



## Effect of Feed Types on Ochratoxin A Disappearance in Goat Rumen Fluid

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**ABSTRACT :** This study was conducted to investigate the effect of feed types on Ochratoxin A (OTA) degradation by Korean native goats. Rumen fluid from cannulated goats fed whole roughage or 50% roughage served as a source of micro-organisms. Experiments were undertaken i) to investigate OTA degradation ability in a 2×4 factorial arrangement with different feed types (100% roughage vs. 50% roughage) and rumen fluid fractions (whole rumen fluid, cells, autoclaved rumen fluid and supernatant) supplemented with OTA ii) to evaluate OTA degradation by the rumen fluid of goats fed two different diets at different time points (0, 3, 6, 9 and 12 h) of feeding iii) to isolate potential rumen microorganisms and iv) to identify elements responsible for OTA degradation. Rumen fluid from goats fed 100% roughage had higher ( $p < 0.05$ ) OTA degradability than 50% roughage diets. OTA degradation based on rumen fluid collection times showed that rumen fluid at 0 h showed significantly higher ( $p < 0.05$ ) degradability. Carboxypeptidase A (CPA) enzyme has been reported to be responsible for OTA degradation. Thus, using real time PCR, primers designed to target the CPA gene from *Bacillus licheniformis* could be amplified using genomic DNA from rumen fluid of goats and sequenced, thus enabling evaluation of the *Bacillus* population under different feeding condition and times. Our findings showed that the *Bacillus* population was significantly higher ( $p < 0.05$ ) before feeding (0 h) in animals which were fed a whole roughage diet, giving indirect evidence of OTA degradation being influenced by *Bacillus* spp. Thus, it can be concluded that OTA degradability is influenced by feed, feeding time and *Bacillus licheniformis* population. (**Key Words :** Ruminants, Ochratoxin A, Degradation, Carboxypeptidase A, *Bacillus*)

### INTRODUCTION

Ochratoxins are a group of secondary metabolites produced by fungi of two genera: *Penicillium* and *Aspergillus*. The primary toxin was identified as ochratoxin A (OTA); its less toxic dechloro analog is ochratoxin B (OTB) (van der Merwe et al., 1965). Ochratoxin A contaminate cereals, coffee beans, grape and other fruits, beer and wine (Halasz et al., 2009). It has a proven toxic property and is primarily known for its nephrotoxicity and carcinogenicity seriously affecting animal performance and well-being (Abrunhosa et al., 2007), as well as it causes deleterious effects on humans (Alexandros and Jean-Pierre, 2002). Among the greatest concern in humans is its implicated role in an irreversible and fatal kidney disease referred to as Balkan endemic nephropathy and its potent carcinogenic effects.

Ochratoxicosis has rarely been found in ruminants. Degradation of OTA to its alpha metabolite (OT $\alpha$ ) is known to be the principal means of detoxification of OTA. Hult et al. (1976) and Kiessling et al. (1984) reported that OTA was degraded to OT $\alpha$  by ruminal microbes, particularly by ruminal protozoa and the overall capacity of rumen fluid to degrade OTA was found to be highest in the rumen fluid shortly after feeding. Xiao et al. (1991) suggested that the type of diet is important: the rate of *in vitro* hydrolysis of OTA was more rapid with ruminal digesta from sheep fed hay than with ruminal digesta from those fed grain. Some reports indicated that the gut microbes lacking protozoa can have significant OTA degradation capacity (Mobashar et al., 2010) and rumen bacterial fractions has also been proved to have significant OTA detoxification ability (Keissling et al., 1984; Schatzmayr et al., 2006) However, the detoxification capacity of the rumen may be exceeded in cases of severe poisoning (Ribelin et al., 1978).

Several strategies have been reported for the decontamination/detoxification of mycotoxins-contaminated grains. This includes physical, chemical and biological

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means depending on the commodities. However, each treatment has its limitations since the treatment could be expensive and may lead to loss of nutritive values and there are also concerns in food and feed safety issues (Marquadt, 1996). In view of the extensive contamination of food and feedstuffs by mycotoxins originating as a secondary metabolite of different fungi, it is imperative to develop cost effective, safe and efficient methods for their decontamination. Biodegradation is a popular and safe technology that utilizes the metabolic potential of microorganisms or enzymes to decontaminate food or feedstuffs (Schatzmayr et al., 2006). Ruminants are potential source of microbes or enzyme for mycotoxin biotransformation (Upadhaya et al., 2010). Among the enzymes, the proteolytic enzyme, carboxypeptidase A was reported to have significant OTA detoxification capacity (Pituot, 1969; Stander et al., 2002). Main objectives of this study were to investigate OTA degradation by the rumen fluid of goats fed two different diets at different time points of feeding, to isolate bacteria or gene from rumen microbial source and to identify the element responsible for OTA degradation.

