South Korea recycles nearly 95% of its food waste by-products for use as fertilizer and animal feed [2]. When different types of cereal grains are processed for consumers, part of the grain is removed and the byproduct is used primarily in animal feed [3]. Most of the cereal byproducts are higher in nutritional value than the original grains in terms of protein, fat, and fiber content. Corn gluten feed (CGF), wheat gluten (WG), and corn germ meal (CGM) have been widely used in the ruminant diets as protein supplements that provide not only adequate nutrition but also riboflavin, vitamin A, and vitamin E to ruminants [4].

Ginseng (*Panax ginseng*) is a traditional herb that is used as a medicine and has been known for its beneficial effects on human health for high blood pressure, stress and as a cancer cure for over 2,000 years, especially in China and Korea [5]. Since the 1990s, approximately 500 MT of ginseng meal have been produced every year in Korea [6], but recent figures are unknown. After the solubles have been extracted with solvents such as water or alcohol (70%–75%), a residue known as ginseng meal is formed. The ginseng meal residue contains 16.06% crude protein (CP), 2.0% ether extract (EE), 45% neutral detergent fiber (NDF), and 60% nitrogen-free extract on a dry matter (DM) basis [3]. It also contains saponins, ginsenosides, essential oils, peptides, vitamins, and polysaccharides [7], and studies have revealed an improvement in the milk yield and milk composition of dairy cows [1] and improved the egg production in laying hens [8] that were fed the residue. Replacement of alfalfa hay (0%–15%) with ginseng meal showed that *in vitro* DM digestibility (IVDMD) was not affected, but CP digestibility decreased without affecting the production of volatile fatty acids (VFAs), indicating that ginseng meal could be used as a ruminant feed resource. Many Korean farmers use medicinal herbs, including ginseng meals, to improve the production performance and product quality in ruminants [6].

In the livestock industry, the use of food byproducts is of great interest, especially if it pertains to a reduction in feed costs [9]. A large proportion of red ginseng byproduct (RGP) is continuously being produced as the market for red ginseng continues to expand in Korea, but most of it is wasted [7]. To the best of our knowledge, this is the first study to explore the potential of RGP as a protein feed resource for ruminant diets. Because RGP includes certain bioactive compounds [8] and has the potential to mitigate $CH_4$ and $CO_2$ production from large volumes of waste each year, we investigated the feed value of RGP as a potential substitute for conventional protein sources, and the effects on *in vitro* rumen fermentation, microflora, $CO_2$, and $CH_4$ production in ruminant diets.

# MATERIALS AND METHODS

Animal use and experimental protocols were studied and accepted by the Animal Research Ethics Committee of Pusan National University (PNU-2019-2239).

## Feed preparation and chemical analysis

The chemical constituents of the protein byproducts of the experimental feeds are presented in Table 1. Before performing the chemical analyses, feed protein byproducts were air dried at 60 ℃ for 96 h and ground through a cyclone mill (Foss Tecator Cyclotec 1093, Foss, Hillerød, Denmark) fitted with a 1 mm screen. The DM (#934.01), CP (#976.05), EE (#920.39), acid detergent fiber (ADF, #973.18), and ash (#942.05) were analyzed according to the relevant Association of Official


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**Table 1. Estimated chemical composition (% dry matter basis or as stated) of experimental diets used in *in vitro* incubation of four protein byproduct raw materials**

| Items | Treatments | | | |
|---|---|---|---|---|
| | RGP | CGF | WG | CGM |
| Chemical composition | | | | |
| DM | 95.95 | 90.61 | 87.97 | 90.98 |
| CP (%DM) | 15.03 | 20.87 | 14.97 | 19.03 |
| NDF (%DM) | 38.62 | 45.50 | 44.40 | 47.30 |
| ADF (%DM) | 31.64 | 9.41 | 11.77 | 12.53 |
| EE (%DM) | 1.22 | 3.62 | 3.60 | 6.93 |
| Ash (%DM) | 3.11 | 5.32 | 4.65 | 1.70 |
| NFC (%DM) | 42.02 | 24.69 | 32.38 | 25.04 |

RGP, red ginseng byproduct; CGF, corn gluten feed; WG, wheat gluten; CGM, corn germ meal; DM, dry matter; CP, crude protein; NDF, neutral detergent fiber analyzed with heat- stable α-amylase; ADF, acid detergent fiber; EE, ether extract; NFC, non-fibrous carbohydrate.

