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In vitro Study of *Urtica cannabina* and *Leymus chinensis* on Rumen Microbial Fermentation and Gas Production

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ABSTRACT

Background: *Urtica cannabina*, an unconventional forage, is widely distributed in northern China. It has high nutritional values that make it suitable for the ruminant's feeding requirments as compared to *Leymus chinensis*. The current study was designed to evaluate varying ratios of *Urtica cannabina* and *Leymus chinensis* in the feeding diet and to see the effects on rumen fermentation and gas production *in vitro*.

Methods: The study was designed into five treatments based on the different ratios of *U. cannabina* and *L. chinensis*: 0:100, 30:70, 50:50, 70:30 and 100:0 categorized into five groups from A-E. To detect the rumen fermentation parameters, the culture medium was collected at 1, 3, 6, 12 and 24 h.

Result: Gas production of groups A and C was increased than other groups at 24h (P<0.05), whereas the rate of gas production (c) was also increased in group A (P<0.05). The pH values at 1, 3, 6 and 24 h were increased in groups A and C with higher values in group C at 24h (P<0.05). The ammonia concentration was increased in groups D and E at 3, 6, 12 and 24 h, with the lower values in group C at 24h (P<0.05). The concentration of bacterial and protozoal proteins was also observed higher in groups A and C at 1 and 24 h, with highest value in group C at 24 h (P<0.05). In summary, as for *Urtica cannabina* to *Leymus chinensis* ratios are concerned, 50:50 is an optimal ratio for rumen fermentation *in vitro*, which increases the gas production and microbial protein synthesis.

Key words: Gas production, *In vitro*, *Leymus chinensis*, Rumen fermentation, *Urtica cannabina*.

INTRODUCTION

High-quality forage is essential for the growth and development of ruminants. Nowadays, the quantity of high-quality forage is insufficient in the ruminant industry in China (Xie *et al*. 2019). Some studies demonstrated that an unconventional forage with high nutritional values can substitute for ruminant feed sources (Iniguez, 2011; Porqueddu *et al*. 2016).

As an unconventional forage, *Urtica cannabina* is widely distributed in Xinjiang, Inner Mongolia and other places because of its strong vitality and adaptability (Zhang *et al*. 2014). Usually, the yield range of this plant is 3.6-7.8 t ha^{-1} without chemical fertilizers, while under good environment and nutrition conditions, the dry matter yield of the natural *U. cannabina* can reach to 14 t ha^{-1} (Zhang and Zhao, 2008). It's stem and leaves are rich in protein, trace elements, cellulose and a variety of active substances (Zhang *et al*. 2020). Medically, it has been used in the treatment of diseases such as rheumatoid arthritis and prostatitis syndrome, *etc*. (Bourgeois *et al*. 2016; Carvalho *et al*. 2017). In recent years, *U. cannabina* has been used in livestock production. It has already been demonstrated by that *U. cannabina* is involved in improving morphological features of gastrointestine and nutrients digestibility in lamb (Jin *et al*. 2018). Humphries and Reynolds (2014) reported that *Urtica dioica haylage* (100 g/kg DM) increased milk production in the diet of lactating dairy cows instead of ryegrass silage. However, little is known about the effects of *U. cannabina* on rumen microorganisms, it may alter rumen microbial activity because it indeed contains many biologically active compounds. So we hypothesized that the addition of *U. cannabina* can increase rumen microbial activity *in vitro*. Therefore, the effects of *U. cannabina* to *L. chinensis* ratios on the rumen fermentation *in vitro* were investigated in the present study.

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MATERIALS AND METHODS

The study was carried out during the period of July 2017 to June 2018, at the Institute of Metabolic Manipulation of Herbivorous Animal Nutrition, College of Animal Science and Technology, Yangzhou University, China.

Animal management

The following experimental procedures, including animal ethics and usage, were approved by the Animal Welfare Committee which is managed by Yangzhou Veterinarians of the Agriculture Ministry of China.

Two-year-old, three healthy Xuhuai goats having permanent rumen fistulas with body weight (BW±SD) of 35±3.1 kg were chosen for the sampling purpose from the experimental farms of Yangzhou University. The diet offered to goats (dry matter amount about 3% BW) was mixed well with following contents including 70% alfalfa, 28% corn kernels and 2% soybean meal. This diet was fed to goats in equal amounts at 07:00 h and 19:00 h. They had free access to mineral licking blocks and fresh drinking water.