## MATERIALS AND METHOD

### Experimental animal and diet

Two Korean native goats (27±3 kg B/wt) served as the donor of rumen fluid. Animals were maintained on 40% concentrates (commercial preparation) having 16.5% crude protein (Corn Beef, Purina) and 60% roughages (Timothy; Feedland, Korea). For evaluation of OTA degradation in different fractions of rumen fluid as well as assessment of the effect of feed types on OTA degradability, a mixture of 50% roughage and 50% concentrate diet and whole roughage (100%) based diet were fed to two goats. One month of adaptation period was maintained in both feeding conditions. The rumen fluids from these animals fed different diets supplemented with purified OTA (Sigma) were used to assay the OTA degradability after incubation at 39°C.

### Ochratoxin

The purified OTA (5 mg powder) was procured from Sigma Aldrich Inc. (Spruce Street, St. Louis, USA) and dissolved in absolute ethanol (Merck, KGaA, Germany). Further dilution was done in sterilized distilled water to prepare the working solution. The concentration of working solution was further diluted so as to have the concentration within the detection limit of the kit (Agra quant Total Ochratoxin kit, 2-40 ng/ml, Romers' Lab, Sigapore). The concentration in ng/ml was then determined using ELISA reader (VERSA max microplate reader, Molecular devices,

Sunnyvale, CA, USA) at 450 nm wavelength filters.

### Rumen fluid collection

The rumen fluid along with the ingesta from the donor animals were collected separately in the morning before feeding into a 500 ml stainless steel vacuum bottles with a lid and immediately transferred to laboratory. The rumen fluid with the ingesta was gassed with oxygen-free CO<sub>2</sub> using the gassing apparatus and homogenized with a mixer (Mini mixer, Hanil, Korea) for 1min. Then the rumen content was strained through the 8 layers of cheese cloth for further experimentation. Fractionation of rumen fluid was done to obtain the supernatant and bacterial fractions using centrifugation at 4°C. The collection of rumen fluid for experiment on OTA degradation based on sampling time after feeding was done in sterilized falcon tubes of 25 ml capacity each in triplicate. Each fraction was immediately inoculated into sterilized Hungate tubes to a total volume of 5 ml, supplemented with OTA into a final concentration of 100 ng/ml, then sealed with screw caps fitted with butyl rubber stoppers (Bellco Glass, USA) in each sampling time after feeding and then incubated at 39°C for different duration.

### Experimental design

*Effects of rumen fluid conditions on OTA degradation :*

i) OTA degradation in different fractions of rumen fluid

The aim of this study was to investigate OTA degradation in different fractions of rumen fluid collected from goats fed different diets. Rumen fluid with the digesta from each goat was collected before feeding. The rumen fluid was filtered with 8 layers of cheesecloth after homogenization with a mini mixer. Four fractions were used in this experiment, whole rumen fluid (WRF), supernatant (SUP), cells (CELLS) and autoclaved rumen fluid (ARF). For preparation of different fractions we used the protocol as mentioned by Lee et al. (2000). After homogenization, the WRF was separated into 2 parts; one part was autoclaved at 121°C for 15 min. Briefly, to prepare fractions of supernatant and cell, WRF was further centrifuged at 500×g for 5 min at 4°C (Supra K21, High Speed centrifuge; Hanil Science Industrial, Korea) to remove the protozoan fractions. The collected supernatants having bacterial fractions were again centrifuged at 12,000×g for 30 min, and then the supernatants and cells were harvested for the fraction study. Each fraction was gassed with CO<sub>2</sub> and then experiment was carried out in anaerobic chamber. Each fraction was spiked with OTA (Sigma Aldrich Inc. Spruce Street, St. Louis, USA) in triplicates with the final OTA concentration of 100 ng/ml. Sampling for OTA degradation assay was done before incubation and after 6 h of incubation. Sterilized distilled

water supplemented with OTA served as the control.

ii) OTA degradation based on sampling time after feeding and pH

Rumen fluid was collected from each goat at 0h (before feeding in the morning), 3 h, 6 h, 9 h and 12 h after feeding, respectively. The rumen fluid was filtered with 8 layers of cheesecloth. The samples from each time point were spiked with OTA (Sigma) in triplicates from each goat, and then incubated in Hungate tubes in a shaking incubator at 39°C for 6 h. The pH was analyzed immediately to determine if it influences OTA degradation.

iii) Effect of pH on OTA degradation in *in-vitro* rumen fluid

Rumen fluid from each goat was collected before morning feeding. The rumen fluid was filtered with 8 layers of cheesecloth after homogenization with a mini mixer. Then pH was adjusted to 5.8, 6.8 and 7.8 by adding hydrochloric acid or sodium hydroxide. After gassing with CO<sub>2</sub>, further experiment was carried out in anaerobic chamber. Each fraction was spiked with OTA (Sigma) in triplicates at the final OTA concentration of 100 ng/ml. Sampling for OTA degradation assay was done before incubation and after 6 h of incubation at 39°C.