Analytical Chemists methods [10]. Kjeldahl analysis yielded the total amount of nitrogen using a nitrogen combustion analyzer (Leco FP-528 Leco, St. Joseph, MI, USA), which was multiplied by 6.25 to determine the CP content. Ash-free NDF (aNDF) and lignin were analyzed [11] to measure the fiber content. Heat-stable amylase ( $\alpha$ -amylase) was used to estimate aNDF and was expressed inclusive of residual ash. Non-fibrous carbohydrate (NFC) of the experimental diets was estimated as follows:

$$NFC = [100 - ash - EE - CP - aNDF] \quad \text{(Eq. 1)}$$

## *In vitro* fermentation

Rumen fluid was used for the *in vitro* incubation and was collected from two fistulated Holsteins (body weight [BW] 450 ± 30 kg), before they were fed the morning feed at the Center for Agriculture Research, Pusan National University (Miryang, Korea). The fistulated cattle were completely adapted to a diet containing 600 g/kg Timothy hay and 400 g/kg of a commercial concentrate mix. Rumen fluid was collected in a thermos flask and immediately transferred to the laboratory within 30 min. Eight layers of cheese cloth were used to filter rumen fluid, which was then thoroughly mixed with 4× volume of *in vitro* rumen buffer solution [12] under strict anaerobic conditions. The ground, dietary protein byproduct feed substrates (0.5 g) were placed into pre-weighed nylon bags (R510, Ankom Technology, Macedon, NY, USA). All the bags were heat-sealed and transferred into empty 125 mL serum bottles. Four bottles were used per dietary protein byproduct treatment, and each bottle contained two bags. Then, 70 mL of the buffered rumen fluid was transferred into the serum bottles, with continuous flushing of $O_2$-free, $CO_2$ gas. The serum bottles were sealed with butyl rubber stoppers and aluminum caps and incubated on a rotary shaker (JSSI-300T, JS Research, Gongju, Korea) at 20 rpm for 48 h incubation at 39℃. After incubation for 48 h, the IVDMD, *in vitro* NDF digestibility (IVNDFD), pH, ammonia nitrogen ($NH_3$-N), and VFA concentrations were analyzed. Gas production was measured at 3, 6, 12, 24, 36, and 48 h by using a pressure transducer (Sun Bee Instrument, Seoul, Korea) as described by Theodorou et al. [13]. Gas samples for $CO_2$ and $CH_4$ were analyzed using a gas chromatograph-mass spectrometer (GS-MS QP2020, Shimadzu, Japan), fitted with Porapak Q packed columns and $CO_2$ and $CH_4$ (electron capture detector [ECD]) detectors. The carrier gas helium was pumped through the column at a flow rate of 4.25 mL/min. The temperatures for the injector, column oven, and ECD were 200℃,

35℃, and 250℃, respectively. The sample dilution for $CO_2$ and $CH_4$ at 200 and 2000 times was done with helium for analyses.

After 48 h of *in vitro* incubation, the bottle caps were removed, and the serum bottles were transferred to ice to stop the microbial fermentation. Subsequently, the nylon bags were then removed from the bottles and rinsed under flowing water until the water ran clear. The washed bags were then dried at 60℃ for 72 h and weighed to measure IVDMD. The aNDF content of the weighed bags was assessed using an improved version of the micro-NDF method to evaluate the IVNDFD. The *in vitro* CP digestibility (IVCPD) was estimated using Kjeldahl nitrogen analysis. The sample fluid (1.8 mL) was centrifuged at 20,000×g for 20 min at 4℃, and then the supernatant was removed. The remaining pellet was stored at −80℃ until microbial DNA extraction for the rumen microbial population analysis. The remaining culture fluid (about 50 mL) was transferred to a centrifugal tube for 15,000×g and 4℃ for 10 min. The supernatant was collected for the determination of pH, VFA concentration, and $NH_3$-N concentration.