Culture substrate and experimental design

The samples of *U. cannabina* were collected early in July 2017 during the flowering stage and the samples of *L. chinensis* were bought from Caodu Co., Ltd. The forages were automatically collected and dried outside in an open environment in Xilinguole, Inner Mongolia region, China.

U. cannabina and *L. chinensis* were dried at 65°C to a constant mass in a force air oven and then ground finely with a Retsch ZM 100 Wiley mill (Retsch GmbH, Haan, Germany) to pass through 1 mm screen. These two kinds of refined grass were mixed in proportion as the *in vitro* culture substrates with the following five ratios: 0:100, 30:70, 50:50, 70:30 and 100:0 categorized into the five groups from A-E respectively. The ingredient and nutritive values of each group are shown in the Table 1.

In vitro gas production test

The rumen fluid samples were collected from different localities through the rumen fistula using a vacuum negative pressure device (self-constructed) before morning feeding. These samples were filtered into a thermos flask containing four layers of gauze. The flask was filled with CO_2 and heated at 39 °C before collecting samples. The artificial saliva salt was configured according to Menke and Steingass (1988). The culture medium for *in vitro* culture of rumen microorganisms was prepared using artificial saliva salt with rumen fluid ratio 2:1. Then CO_2 was introduced for saturation purpose. The substrate of each group weighed 0.5000 g (±0.0500g) accurately in a 150 mL fermentation bottle and 50mL of artificial saliva and 25mL of rumen fluid were added later on. Each treatment in sampling time was replicated three times. The culture bottle was connected to the 64-way microbial fermentation micro-product gas automatic recorder (AGRS-III, Beijing, China). The incubation temperature was set to 39°C and incubated for 24 h at constant temperature under anaerobic conditions.

Sample collections and chemical analysis

Gas production was recorded at 1, 3, 6, 12 and 24 h of incubation. The kinetics of gas production *in vitro* were calculated according to the exponential function model proposed by Ørskov and McDonald (1979). The culture medium at each time point was filtered through the four layers of gauze and dispensed in 5 mL centrifuge tubes. The pH value of the rumen fluid was measured immediately using an electrode pH meter (pHS-3C, Shanghai, China). The rumen fluid was immediately stored for analysis of ammonia nitrogen, bacterial and protozoal proteins at -20°C. The ammonia nitrogen concentration was measured by the phenol-sodium hypochlorite colorimetric method (Weatherbum, 1967). The bacterial and protozoal protein concentrations in rumen fluid were determined according to the method as described by Hall *et al*. (2001).

Statistical analysis

Statistical analysis was performed by SPSS software (version 16.0, SPSS Inc., Chicago, USA) using ANOVA procedure with posthoc tukey's multiple comparison test. The fixed effect was treatment and sampling time, whereas random effect in the ANOVA model was repetition. Significance was declared at p-value < 0.05.

RESULTS AND DISCUSSION

Effects on the kinetics of gas production *in vitro*

In our study, gas production of different groups increased with fermentation time (Fig 1). Whereas gas production was increased in the groups A and C at 24 h (P<0.05). The data in Table 2 show that the gas produced from soluble fraction (***a***) was higher (P<0.05) in groups D and E (1.83 and 2.10mL/g DM), whereas the gas produced from insoluble but fermentable fraction (***b***) produced was lower (P<0.05) in group E (41.01mL/g DM). The highest value was registered

Table 1: Ingredient composition and nutritive value of substrate.

Item	Group treatment				
	A	B	C	D	E
Ingredient (%)					
U. cannabina	0	30	50	70	100
L. chinensis	100	70	50	30	0
Total	100	100	100	100	100
Nutritive level (g/kg)[1]					
CP	87.3	110.61	126.15	141.69	165
EE	25.4	26.48	27.2	27.92	29
NDF	670	583.6	526	468.4	382
ADF	302	301.7	301.5	301.3	301
NFC	153	177.6	194	210.4	235
Lignin	86.5	71.98	62.3	52.62	38.1
Ash	63.8	101.36	126.4	151.44	189
Ca	5.1	13.89	19.75	25.61	34.4
P	1.2	5.64	8.6	11.56	16

[1]Nutritional value was measured; CP=Crude protein; EE=Ether extract; NDF=Neutral detergent fibre; ADF=Acid detergent fibre; NFC=Non-fibre carbohydrates = 1000 - (CP + EE + NDF + ash); Ca=calcium; P=phosphorus.

