Effects of *Andrographis paniculata* and *Orthosiphon stamineus* Supplementation on *in-vivo* Rumen Fermentation Parameters and Microbial Population in Goats Fed Urea-treated Rice Straw

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Abstract

Four fistulated Boer cross-bred bucks with 25 kg average body weight was used to test the effects of dietary treated rice straw supplemented with *A. paniculata* and *O. stamineus* on *in-vivo* rumen parameters and microbial population in goats. The study was conducted in 4 periods (4 x 4 Latin square design), where each period was for a duration of 22 d; 10 dof adaptation period, 5 dof sampling and 7 dof change-over. The animals were fed once daily at 0800 (3% body weight) with 60% of urea-treated rice straw and 40 % of one of four concentrate diets: T1-basal diet + 1% *A. paniculata*, T2-basal diet + 1% *O. stamineus*, T3-basal diet + 0.5% of *A. paniculata* and 0.5% *O. stamineus* (AO) and T4-basal diet without supplementation of herbs. Clean water was provided *ad libitum* and the animals were individually penned. Rumen contents were sampled at 0, 2, 4, 6 and 12 hafter the onset feeding and the pH was recorded. Rumen pH, VFA's, concentration of ammonia and microbial population in the rumen fluid were measured. The mean rumen pH was the highest (P<0.05) at 2 h in T3 after the onset feeding while the mean concentration (mg/L) of ammonia in the rumen fluid was the lowest at 6 and 12 h in T2 (P<0.05). The molar proportion of valerate was higher (P<0.05) at 6 h in T1. Meanwhile, the acetate to propionate ratio was affected by time where it was significantly higher at 12 h in T3. Significant reduction of total protozoa, methanogens, *F. succinogens* and *R. albus* number was observed in the herb-supplemented groups (P<0.05). The results suggest that urea-treated rice straw with herbs supplementation can be fed to goats without impairing their performance. However, further study could be done by increasing the supplementation of herbs in order to observe more effective results.

Keywords: rumen fermentation, microbial population, *Andrographis paniculata*, *Orthosiphon stamineus*, small ruminants

Introduction

The ruminant industry in Malaysia is not well developed in spite of the emphasis and priority it has received from the Government in its development plans (Wan Zahari *et al*., 2013). Currently, the self sufficiency level for beef in Malaysia is only 28% and less than 10% in milk production. The issues related to the feed supply, nutrition and low productivity are the attributing factors that slow the ruminant industry in Malaysia. To overcome this limitation, the use of additives and growth promotants to improve efficiency of feed utilization has been widely practiced by livestock farmers in tropical countries (Khejornsart and Wanapat, 2011). In the past, antibiotics were commonly used as a growth

enhancer, but this has been banned worldwide due to risk to human health (Chiquette, 2009; Rolfe, 2000). Other additives such as probiotics, prebiotics and herbs are being investigated. *Andrographis paniculata* and *Orthosiphon stamineus* are the examples of herbs that have potential as growth promoters (Karami *et al.*, 2010; Yusuf *et al.*, 2014).

A. paniculata has been used for centuries in Asia for treating various ailments in humans, notably common colds, influenza, upper respiratory infections (Akbar, 2011; Deng, 1978). *A. paniculata* has been identified as one of the herbs that contain diterpenoids and natural polyphenols such as flavanoids and tannins (Chao and Lin, 2010). These secondary metabolites are believed to improve the rumen fermentation and microbial activity of goats. In a study conducted by Yusuf *et al.* (2014), the inclusion of *A. paniculata* in Boer goats' diet had significantly improved the feed intake, weight gain, feed efficiency and live weight. Besides, the ratios of carcass to fat, lean to bone, lean to fat, and composition of meat were also improved.

On the other hand, Adnyana *et al.* (2013) reported that *O. stamineus* contains more than a hundred chemical compounds which include monoterpenes, diterpenes, triterpenes, saponins and flavonoids. In a study conducted by Malahubban *et al.* (2013), *A. paniculata* and *O. stamineus* were fed in the diet of broiler chickens to observe on their growth performance and carcass characteristics. It was found that, weight gain of broiler chickens fed on a diet supplemented with powdered *O. stamineus* was improved significantly over birds raised on a control diet. Further, the abdominal fat component of broiler chickens was also reduced by *O. stamineus*. Thus *A. paniculata* and *O. stamineus* were shown to be beneficial as a supplement in the broiler chicken diet and also in goats that they have the potential of replacing other conventionally used inorganic feed additives that may be unsustainable or hazardous.