The pH of the culture fluid was measured using a pH meter (FP20, Mettler Toledo, Worthington, OH, USA). The supernatant for the VFA analysis was acidified with 200 μL of 25% meta-phosphoric acid, and the supernatant for the $NH_3$-N analysis was acidified with 200 μL of 0.2 M sulfuric acid; both were stored at −20℃ until VFA and $NH_3$-N analyses. Then, 200 μL of the supernatant was diluted with 800 μL of anhydrous ethyl alcohol (4023-2304, Daejung Chemicals, Siheung, Korea) after 15 min of centrifugation at 20,000×g. VFAs were measured via gas chromatograph (Agilent 7890A, Agilent Technology, Santa Clara, CA, USA) equipped with a flame ionization detector and capillary column (Nukol ™ Fused silica capillary column, 30 m × 250 μm × 0.25 μm, Supelco, Bellefonte, PA, USA). The temperature of the oven, injector, and detector was set at 90℃, 90℃–200℃, and 230℃, respectively. Nitrogen was used as the carrier gas at a flow rate of 30 mL/min. The $NH_3$-N concentration was analyzed with several modifications [14]. Briefly, the supernatant of centrifuged rumen fluid (2 μL) at 20,000×g for 20 min at 4℃ was mixed with phenol color reagent (100 μL, 50 g of phenol, 0.25 g of sodium nitroferricyanide, and 1 L of distilled water) and alkali hypochlorite (100 μL, 25 g of sodium hydroxide, 16.8 mL of sodium hypochlorite, and 1 L of distilled water). The mixture was then incubated at 37℃ for 15 min in a water bath. The $NH_3$-N concentration was determined by measuring the optical density at 630 nm using a microplate reader (iMARK, Bio-Rad, Hercules, CA, USA).

## Microbial genomic DNA extraction and quantitative polymerase chain reaction

Total genomic DNA was extracted from the pellet stored at −80℃ using the repeated bead beating plus column (RBB+C) method [15]. Genomic DNA was treated with RNase A and proteinase K and purified using columns from the DokDo-Prep Genomic DNA Kit (Elpis-Biotech, Daejeon, Korea). The DNA concentration and purity were evaluated with a NanoDrop (ND-1000, Thermo Fisher, Waltham, MA, USA). Quantitative PCR assays were performed on a CFX 96 Touch system (Bio-Rad Laboratories). The primers used in this study are represented in Table 2 and information on primer sequences for rumen microbes was collected from previous studies [16,17]. Each reaction mixture volume was 20 μL containing 10× buffer (2 μL, BioFACT, Daejeon, Korea), 10 mM dNTP mixture (0.5 μL, BioFACT, Daejeon, Korea), 10-fold diluted genomic DNA (1 μL), 10 μM primer-set (each 1 μL), taq polymerase (0.1 μL, BioFACT, Daejeon, Korea), Evagreen (1 μL, SolGent, Daejeon, Korea), and bio-grade water (13.4 μL). All the reactions were conducted in triplicate. The protocol conditions of qPCR were as follows: initial denaturation of DNA at 95℃ for 10 min, and 40 cycles of denaturation at 95℃ for 30 s, annealing at 60℃ for 30 s, and extension at 72℃ for 30 s, followed by a final extension at 72℃ for 5 min. At the end of the extension stage, fluorescence was noted. Amplicon specificity was checked with the dissociation curve by increasing


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**Table 2.** Primers used for quantitative PCR

| Target species | Primer | Sequence (5' → 3') | Size (bp) | Efficiency[1)] | References |
|---|---|---|---|---|---|
| General bacteria | F | CGGCAACGAGCGCAACCC | 130 | 1.90 | [16] |
| | R | CCATTGTAGCACGTGTGTAGCC | | | |
| Ciliate protozoa | F | GCTTTCGWTGGTAGTGTATT | 223 | 1.89 | [35] |
| | R | CTTGCCCTCYAATCGTWCT | | | |
| Methanogenic archaea | F | GAGGAAGGAGTGGACGACGGTA | 232 | 1.92 | [35] |
| | R | ACGGGCGGTGTGTGCAAG | | | |
| *Prevotella ruminicola* | F | GCGAAAGTCGGATTAATGCTCTATG | 78 | 1.91 | [35] |
| | R | CCCATCCTATAGCGGTAAACCTTTG | | | |

[1)]Efficiency is calculated as [$10^{-1/slope}$].

