The effect of Sunphenon 30S-O on methane emission, nutrient intake, digestibility and rumen fermentation

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Abstract

Sunphenon 30S-O is obtained from the leaves of traceable green tea (*Camellia sinensis*) and standardized for its catechin content (205 g/kg DM). This experiment was conducted to evaluate the effect of supplementation with different concentrations of Sunphenon 30S-O on methane emissions, nutrient intake, digestibility, protozoa abundance and the concentrations of volatile fatty acids (VFA) and ammonia-N (NH_3-N) in sheep. Four Corriedale wethers with an average body weight of 64.25±3.86 kg were arranged in a 4 x 4 Latin square and fed a basal diet of Guinea grass (*Panicum maximum*) hay at the maintenance level with four different concentrations of Sunphenon 30S-O (0, 10, 25 and 40 g/Kg DM intake). The experiment was conducted over 84 days in four 21-day periods that consisted of 14 days of acclimatization, five days of measurement and two 24-h runs in open-circuit respiration chambers to measure gas exchange. A second study was also conducted using an *in vitro* continuous gas quantification system and *in vitro* digestion techniques. All of the data were

Abbreviations

ADF: acid detergent fibre; CP: crude protein; CT: condensed tannin; DE: digestible energy; DM: dry matter; EC: epicatechin; ECG: epicatechin gallate; EE: ether extract; EGC: epigallocatechin; EGCG: epigallocatechin gallate; ER: energy retention; GE: gross energy; GHG: greenhouse gas; HP: heat production; IVDMD: *in vitro* dry matter degradability; IVOMD: *in vitro* organic matter degradability; ME: metabolizable energy; NDF: neutral detergent fibre; NH_3-N: ammonia-N; OM: organic matter; ORP: oxidation reduction potential; IVRCPD: *in vitro* rumen crude protein degradability; VFA: volatile fatty acid

subjected to polynomial regression analysis. Dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF) and gross energy (GE) intake all declined linearly ($P<0.01$) and quadratically ($P<0.05$) with increasing concentrations of Sunphenon 30S-O. Conversely, the apparent nutrient digestibility remained similar among treatments regardless of the concentration of Sunphenon 30S-O in the ration. *In vivo* methane emission (l/kg digestible OM intake) declined linearly ($P<0.05$) by 7.4-13.5% with increasing concentrations of Sunphenon 30S-O, and a similar trend was observed in *in vitro* methane emissions. Urinary and methane energy decreased linearly ($P<0.01$) from 17.4% to 11.2% and from 7.3% to 6.2% of the gross energy intake, respectively, with increasing supplement concentrations, and the *in vitro* VFA (mmol/L) and NH_3-N concentrations (mg/ml) were also reduced (linear $P<0.01$; quadratic $P<0.01$). The total abundance of the protozoa population also declined linearly and quadratically ($P<0.01$), and the *in vitro* DM degradability (IVDMD) was reduced (linear $P<0.01$; quadratic $P<0.01$) with increasing concentrations of Sunphenon 30S-O. The findings of this study indicated that the addition of Sunphenon 30S-O reduced *in vivo* methane emissions without affecting total tract nutrient digestibility, and energy and protein retention were not affected despite the reduction in total nutrient intake. Thus, to achieve optimum reduction of methane emissions and the concomitant saving of dietary energy without any negative impacts on total-tract digestibility and nutrient balance, Sunphenon 30S-O supplementation up to 40 g/kg DM could be an option.

1. Introduction

Climate change is one of the greatest obstacles facing the world today, and its association with the emission of greenhouse gases (GHGs), such as CO_2 and CH_4, is well known. While ruminants play an important role as an essential source of high-quality protein

47 in human diets, they are also a major source of GHGs. According to FAO estimates (Opio et
48 al., 2013), the greatest source of CH_4 in ruminant production is enteric fermentation, which
49 accounts for approximately 47% of the sector's GHG emissions and more than 90% of total
50 CH_4 emissions. As a GHG, CH_4 is 25 times stronger than CO_2 (Opio et al., 2013), and its
51 effect will become more pronounced in the short term because ruminant production is
52 increasing worldwide to meet an ever-increasing demand for milk and meat (Becker et al.,
53 2013). Therefore, reducing CH_4 emissions from ruminant livestock will play a significant
54 role in decreasing environmental pollution, provided that nutrient utilization efficiency is not
55 affected. Modifying the composition of animal diets is often regarded as an option to
56 minimize ruminal CH_4 emissions (Becker et al., 2013), and condensed tannin-containing
57 legume forages (Animut et al., 2008 with 50-151 g CT/kg DM; Min et al., 2002b with 32 g
58 CT/kg DM; Tavendale et al., 2005 with 91-107 g CT/kg DM; Williams et al., 2011 with 5-49
59 g CT/kg DM; Woodward et al., 2001 with 26 g CT/kg DM) and tannin extracts (Beauchemin
60 et al., 2007 with 18 g CT/kg DM; Carulla et al., 2005 with 25 g/kg DM; Hess et al., 2006
61 with 25 g CT/kg DM; Pellikaan et al., 2011 with 100 g CT/kg DM; Tan et al., 2011 with 20-
62 60 g CT/kg DM) have been extensively investigated for their ability to inhibit ruminal CH_4
63 production. Tannins reduce methane emissions by suppressing protozoa and other hydrogen-
64 producing microbes thus interfering with methanogenesis (Patra, 2010; Tavendale et al.,
65 2005).

66 Tea is one of the most popular beverages in the world (Khokhar and Magnusdottir, 2002);
67 annual production totals approximately 4 million tons (Bordoloi, 2012). As part of the
68 production of ready-made tea drinks packaged in bottles, packs and cans, beverage
69 companies discard a large amount of tea grounds annually (Wang and Xu, 2013). Green tea
70 extracts contain polyphenolic compounds that account for 30% of the dry weight of leaves
71 (Mukhtar and Ahmad, 2000), and *in vivo* and *in vitro* studies (Mitsumoto et al., 2005; Wang

and Xu, 2013; Zhong et al., 2009) have indicated that green tea polyphenols improve growth performance, meat quality and shelf life due to their antioxidant properties in cattle, sheep and goats. Flavanols, generally known as catechins, are the most abundant polyphenols in green tea leaves and account for nearly 80-90% of the total polyphenol content (Htay et al., 2008; Riemersma et al., 2001). The physiological effects of green tea depend on a variety of catechins, including epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG), all of which are usually present in high concentrations in tea leaves (Spencer, 2003). The structural formation of EGCG (also known as condensed tannin) is believed to be responsible for the pronounced physiological activity of tea, including its antioxidant effects (Htay et al., 2008).

Sunphenon 30S-O is obtained from green tea (*Camellia sinensis*) leaves, and it is standardized for its catechin content. Catechin is the only polyphenol present in Sunphenon 30S-O, and the inclusion of such catechin-containing natural plant extracts in ruminant rations might influence CH_4 emissions, nutrient intake, digestibility and other rumen fermentation parameters. To the best of our knowledge, there is no information available on the effect of Sunphenon 30S-O on rumen fermentation, so this experiment was conducted to investigate the influence of Sunphenon 30S-O (containing a standardized level of catechin, 205 g/kg DM) on nutrient intake, digestibility, CH_4 emissions, VFA concentrations, NH_3-N concentrations, the protozoa population and rumen degradability.