*Isolation of rumen bacteria having OTA degrading potentiality* : Rumen fluid from goat was collected before morning feeding. The rumen fluid was filtered with 8 layers of cheesecloth after homogenization with a mini mixer. The strained rumen fluid was gassed with CO<sub>2</sub> and serial dilution was done in Rumen-Glucose-Cellobiose (RGC) liquid media (Bryant and Burky, 1953) in an anaerobic chamber (COY laboratory products Inc., Grass Lake, Michigan). Then bacterial colonies were isolated in RGC agar media. Screening of bacterial isolate for OTA degradation was done by incubating the culture in a liquid medium spiked with OTA and degradation assay done by ELISA kit (Romers' Lab, Singapore).

*Amplification of carboxypeptidase A gene from genomic DNA of rumen fluid* : Various reports indicated that carboxypeptidase A is responsible for OTA degradation (Pitout, 1969; Deberghes et al., 1995; Stander et al., 2001). Primers for amplification of carboxypeptidase A gene from genomic DNA of rumen fluid was designed from different bacteria and insects sequences available from Genbank database. Among different primers, those designed from carboxypeptidase of *Bacillus licheniformis* (ATCC 14580) could be amplified. The amplified product was purified using QIA quick spin Gel extraction kit (Qiagen Korea Ltd.) as per manufacturer's protocol and cloned to pGem Teasy vector (Promega, USA) followed by transformation to *E. coli* (Top 10). Plasmid DNA from the transformants was obtained using Plasmid DNA purification kit (iNtron

Biotechnology Inc. Korea) as per the manufacturer's instruction. 16SrDNA of the sequence of the inserts in *E. coli* clones were determined by cycle sequencing.

*Real time PCR for Bacillus population determination under two feeding conditions* : Rumen contents from cannulated Korean native goat fed 100% roughage, and mixture of 50% roughage and 50% concentrate were collected in Corning tube, put into an icebox and transported to laboratory. Then the rumen fluid was filtered by using the 8 layers of cheese cloth. DNA extraction of rumen fluid was done by using cetyl-trimethyl ammonium bromide (Allen et al., 2006).

Sample derived standard was prepared from total genomic DNA of pooled rumen fluid from two goats in similar way as Wanapat and Cherdhong (2009). For amplification of partial DNA sequence of *Bacillus*, primers developed by De Clerck et al. (2004), targeting 16S rDNA were used. The amplified PCR product (267 bp) was gel purified and quantified using Nano Vue spectrophotometer (GE Life Sciences, Piscataway, NJ, USA) and used as standard for real time PCR. Sample derived standard, copy number concentration was calculated based on the length of PCR product and the mass concentration. Ten-fold dilutions were prepared in sterilized distilled water prior to real time PCR.

Genomic DNA from rumen fluid of goats fed two different diets was used as template. Real-time PCR amplification and detection was performed using a SYBR 490 system. The reaction was conducted in a final volume of 25 µl containing the following: 12.5 µl 2×SYBR Green super mix (IQ SybrGreen Supermix; Bio-Rad Inc. USA), 1 µl forward primer (10 pmol), 1 µl reverse primer (10 pmol), 9.5 µl autoclaved distilled water and 1 µl of DNA solution. PCR condition was as follows: One cycle of initial denaturation at 95°C for 3 min followed by 50 cycles of denaturation at 95°C for 30 s and annealing at 58°C for 30 s followed by extension at 72°C for 30 s.

#### Sample assay procedure

One hundred µl of ochratoxin test kit standards (0, 2, 5, 20 and 40 ng/ml) as well as the samples to be analyzed were each mixed with 200 µl of conjugate in individual dilution wells, and then 100 µl from each dilution well was transferred to a respective antibody-coated microwell. After 10 min incubation at room temperature, the plates were washed 5 times with distilled water and tapped dry into several layers of absorbent papers. Then 100 µl of enzyme substrate was added to each well and allowed to incubate for an additional 5 min. Stop solution (100 µl for each well) was then added and the intensity of the resulting yellow color was measured optically with a microplate reader at a wavelength of 450 nm. The total incubation time of the test

**Table 1.** OTA degradability (%) as influenced by feed types in different fractions of rumen fluid

Feed	Fractions					Mean	F	p-value
	Fn1	Fn2	Fn3	Fn4				
F100	2 <sup>a</sup>	68 <sup>b</sup>	5.2 <sup>a</sup>	100 <sup>c</sup>	44.031	F	0.0035	
F50	14 <sup>d</sup>	57 <sup>e</sup>	24.8 <sup>d</sup>	50 <sup>f</sup>	36.789	Fn	>0.0001	
Mean	8	62	15	75	-	F*Fn	>0.0001	

Means in the same row with different superscript differ significantly at  $p < 0.05$ .

Means in the same column with different superscript differ significantly at  $p < 0.05$ .

Fn1 = Supernatant, Fn2 = Bacterial fractions; Fn3 = Autoclaved rumen fluid; Fn4 = Whole rumen fluid F, Feed; Fn, fractions F\*Fn, Feed and fraction interaction, Feed 100; animals fed 100% roughage F50; animals fed 50% roughage.

kit assay was 20 min.