However, both of *A. paniculata* and *O. stamineus* had never been tested to enhance the microbial population of goats. Therefore this study was conducted to examine the changes in terms of microbial population, volatile fatty acids (VFAs) concentrations and digestion of fibre that occur in the rumen of goats as a result of *A. paniculata* and *O. stamineus* herbal supplementation.

Materials and Methods

Rice Straw Treatment

Rice straws were chopped to 4-6 cm in length and treated with 3% of urea. The amount of water added was 50% from the total weight of rice straw used. Treated rice straws were ensiled in drums and were opened for feeding trial after 21 d.

Experimental Animals and Diets

Four fistulated Boer cross-bred bucks with mean body weight of 25 kg were used in this study conducted in 4 periods using 4 x 4 Latin square design. Each period consisted of 22 d; where 10 d were used for adaptation period, 5 d of sampling and the remaining 7 d as a change-over period. Clean water was provided *ad libitum* and the animals were individually penned. The animals were fed once daily at 0800 at 3% body weight with 60% of urea-treated rice straw and 40% of one of four concentrate diets: basal diet + 1% *A. paniculata* (T1), basal diet + 1% *O. stamineus* (T2), basal diet + 0.5 % of *A. paniculata* and 0.5 % *O. stamineus* (T3) and a basal diet without supplementation of herbs (T4).

Table 1. Ingredients and chemical composition of diets with supplements and urea treated rice straw

Items	Supplements				Urea treated rice straw
	AP	OS	AO	BD	
Ingredients (%)					
Maize	40	40	40	40	-
SBM	35	35	35	35	-
PKC	22	22	22	22	-
$CaCO_3$	1	1	1	1	-
Vitamin mineral mixture	1	1	1	1	-
NaCl	1	1	1	1	-
Herbs	1	1	1	0	-
Chemical composition (%)					
Dry matter	90.50	90.83	90.79	89.87	96.03
Organic matter	34.67	34.36	30.43	24.10	13.38
Crude protein	18.01	19.26	18.48	17.02	5.72
NDF	45.18	35.82	45.55	33.14	76.91
ADF	14.80	12.39	13.47	11.84	55.81
ADL	11.89	8.04	6.51	8.40	8.38

AP: Basal diet + 1% *A. paniculata*, OS: Basal diet + 1% *O. stamineus*, AO: Basal diet + 0.5% of *A. paniculata* and 0.5% *O. stamineus*, BD: Basal diet without supplementation of herbs, NDF: Neutral detergent fibre, ADF: Acid detergent fibre, ADL: Acid detergent lignin.

Sampling Procedure

Rumen contents were collected at 0, 2, 4, 6 and 12 h every day after the onset of feeding. A small proportion of rumen content from each animal was squeezed through one layer of cheesecloth and the ruminal fluid was used for a measurement of pH and quantification of rumen microbial population. Approximately 10 mL of fresh rumen content was stored at -20 ^{o}C and was used for quantification of total bacteria, methanogens, protozoa and three major fibre-degrading bacteria; *Fibrobacter succinogens*, *Ruminococcus albus* and *Ruminococcus flavefaciens* using real-time PCR techniques.

The remaining rumen content was squeezed through four layers of cheesecloth, mixed with 2 drops of concentrated sulphuric acid and kept frozen at -20 ^{o}C for the analysis of volatile fatty acids (VFA's) and determination of ammonia.

Chemical Analysis

For the analysis of VFA, the frozen samples were thawed at 4 ^{o}C and mixed with 0.4 mL of 25% (w/v) meta-phosphoric acid. It was then centrifuged at 3000 g for 10 min. The supernatant (0.5 mL) was mixed with the same volume of 20 mM 4- methyl N-valeric acid as an internal standard. Ruminal VFA profile was assessed by gas chromatography (Agilent 6890, Mississauga, ON, Canada) as described by Cottyn and Boucque (1968).

