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Low pH Enhances Rumen Absorption of Aflatoxin B1 and Ochratoxin A in Sheep

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Abstract: The objective of this study was to determine whether the ruminal disappearance rate of aflatoxin B1 (AFB1), ochratoxin A (OTA) and fumonisin B1 (FB1) is affected by acidic rumen pH conditions. Disappearance was measured using a temporally isolated rumen model. A buffered solution containing AFB1, OTA and FB1 at pH 5 or 7 was incubated for up to 2 h in the rumen of three adult rumen-cannulated sheep. The mean pH of the solution during the 2-h incubation in the rumen was 6.8±0.15 and 5.7±0.25 for the neutral and acid conditions, respectively. AFB1 and OTA were readily absorbed in the rumen, particularly at acid pH. The disappearance rates at acid and neutral pH for AFB1 were, respectively, 1.98±0.52 and 1.42±0.57 ng/h ($p<0.019$) and for OTA they were 0.16±0.10 and 0.06±0.03 ng/h ($p<0.058$). OTA disappearance from the rumen was followed by a concomitant increase of OTA concentration in plasma throughout the 2-h incubation. In contrast, FB1 was not absorbed in the rumen. In conclusion, acid pH in the rumen increases the absorption of AFB1 and OTA, potentially contributing to an exacerbated toxic risk.

Key words: Ruminal Acidosis · Mycotoxins · Ruminal Absorption · Sheep

INTRODUCTION

Mycotoxins are major contaminants of cereals used as animal feed and human food. Ingestion of contaminated feeds by ruminants may represent a health hazard to consumers as some feed mycotoxins are excreted into animal products. In addition, these mycotoxins can have genotoxic, teratogenic, carcinogenic and immunosuppressive effects thus affecting animal performance and health [1]. In intensive dairy and beef production systems, high concentrate diets, often used to maximize performances, may induce ruminal acidosis. This digestive disorder can have a negative impact on the economy of the farm. The losses can range from a simple decrease in production up to more serious health problems [2]. Under normal conditions, the pH of the rumen is maintained in the range of 6 to 6.9. However, when rapidly fermentable carbohydrates contained in cereal-rich diets are fed to animals there is a higher production of organic acids that readily decrease ruminal pH [2, 3]. Ruminal acidosis can also affect mycotoxins' toxicokinetics and detoxification by altering, respectively, absorption and microbial diversity in the rumen. For instance, protozoa that are responsible of the degradation of some mycotoxins [4] usually decrease in numbers under this dietary conditions [2] and in animals fed high concentrate diets an increase of the systemic availability of OTA has been described [5, 6].

In ruminants, the rumen represents an important site of absorption due to its epithelial mucosal surface and the long residence time of feeds in the rumen. However, there is scarce information on the rate and extent of mycotoxin absorption across the rumen wall. The rapid apparition of AFB1 and OTA in body fluids of ruminants Boudra and Morgavi [7] suggests that absorption starts in the rumen compartment. Most xenobiotics are absorbed in the digestive tract by passive diffusion of the non-ionized form. Therefore, the extent to which a compound is in its

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ionized or non-ionized form at a certain pH is an indicator of its potential absorption through biological membranes. At low pH, weak acid compounds are non-ionized and hence are well absorbed through the membrane while weak base compounds are poorly absorbed. Under normal conditions, the pH of the rumen is maintained in the range of 6 to 6.9. The objective of this work was to examine the effect of acid and neutral pH on the disappearance of relevant mycotoxins, OTA, AFB1 and FB1from the rumen using temporally isolated rumen as a model.

MATERIALS AND METHODS

Reagents: Pure AFB1, OTA and FB1 were purchased from Sigma (St Quentin, France). Experimental solutions (ES) for use in temporally isolated rumens were prepared as described before [8]. ES at pH 5 or 7 were based on citrate and phosphate buffer and contained 60 mM of acetate,15mM of propionate and 10 mM of butyrate as markers of absorption and 0.01 mM of Cr-EDTA as a marker of volume. The osmotic pressure was between 280 and 310 mOsm/L. ES (2 L) were freshly prepared the day of the experimentation, kept at 39 °C and bubbled with CO_2 gas for at least 2 h before used. The concentration of mycotoxins used in this study was 0.2 µg/ml for AFB1 and OTA and 1 µg/ml for FB1. AFB1 is carcinogen and OTA and FB1 are suspect carcinogens in humans and all personnel wore disposable gloves, protective masks and goggles and coats. For analysis, appropriate laboratory safety precautions for handling and decontamination of mycotoxins were followed [9].