2. Materials and Methods

2.1. Sunphenon 30S-O.

Sunphenon 30S-O, which is standardized for catechin content (205 g/kg DM), was obtained from the leaves of traceable green tea (*Camellia sinensis*) via extraction by water infusion and decaffeination using approved food-grade solvents. Catechin is the only polyphenol present in Sunphenon 30S-O, which contains water soluble fibres as filler and

whose chemical composition and major catechin components are presented in Table 1. Samples of Sunphenon 30S-O were purchased from Taiyo Kagaku Co., Ltd., Japan; Sunphenon® extracts are food grade and approved by the Japanese Foundation for Health and Nutrition for specific medical uses. They are certified organic and possess an excellent tea taste and maintain good stability in beverages.

2.2. Rumen fluid sampling

Two ruminally fistulated, non-lactating Holstein cows (600 kg average BW) were used as rumen fluid donors. The cows were maintained on a daily diet of 10 kg orchard grass hay (OM, 980 g/kg; CP, 132 g/kg; NDF, 701 g/kg; ADF, 354 g/kg; lignin, 40 g/kg; and GE, 18.02MJ/kg; DM basis) with free access to clean drinking water and mineral blocks (Fe, 1836 mg; Cu, 377 mg; Co, 66 mg; Mg, 1046 mg; Zn, 1235 mg; I, 77 mg; Se, 33 mg; vit E, 5000 mg; Na, 962 g/~~3~~ 1 kg). Rumen liquor was collected from the two cows just before feeding (0 h) using a vacuum line and strained through a woven nylon cloth into a thermos flask that had been pre-heated (39ºC) with hot water. All animal management and sampling procedures were approved by the Obihiro University of Agriculture and Veterinary Medicine Animal Care and Use Committee.

2.3. Experimental in vitro *fermentation treatments*

The experimental samples were oven-dried at 60°C for 48 h and stored in sealed containers under dry, cool conditions prior to use. Four treatments were prepared that consisted of different concentrations of Sunphenon 30S-O and Guinea grass (*Panicum maximum*) hay as follows: 10 g of Guinea grass hay (Control, T1); 9.8 g of Guinea grass hay + 0.2 g of Sunphenon 30S-O (T2); 9.6 g of Guinea grass hay + 0.4 g of Sunphenon 30S-O (T3) and 9.5 g of Guinea grass hay + 0.5 g of Sunphenon 30S-O (T4). The effects of each treatment on CH_4 production, VFA concentrations, NH_3-N concentrations, pH, oxidation reduction potential (ORP) and the protozoa populations were tested *in vitro* for 24 h at 39°C

using a continuous gas quantification system, as previously described by Sar et al. (2005). Briefly, samples of rumen fluid were obtained from two non-lactating Holstein cows and strained and combined in equal volumes. The buffer was prepared according to McDougall (1948), sterilized by autoclaving and flushed with CO_2 for 1 h prior to being dispensed into fermentation vessels. Fermentation was allowed to continue for 24 h at 39 °C, and rumen fluid was added to the buffer in a ratio of 1:4. The source of replication (n=4) in the experimental model was provided by rumen fluid inocula collected on separate occasions, and the treatments were randomly assigned to incubation vessels for each incubation period. The gas output from each fermentation vessel was measured for 10 minutes at 30-min intervals. Samples of the incubation medium were collected after 24 h of incubation and were stored at -20°C for the analysis of NH_3-N and VFA, and at the end of each 24-h incubation period, all incubations were stopped, the contents were discharged, and the fermenters were thoroughly washed and autoclaved. The fermenters were then re-charged with fresh buffer and inoculum to begin the next 24-h incubation period.

2.4. Analysis of methane, carbon dioxide and volatile fatty acids

Methane production from each fermentation vessel was measured continuously with automatic infrared CH_4 (EXA IR, Yokogawa Electric Corporation, Tokyo, Japan) and CO_2 (Model RI-555, Riken Keiki Co. Ltd, Tokyo, Japan) analysers installed in the *in vitro* continuous gas quantification system (Takasugi Seisakusho Co. Ltd, Tokyo, Japan). Total VFA and its components were determined with a gas chromatograph (GC-2014, Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and a capillary column (ULBON HR-52, 0.53 mm ID × 30 m, 3.0 μm) using 2-ethyl-n-butyric acid as an internal standard; samples were prepared for analysis according to Sar et al. (2005). The pH and ORP of the fermentation media were monitored in each vessel at 1-min intervals (HP-21P, Toa electronics Ltd., Tokyo, Japan). All data were pooled and stored on a computer via an

147 interface with the analysers.

148 *2.5.* In vitro *nutrient degradability*

149 *In vitro* nutrient degradability was estimated by following the first stage of the
150 digestion technique described by Tilley and Terry (1963). Triplicate 0.5 g samples of Guinea
151 grass hay (control, T1), 0.49 g of Guinea grass hay + 0.01 g Sunphenon 30S-O (T2), 0.48 g
152 of Guinea grass hay + 0.02 g of Sunphenon 30S-O (T3) and 0.475 g of Guinea grass hay +
153 0.025 g of Sunphenon 30S-O (T4) were weighed and placed into a 100-ml plastic bottles, and
154 40 ml of McDougall's buffer (McDougall, 1948) was added to each bottle and pre-warmed to
155 39°C. Then, 10 ml of strained rumen fluid was dispensed into each bottle and sealed under a
156 continuous supply of CO_2 gas. The mixture was incubated at 39 ºC for 24 h and carefully
157 shaken occasionally. After incubation termination, the contents were filtered through pre-
158 weighed Gooch crucibles; the amount of residual DM was determined, and the loss in weight
159 was considered the IVDMD. This was followed by ashing of the residues for the estimation
160 of *in vitro* OM degradability (IVOMD), and *in vitro* rumen CP degradability (IVRCPD) was
161 estimated at the end of the incubation period by filtering the contents through laboratory-
162 grade filter paper (Grade 1, 100 circles/125 mm, Toyo Roshi, Ltd, Japan). The amount of N
163 in the residues was analysed by the Kjeldahl method (AOAC 984.13), and the disappearance
164 was calculated from the differences in the protein content of the sample before and after
165 incubation. *In vitro* rumen degradability experiments were repeated four times.

166

167 *2.6. Animals, diets and supplements*

168 Four Corriedale wether sheep with body weights of 64.25±3.86 kg were used in a 4×4
169 Latin square design. The wethers were kept in an individual metabolic cages equipped with a
170 ventilated respiratory collection hood and fed a maintenance-level (55 g DM/kg $BW^{0.75}$/ day)
171 basal diet of Guinea grass (*Panicum maximum*) hay twice daily (08:30 and 16:30), and all had

free access to clean drinking water and a mineral block. The treatments were as follows: 1. control (100% Guinea grass hay); treatments 2, 3 and 4 contained 10, 25 and 40 g Sunphenon 30S-O per kg DM, respectively, in addition to the amount contained in the control diet. Sunphenon 30S-O was thoroughly mixed with 50 g of concentrate mixture in each treatment to facilitate intake and to avoid loss; the control group was also supplemented with 50 g of the concentrate mixture.