The absorbance obtained from the ELISA (Enzyme linked Immunosorbent assay) plate reader was interpolated to the Romers lab<sup>®</sup> data reduction spread sheet for the calculation of OTA concentration in samples ng/ml.

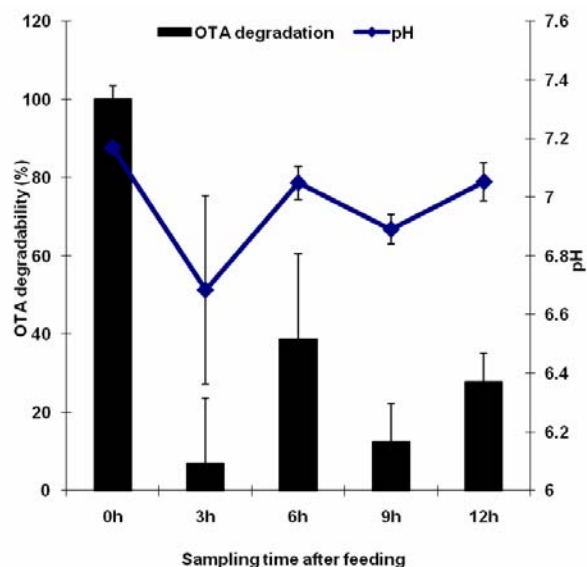
### Statistical analysis

Data generated were subjected to analysis of variance (ANOVA) procedure and mixed procedure of SAS (2002, SAS Inst. Inc. Cary, NC, USA). Differences among means were tested using least significant difference procedure (2002). Significance was declared at  $p < 0.05$  and  $p < 0.001$  was declared highly significant.

## RESULTS

### OTA degradation in different fractions of rumen fluid

In an *in vitro* experiment for OTA degradation using the

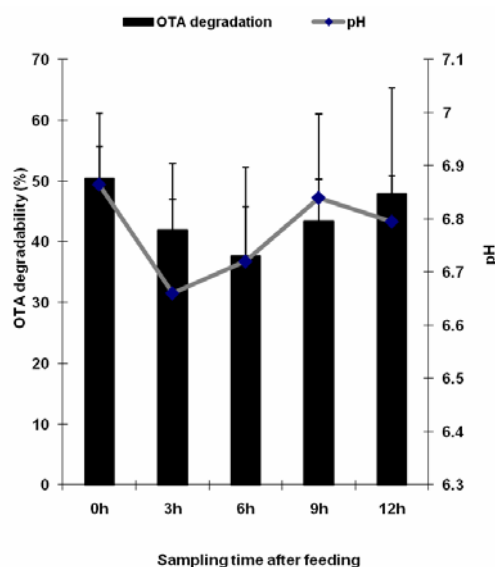


**Figure 1.** Changes in OTA degradability (%) and rumen pH during 0, 3, 6, 9 and 12 h after feeding 100% roughage diet. OTA = Ochratoxin A; Rumen fluid supplemented with OTA so as to have the final concentration of 100 ng/ml. Incubation was done at 39°C. for 6 h after each rumen fluid collection times of 0, 3, 6, 9, and 12 h after feeding the animal. Ochratoxin A degradation assay done by ELISA.

different fractions of rumen fluid obtained from goats fed a mixture having 50% roughage and 50% concentrate, it was found that 50% OTA was degraded in whole rumen fluid and about 60% OTA degraded by the cells. However, the degradation was much higher in goats fed 100% roughage diet (Table 1). In the present study, WRF from goats fed both diet had significantly higher OTA degradation compared to other fractions ( $p < 0.05$ ). Cell (bacterial cells) also degraded OTA significantly.

### Effects of sampling time after feeding on OTA degradation

The influence of feeding time on OTA degradation showed that in the rumen fluid of goat fed 100% roughage, OTA degradation was the highest before feeding and lowest after feeding (Figure 1). However, in the rumen fluid from goat fed 50:50 roughage:concentrate, OTA degradation was not much influenced by feeding times (Figure 2). Time after



**Figure 2.** Changes in OTA degradability (%) and rumen pH during 0, 3, 6, 9 and 12 h after feeding 50% roughage diet. OTA = Ochratoxin A; Rumen fluid supplemented with OTA so as to have the final concentration of 100 ng/ml. Incubation was done at 39°C for 6 h after each rumen fluid collection times of 0, 3, 6, 9, and 12 h after feeding the animal. Ochratoxin A degradation assay done by ELISA.

**Table 2.** Effect of pH on OTA degradation (%) in *in-vitro* rumen fluid media from the donor animals fed 100% roughage diet and 50% roughage diet

F100			F50			SEM	Significance		
5.8	6.8	7.8	5.8	6.8	7.8		Feed	pH	F*pH
76.1	99.15	99.29	47	71.69	70.36	4.41	**	**	NS

\*\* Highly significant at  $p < 0.001$ ; NS = Non significant.