Animals and Experimental Procedures: Animals were cared for in accordance with the guidelines for animal research of the French Ministry of Agriculture and applicable European guidelines and regulations for experimentation with animals (http://www2.vet-lyon.fr/ens/expa/acc_regl.html). The experimental protocol was approved by the Regional Ethics Committee of Animal Experimentation (No CE31-11). Three adult Texel wethers averaging 65±3 kg BW and fitted with ruminal cannula (diameter 75 mm) made of plastisol were used in a completely randomised design experiment. Three absorption measurements were assayed at acid and neutral ES pH, using one animal per experimental solution per day. Each wether was used twice with a minimum of two days washout time between measurements to test the ES at different pH. The disappearance was measured using temporarily isolated rumens [8]. Briefly, rumen contents were emptied through the cannula and the empty rumen was washed abundantly with warm saline solution until the effluent was clear. The reticulo-omasal orifice was closed with a balloon catheter, while the oesophagus was closed with a custom-made balloon catheter that allowed the collection of saliva through a nasal tube connected to a vacuum pump. Two litres of ES were introduced into the rumen through the cannulae and maintained under anaerobic conditions by infusing CO_2 gas via a tube that went through the cannulae's lid. Ruminal pH was measured every 15 min over the 2-h incubation using indwelling e-Cow boluses as described by Phillips *et al*. [10]. At the end of incubation, ES were taken out with vacuum pump and disposed following decontamination procedures for mycotoxins and catheters were withdrawn and decontaminated. Rumen walls were washed with saline solution and ruminal contents were returned to the rumen.

Sample Collection: Samples (10 ml) of ES were taken with the help of a 25-ml syringe at 0, 0.5, 1, 1.5 and 2 h after introduction into the rumen. For mycotoxin analysis, 2 ml were collected in polypropylene tubes. For volatile fatty acids (VFA), 0.8 ml were transferred to a microcentrifuge tube containing 0.5 mL of a 0.5 N HCl solution containing 2% (w/v) metaphosphoric acid and 0.4% (w/v) crotonic acid. For Cr-EDTA analysis,3 ml were stored in polypropylene tubes. Blood samples were collected via the jugular vein in heparinised tubes (Vacutainer, Frankin Lakes, USA) at the same time as for ES samples. Tubes were immediately centrifuged (5000 x g, 10 min) and plasma was transferred to clean tubes. All samples were stored at -20°C until analysis.

Sample Analysis: ES samples were thawed at room temperature and 100 µl were transferred into a 1.5 ml micro centrifuge tube and mixed with an equal volume of acetonitrile-distilled water solution (1:1) containing 5 µg/ml of alpha zearalenol as internal standard. Samples were vortex-mixed for 10 s and centrifuged at 10000 g for 10 min. Ten µl supernatant were injected into a LC-MS/MS system. The chromatographic system was an Alliance 2695 module (Waters Corporation, St-Quentin-en-Yvelines, France). Separation was performed at room temperature on a Kinetex C_{18} column (50 × 2 mm, 2.6 µm Phenomenex, Paris, France) using a gradient solvent system (solvent A= 0.1% formic acid ammonium acetate 0.5 mM adjusted to pH 3.5 and solvent B= acetonitrile-0.1% formic acid). The gradient conditions were as follows: 8% of solvent B was held for 4 min, increased to 80% in 12 minutes and then lowered to the initial 8%

percentage in 0.1 min. This concentration was maintained for 6 minutes to re-equilibrate the column prior to the next injection. The flow rate was 0.3 ml/min. Electrospray mass spectrometric (ESI-MS/MS) analyses were performed on a Quattro MicroTM triple quadrupole mass spectrometer (Waters Corporation, St-Quentin-en-Yvelines, France) equipped with an electrospray source operating in positive and negative ion mode. Capillary voltage was set to 4 kV, source temperature to 120°C and desolvatation temperature to 350 °C. The cone and nebulisation gas flows (both nitrogen) were set at 50 and 500 L/h respectively. Data were acquired using the multiple reaction monitoring (MRM) scanning mode. The tune parameters were optimized by infusing separately a 10 µg/ml in mobile phase solution of each mycotoxin at a flow rate of 10 µl/min. The MRM transitions and the applied cone voltages and collision energies are summarised in Table 1. The calibration curve was included in each sample series by using a series of standard solutions containing different levels of the three tested mycotoxins. The range of calibration standard solutions was 0.1-15 ng/ml for AFB1 and OTA and 31-500 ng/ml for FB1. Ochratoxin A in plasma was analyzed as previously described [7]. Samples for VFA were analysed by gas chromatography according to Morgavi *et al*.[11]. The concentration of Cr-EDTA was determined by atomic absorption spectrometry using a Perkin-Elmer Model 2380 spectrophotometer as described by Uden *et al*. [12].