2.7 Experimental procedure

The experiment lasted for 84 days with each 21-day period consisting of 14 days of acclimatization followed by a 5-day digestion trial and two 24-h runs in open-circuit respiration chambers to measure gas exchange. Samples of the offered feed, refusal, faeces and urine were collected and analysed for nutrient content following standard procedures. Oxygen consumption and carbon dioxide and methane emissions were quantified by an open-circuit respiratory system using a hood over the heads of the wethers as described by Takahashi et al. (1999). Data were collected and entered into a computer through an interface with the analysers at 1-min intervals and then automatically standardized at 0 °C, 1013 hpa and zero water vapour pressure.

2.8. Calculation of energy balance

The total methane gas volume obtained from the open-circuit respiratory system was converted to its gross energy (GE) value using a conversion factor of 39.54 kJ/l (Brouwer, 1965). Digestible energy (DE) was calculated as the difference between energy intake and faecal energy; energy lost as methane was the methane emitted in l/day x 39.54kJ/l (Brouwer, 1965); metabolizable energy (ME) was the difference between DE and the sum of the energy in urine and methane; and energy retention (ER) was the difference between ME and heat production (HP). Heat production (kJ/day) was calculated using the equation: 16.18 O_2 (l/day) + 5.02 CO_2 (l/day) – 2.17 CH_4 – 5.99 N (g/day) (Brouwer, 1965).

197

198 *2.9. Faeces and urine collection and preparation*

199 Faeces and urine were collected for 5 days during each period, and the faecal
200 samples from each treatment were thawed, bulked, mixed and sub-sampled. Sub-samples
201 were dried at 60°C for 48 h in a forced-air oven and ground to pass through a 1-mm sieve for
202 subsequent laboratory analysis. Urine was collected into buckets containing 100 ml of 100
203 ml/l (v/v) sulphuric acid to reduce the pH below 3.0 and to prevent bacterial degradation of N
204 compounds. Approximately 50 ml/l of the urine sample was sub-sampled and stored at -20 °C
205 until the nitrogen analysis.

206 *2.10. Laboratory analysis*

207 Experimental samples were analysed for DM by drying at 135 °C for 2 h (930.15),
208 and OM, total ash (942.05) and ether extract (EE) (920.39) were determined according to the
209 procedures of AOAC (1995). N was determined by the Kjeldahl method (984.13) (AOAC,
210 1995) using an electrical heating digester (FOSS Tecator^{TM} Digester, Tokyo, Japan) and an
211 automatic distillation apparatus (FOSS Kjeltec^{TM} 2100, Tokyo, Japan), and crude protein
212 (CP) was then calculated as the amount of N × 6.25. Neutral detergent fibre (NDF) was
213 estimated without amylase and expressed inclusive of residual ash according to the method
214 described by Van Soest et al. (1991), which was also used to determine acid detergent fibre
215 (ADF) and lignin. ADF was expressed inclusive of ash, and lignin was determined by the
216 solubilization of cellulose with sulphuric acid. The gross energy (GE) content of the samples
217 was analysed in a Shimadzu auto-calculating bomb calorimeter (CA-4AJ, Shimadzu
218 Corporation, Japan), and the NH_3-N concentration was analysed according to Conway and
219 O'Malley (1942).

220 The components of the total catechins in Sunphenon 30S-O were analysed by Japan
221 Food Research Laboratories using HPLC (Shimadzu LC-MS with an LC-20AD column and a

SPD-20A detector). EC, EGC, EGCG, and ECG were separated by a reverse phase mechanism on a C18 column containing water, methanol and 0.02-mol/L phosphate buffer (pH=3.0) as the mobile phase gradient. EC was detected and quantified by fluorescence with excitation at 280 nm and measured at 315 nm with a flow rate of 1.0 ml/min. EGC, EGCG and ECG were detected by ultraviolet light at 270 nm with a 1 ml/min flow rate, and mass spectra were collected by Shimadzu LC-MS and electrospray ionization mass spectrometry ES/MS). Catechin was separated on an Atlantis T3 2.1-mm*150-mm column with acetonitrile, acetic acid and water linear gradient ionization.

2.11. Statistical analysis

Data obtained from the *in vivo* study were subjected to ANOVA in a 4 x 4 Latin square design using a polynomial regression analysis (REG procedure) available in SAS (2010) with the model: $Yij = \mu + Ti + eij$, where *Yij* is the dependent variable; μ is the overall mean; *Ti* is the fixed treatment effect; and *eij* is the residual. The experimental unit was the individual animal. For the *in vitro* study, the effects included in the PROC REG for each variable were the treatment effects and replicates (rumen fluid inocula) with the model $Yij = \mu + Ti + Rj + eij$, where *Yij* is the dependent variable; μ is the overall mean; *Ti* is the fixed treatment effect (i=4); *Rj* is the random effect of the vessels (rumen inocula) (j=4); and *eij* is the residual. The statistical unit was the average of the *in vitro* bottles/vessels within a run, and the linear, quadratic and cubic contrasts of the treatment means were assessed (PROC REG). Differences among the means were identified using Tukey's multiple comparisons, and effects were considered significant when $P<0.05$ while trends were discussed at $0.05<P<0.10$. The standard error of the means was determined using the least squares means procedure (lsmeans option) in SAS (2010).

3. Results

247 *3.1. Chemical composition of the experimental feeds*

248 Sunphenon 30S-O contained concentrations of CP, OM, GE and total ash
249 comparable to Guinea grass hay (Table 1), and the total catechin content of the Sunphenon
250 30S-O used in this study was 205 g/kg DM. The components of the catechins contained in
251 Sunphenon 30S-O are indicated in Table 1; EGCG and EGC were the major components (80
252 g/100 g DM).

253 *3.2.* In vitro *methane emissions and rumen degradability*

254 Methane production (ml 24 h^{-1}) was reduced linearly ($P<0.01$) with increasing
255 concentrations of Sunphenon 30S-O (Table 2), and CO_2 production (ml 24 h^{-1}) followed the
256 same trend, declining linearly ($P<0.01$) and quadratically ($P<0.01$). *In vitro* rumen DM
257 degradability was reduced linearly and quadratically ($P<0.01$) at an increasing rate with
258 higher concentrations of Sunphenon 30S-O. A similar linear ($P<0.01$) and quadratic ($P<0.01$)
259 trend was observed for IVOMD, and IVRCPD was reduced linearly ($P<0.01$) but tended to
260 decrease quadratically ($P=0.06$).

261 *3.3. The effects of Sunphenon 30S-O on* in vitro *fermentation*

262 The total concentrations of VFA (mmol/L) and NH_3-N (mg/ml) decreased at an
263 increasing rate (linear $P<0.01$; quadratic $P<0.01$) with increasing concentrations of
264 Sunphenon 30S-O (Table 3). The acetate-to-propionate ratio decreased linearly ($P<0.01$) and
265 quadratically ($P<0.05$). The molar proportions of acetate were not affected ($P>0.05$), whereas
266 the proportion of propionate increased linearly ($P<0.01$) and quadratically ($P<0.05$). Valeric
267 acid tended to decrease linearly ($P<0.10$), and the molar proportion of butyrate declined
268 (linear $P<0.01$; quadratic $P<0.01$) with increasing concentrations of Sunphenon 30S-O. The
269 protozoa population also declined linearly and quadratically ($P<0.01$) at an increasing rate
270 with increasing concentrations of Sunphenon 30S-O. The addition of Sunphenon 30S-O did
271 not have any significant effects on ORP ($P>0.05$) and pH ($P>0.05$).