PCR, polymerase chain reaction; bp, base pair.

the temperature at a rate of 1℃ per 30 s, from 60℃ to 95℃. A standard plasmid having the respective target sequence was used for absolute quantification of each microbe. The copy number of each standard primer was calculated as described by [18] and diluted in 10-fold serial dilutions. The CFX manager software (Bio-Rad) was used to compare microbe quantifications with the standard curve.

### Statistical analysis

Experimental protein byproduct treatments were evaluated using a complete randomized block design, with treatment as the main effect. The four experimental protein byproduct treatments were as follows: (1) RGP, (2) CGF, (3) WG, and (4) CGM. Statistical data analysis was carried out using the PROC GLIMMIX procedure (SAS 9.3; SAS Institute, Cary, NC, USA). Tukey's range test was used to compare differences among treatments. Statistical significance was set at $p < 0.05$, and a trend was assumed at $0.05 < p < 0.10$.

## RESULTS

*In vitro* fermentation resulted in higher total gas production ($p < 0.01$) for RGP and CGM than for CGF and WG during the 48 h of incubation. The CGF had a higher ($p < 0.01$) gas production at 3 h, and WG was higher ($p < 0.01$) from 3 to 24 h. RGP and CGM had higher ($p < 0.01$) and CGF and WG had lower ($p < 0.01$) $CO_2$ production values at 48 h. Similarly, the production of $CH_4$ was higher ($p < 0.01$) for RGP than CGM but comparable with that for CGF and WG after 48 h incubation. The $CO_2$ and $CH_4$ ratios of the total production of gas during the *in vitro* incubation and the $CO_2$ ratio for all treatments was similar ($p = 0.45$). However, the $CH_4$ ratio is the relative value based on total gas production, was higher ($p < 0.05$) for WG than for the other treatments (Table 3). The RGP had significantly lower IVDMD than WG ($p < 0.01$) but was similar to that of CGF and CGM ($p < 0.01$). The IVNDFD of RGP and CGF was higher than that of WG but lower than that of CGM ($p < 0.01$). The IVCPD and $NH_3$-N of RGP were the lowest ($p < 0.01$) among the treatments. Similarly, pH of RGP was higher ($p < 0.01$) than CGM but lower ($p < 0.01$) than WG and CGF. Moreover, RGP had the lowest ($p < 0.05$) total VFA concentration, butyrate, iso-butyrate, valerate, and iso-valerate proportions ($p < 0.01$) and had the highest (both; $p < 0.01$) acetate proportion and acetate to propionate ratio among the treatments (Table 4). In the microbial population, there were no changes in the total bacteria ($p = 0.31$) or ciliate protozoa ($p = 0.19$). The use of RGP decreased ($p < 0.01$) the absolute abundance of methanogenic archaea, whereas *Prevotella ruminicola* was higher ($p < 0.01$) in RGP than in WG (Table 5).