Legends: Ratio of *U. cannabina* to *L. chinensis* in groups A-E are 0:100, 30:70, 50:50, 70:30 and 100:0 respectively.

Table 2: Effects of the kinetics of gas production of each group *in vitro.*

Items	Group treatment					SEM	*P*-value
	A	B	C	D	E		
a [1]	1.36[b]	1.22[b]	1.67[ab]	1.83[a]	2.10[a]	0.13	<0.01
b [2]	62.76[a]	54.51[a]	57.03[a]	55.17[a]	41.01[b]	2.17	<0.01
c [3]	0.201[a]	0.128[c]	0.167[b]	0.095[d]	0.029[e]	0.01	<0.01
a+b [4]	64.12[a]	55.71[a]	58.70[a]	56.99[a]	43.10[b]	2.09	<0.01

1 = gas produced from soluble fraction (mL/g DM).

2 = gas produced from insoluble but fermentable fraction (mL/g DM).

3 = rate constant of gas production during incubation (mL/h^{-1}).

4 = potential gas production (mL/g DM).

Legends: Ratio of *U. cannabina* to *L. chinensis* in groups A-E are 0:100, 30:70, 50:50, 70:30 and 100:0 respectively.

[a-e]: values in the same row with different small superscript letters are different from each other significantly (*P* <0.05).

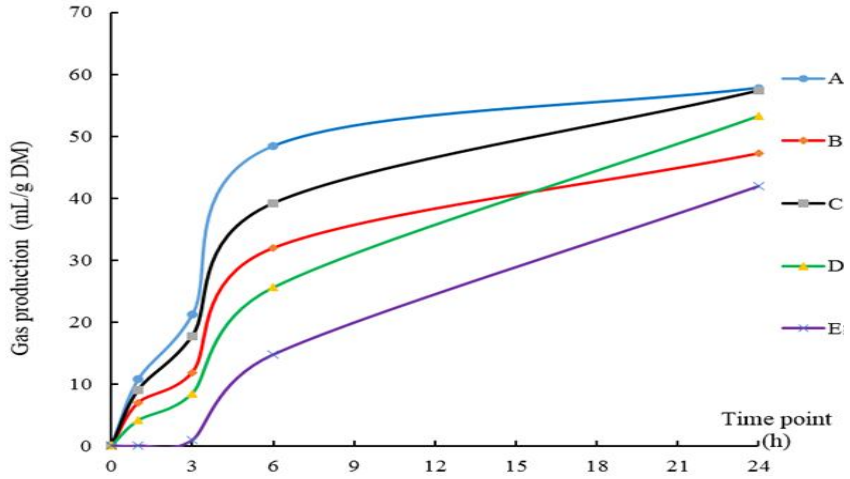

Fig 1: Cumulative gas release of different ratios of *U. cannbinau* to *L. chinensis in vitro*(mL/g DM).

Legends: Ratio of *U. cannabina* to *L. chinensis* in groups A-E are 0:100, 30:70, 50:50, 70:30 and 100:0 respectively.

for group A (62.76mL/g DM). The rate of gas production (***c***) was higher (*P*<0.05) in group A (0.201mL/g DM), whereas the lowest value for this fraction was observed in group E (0.029mL/g DM). The reason might be that *L. chinensis* was a high-quality forage with a higher NDF content (Table 1) and the rapid degradation of soluble carbohydrates increased the gas production rate in the early stages (Cone and van Gelder, 1999). The rate of gas production (*c*) in group C was higher than groups B, D and E, This could be due to the reason that the proper addition of *U. cannabina* can promote the balance of energy and protein in the substrate, which further promote the reproduction of rumen microorganisms (Tang *et al*. 2005) and thus increase the gas production rate.

Similarly, the potential gas production (*a* + *b*) was higher for group A than group E (*P*<0.05). Khazaal *et al.* (1993) pointed out that feed intake is mainly explained by the rate of gas production (*c*), which affects the pass rate of feed through the rumen and the potential gas production (*a* + *b*) is related to the degradability of the feed. Therefore the higher values of potential gas production in groups A and C might indicate a better nutrient availability for rumen microorganisms (Nsahlai *et al*. 1994).