272 *3.4. Nutrient intake, digestibility and loss*

273 Increasing the concentration of Sunphenon 30S-O to 40 g/kg DM resulted in a linear
274 ($P<0.01$) and quadratic ($P<0.05$) decrease in DM, OM, CP, NDF and ADF intake, whereas
275 the nutrient digestibility (DM, OM, CP, NDF and ADF) was not influenced by
276 supplementation (Table 4). GE intake (MJ/d) and DE intake (MJ/d) were reduced linearly
277 ($P<0.01$) with increasing concentrations of Sunphenon 30S-O, but ME intake was not
278 affected ($P>0.05$) (Table 5). Energy losses through urine and methane were reduced linearly
279 ($P<0.01$) with increasing concentrations of Sunphenon 30S-O, but energy loss through the
280 faeces was not affected ($P>0.05$). Heat production and ER did not differ among treatments
281 ($P>0.05$). Crude protein loss through urine was reduced linearly ($P<0.01$), but there was no
282 influence on CP loss through faeces ($P>0.05$). Crude protein retention was not affected
283 ($P>0.05$) by the addition of Sunphenon 30S-O.

284 *3.5. Effect on methane and carbon dioxide emissions*

285 *In vivo* methane emissions (L/d) decreased linearly ($P<0.01$) in a dose-dependent
286 manner when Sunphenon 30S-O was added (Table 5). Methane emissions (l/kg digestible
287 OM intake) also decreased linearly ($P<0.05$) as the level of Sunphenon 30S-O increased.
288 Carbon dioxide production (L/d) decreased linearly ($P<0.05$) and quadratically ($P<0.05$) as
289 the level of supplementation increased.

290 **4. Discussion**

291 4.1. *Nutrient intake and digestibility*

292 In small ruminants, the level of food intake is inversely related to the concentration
293 of CT in the food (Landau et al., 2002; Silanikove et al., 1994; Silanikove et al., 1997a). In
294 the present experiment, daily nutrient intake (DM, OM, CP, NDF or ADF) was not affected
295 by supplementation with 10-25 g of Sunphenon 30S-O per kg of DM, but when the
296 concentration was increased to 40 g/kg DM, the intake of DM, OM, NDF and ADF was

297 reduced by 15.6% and that of CP by 13.9%. Dschaak et al. (2011) reported that
298 supplementation with CT extract (30 g/kg DM) decreased DM, OM, CP, NDF, and ADF
299 intake. The presence of catechin (a precursor of CT) in Sunphenon 30S-O could have
300 affected nutrient intake due to its astringency as in previous studies in which CT from
301 quebracho (*Aspidosperma quebracho*) was shown to negatively affect the intake of Holstein
302 heifers (Landau et al., 2000). Additionally, the relative amounts of consumed plant secondary
303 compounds affect intake and forage preference of herbivores (Mote et al., 2007). Conversely,
304 the inclusion of Sunphenon 30S-O at different concentrations did not affect the total-tract
305 digestibility of DM, OM, CP, NDF and ADF, and the overall CP digestibility coefficient was
306 0.80, 0.80, 0.79 and 0.79 for concentrations of 0, 10, 25 and 40 g/kg DM of Sunphenon 30S-
307 O, respectively. This is consistent with the findings by Dschaak et al. (2011), who found that
308 although supplementation with CT extract in the diet (30 g/kg of DM) decreased feed intake,
309 total–tract digestibility of DM, OM, CP and ADF was not affected. Metabolizable energy
310 intake, HP and ER were also not affected ($P>0.05$), which could be due to the increased
311 nutrient utilization efficiency resulting from the addition of Sunphenon 30S-O. A previous
312 study by Hess et al. (2006) indicated that, despite increased ($p<0.01$) total energy losses,
313 tannins affected neither ($p>0.05$) energy expenditure nor body energy retention.
314 Feeding forages containing condensed tannin (CT) have been reported to decrease ruminal
315 protein degradation and depress the feeding value of the diet. Inclusion of CT (32 g/kg DM)
316 from *Lotus corniculatus* reduced nitrogen degradability by 10% (Min et al., 2002a), and the
317 addition of CT (25 g/kg DM) from the bark of *Acacia mearnsii* reduced ($P<0.05$) apparent
318 digestibility of all nutrients except hemicelluloses (Hess et al., 2006). In our *in vitro* study,
319 ruminal DM and OM degradability declined by 2-7% and 3-9%, respectively, due to the
320 inclusion of Sunphenon 30S-O (20-50 g/kg DM). This finding agrees with previous work by
321 Oskoueian et al. (2013), who indicated that a 4.5% (w/w) dose of catechins decreased DM

degradability ($p<0.05$) by 6.7% compared to the control. Our findings are also consistent with those of Tan et al. (2011), who stated that *in vitro* DM degradability and N disappearance declined by 7% and 15%, respectively, with the addition of CT (30 g/kg DM).

4.2. The effect of Sunphenon 30S-O on methane emissions and energy balance

The inclusion of natural feed additives should be considered from the perspective of their effect on environmental safety and nutrient utilization efficiency. The findings of our *in vivo* study indicated that Sunphenon 30S-O (20-40 g/kg DM) supplementation decreased methane emissions (l/kg digestible OM intake) by 7.4-13.5% compared to the control, and our *in vitro* study similarly confirmed that supplementation (40-50 g/kg DM) reduced CH_4 emissions by 9.5 to 14.5% while CO_2 production declined by 6.4-13.8% compared to the control. It has been indicated that supplementation with *Acacia mearnsii* tannin (25 g/kg dietary DM) decreased methane emissions by 0.13 of GE intake (Hess et al., 2006). Similarly, a study by Tan et al. (2011) indicated that CT extracts from *Leucaena leucocephala* hybrid-Rendang (20-60 g/kg DM of the diet) reduced methane emissions by 0.33-0.63 of the DM. Methane emissions from dairy cows were reduced by 0.23 of digestible DM when fed silage made from *Lotus corniculatus* (CT 26 g/kg DM) compared to silage from pasture (Woodward et al., 2001). In the present study, 1 mol of catechin in Sunphenon 30S-O (1.0% of the substrate on a DM basis) reduced the emission of methane by 1.8 mol, while the findings of Becker et al. (2013) suggested that catechins decreased CH_4 production in a dose-dependent manner, where 1.0 mol of catechin prevented the emission of 1.2 mol of CH_4.

In the control animals, energy wasted as urine and methane represented 17.4 and 7.3% of the GE intake, respectively, where it only represented 11.2 and 6.2% of the GE intake in the treated animals (supplemented with Sunphenon 30S-O at the 40 g/kg DM dose). This indicates that, compared to the control, the urinary and methane energy emissions decreased significantly, but the basal metabolism remained unchanged because GE intake was reduced

347 by 16%.