Determination of ammonia-N content in the rumen sample was done by determining the regression equation from blank and standard samples (Parson *et al.*, 1984). Standard solutions of 0.2, 0.5, 1.0 and 2.0

ppm were prepared by dissolving ammonium chloride in distilled water. Approximately 5 ml of rumen sample were added with 0.2 mL of phenol and swirled. In sequence, 0.2 mL of nitroprusside and 0.5 mL of oxidizing solution were added and swirled again. It was allowed to stand for one hand the absorbance was determined at 640 nm.

Feeds were analyzed for DM, OM and CP according to procedure of AOAC (1990) while ADF and NDF were determined using the method of Van Soest *et al.* (1991).

Real-time PCR

The DNA was isolated from the rumen samples using CTAB DNA extraction as described by Nettmann *et al.* (2008). The targeted rumen microbial populations, primer sequences, annealing temperature and literature references in this study are presented in Table 2. The real-time PCR was conducted using BioRad CFX96 real-time PCR system (BioRad, USA) complete with optical grade plates. A total volume of 25 µl QuantiFast® SYBR® Green PCR kit (Qiagen Inc., Valencia, USA) which included 12.5 µl of 2 x SYBR Green Master Mix, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 2 µl of DNA sample and 8.5 µl nuclease-free water for each reaction, the RT-PCR was carried out. The samples were analyzed in triplicates reactions. To ensure that there was no cross-contamination, a no-template control was established in the real-time PCR amplication to rule. The real-time cycling conditions were set up as follows: 94 ^{o}C for 5 min for initial denaturation, 40 cycles of 94 ^{o}C for 20 sec for primer annealing of total bacteria, methanogens, total protozoa, *F. succinogens* and *R. albus* while *R. flavefaciens* was at 60 ^{o}C and the extension at 72 ^{o}C for 20 sec (Navidshad *et al.*, 2012).

Table 2. The sequence of primers used targeting total bacteria, methanogens, protozoa, *F. succinogens, R. albus* and *R. flavefaciens*

Target bacteria	Sequence 5'-3'	Annealing temperature (^{o}C)	References
Total bacteria	F-CGGCAACGAGCGCAACCC R-CCATTGTAGCACGTGTGTAGCC	55	Koike and Kobayashi (2001)
Methanogens	F-CCGGAGATGGAACCTGAGAC R-CGGTCTTGCCCAGCTCTTATTC	55	Zhou *et al.* (2009)
Total protozoa	F-CTTGCCCTCYAATCGTWCT R-GCTTTCGWTGGTAGTGTATT	55	Sylvester *et al.* (2004)
F. succinogens	F-GTTCGGAATTACTGGGCGTAAA R-CGCCTGCCCCTGAACTATC	55	Lane (1991)
R. albus	F-CCCTAAAAGCAGTCTTAGTTCG R-CCTCCTTGCGGTTAGAACA	55	Koike and Kobayashi (2001)
R. flavefaciens	F-TCTGGAAACGGATGGTA R-CCTCCTTGCGTTAGAACA	60	Koike and Kobayashi (2001)

Preparation of Standard Curve

The real-time PCR standard curve method was conducted to quantify the rumen microbial populations of goats. Using the DNA extracted from pure culture of each target rumen microbe, the standard curves were plotted and the amplication of bacterial DNA was carried out by the normal PCR. The PCR products of the observed bacteria were run in 1% agarose gel while the specific bands on the other hand were purified using the MEGAquick-spin™ purification kit (iNtRON Biotechnology, Korea). A Nanodrop ND-1000 spectrophotometer (Implen NanoPhotometer™, Germany) was used to measure the purity and concentration of 16S real-time PCR gene. Using the following formula that is available online in Genomics and Sequencing Center web-based calculator of University of Rhode Island, the number of copies of the 16S real-time PCR gene per mL of elution buffer was calculated.

$$No.of\ copies = \frac{Amount\ of\ DNA\ \left(\frac{g}{ml}\right) \times 6.022 \times 10^{23}}{Length\ (bp) \times 10^{9} \times 650}$$

The slope value of linear regression of each standard curve was used to determine the amplication of efficiency (E) of each primer-template combination due to the probability of variation in the efficiency of amplication between primers and templates. The E was calculated by the following equation:

$$E\ (\%) = \left[10^{\left(-\frac{1}{slope}\right)} - 1\right] \times 100$$

In this equation, E was 100 % if a 10-fold dilution of DNA template results in a Cq (quantification cycle) difference of 3.32 upon completion of the amplication, the specificity of the amplified product was confirmed by melting curve analysis. The real-time PCR products were incubated by raising the temperature from 70 ^{o}C to 95 ^{o}C in 0.5 ^{o}C increments with a hold of 5 sec at each increment.