**Table 3.** Gas production after *in vitro* incubation of treatments using buffered rumen fluid

| Item | Treatment | | | | SEM | *p*-value |
|---|---|---|---|---|---|---|
| | RGP | CGF | WG | CGM | | |
| Total gas (mL/g DM) | | | | | | |
| 3 h | 10.7$^{b}$ | 14.2$^{a}$ | 13.5$^{a}$ | 9.0$^{c}$ | 0.41 | < 0.01 |
| 6 h | 34.5$^{a}$ | 28.6$^{b}$ | 33.8$^{a}$ | 27.4$^{b}$ | 0.56 | < 0.01 |
| 12 h | 78.4$^{a}$ | 55.6$^{c}$ | 75.3$^{ab}$ | 72.2$^{b}$ | 1.91 | < 0.01 |
| 24 h | 152.0$^{a}$ | 118.9$^{b}$ | 145.0$^{a}$ | 152.7$^{a}$ | 3.44 | < 0.01 |
| 36 h | 199.2$^{a}$ | 170.4$^{c}$ | 187.2$^{b}$ | 200.7$^{a}$ | 2.79 | < 0.01 |
| 48 h | 224.9$^{a}$ | 207.8$^{b}$ | 211.9$^{b}$ | 227.2$^{a}$ | 2.48 | < 0.01 |
| $CO_2$ (mL/g DM) | | | | | | |
| 12 h | 62.54$^{a}$ | 48.02$^{b}$ | 65.25$^{a}$ | 61.25$^{a}$ | 3.592 | < 0.01 |
| 24 h | 126.81$^{a}$ | 98.94$^{b}$ | 121.25$^{a}$ | 128.25$^{a}$ | 5.783 | < 0.01 |
| 36 h | 156.06$^{a}$ | 130.64$^{b}$ | 145.62$^{a}$ | 153.08$^{a}$ | 4.022 | < 0.01 |
| 48 h | 171.59$^{a}$ | 152.48$^{b}$ | 160.69$^{ab}$ | 171.58$^{a}$ | 4.448 | < 0.01 |
| $CO_2$ ratio[1)] | 76.28 | 73.36 | 75.83 | 76.35 | 2.033 | 0.45 |
| $CH_4$ (mL/g DM) | | | | | | |
| 12 h | 4.02$^{a}$ | 2.5$^{b}$ | 4.04$^{a}$ | 3.34$^{a}$ | 0.247 | < 0.01 |
| 24 h | 8.82$^{a}$ | 6.62$^{b}$ | 9.18$^{a}$ | 8.62$^{a}$ | 0.483 | < 0.01 |
| 36 h | 11.83$^{a}$ | 10.02$^{b}$ | 11.71$^{a}$ | 10.89$^{ab}$ | 0.382 | < 0.01 |
| 48 h | 13.40$^{a}$ | 12.26$^{ab}$ | 13.13$^{ab}$ | 12.11$^{b}$ | 0.439 | < 0.05 |
| $CH_4$ ratio[1)] | 5.95$^{b}$ | 5.90$^{b}$ | 6.19$^{a}$ | 5.53$^{b}$ | 0.156 | < 0.05 |

$^{a-d}$Values in the same row with different letters differ significantly ($p < 0.05$).

[1)]$CO_2$ and $CH_4$ ratio as portion of total gas produced.

RGP, red ginseng byproduct; CGF, corn gluten feed; WG, wheat gluten; CGM, corn germ meal.

**Table 4.** Fermentation characteristics after *in vitro* incubation of treatments using buffered rumen fluid

| Item | Treatment | | | | SEM | *p*-value |
|---|---|---|---|---|---|---|
| | RGP | CGF | WG | CGM | | |
| IVDMD (%) | 80.31$^{bc}$ | 81.77$^{b}$ | 90.54$^{a}$ | 77.36$^{c}$ | 1.538 | < 0.01 |
| IVNDFD (% NDF) | 72.52$^{b}$ | 68.98$^{b}$ | 59.40$^{c}$ | 81.47$^{a}$ | 2.719 | < 0.01 |
| IVCPD (%CP) | 83.72$^{c}$ | 94.55$^{a}$ | 93.57$^{a}$ | 90.20$^{b}$ | 1.015 | < 0.01 |
| pH | 6.33$^{c}$ | 6.41$^{b}$ | 6.49$^{a}$ | 6.25$^{d}$ | 0.013 | < 0.01 |
| $NH_3$-N (mg/100 mL) | 15.54$^{d}$ | 36.05$^{a}$ | 34.09$^{b}$ | 22.58$^{c}$ | 0.445 | < 0.01 |
| TVFA (mM) | 77.8$^{c}$ | 82.6$^{ab}$ | 80.3$^{bc}$ | 83.5$^{a}$ | 1.00 | < 0.05 |
| Acetate | 572.7$^{a}$ | 544.8$^{b}$ | 506.5$^{d}$ | 519.6$^{c}$ | 2.26 | < 0.01 |
| Propionate | 297.5$^{c}$ | 286.5$^{d}$ | 325.6$^{b}$ | 333.2$^{a}$ | 1.91 | < 0.01 |
| Butyrate | 90.1$^{d}$ | 98.1$^{c}$ | 107.1$^{a}$ | 102.3$^{b}$ | 0.82 | < 0.01 |
| Iso-butyrate | 8.2$^{d}$ | 16.1$^{a}$ | 13.7$^{b}$ | 9.3$^{c}$ | 0.10 | < 0.01 |
| Valerate | 22.0$^{c}$ | 32.2$^{a}$ | 28.5$^{b}$ | 24.0$^{c}$ | 0.74 | < 0.01 |
| Iso-valerate | 9.5$^{d}$ | 22.2$^{a}$ | 18.7$^{b}$ | 11.7$^{c}$ | 0.23 | < 0.01 |
| A:P ratio | 1.93$^{a}$ | 1.90$^{a}$ | 1.56$^{b}$ | 1.56$^{b}$ | 0.017 | < 0.01 |

$^{a-d}$Values in the same row with different letters differ significantly ($p < 0.05$).