348 Energy retention was not affected by the addition of different concentrations of
349 Sunphenon 30S-O. Dietary energy loss through methane emissions by sheep was 0.06 – 0.07
350 of the total GE intake, which agrees with the findings by Sauvant and Giger-Reverdin (2007),
351 who reported methane losses of 0.06 – 0.07 of GE with a lower proportion of concentrate in
352 the diet. Animut et al. (2008) also reported methane emissions by meat goats of 0.09 of GE
353 intake with ad libitum consumption of sorghum-sudangrass.

354 *4.3. The effect of Sunphenon on protein utilization efficiency*

355 In this study, protein intake was reduced by up to 0.16 of the total CP intake at the
356 highest level of Sunphenon 30S-O supplementation (40 g/kg DM). Crude protein loss
357 accounted for 0.46–0.57 of the total CP intake, and most of the loss (0.54–0.64 of the total CP
358 loss) was through urine, which was reduced by 17-41% under supplementation. Despite the
359 reduction in total CP intake, retained CP was not affected by Sunphenon 30S-O
360 supplementation, and this could be attributed to the effect of supplementation, which reduced
361 CP loss through urine and increased CP absorption efficiency in the lower tract. This is
362 consistent with Priolo and Ben Salem (2004), who stated that low concentrations of CT
363 appear to reduce protein degradation in the rumen and enhance the availability and absorption
364 of amino acids from the small intestine. Moderate levels of CT (20-40 g/kg DM) bind to
365 protein in the rumen to form a CT-protein complex but then dissociate, and the protein
366 becomes available in the abomasums (Barry et al., 2001). Makkar (2003) also indicated that
367 CT protects dietary protein from rumen microbial degradation and increases the supply of
368 amino acids to the intestine for greater absorption.

369 *4.4. Effects of Sunphenon 30S-O on the* in vitro *protozoa population and concentration of*
370 *NH_3-N*

371 Tannins either suppress methanogenesis directly or by reducing the protozoa

population, thereby reducing the amount of symbiotically associated methanogens (Bhatta et al., 2009). The present *in vitro* study indicated that the protozoa population was reduced by 2-19% when Sunphenon 30S-O was included at concentrations of 20-50 g/kg DM of the substrate. Consistent with our findings, previous work by Tan et al. (2011) indicated that the total protozoa population declined by 15-30% with the addition of CT at doses of 20-60 g/kg DM of the diet. Similarly, Animut et al. (2008) found that the protozoa population decreased by 42% when CT was included at a dose of 52 g/kg DM in the diet of goats and was further reduced as the concentration increased. In the present study, the inhibitory effects of Sunphenon 30S-O on the protozoa population were more pronounced as the concentration increased. The use of CT-containing forages may substantially improve environmental sustainability by reducing N excretion (Williams et al., 2011). In this study, *in vitro* NH_3–N concentrations decreased with increasing concentrations of Sunphenon 30S-O and were 2 and 8% lower at 40 and 50 g /kg DM of the substrate, respectively, compared to the control. The observed decrease in the rumen ammonia concentration was due to a decrease in the numbers of protozoa (Wina et al., 2005a; Wang et al., 2012) and the nitrogen-binding effect of CT (Beauchemin et al., 2007). Previous works by Min et al. (2000, 2002) also indicated that CT in the diet reduced protein degradation and rumen NH_3-N concentrations.

4.5. Effect on the in vitro *volatile fatty acid concentration*

In our study, total VFA concentration was reduced by 8.6-15.9% with increasing concentrations of Sunphenon 30S-O (20-50 g/kg DM), and our data agree with Dschaak et al., 2011, who stated that CT extract supplementation (30 g/kg DM) of lactating cows fed a high-forage diet reduced the total VFA concentration by 6%. Tan et al. (2011) found that the total VFA concentration (mmol/L) decreased by 17-23% with increasing levels of CT (20-60 g/kg DM), and Kondo et al. (2004) also indicated that the addition of green tea grounds (CT 23

g/kg DM of the diet) reduced total VFA production. The proportion of propionate increased by 4.3%; the acetate-to-propionate ratio decreased by 4.3%; and butyrate declined by 15.6 % when Sunphenon 30S-O was included at a dose of 50 g/kg DM. In support of our findings, Bhatta et al. (2009) reported that *in vitro* propionate production increased when the CT extract from either quebracho (*Schinopsis loentzii*) or mimosa (*Acacia mearnsii*) was added, and a decrease in the acetate-to-propionate ratio was observed when *Acacia mearnsii* extract was supplemented as a source of CT (Khiaosa-Ard et al., 2009). Conversely, the findings of Oskoueian et al. (2013) indicated that the inclusion of catechins did not have a significant effect on total VFA production.

5. Conclusion

EGCG and EGC are the major constituents of catechin present in Sunphenon 30S-O, which could be responsible for influencing methane emission and other rumen fermentation parameters. Both *in vitro* and *in vivo* studies confirm that the addition of Sunphenon 30S-O reduced CH_4 emissions in a dose-dependent manner. Although supplementation reduced feed intake in sheep, the total-tract digestibility of nutrients was not affected regardless of the Sunphenon 30S-O concentrations in the diet. This study found that a relatively high concentration of Sunphenon 30S-O (50 g/kg of the substrate) exerted a negative effect on *in vitro* nutrient degradability, the protozoa population and NH_3-N and VFA concentrations. Thus, to achieve optimum CH_4 reduction and save dietary energy without any negative effect on whole-tract nutrient digestibility, Sunphenon 30S-O supplementation up to 40 g/kg DM of the diet could be a possible option.

Conflict of interest

The authors declare no conflicts of interest

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References

AOAC, 1995. Official Methods of Analysis, vol 1, 16th ed. Association of Official Analytical Chemists, Arlington, VA, USA.

Animut, G., Puchala, R., Goetsch, A.L., Patra, A.K., Sahlu, T., Varel, V.H., Wells, J., 2008. Methane emission by goats consuming diets with different levels of condensed tannins from lespedeza. Anim. Feed Sci. Technol. 144, 212–227.

Barry, T.N., McNeill, D.M., McNabb, W.C., 2001. Plant secondary compounds; their impact on forage nutritive value and upon animal production. In: Proc. XIX Inter. Grassl. Conf., Sao Paulo, Brazil, pp. 445–452

Beauchemin, K.A., McGinn, S.M., Martinez, T.F., McAllister, T.A., 2007. Use of condensed tannins extract from quebracho trees to reduce methane emissions from cattle. J. Anim. Sci. 85, 1990–1996.

Becker, P.M., van Wikselaar, P.G., Franssen, M.C.R., de Vos, R.C.H., Hall, R.D., Beekwilder, J., 2013. Evidence for a hydrogen-sink mechanism of (+) catechin-mediated emission reduction of the ruminant greenhouse gas methane. Metabolomics. 10, 179-189.

Bhatta, R., Uyeno, Y., Tajima, K., Takenaka, A., Yabumoto, Y., Nonaka, I., Enishi, O., Kurihara, M., 2009. Difference in the nature of tannins on *in vitro* ruminal methane and volatile fatty acid production and on methanogenic archaea and protozoal populations. J. Dairy Sci. 92, 5512–5522.