Statistical Analysis

All the experiments were run in triplicates and the data were subjected to General Linear Model (GLM) procedure of SPSS 17 (StatSoft Inc., Tulsa, Oklahoma). Mean treatment differences were determined by Duncan multiple range test (DMRT) (Steel & Torrie, 1980) at $P<0.05$.

Results and Discussion

Rumen pH

The mean rumen pH value ranged from 6.1 to 7 and was affected by hour of sampling. The treatment diets of the present study, however, did not show any significant effect on the ruminal pH of goats. The pH values were relatively low before the animals started consuming the diets and started to increase shortly after they started to consume the diets. However, the pH then continuously decreased until the end of sampling hour. The result of the present study was in agreement with Canesin *et al.* (2014) where the supplementation of citrus pulp and cotton seed meal was similar among the supplementation treatments, while significant differences were observed at 0 h (before supplementation) and from 2 h after supplementation to 8 h after supplementation. According to Stakelum (1993), the rapid decline in rumen pH could be associated with feeding concentrates and was due to the rapid fermentation of starch. This was because, as the rumen pH continuously decreased throughout the day, the fermentation activity in the rumen might have decreased too. Rumen microbes could not grow well because nutrients might be

used for maintenance function rather than for growth. Hoover (1986) reported that, when rumen pH was approximately 6, bacterial cellulolytic activity was moderately depressed but the number of cellulolytic bacteria was not affected.

Rumen Fermentation Characteristics

In ruminants most of the energy used for maintenance and production performance is provided by VFAs (Allen, 1997). The most important VFAs have been formed in rumen during the fermentation of acetate, propionate and butyrate (Van Houtert, 1993). In the present study, there was no significant difference observed in the total VFA content and proportions of other VFAs such as acetate, propionate and butyrate production among the treatment diets (Table 3). The present results are in agreement with those presented by Zhu *et al.* (2012) where the inclusion of garlic in goats diet did not showany significant effect on total VFA concentration and individual VFA molar proportions. Besides that, Singh *et al.* (2011) also showed no significant effect on the total VFAs concentration and molar proportions of acetate, propionate, butyrate and acetate to propionate ratio when *Ficus infectoria* leaves were supplemented in goats diet. In contrast, Yusuf *et al.* (2014) observed that the supplementation of both 1.5% (w/w) leaf powder of *A. paniculata* and 1.5% (w/w) whole plant of *A. paniculata* of herbs had increased significantly the total rumen VFA and ruminal pH values in Boer bucks' diet. The higher level of *A. paniculata* supplementation of the study conducted by Yusuf *et al.* (2014) compared to the level of supplementation in the present study could be a reason of the increment in total rumen VFA and ruminal pH of the rumen fluid.

Table 3. Effects of dietary treatments on rumen fermentation characteristics of goats

Item	Diet					Effect		
	AP	OS	AO	BD	SE	Diet	Hour	Diet X Hour
pH	6.55	6.54	6.61	6.53	0.04	NS	*	NS
NH_3-N(mg/L)	0.92	0.92	0.85	0.90	0.08	NS	*	NS
Total VFA (mmol)	93.42	91.78	90.44	95.38	11.62	NS	NS	NS
VFA (mol/100 mol)								
Acetate	79.65	74.28	70.11	74.41	10.54	NS	NS	NS
Propionate	19.44	17.42	14.51	19.85	1.85	NS	NS	NS
Butyrate	13.59	11.16	10.54	15.51	1.69	NS	NS	NS
Valerate	1.76	2.60	2.65	2.85	0.08	*	*	NS
Acetate:Propionate	4.34	4.68	4.88	4.56	0.16	NS	*	NS

Significantly different at 5% ($P<0.05$)

AP: Basal diet + 1 % *A. paniculata*, OS: Basal diet + 1 % *O. stamineus*, AO: Basal diet + 0.5 % of *A. paniculata* and 0.5 % *O. stamineus*, BD: Basal diet without supplementation of herbs.