F 100; animals fed 100% roughage F50; animals fed 50% roughage. F\*pH; Feed and pH interaction.

feeding significantly influenced pH in the rumen of goat fed 100% roughage (Figure 1) but not much change in pH was observed in the rumen fluid of goats fed 50:50 roughage: concentrate in different time points (Figure 2). The pH generally decreased after feeding and did not increase again until after 4 h.

#### Effect of pH on OTA degradation in *in-vitro* rumen fluid media

We conducted *in vitro* incubation study to see the effect of different pH on OTA degradation by adjusting pH of incubation media. It was found that rumen fluid obtained from goat fed both types of diets had significantly lower ( $p < 0.05$ ) OTA degradation when pH was adjusted to acidic (pH 5.8) (Table 2). The rumen fluid of goats fed 100% roughage had significantly higher ( $p < 0.05$ ) OTA degradation ability compared to 50% concentrate feeding ( $p < 0.05$ ) regardless of media pH. However, the interaction between feed and pH was non significant.

#### Isolation and screening of bacteria having OTA degrading potentiality

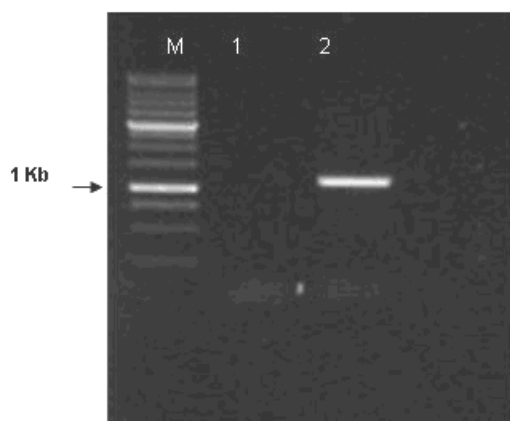
About 200 bacterial isolates were isolated from the rumen anaerobically using RGC media and screened for OTA degradation using ELISA (data not shown). Among the bacteria isolated, none of them were found to have high

OTA degrading ability. So identification of and characterization of isolates was not done and the study was focused to find the gene responsible for OTA degradation from the rumen of goats.

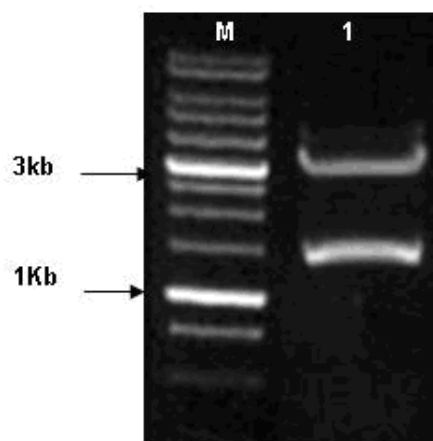
#### Amplification of carboxypeptidase A gene from genomic DNA of rumen fluid

Carboxypeptidase A gene from the genomic DNA of rumen fluid of goat was amplified with primers designed from CPA gene of *Bacillus licheniformis* with the expected product size of 1.2 kb. Electrophoresis analyses of amplified PCR products with carboxypeptidase A primers is shown in Figure 3. Purified PCR product was cloned into p GEM T easy Vector (Promega, USA) to transform *E. coli* (Top 10) and the plasmid DNA of recombinant clone were digested with *ECORI* (Bio labs, England) for the confirmation of the presence of inserts. After confirmation of the presence of inserts (Figure 4), plasmid DNA was sequenced. Blasting the sequence result in genbank database showed it had 100% sequence identity with carboxypeptidase A (CP 00000.2) from *Bacillus licheniformis* (ATCC 14580). This led us to investigate on the population study of *Bacillus spp.* using real time PCR to analyze the copy number of *Bacillus* in the rumen under different feeding conditions.

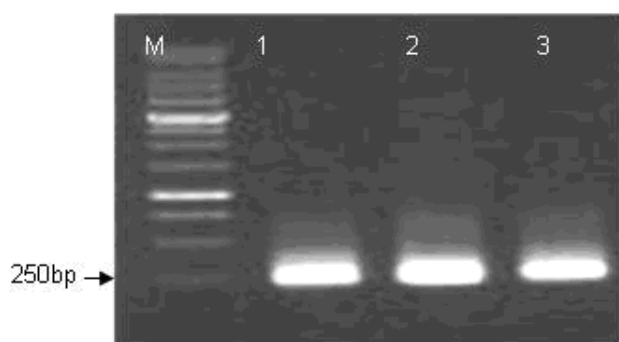
#### Real time PCR for *Bacillus* population determination



**Figure 3.** Electrophoretic analysis of amplified PCR products with carboxypeptidase A primers. M = Marker, 1 kb ladder; Lane 1 = control, Lane 2 = PCR amplified product with primer designed from Carboxypeptidase A gene of *Bacillus licheniformis* using genomic DNA of rumen fluid as a template.