Effects on the fermentation parameters *in vitro*

In Fig 2(a), the pH value in all groups was within the normal range during the 24h fermentation. However, the pH values at 1 h, 3 h and 6 h were increased in groups A and C; and at 24 h were observed highest in group C (*P* < 0.05). The reason might be that the crude protein of *U. cannabina* was higher and the digestible protein was up to 70 %. A certain addition of *U. cannabina* can increase the digestible protein in the fermentation substrate and a large amount of protein degradation promotes the increase of ammonia nitrogen concentration and finally lead to the increased the pH value. It was found that addition of *U. cannabina* (100 mg/g) can prevent the effects of rumen acidosis effectively, by increasing the pH value of rumen fluid to 30% *in vitro* (Kleim *et al*. 2017). Similarly, Arroquy *et al.* (2004) reported that as the ratio of rumen degradable protein (RDP) increased, the pH value was also increased linearly within the normal range.

In Fig 2(b), the ammonia nitrogen concentration in all groups was all within the normal range during the 24h fermentation. However, the ammonia nitrogen in groups D and E was higher at 3 h, 6 h, 12 h and 24 h. The concentration of ammonia nitrogen is determined by the rumen microbial

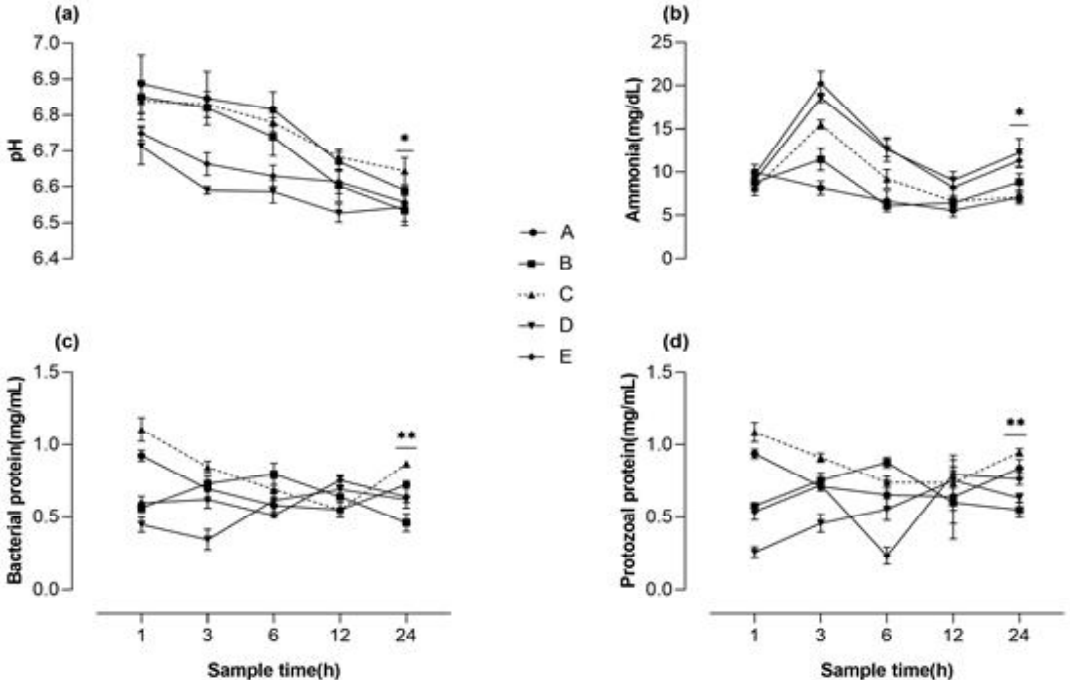

Fig 2: The effect of *U. cannbinau to L. chinensis* ratios on pH value(a), ammonia(b), bacterial protein(c), protozoal protein (d) *in vitro.*
Legends: Ratio of *U. cannabina* to *L. chinensis* in groups A-E are 0:100, 30:70, 50:50, 70:30 and 100:0 respectively.
*indicates differences between treatments at $p < 0.05$; ** indicates differences between treatments at $p < 0.01$.

decomposition of proteins and the rate at which ammonia is used to synthesize microbial proteins. Groups D and E contained higher CP and NFCs contents (Table 1), which lead to higher ammonia nitrogen synthesis. At 24h, the ammonia nitrogen concentration in group C decreased significantly ($P<0.05$), due to the increased ratio of NDF and NFCs and decreased lignin concentration (Table 1) leading to suitable conditions for microbial growth and protein synthesis (Shen *et al*. 2017).