*$P < 0.05$

Among the VFA molar proportions in the present study, the production of valerate was found to have significant effect on the treatment diet and hour of sampling where the lowest proportion of valerate was observed in treatment AP. Compared to acetate, propionate and butyrate, which are involved in the fermentation of fibre and

starch, valerate does not have the clear role in the fermentation process (Bergman, 1990). Thus, the decrement of valerate in the study was probably due to the experimental conditions including animal condition, diets/substrate and adaptation time used.

In the present study, the reduction of ammonia concentration over time could be seen at its lowest time during 6 and 12 h of incubation. There was no significant difference among the treatment diets observed and the results is in contrast with Yusuf *et al.* (2014) where the supplementation of both leaf powder and whole plant of *A. paniculata* had significantly reduced the concentration of ammonium nitrogen in Boer bucks' diet. Wanapat *et al.* (2013) however, observed that, the supplementation of lemon grass meal, peppermint powder and garlic had no significant effect on ammonia. Ivan *et al.*, (2009) reported that the reduction of ammonia in the rumen fluid could be significantly observed in defaunated ruminants with reduced fauna. Reduced recycling of bacterial protein due to lower protozoal predation and higher bacterial population to utilize ammonia is also the main cause of lower rumen ammonia concentration in the animals (Wallace *et al.*, 1987). Microbes become less efficient at using ammonia if energy is limited. Thus, instead of being converted into microbial protein, the ammonia is then converted to urea. Most of this urea is excreted in the urine although some is recycled back into the rumen as non-protein nitrogen in the saliva (Moran, 2005). In this study the decreasing ammonia concentration over time could be due to this effect.

Rumen Microbial Population

The information on the population of microorganisms in the rumen of goats fed with by-products like rice straw supplemented with herbs is still lacking. This is possibly due to the lack of knowledge about the nutrients contained in the local herbs among the farmers in Malaysia. The data presented in this study showed that both *A. paniculata* and *O. stamineus* supplementation in urea-treated rice straw diet had significantly reduced the number of protozoa, methanogens, *F. succinogens* and *R. albus* except total bacteria and *R. flavefaciens.*

Table 4. Quantification of total bacteria, total protozoa, methanogens and fibre-degrading bacteria (Log^{10} copy number per g)

Item	Diet				SE	Effect		
	AP	OS	AO	BD		Diet	Hour	Diet X Hour
Total bacteria	8.87	8.6	8.91	8.97	0.22	NS	NS	NS
Protozoa	5.8^{a}	5.79^{a}	5.55^{a}	6.15^{b}	0.07	*	*	*
Methanogens	6.71^{ab}	6.93^{bc}	6.56^{a}	7.18^{c}	0.07	*	NS	NS
F. succinogens	5.21^{a}	5.37^{b}	5.31^{ab}	5.73^{c}	0.02	*	*	*
R. albus	7.07^{ab}	7.25^{b}	6.85^{a}	7.26^{b}	0.06	*	NS	*
R. flavefaciens	4.01	3.89	4.05	4.06	0.22	NS	*	NS

Significantly different at 5% ($P<0.05$)

^{abc}Means with different letter within a row differed significantly.

AP: Basal diet + 1% *A. paniculata*, OS: Basal diet + 1% *O. stamineus*, AO: Basal diet + 0.5% of *A. paniculata* and 0.5% *O. stamineus*, BD: Basal diet without supplementation of herbs.

The reduction in the number of protozoa, *F. succinogens* and *R. albus* in rumen of goats fed urea-treated rice straw supplemented with *A. paniculata* and *O. stamineus* was in line with the results of Singh *et al.* (2011) and Chaturvedi *et al.* (2015). Previously, it was observed that supplementation of *F. infectoria* leaves in goats diet resulted in the reduction of the number of protozoa and the three groups of fibre-degrading bacteria. Similar result was obtained in the present study as Singh *et al.* (2011) where the number of total bacteria in the rumen of goats remained unchanged for both the control and supplemented groups. In contrast, Chaudhary *et al.* (2011) observed that the supplementation of herbs in goat's diet did not show any significant change in the population density of total bacteria, *R. flavefaciens, F. succinogenes* and methanogens. Moreover, Wanapat *et al.* (2008) reported no effects of supplemented herbs on the number and composition by total bacteria and genera of ciliate protozoa populations. On a side note, most studies showed significant effects on the number of methanogens in goats supplemented with herbs (Belanche *et al.*, 2014; Jahromi *et al.*, 2012; Kumar *et al.*, 2009).