**Figure 4.** Electrophoretic analysis of plasmid DNA of recombinant clone after *EcoRI* digestion. M = Marker, 1 kb ladder; Lane 1 = *EcoRI* digested plasmid DNA from clone harboring CPA gene of 1.12 kb product size.



**Figure 5.** Electrophoretic analysis of PCR product amplified with bacillus primers. M = marker, 1 kb; Lane 1, 2 and 3 = PCR product amplified with Bacillus primer using genomic DNA of goat rumen fluid as a template.

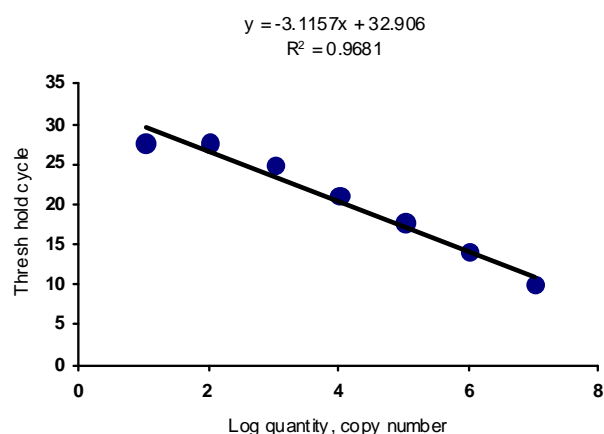
### pre and post feeding with two different diets

External standards for real time PCR was prepared from rumen genomic DNA by PCR amplification using Bacillus primer. The amplified PCR product (Figure 5) was gel purified and used as standard for real time PCR. Linear regression derived from the threshold cycle [C<sub>t</sub>] of each DNA dilution versus the log quantity was calculated. Logarithms of the DNA concentration (copies/μl) were plotted against the calculated means, obtaining a straight line of equation:  $y = -3.115x + 32.906$  (where y is the C<sub>t</sub> and x is the log of DNA concentration (Figure 6). The equation was used to quantify DNA from rumen fluid of goats fed two different diets.

The real time PCR results showed that the *Bacillus* population (DNA concentration) fed 100% roughage diet was significantly higher ( $p < 0.05$ ) than that in rumen fluid of goats on 50% concentrate diet and in both feeding conditions, the DNA concentration of *Bacillus spp.* was significantly higher ( $p < 0.001$ ) in rumen fluid collected before morning feeding (Table 3) compared to the rumen fluid collected at 3 h after morning feeding.

## DISCUSSION

The study on the effect of feed types on OTA degradability by ruminal fluid showed that the OTA degradation was highest in the ruminal fluid from goats fed with 100% roughage when rumen fluid was sampled before



**Figure 6.** Standard curve of real time PCR. Standard curve obtained by plotting the logarithm of the DNA concentration for Bacillus versus threshold cycle (C<sub>t</sub>) mean values. The curve was constructed using the data from all the seven triplicate standards amplifications.

feeding compared to goats fed 50% concentrate. In the previous studies Xiao et al. (1991) also demonstrated that upon incubation of OTA in the ruminal fluid of animals fed with hay, five times as much OTA was degraded than in the ruminal fluid from sheep fed with cereals. Similarly, Hohler et al. (1999) indicated that hydrolysis of OTA was less in sheep fed concentrate rich diets. Upadhaya et al. (2009) also reported that feed types influences aflatoxin B<sub>1</sub> degradation by rumen fluid of Korean native goat. Hult et al. (1976) and Kiessling et al. (1984) in previous studies have indicated that OTA was degraded to OT $\alpha$  by ruminal microbes, particularly by ruminal protozoa. In the rumen fluid obtained from goats fed 50% concentrate, OTA degradation was not much influenced by rumen fluid sampling times. Unlike in rumen fluid obtained from goat fed 50% concentrate, the OTA degradation as well as pH in rumen fluid obtained from goat fed 100% roughage, was lowered after 3 h of feeding. The lower degradation in the rumen fluids of goat fed 100% roughage after feeding could be due to the incubation of rumen fluid without any energy substrate leading to limited bacterial activity.

In the present study OTA degradation was also seen in bacterial fraction. On the other hands, supernatant fraction containing no bacteria or protozoa showed almost no degradation ability, indicating that OTA was degraded

**Table 3.** Comparative quantities of *Bacillus licheniformis* bacterial DNA concentration from rumen fluid of goat at different feeding times and types using real-time PCR

Feeding type	DNA concentration (copies $\pm$ SD/ $\mu$ l)		
	Pre feeding (0 h)	Post feeding(3 h)	p-value
F100	1.56 $\pm$ 0.11( $\times 10^6$ )	1.07 $\pm$ 0.02( $\times 10^6$ )	0.0016
F50	2.66 $\pm$ 0.51( $\times 10^5$ )	1.83 $\pm$ 0.14( $\times 10^5$ )	0.0019
p-value	<0.0001	<0.0001	

F 100; animals fed 100% roughage F50; animals fed 50% roughage and 50% concentrate.

mainly by the microbes and that the microbial enzymes degrading OTA was cell-associated. In our study the autoclaved rumen fluid also showed significantly low degradation although some studies indicated that the dead cells were also able to adsorb OTA (Peteri, 2007). Possibly the dead cells in autoclaved rumen fluid in our study could have adsorbed OTA to some extent leading to significantly low degradation.