RGP, red ginseng byproduct; CGF, corn gluten feed; WG, wheat gluten; CGM, corn germ meal; IVDMD, *in vitro* dry matter digestibility; IVNDFD, *in vitro* neutral detergent fiber digestibility; IVCPD, *in vitro* crude protein digestibility; $NH_3$-N, ammonia nitrogen; TVFA, total volatile fatty acids; A:P ratio, acetate to propionate ratio.


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**Table 5.** Microbial abundance after *in vitro* incubation of treatments using buffered rumen fluid

| Items | Treatment | | | | SEM | *p*-value |
|---|---|---|---|---|---|---|
| | RGP | CGF | WG | CGM | | |
| Absolute abundance[1)] | | | | | | |
| General bacteria | 11.25 | 11.27 | 11.37 | 11.30 | 0.064 | 0.31 |
| Ciliate protozoa | 8.40 | 8.32 | 8.63 | 8.08 | 0.237 | 0.19 |
| Methanogenic archaea | 9.50$^{c}$ | 9.70$^{bc}$ | 9.94$^{a}$ | 9.93$^{ab}$ | 0.079 | < 0.03 |
| *Prevotella ruminicola* | 10.44$^{a}$ | 10.24$^{ab}$ | 10.16$^{b}$ | 10.28$^{ab}$ | 0.069 | < 0.01 |

$^{a–d}$Values in the same row with different letters differ significantly ($p < 0.05$).

[1)]The absolute abundance of each bacteria expressed as follows: $\log_{10}$/mL of rumen fluid.

RGP, red ginseng byproduct; CGF, corn gluten feed; WG, wheat gluten; CGM, corn germ meal.

## DISCUSSION

To the best of our knowledge, there is no recent evidence of the use of RGP as a resource for animal feed, particularly in ruminant nutrition. The key criteria for selecting RGP were increased production of the resource and bio-industrial waste, high nutrient content, and additional bioactive compounds. The RGP contains saponins, phenols, peptides, polysaccharides, alkaloids, lignans, and polyacetylenes, of which saponins are the main bioactive ingredient [19]. The bioactive phenolic compounds found in this byproduct are expected to modulate microbial behavior, fermentation characteristics, and emissions of $CO_2$ and $CH_4$ from the rumen. In this *in vitro* analysis, RGP and CGM resulted in a higher total gas production than WG and CGF after 48 h of incubation. Higher *in vitro* gas production in RGP indicates the rate and extent of IVDMD, which was higher than that in CGM, similar to CGF, and lower than WG. Similarly, the higher IVDMD of RGP makes it more favorable to microbial growth and accessible to microbial enzymes [20]. In a previous *in vitro* test, Alam et al. [21] used red ginseng barn powder as a feed additive in swine diets and recorded higher gas production for up to 12 h of incubation. Meanwhile Kim et al. [6] reported that the IVDMD of ruminant diets containing dried ginseng meal did not significantly improve during the 48 h incubation period. Generally, cereal grain byproducts creates a higher fraction of gas primarily from the fraction of carbohydrates and offer kinetic data to determine energy values and fermentation characteristics in ruminants [22].

The use of RGP did not decrease the $CO_2$ and $CH_4$ production. Except for CGM, a significant difference in $CH_4$ concentrations among treatments was not observed. However, the $CH_4$ ratio as an indicator of the total gas relative value in RGP, CGF, and CGM was lower than that in WG (Table 3). The ratio indicates a lower $CH_4$ yield per total gas production in RGP and has the potential to replace WG as a protein ingredient to minimize $CH_4$ production in ruminants. Moreover, RGP contains saponins, essential oils and other bioactive compounds [7], which may have the ability to affect microbial rumen diversity and fermentation and, thus, inhibit methanogenesis in ruminants. In ruminants, the production of gas is directly proportional to the microbial degradation of the feed and indirectly related to the buffering of acids produced as fermentation products [23]. Contrary to this, feeding ruminants a determined quantity of byproducts could minimize the risk of greenhouse gas emissions and fermentation products but could lead to a displaced abomasum and acidosis [24].