Bordoloi, P.K., 2012. Global tea production and export trend with special reference to India. Research Paper. Two and a Bud. 59(2), 152-156.

447 Brouwer, E., 1965. Report of sub-committee on constants and factors. In: Blaxter, K.E. (Ed.),
448 Proceedings of the Third Symposium on Energy Metabolism, EAAP, Academic
449 Press, London. 11, 441–443.
450 Carulla, J.E., Kreuzer, M., Machmuller, A., Hess, H.D., 2005. Supplementation of *Acacia*
451 *mearnsii* tannins decreases methanogenesis and urinary nitrogen in forage fed sheep.
452 Aust. J. Agric. Res. 56, 961-970.
453 Conway, E.J., O'Malley, E., 1942. Micro diffusion methods: ammonia and
454 urea using buffered absorbent (revised methods for ranges greater than
455 10 μg N), Biochem. J. 36, 655-661.
456 Dschaak, C.M., Williams, C.M., Holt, M.S., Eun, J.S., Young, A.J., Min, B.R., 2011. Effect of
457 supplementing condensed tannin extract on intake, digestion, Ruminal fermentation,
458 and milk production of lactating dairy cows. J. Dairy Sci. 94, 2508-2519.
459 Hess, H.D., Tiemann, Noto, F., Carulla, J.E., Kreuzer, M., 2006. Strategic use of
460 tannins as means to limit methane emission from ruminant livestock.
461 International congress series. 1293, 164-167.
462 Htay, H.H., MacNaughton, L.E., Kapoor, M.P., Juneja, L.R., 2008. Functional behavior of tea
463 polyphenols in cardiovascular disease. In：Economic crisis in tea industry. Stadium
464 press LLC, USA. pp. 256-273.
465 Khiaosa-Ard, R., Bryner, S.F., Scheeder, M.R.L., Wettstein, H.R., Leiber, F., Kreuzer, M.,
466 Soliva, C.R., 2009. Evidence for the inhibition of the terminal step of ruminal
467 linolenic acid biohydrogenation by condensed tannins. J. Dairy Sci. 92, 177–188.
468 Khokhar, S., Magnusdottir, S., 2002. Total phenol, catechin, and caffeine contents of teas
469 commonly consumed in the United Kingdom. J. Agric food Chem. 50, 567-570.
470 Kondo, M., Kita, K., Yokata, H., 2004. Feeding value to goats of whole-crop oat
471 ensiled with green tea waste. Anim. Feed Sci. Technol. 113, 71-81.

472 Landau, S., Silanikove, N., Nitsan, Z., Barkai, D., Baram, H., Provenza, F.D., Perevolotsky,
473 A., 2000. Short-term changes in eating patterns explain the effects of condensed
474 tannins on feed intake in heifers. Appl. Anim. Behav. Sci. 69, 199-213.
475 Landau, S., Silanikove, N., Nitsan, Z., Provenza, F.D., Perevolotsky, A., 2002. Polyethylene-
476 Glycol affects goats' feeding behavior in a tannin-rich environment. J. Range Manag.
477 55, 598–603
478 Makkar, H.P.S., 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins,
479 and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small
480 Rumin. Res. 49, 241-256
481 McDougall, E.I., 1948. "Studies on ruminant saliva". I. The composition and output of
482 sheep's saliva. Biochem. J. 43, 99-109.
483 Min, B.R., Attwood, G.T., Barry, T.N., McNabb, W.C., 2002b. The effect of condensed
484 tannins from *Lotus corniculatus* on the proteolytic activities and growth of rumen
485 bacteria. J. Anim. Sci., 80 (Suppl. 1) (2002), p. 1602
486 Min, B.R., Attwood, G.T., Reilly, K., Sun, W., Peters, J.S., Barry, T.N., McNabb, W.C., 2002a.
487 *Lotus corniculatus* condensed tannins decrease in vivo populations of proteolytic
488 bacteria and affect nitrogen metabolism in the rumen of sheep. Can. J. Microbiol. 48,
489 911–921.
490 Min, B.R., McNabb, W.C., Barry, T.N., Peters, J.S., 2000. Solubilization and
491 degradation of ribulose-1,5-bisphosphate carboxylase/ oxygenase (EC 4.1.1.39;
492 Rubisco) protein from white clover (*Trifolium repens*) and *Lotus corniculatus* by
493 rumen microorganisms and the effect of condensed tannins on these processes. J.
494 Agric. Sci. 134, 305–317.
495 Mitsumoto, M., O'Grady, M.N., Kerry, J.P., Buckley, D.J., 2005. Addition of tea
496 catechins and vitamin C on sensory evaluation, colour and lipid stability during

497 chilled storage in cooked or raw beef and chicken patties. Meat Science. 69, 773–
498 779.

499 Mote, T.E., Villalba, J.J., Provenza, F.D., 2007. Relative availability of tannin and terpend-
500 containing foods affects food intake and preference by lambs. J. chem. Ecol. 33,
501 1197-1206.

502 Mukhtar, H., Ahmad, N., 2000. Tea polyphenol: Prevention of cancer and ptimizing health.
503 The American Journal of Clinical Nutrition. 71, 1698S–1702S.

504 Opio, C., Gerber, P., Mottet, A., Falcucci, A., Tempio, G., MacLeod, M., Vellinga, T.,
505 Henderson, B., Steinfeld, H., 2013. Greenhouse gas emissions from ruminant
506 supply chains – A global life cycle assessment. Food and Agriculture Organization
507 of the United Nations (FAO), Rome. E-ISBN 978-92-5-107945-4

508 Oskoueian, E., Abdullah, N., Oskoueian A., 2013. Effects of flavonoids on rumen
509 fermentation activity, methane production, and microbial population. BioMed
510 Res. Int. doi:10.1155/2013/349129.

511 Patra, A.K., 2010. Meta-analysis of effects of phytochemicals on digestibility and rumen
512 fermentation characterstics associated with methanogenesis. J. Sci. Food Agric. 90,
513 2700-2708

514 Pellikaan, W.F., Stringano, E., Leenaars, J., Bongers, D J.G.M., Schuppen, S.V.L.V., Plant, J.,
515 Mueller-Harvey, I., 2011. Evaluating effects of tannins on extent and
516 rate of in vitro gas and CH_4 production using an automated pressure evaluation
517 system (APES). Anim. feed Sci.technol.166-167, 377-390.

518 Priolo A., Ben Salem, H., 2004. Effect of dietary condensed tannins on small ruminant
519 productions.Ben Salem H. (ed.), Nefzaoui A. (ed.), Morand-Fehr P. (ed.). Nutrition
520 and feeding strategies of sheep and goats under harsh climates. CIHEAM. 209-213

521 Riemersma, R.A., Rice-Evens, C.A., Tyrrell, R.M., Clifford, M.N., Lean, M.E.J.,

2001. Tea flavonoids and cardiovascular health. QJ. Med. 94, 277-282.

SAS Institute, 2010. SAS version 9.3. SAS Inst. Inc., Cary, NC. USA.