Effects on the contents of bacterial and protozoal proteins *in vitro*

As it can be seen in Fig 2 (c,d), the concentration of bacteria and protozoa protein was higher in groups A and C at 1 h, 3 h and 6 h. The reason might be that *L. chinensis* has high-quality forage contents which are more conducive for microbial growth. Stern *et al*. (1994) reported that energy and nitrogen balance and simultaneous release of them in the diet determine the growth of rumen microbes, which could change the synthesis efficiency of MCP (bacteria and protozoa protein). In the beginning, the supply of energy and nutrients in the substrate was sufficient to meet the needs of the microorganisms. Fermentation produced a large amount of energy and precursors required for the synthesis of self-proteins by microorganisms such as ammonia, volatile fatty acids and ATP (Van Soest, 1994). As time went on, the gradual consumption of nutrients and energy and the proliferation of bacteria had reached a stationary stage. In addition, the phagocytosis of protozoa was enhanced, the production of bacterial proteins in the rumen began to decrease and it might also be caused by bacteriolysis (Sherwood *et al*. 2012).

The comparison among the groups showed that with the increased ratio of *U. cannabina* and the decreased ratio of *L. chinensis,* the bacterial and protozoal protein concentrations were decreased. However, this concentration was observed highest in group C at 24 h. This could be due to the certain complementary interaction effect of *U. cannabina* and *L. chinensis* nutrients (Haddad, 2000), which promotes the balance of energy and protein in the substrate by influencing the overall increased microbial protein synthesis. It can also be inferred that with the passage of culture time the protozoal protein concentration fluctuated greatly in the groups D and E. It is speculated that some pharmacological effects of *U. cannabina* changes the rumen microbiota (Yang *et al*. 2013).

CONCLUSION

Based on the results of current experiment, the 50:50 ratio of *Urtica cannabina* and *Leymus chinensis* increased the gas production in goat rumen microorganisms *in vitro*. Besides, this group increased the pH value and microbial protein contents; and decreased the ammonia nitrogen concentration in culture solution. Therefore, the 50:50 ratio of *Urtica cannabina* and *Leymus chinensis* could potentially be favorable for fermentation and gas production of rumen microorganisms as shown in the *in vitro* studies.

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REFERENCES

Arroquy, J.I., Cochran, R.C., Wickersham, T.A., Llewellyn, D.A., Titgemeyer, E.C., Nagaraja, T.G. and Johnson, D.E. (2004). Effects of type of supplemental carbohydrate and source of supplemental rumen degradable protein on low quality forage utilization by beef steers. Animal Feed Science and Technology. 115: 247-263.

Bourgeois, C., Leclerc, É.A., Corbin, C., Doussot, J., Serrano, V., Vanier, J.R., Seigneuret, J.M., Auguin, D., Pichon, C., Lainé, É., Hano, C. (2016). Nettle (*Urtica dioica L.*) as a source of antioxidant and anti-aging phytochemicals for cosmetic applications. Comptes Rendus Chimie. 19: 1090-1100.

Carvalho, A.R., Costa, G., Figueirinha, A., Liberal, J., Prior, J.A.V., Lopes, M.C., Cruz, M.T. and Batista, M.T. (2017). *Urtica* spp.: Phenolic composition, safety, antioxidant and anti-inflammatory activities. Food Research International. 99: 485-494.

Cone, J.W. and van Gelder, A.H. (1999). Influence of protein fermentation on gas production profiles. Animal Feed Science and Technology. 76: 251-264.

Haddad, S.G. (2000). Associative effects of supplementing barley straw diets with alfalfa hay on rumen environment and nutrient intake and digestibility for ewes. Animal Feed Science and Technology. 87: 163-171.

Hall, M.B. and Herejk, C. (2001). Differences in yields of microbial crude protein from *in vitro* fermentation of carbohydrates. Journal of Dairy Science. 84: 2486-2493.