Higher IVNDFD in RGP than in WG resulted in a higher acetate and acetate to propionate ratio, and a similar pattern was observed for CGF as a protein byproduct during *in vitro* incubation. The NDF of RGP might be more degradable because the process of making red ginseng entails boiling and extracting soluble contents [25]. IVNDFD of RGP could be a better measure of DM

intake (DMI) than IVNDFD digestibility because in ruminant feeds, higher IVNDFD might mean a shorter rumen retention time, facilitating greater DMI at the expense of IVNDFD [26]. The partial replacement of ruminant feed with agricultural byproducts (rich in NDF) may serve as an excellent strategy to lower feed costs without affecting the characteristics of rumen fermentation [27].

The IVCPD of RGP was the lowest and can be attributed to the Maillard reaction, which involves binding of amino groups to the reducing-sugar carbonyl group, such as those found in glucose and lactose. It not only increases the amino acid losses but also deteriorates the protein quality [28]. However, IVCPD of RGP was more than 80% but significantly lower than that of CGF, WG, and CGM after 48 h of incubation. Kim et al. [6] reported that IVCPD was significantly reduced in alfalfa basal diets partially replaced with ginseng meal at different levels (0%, 5%, 10%, and 15%) incubated for 48 h. This variability in IVCPD is owing to the disparity in industry byproduct extraction methods and consequently in non-standardized meals, which are available for cattle rearing [29]. Similarly, $NH_3$-N concentration of RGP was the lowest, and CGF and WG had higher IVCPD and $NH_3$-N than CGM.

Total VFA production for RGP was lower than other treatments, and in similar research, ginseng meal supplementation did tend to increase the production of VFAs but not to any significant extent [6]. However, acetate and acetate to propionate ratio were higher for RGP than for the other treatments. Acetate is the main energy-producing substrate and has a dominant effect on the overall efficiency of feed usage in ruminants [30]. These findings can be because ruminal fermentation end products are based on diet formulation [31], and the ratio of acetate to propionate for cereal grains is usually low. The anti-methanogenic effect of certain medicinal byproducts may not be related to rumen microbial changes but rather to an alteration of rumen fermentation favoring propionate formation, lowering the acetate to propionate ratio [32]. Individual VFAs (valerate and iso-valerate) were higher for CGF and WG owing to branched chain amino acid fermentation and resulted in higher IVCPD of these protein byproducts than in CGM and RGP (Table 4). Getachew et al. [24] reported a strong positive correlation between CP digestibility and valerate and iso-valerate production in ruminants.

The ruminant forestomach contains diverse microbial populations, bacteria, fungi, protozoa, archaea, and phages which exist in mutualistic association with the host animal [32]. Absolute abundance of general bacteria and ciliate protozoa was similar among the treatments. However, methanogenic archaea were lower in RGP than in WG because of bioactive compounds and inhibited methanogenesis resulting in a lower $CH_4$ ratio than in WG (Table 3). Similarly, *Prevotella ruminicola* was lower in WG than in RGP, CGM, and CGF, indicating utilization of byproducts in the rumen. This species can hydrolyze xylan and pectin, plays an important role in the metabolism of protein and peptides, is actively proteolytic, and possesses a dipeptidyl peptidase activity that is detectable in rumen contents [33]. Microbial activity in the rumen liberates nutrients from low quality, high fiber diets, resulting in the production of VFAs and microbial proteins, providing the animal with sources of energy and highly digestible proteins, respectively [34].

Different types of industrial protein byproducts can be used in adequate amounts for ruminant feeding. This *in vitro* study indicated that RGP can be used as an alternative to conventional protein byproducts in ruminants diet based on its nutritive value, IVDMD, low $NH_3$-N, and decreased methanogenic archaea to mitigate $CH_4$ emissions without affecting the rumen fermentation characteristics.


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