Sar, C., Mweny, B., Santoso, B., Takaura, K., Morikawa, R., Isogai, N., Asakura, Y., Toride, Y., Takahashi, J., 2005. "Effect of Escherichia coli W3110 on ruminal methanogenesis and nitrate/nitrite reduction *in vitro*," Anim. Feed Sci. Technol., 118, 295-306.

Sauvant, D., Giger-Reverdin, S., 2007. 'Empirical modeling meta-analysis of digestive interactions and CH_4 production in ruminants,' in Energy and Protein Metabolism and Nutritoin, I. Ortigues-Marty, N. Miraux, and W. Brand-Willianms, Eds., Wageningen Academic. 561-563..

Silanikove, N., Gilboa, N., Nitsan, Z., 1997a. Interactions among tannins, supplementation and polyethylene-glycol in goats given oak leaves: effects on digestion and food intake. Anim. Sci. 64, 479–483.

Silanikove, N., Nitsan, Z., Perevolotsky, A., 1994. Effect of a daily supplementation of polyethylene glycol on intake and digestion of tannin-containing leaves (*Ceratonia siliqua*) by sheep. J. Agric. Food Chem. 42, 2844–2847.

Spencer, J.P., (2003). Metabolism of tea flavonoids in the gastrointestinal tract. J.Nutr. **133**, 3255S-3261S.

Takahashi, J., Chaudhry, A.S., Beneke, R.G., Young, B.A., 1999. An open-circuit hood system for gaseous exchange measurements in small ruminants. Small Rumin. Res. 32, 31-36.

Tan, H.Y., Sieo, C.C., Abdullah, N., Liang, J.B., Huang, X.D., Ho, Y.W., 2011. Effects of condensed tannins from Leucaena on methane production, rumen fermentation and populations of methanogens and protozoa *in vitro*. Anim. Feed Sci. Technol. 169, 185-193

547 Tavendale, M.H., Meagher, L.P., Pacheco, D., Walker, N., Attwood, G.T., Sivakumaran, S.,
548 2005. Methane production from *in vitro* rumen incubations with *Lotus pedunculatus*
549 and *Medicago sativa*, and effects of extractable condensed tannin fractions on
550 methanogenesis. Anim. Feed Sci. Technol. 123/124, 403–419.
551 Tilley, J.M. A., Terry, R.A., 1963. A two stage technique for *in vitro* digestion of forage
552 crops. J.Br. Grassland Soc.18, 104-111.
553 Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral
554 detergent fiber and non-starch polysaccharides in relation to animal nutrition. J.
555 Dairy Science. 74, 3583-3597.
556 Wang, H., Xu, C., 2013. Utilization of tea grounds as feedstuff for ruminants. Journal of
557 Animal Science and Biotechnology. 4, 54. doi:10.1186/2049-1891-4-54
558 Wang, J.K., Ye, J.A., Liu, J.X., 2012. Effect of tea sapponins on rumen
559 microbiota, rumen fermentation, methane production and growth
560 performance. Trop Anim Health prod.44, 697-706.
561 Williams, C.M., Eun, J.S., MacAdam, J.W., Young A.J., Fellner, V., Min, B.R.,
562 2011. Effect of forage legumes containing condensed tannins on
563 methane and ammonia production in continuous cultures of mixed
564 ruminal microorganisms. Anim. Feed Sci. and Technol. 166-167, 364-372.
565 Wina, E., Muetzel, S., Becker, K., 2005a. The impact of saponins or saponin-
566 containing plant materials on ruminant production-a review, Journal of
567 Agricultural and Food Chemistry. 53, 8093-8105.
568 Woodward, S.I., Waghorn, G.C., Ulyatt, M.J., Lassey, K.R., 2001. Early indications that
569 feeding Lotus will reduce methane emissions from ruminants. Proc. N.Z. Soc. Anim.
570 Prod. 61, 23–26.
571 Zhong, R.Z., Tan, C.Y., Han, X.F., Tang, S.X., Tan, Z.L., Zeng, B., 2009. Effect of dietary

572 tea catechins supplementation in goats on the quality of meat kept under
573 refrigeration. Small Ruminant Research. 87, 122 –125.

Table 1.
Chemical composition of experimental feeds

Item [a]	Guinea grass hay	Concentrate mixture	sunphenon 30S-O
DM (g/kg)	956	958	944
OM (g/kg DM)	909	918	913
Ash(g/kg DM)	84.4	76.5	85.6
CP (g/kg DM)	147	165	137
EE (g/kg DM)	16.0	33.6	6.20
GE (MJ/kg DM)	17.1	16.7	15.9
NDF (g/kg DM)	609	232	20.0
ADF (g/kg DM)	303	78.3	9.00
ADL(g/kg DM)	36.2	35.6	2.00
(+)-catechin (g/kg DM)	-	-	3.00
EC (g/kg DM)	-	-	21.0
EGC (g/kg DM)	-	-	79.0
EGCG (g/kg DM)	-	-	84.0
ECG (g/kg DM)	-	-	18.0

[a]DM: dry matter; OM: organic matter; CP: crude protein; EE: ether extract; GE: gross energy; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL acid detergent lignin; EC epi catechin; EGC epi galo catechin; EGCG epi galo catechin galate; ECG epi catechin galate

Table 2

The effect of sunphenon 30S-O on *in vitro* CH_4 emission, CO_2 production and nutrient degradability

Item[1]	Level of Sunphenon 30S-O (g/kg DM)				SEM	Contrasts[2]		
	0.0	20	40	50		L	Q	C
CH_4(ml24h-^{1})	36.0^{a}	36.4^{a}	32.6^{b}	30.8^{b}	0.398	<0.001	0.241	0.113
CH_4(ml/g digestible DM)	6.59a	6.56a	6.00b	5.68c	0.046	<0.001	0.079	0.015
CH_4(ml/g digestible OM)	8.27^{a}	8.27^{a}	7.61^{b}	7.43^{b}	0.040	<0.001	0.353	<0.001
CO_2(ml24h-^{1})	396^{a}	391^{a}	370^{b}	341^{c}	1.883	<0.001	0.006	0.581
In vitro rumen degradability(24 h)								
IVDMD	0.51^{a}	0.51^{a}	0.50^{a}	0.47^{b}	0.002	<0.001	<.001	0.469
IVOMD	0.45^{a}	0.46^{a}	0.44^{a}	0.42^{b}	0.002	<0.001	0.004	0.731
IVRCPD	0.57^{a}	0.57^{a}	0.55^{b}	0.54^{b}	0.002	<0.001	0.056	0.273

[1]$CH_{4:}$ methane; IVDMD: *in vitro* dry matter degradability; IVOMD: *in vitro* organic matter degradability; IVRCPD: *in vitro* rumen crude protein degradability;

$^{a-c}$ Means within a raw with different superscripts differ($P<0.05$),

[2] L= linear, Q= quadratic, C= cubic,

Table 3

The effect of sunphenon 30S-O on *in vitro* rumen fermentation and protozoa count after 24 hours of incubation