Humphries, D.J. and Reynolds, C.K. (2014). The effect of adding stinging nettle (*Urtica dioica*) haylage to a total mixed ration on performance and rumen function of lactating dairy cows. Animal Feed Science and Technology. 189: 72-81.

Jin, Y.M., Jiang, C., Zhang, X.Q., Shi, L.F. and Wang, M.Z. (2018). Effect of dietary *Urtica cannabina* on the growth performance, apparent digestibility, rumen fermentation and gastrointestinal morphology of growing lambs. Animal Feed Science and Technology. 243: 1-9.

Khazaal, K., Markantonatos, X., Nastis, A. and Ørskov, E.R. (1993). Changes with maturity in fibre composition and levels of extractable polyphenols in Greek browse: Effects on *in vitro* gas production and in sacco dry matter degradation. Journal of the Science of Food and Agriculture. 63: 237-244.

Kliem, K.E., Morgan, R. and Mould, F.L. (2017). The effect of *Lactuca sativa* and *Urtica dioica* on *in vitro* acidosis. Proceedings of the British Society of Animal Science. 2005: 226-226.

Menke, K.H. and Steingass, H. (1988). Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. Animal Research and Development. 28: 7-55.

Nsahlai, I.V., Siaw, D. and Osuji, P.O. (1994). The relationships between gas production and chemical composition of 23 browses of the genus Sesbania. Journal of the Science of Food and Agriculture. 65: 13-20.

Ørskov, E.R. and McDonald, I. (1979). The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. Journal of Agricultural Science. 92:499-503.

Shen, H., Lu, Z.Y., Xu, Z.H., Chen, Z. and Shen, Z.M. (2017). Associations among dietary non-fiber carbohydrate, ruminal microbiota and epithelium G-protein-coupled receptor and histone deacetylase regulations in goats. Microbiome. 5: 123.

Sherwood, L., Klandorf, H. and Yancey, P. (2012). Animal physiology: from genes to organisms, 2nd edn. Belment, California.

Stern, M.D., Varga, G.A., Clark, J.H., Firkins, J.L., Huber, J.T. and Palmquist, D.L. (1994). Evaluation of Chemical and Physical Properties of Feeds That Affect Protein Metabolism In the Rumen. Journal of Dairy Science. 77: 2762-2786.

Tang, S.X., Jiang, H.L., Zhou, C.S. and Tan, Z.L. (2005). Effects of different forage species on *in vitro* gas production characteristics. Acta Prataculturae Sinica. 14: 72-77.(in Chinese).

Van Soest, P.J. (1994). Nutritional Ecology of the Ruminant, 2nd edn. Cornell University, Ithica, New York.

Weatherburn, M.W. (1967). Phenol-hypochlorite reaction for determination of ammonia. Analytical Chemistry. 39: 971-974.

Xie, F., Zhang, L., Jin, W., Meng, Z., Cheng, Y., Wang, J. and Zhu, W. (2019). Methane Emission, Rumen Fermentation and Microbial Community Response to a Nitrooxy Compound in Low-Quality Forage Fed Hu Sheep. Current Microbiology. 76: 435-441.

Yang, C.L.H., Or, T.C.T., Ho, M.H.K. and Lau, A.S.Y. (2013). Scientific Basis of Botanical Medicine as Alternative Remedies for Rheumatoid Arthritis. Clinical Reviews in Allergy and Immunology. 44: 284-300.

Zhang, X.O. and Zhao, S.Z. (2008). Nutritional function and silage characteristics of *Urtica cannabina*. Animal Husbandry and Feed Science. 4: 11–13. (in Chinese).

Zhang, X.Q., Jin, Y.M., Wang,Y.Q., Gao,F.Q. and Qi,J. (2014). Effects of inoculants and molasses on silage quality of nettle (*Urtica cannabina*). Journal of Animal and Veterinary Advances. 16: 33-40.

Zhang, X.Q., Jin, Y.M., Zhang, Y.J., Yu, Z. and Yan, W.H. (2014). Silage quality and preservation of *Urtica cannabina* ensiled alone and with additive treatment. Grass and Forage Science. 69: 405-414.

Zhang, Z., Wang, S., Wang, M., Shahzad, K., Zhang, X., Qi, R. and Shi, L. (2020). Effects of *Urtica cannabina* to *Leymus chinensis* ratios on ruminal microorganisms and fiber degradation *in vitro*. Animals. 10: 335.