In this study, the pH generally decreased after feeding in the rumen fluid from goat fed 100% roughage and did not increase again until after 4hr. Our results agreed with Schaadt et al. (1969) who observed that in sheep fed corn silage, pH in the rumen fluid obtained before feeding was the highest and reduced until after four hours of feeding. In this study the rumen fluid was sampled at different times before and after feeding the goats and tested for OTA degrading activity. The highest ability was found just before feeding and OTA degradation reduced immediately after feeding, which indicates that rumen condition after feeding contribute to decline of OTA degradation ability. Similar result was found by Kiessling et al. (1984), who reported lowest degrading activity after 1hr onwards. In case of the 50% concentrate feeding, no significant difference was observed in different sampling times. The mechanism for this is not apparent from data of present study. This could be due to the fact that with intermediate level of concentrates, the protozoa density is highest favoring OTA degradation to about 50% at all sampling times. Differences in OTA degradation between two feeds may also be explained by different types and number of microbial population and to some extent the ruminal pH. Perhaps at higher ruminal pH (before feeding and with 100% roughage feeding), microorganisms responsible for OTA degradation proliferated more or enzymes degrading OTA became more active. The higher OTA degrading ability at higher pH was confirmed by our study on the effect of pH on OTA degradation in *in-vitro* rumen fluid media by adjusting pH to 5.8, 6.8 and 7.8.

The bacteria isolated from rumen in the present study were not found to have high OTA degrading ability. The possible reason could be either the potential bacteria could not be cultivated under laboratory conditions or the synergistic effects of different bacteria in bacterial cell fractions or co-cultures might have led to OTA degradation rather than a single pure isolate.

Research findings demonstrated that carboxypeptidase A gene is responsible to hydrolyze OTA to non toxic metabolite (Pitout, 1969; Abrunhosa et al., 2006; 2007). Some researchers showed that *Bacillus licheniformis* could degrade OTA (Bohm et al., 2000; Petchkongkaew et al., 2008). Our finding that carboxypeptidase A being amplified from the genomic DNA of rumen fluid has 100% match with *Bacillus licheniformis*, is in accordance with the report

that *B. licheniformis* possess OTA degrading capacity (Petchkongkaew et al., 2008). The real time PCR results showed that the *Bacillus* population (DNA concentration) fed 100% roughage diet was significantly higher than that in rumen fluid of goats on 50% concentrate diet and in both feeding conditions the *Bacillus* population was higher just before feeding. From this study, it can be concluded that among rumen bacteria, *Bacillus spp.* having CPA gene might have played a key role in OTA degradation. The population number of *Bacillus* species was found to be influenced under different feeding condition and feeding time. This change in population of *Bacillus* species seems to have influenced the OTA degradation under different feeding conditions and feeding time. The role of *Bacillus* species in OTA degradation has been proposed previously, although the niche was not rumen (Petchkongkaew et al., 2008).

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## REFERENCES

- Abrunhosa, L., S. Lucia and V. Armando. 2006. Degradation of ochratoxin A by proteases and by crude enzyme of *Aspergillus niger*. Food Biotechnol. 20:231-242.
- Abrunhosa, L. and V. Armando. 2007. Isolation and purification of an enzyme hydrolyzing ochratoxin A from *Aspergillus niger*. Biotechnol. Lett. 29:1909-1914.
- Allen, G. C., M. A. Flores-Vergara, S. Krasnyanski, S. Kumar and W. F. Thompson. 2006. Modified protocol for rapid DNA isolation from plant tissues using cetyl trimethyl ammonium bromide. Nat. Protoc. 1:2320-2325.
- Alexandros, Y. and J. Jean-Pier. 2002. Mycotoxins in feeds and their fate in animals: a review. Anim. Res. 51:81-99.
- Bohm, J., J. Grajewski, H. Asperger, B. Cecon, B. Rabus and E. Razzazi. 2000. Study on bio-degradation of some B-trichothecenes and ochratoxin A by use of probiotic microorganisms. Mycotoxin Res. 16A:70-74.
- Bryant, M. P. and L. A. Burkey. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. 36:205-216.
- De Clerck, E., K. Van Mol, G. Jannes, R. Rossau and P. DeVos. 2004. Design of a 5' exonuclease-based real-time PCR assay for simultaneous detection of *Bacillus licheniformis*, members of the "*B. cereus* group" and *B. fumarioli* in gelatin. Lett. Appl. Microbiol. 39:109-115.
- Deberghes, P., A. M. Betbeder, F. Boisard, R. Blanc, J. F. Delably, S. Krivobok, R. H. Steiman, M. F. Seigle and E. E. Creppy. 1995. Detoxification of ochratoxin A, a food contaminant: prevention of growth of *Aspergillus ochraceus* and its production of ochratoxin A. Mycotoxin Res. 11:37-47.
- Halasz, A., R. Latszity, T. Abonyi and A. Bata. 2009.