Item[1]	Levels of Sunphenon 30S-O (g/kg DM)				SEM	Contrasts[2]		
	0.0	20	40	50		L	Q	C
Volatile fatty acids (mol/100mol)								
Acetate (A)	70.0	70.0	70.0	70.0	0.124	0.608	0.765	0.912
Propionate (P)	21.7^{b}	21.7^{b}	21.8^{b}	22.6^{a}	0.07	0.003	0.019	0.342
Butyrate	7.44^{a}	7.42^{a}	7.19^{a}	6.28^{b}	0.07	<0.001	0.007	0.493
Valeric acid	0.93	0.90	0.90	0.90	0.006	0.096	0.445	0.677
Total VFA (mmol/L)	38.5^{a}	37.9^{a}	35.2^{b}	32.4^{c}	0.158	<0.001	0.004	0.156
A:P ratio	3.23^{a}	3.23^{a}	3.21^{a}	3.09^{b}	0.012	0.003	0.024	0.371
pH(mean)	7.07	7.02	6.99	6.98	0.013	0.195	0.579	0.897
ORP (mV)	-412	-411	-412	-413	0.850	0.619	0.847	0.502
Total Protozoa (cell/l*10^{6})	3.40^{a}	3.35^{a}	3.23^{a}	2.75^{b}	0.022	<0.001	<0.001	0.182
NH_3-N (mg/ml)	27.5^{a}	27.3^{a}	27.0^{a}	25.3^{b}	0.107	<0.001	0.004	0.272

[1]VFA: volatile fatty acid; A:P: acetate to propionate ratio; ORP: oxidation reduction potential; NH_3-N, Ammonia N,

[a-c]Means within a raw with different superscripts differ($P<0.05$)

[2] L = linear, Q = quadratic, C = cubic

Table 4.

Intake and digestibility of nutrients by sheep supplemented with different concentration of sunphenon 30S-O

Item[1]	Sunphenon 30S-O concentrations (g/kg DM)				SEM	Contrasts[2]		
	0	10	25	40		L	Q	C
DM								
intake (g/d)	1016[a]	1014[a]	971[a]	857[b]	12.24	<.001	0.041	0.812
digested (g/d)	637[a]	636[a]	591[ab]	532[b]	8.728	0.001	0.135	0.741
digestibility	0.63	0.63	0.63	0.61	0.007	0.688	0.554	0.394
OM								
intake (g/d)	923[a]	922[a]	882[a]	779[b]	11.13	<.001	0.041	0.812
digested (g/d)	589[a]	589[a]	544[ab]	493[b]	7.948	<.001	0.150	0.618
digestibility	0.64	0.64	0.62	0.64	0.006	0.718	0.466	0.264
CP								
intake (g/d)	150[a]	149[a]	143[a]	126[b]	1.803	<.001	0.041	0.812
digested (g/d)	120[a]	120[a]	113[a]	99.4[b]	1.414	<.001	0.039	0.981
digestibility	0.80	0.80	0.79	0.79	0.004	0.169	0.85	0.49
NDF								
intake (g/d)	619[a]	618[a]	591[a]	522[b]	7.46	<.001	0.041	0.812
digested (g/d)	405[a]	407[a]	373[ab]	337[b]	5.350	<.001	0.103	0.515
digestibility	0.66	0.66	0.63	0.65	0.006	0.486	0.605	0.170
ADF								
intake (g/d)	308[a]	307[a]	294[a]	260[b]	3.710	<.001	0.041	0.812
digested (g/d)	178[a]	177[a]	165[ab]	148[b]	2.655	<.001	0.186	0.847
digestibility	0.58	0.58	0.58	0.56	0.007	0.716	0.570	0.557

[1]DM: dry matter; OM: organic matter; CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre;

[a-c]Means within a raw with different superscripts differ($P<0.05$)

[2] L = linear, Q = quadratic, C = cubic.

Table 5.
Methane emission and energy balance by sheep supplemented with different concentrations of sunphenon 30S-O

	Sunphenon 30S-O concentrations (g/kg DM)				SEM	Contrasts[2]		
Item[1]	0	10	25	40		L	Q	C
Methane emission								
CH_4 (l/d)[1]	34.7[a]	32.3[ab]	30.2[b]	24.4[c]	0.484	<.001	0.120	0.379
CH_4 (g/d)	24.9[a]	23.1[ab]	21.7[b]	17.5[c]	0.347	<.001	0.120	0.379
CH_4 E (MJ/d)	1.27[a]	1.18[ab]	1.12[b]	0.91[c]	0.031	<.001	0.372	0.494
CH_4 (l/kg DMI)	34.2[a]	31.8[a]	31.2[ab]	28.5[b]	0.492	0.001	0.879	0.448
CH_4 (g/kg DMI)	24.5[a]	22.8[a]	22.3[ab]	20.4[b]	0.352	0.001	0.879	0.448
CH_4 (g/kg DDMI)	39.3[a]	36.5[ab]	38.1[ab]	34.2[b]	0.601	0.025	0.667	0.099
CH_4 (l/kg DDMI)	54.9[a]	51.0[ab]	53.3[ab]	47.7[b]	0.84	0.025	0.667	0.099
CH_4 (g/kg DOMI)	42.5[a]	39.4[ab]	41.4[ab]	36.8[b]	0.647	0.022	0.622	0.074
CH_4 (l/kg DOMI)	59.4[a]	55.0[ab]	57.8[ab]	51.4[b]	0.903	0.022	0.622	0.074
Energy balance (MJ/d)								
GE Intake	17.3[a]	17.3[a]	16.6[a]	14.6[b]	2.090	<.001	0.041	0.812
Fecal	6.27	6.45	6.36	5.53	0.142	0.105	0.109	0.733
DE	11.1[a]	10.9[a]	10.2[ab]	9.10[b]	1.481	<.001	0.181	0.995
Methane	1.27[a]	1.18[ab]	1.11[b]	0.91[c]	0.291	<.001	0.422	0.592
Urinary	3.01[a]	2.28[ab]	2.04[b]	1.63[b]	0.102	<.001	0.496	0.533
ME	7.92	8.44	8.04	7.37	1.841	0.275	0.150	0.722
HP	7.75	8.24	7.90	7.26	0.118	0.414	0.755	0.681
ER	0.17	0.20	0.14	0.12	0.097	0.491	0.860	0.995

[1]CH_4: methane; DMI: dry matter intake; DDMI: digestible dry matter intake; DOMI: digestible organic matter intake; GE: gross energy; DE: digestible energy; ME: metabolizable energy; HP: heat production; ER: energy retained

[a-c]Means within a raw with different superscripts differ($P<0.05$)

[2] L = linear, Q = quadratic, C = cubic

Table 6
The effect of Sunphenon 30S-O on urinary and fecal crude protein losses

Item[1]	Sunphenon 30S-O concentrations (g/kg DM)				SEM	Contrasts[2]		
	0	10	25	40		L	Q	C
Intake (g/d)	150[a]	149[a]	143[a]	126[b]	1.803	<0.001	0.041	0.812
Feces (g/d)	29.7	29.8	30.2	26.9	0.671	0.233	0.252	0.562
Urine (g/d)	53.5[a]	44.5[ab]	41.6[ab]	31.4[b]	1.880	0.004	0.895	0.482
CP retained (g/d)	66.4	75.0	71.2	67.9	2.234	0.976	0.237	0.561

[1] CP： crude protein
[a-b]Means within a raw with different superscripts differ(P<0.05)
[2] L = linear, Q = quadratic, C = cubic