The main factors affecting the population and the abundance of the bacterial community, especially fibre-degrading bacteria in the rumen were heavily influenced by the dietary conditions (Samsudin *et al.*, 2014). This theory is supported by Cardozo *et al.* (2004), where in a study conducted previously showed herbs supplementation may affect the microorganism population in the rumen, and also effective in enhancing beneficial bacteria or reducing pathogenic bacteria. In the present study, reduction of protozoa, methanogens, *F. succinogens* and *R. albus* number were observed when *A. paniculata* and *O. stamineus* were introduced to the goats compared to control group. Varel *et al.* (1991) stated that this situation could have been due to the presence of secondary metabolite compound in the herbs. Tang and Eisenbrand (1992) found that *A. paniculata* contained various chemical constituents which included diterpenoids, flavonoids, glycosides, alkaloids and saponin which are believed to be responsible for the biological activities of the plant which alter and improve the rumen microbial activity of goats. On the other hand, Tezuka *et al*. (2000) reported that *O. staminues* contained several active chemical compounds such as terpenoids (diterpenes and triterpenes), polyphenol (lipophilic flavonoids and phenolic acids) and sterols, besides possessing high antioxidant properties in the leaves due to greater phenolic fraction compared with the other parts of the plant. These secondary metabolites in both of the herbs are believed to be toxic to protozoa and have been identified as possible defaunating agents (Newbold *et al.*, 1997). Besides that, Francis *et al.* (2002) added that the antiprotozoal effects in the plant herbs may be due to the presence of saponins which has the capacity to form irreversible complexes with the cholesterol in protozoal cell membrane causing break down in the membrane leading to cell lysis and death, thus reducing the number of protozoa as in the present study. Moreover, the secondary metabolites in the supplemented herbs may also have the ability to reduce methane production. This is supported by Bunglavan *et al.* (2010) where the inhibitory action of methanogens in the study showed that supplementation of *Psidium guajava* leaves had reduced the number of methanogens due to presence of phytochemical constituents; i.e. alkaloids, saponins, steroidal rings and deoxy sugars. Last but not least, the secondary metabolites in the herbs are also believed to have an inhibitory effect on the growth of fibre-degrading bacteria, thus inhibiting the attachment of the fibrolytic

bacteria to fibre particles, thus reducing the number of *F. succinogens* and *R. albus* in the present study.

Due to the effects of global warming and climate change caused by greenhouse gas emissions, it has led to a great concern worldwide (Solomon *et al.,* 2007). The world's population of ruminants is estimated to produce around 15% of total methane emission (Moss *et al.,* 2000). Loss of gross energy intake ranging from 2-15% during the methane production of anaerobic fermentation has led to the reduction of potential conversion of feed energy to metabolizable energy. There are many efforts being made to reduce the metabolizable energy loss which include the inhibition or reduction of methane production (Johnson *et al.,* 1991; Van Nevel and Demeyer, 1995). According to Demeyer (1981), Jouany *et al.* (1988) and Tokura *et al* (1999), the methanogenic bacteria in the rumen have a close association with protozoa where methanogens will further utilizethe hydrogen produced by protozoa for methane formation (Hungate, 1966; Williams and Coleman, 1988**).** Thus, with the reduction in the number of protozoa and methanogens, and the help from fibre-degrading bacteria, formation of methane can be reduced and subsequently reducing the green house effect.

Conclusion

In conclusion, the results of the present study indicated that feeding diets supplemented with *A. paniculata*, *O. stamineus* and their combination was not able to enhance the ruminal fermentation characteristics in the goats' rumen in restricted diet. However, *A. paniculata* and *O. stamineus* managed to reduce the population of total protozoa, methanogens and two of fiber-degrading bacteria; *F. succinogens* and *R. albus* in the rumen of goats, quantified using RT-PCR. The results suggest that, urea-treated rice straw with herbs supplementation can be fed to goats without impairing their performance.

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