- Decontamination of Mycotoxin containing food and feed by biodegradation. Food Res. Int. 25:284-298.
- Hohler, D., K. H. Sudekum, S. Wolfram, A. A. Frohlich and R. R. Marquardt. 1999. Metabolism and excretion of ochratoxin A fed to sheep. J. Anim. Sci. 77:1217-1223.
- Hult, K., A. Teiling and S. Gatenbeck. 1976. Degradation of ochratoxin A by a ruminant. Appl. Environ. Microbiol. 32:443-444.
- Kiessling, K. H., H. Pettersson, K. Sandholm and M. Olsen. 1984. Metabolism of aflatoxin, ochratoxin, zearalenone, and three trichothecenes by intact rumen fluid, rumen protozoa, and rumen bacteria. Appl. Environ. Microbiol. 47:1070-1073.
- Lee, S. S., J. K. Ha and K. J. Cheng. 2000. Relative contributions of bacteria, protozoa, and fungi to *in vitro* degradation of orchard grass cell walls and their interactions. Appl. Environ. Microbiol. 66:3807-3813.
- Marquardt, R. R. 1996. Effects of molds and their toxins on livestock performance: a western Canadian perspective. Anim. Feed Sci. Technol. 58:77-89.
- Mobashar, M., J. Hummel, R. Blank and K. H. Sudekum. 2010. Ochratoxin A in ruminants-A review on its degradation by gut microbes and effects on animals. Toxins 2:809-839.
- Petchkongkaew, A., P. Taillandier, P. Gasaluck and A. Leбриhi. 2008. Isolation of *Bacillus spp.* from Thai fermented soybean (Thua-nao): screening for aflatoxin B<sub>1</sub> and ochratoxin A detoxification. J. Appl. Microbiol. 104:1495-1502.
- Peteri, Z. J. Terenb, C. Vagvo Igyia and J. Varga. 2007. Ochratoxin degradation and adsorption caused by astaxanthin- producing yeasts. Food Microbiol. 24:205-210.
- Pitout, M. J. 1969. The hydrolysis of ochratoxin A by some proteolytic enzymes. Biochem. Pharmacol. 18:485-491.
- Ribelin, W. E., K. Fukushima and P. E. Still. 1978. The toxicity of ochratoxin A to ruminants. Can J. Comp. Med. 42:172-176.
- SAS. 2002. SAS user guide (Ver 9.1).SAS Inst.Inc. Cary, NC, USA.
- Schaadt, H. Jr. and R. R. Johnson. 1969. VFA production in the rumen of sheep fed limestone and urea treated corn silages. J. Anim. Sci. 29:839-847.
- Schatzmayr, G., F. Zehner, M. Taubel, D.Schatzmayr, A. Klimitsch, A. P. Loibner and E. M. Binder. 2006. Microbiologicals for deactivating mycotoxins. Mol. Nutr. Food Res. 50:543-551.
- Stander, M. A., P. S. Steyn, F. H.van der Westhuizen and B. E. Payne. 2001. A kinetic study into the hydrolysis of the ochratoxins and analogues by carboxypeptidase A. Chem. Res. Toxicol. 14:302-304.
- Upadhaya, S. D., H. G. Sung, C. H. Lee, S. Y. Lee, S. W. Kim, K. J. Jo and J. K. Ha. 2009. Comparative study on the aflatoxin B<sub>1</sub> degradation ability of rumen fluid from Holstein steers and Korean native goats. J. Vet. Sci. 10(1):29-34.
- Upadhaya, S. D., M. A. Park and Jong K. Ha. 2010. Mycotoxins and their biotransformation in the rumen: A review. Asian-Aust. J. Anim. Sci. 23(9):1250-1260.
- Van der Merwe, K. J., P. S. Step and L. Fourie. 1965. In: Effect of a hay and a grain diet on the rate of hydrolysis of ochratoxin A in the rumen of sheep (Ed. H. Xiao, R. R. Marquardt, A. A. Frohlich, G. D. Phillips and T. J. Vitti). J. Anim. Sci. 69:3706-3714.
- Wanapat, M. and A. Cherdthong. 2009. Use of real time PCR technique in studying rumen cellulolytic bacterial population as affected by level of roughage in swamp buffalo. Curr. Microbiol. 58:294-299.
- Xiao, H., R. R. Marquardt, A. A. Frohlich, G. D. Phillips and T. G. Vitti. 1991. Effect of a hay and a grain diet on the rate of hydrolysis of ochratoxin A in the rumen of sheep. J. Anim. Sci. 69:3706-3714.