Disappearance Rate Measurements and Statistical Analysis: The rate of disappearance of mycotoxins (b, ng/h) is the slope of the following equation: log $Q = \log Q_0 + bt$, where Q_0 is the initial amount of AFB1, OTA and FB1 in the ES (ng), t is the incubation time. Volume of ES was measured using Cr-EDTA concentration as marker.

Data were analysed using the Mixed procedure of SAS (version 9, SAS Institute Inc., Cary, USA) with the following mixed linear model: $Y_{ij} = \mu + P_j + A_{ij} + e_{ij}$, where Y is the dependent variable, µ is the overall mean, P is the fixed effect of pH (neutral and acid), A is the random effect of animal tested within P and e_{ij}, is the residual error.

RESULTS AND DISCUSSION

In this study the rumen disappearance of three mycotoxins, commonly found in ruminant feeds, was monitored at two different pH environments using a temporarily isolated rumen model in sheep. The absence of variation in the volume of ES measured over the 2-h incubation period ($p>0.05$) (Figure 1) together with the disappearance rates of VFA that were in accord with previous reports [13-15] confirmed that the set-up of the isolated rumen model was correct. The disappearance rates of total VFA tended to be higher in acid pH (1.07±0.3 mM/h) than in neutral pH (0.58±0.3 mM/h) ($p=0.1$). Throughout the 2-h incubation period the average pH of the rumen was 5.7±0.25 and 6.8±0.15 for the acid and neutral ES, respectively. While the pH of the neutral ES remained stable throughout the incubation, it increased significantly for the acid ES, from a minimal pH of 5.2±0.36 up to 6.3±0.27. The difference between the two pH solutions, however, was still physiologically relevant and was stable throughout the 2 h incubation time (Figure 2). The increase in pH for the acid solution was gradual and could be due to a progressive loss of the buffering capacity of the ES. The CO_2 in the gas phase that combines with water to form $HCO3^-$[16] and small remaining debris in the washed rumen could be responsible for the increase in pH. It is noted that if the rumen is not isolated, the flow of saliva rapidly increase the pH in less than 1 h to values above 7 [14].

Table 1: Transition reactions monitored by LC-ESI-MS/MS, cone and collision voltages

Metabolites	MW[1]	Precursor ion	Daughter ion	Cone voltage (V)	Collision energy (eV)
Aflatoxin B1	312	313.0	128.0	45	60
			285.1	45	21
Fumonisin B1	721	722.0	81.0	50	57
			334.0	50	37
Ochratoxin A	403	404.1	239.2	25	23
			358.2	25	13
Azearalenol (IS)[1]	322	323.0	123.0	15	21
			305.0	15	7

Transition in bold is used for quantification [1]Internal standard

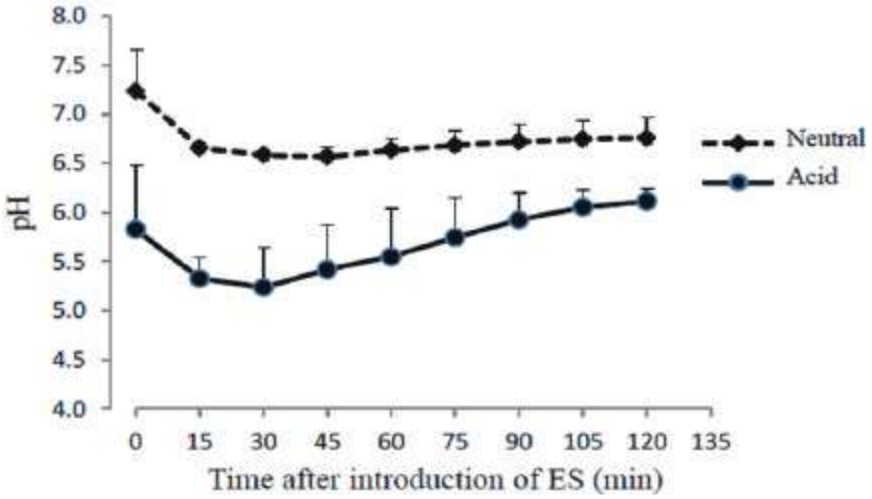

Fig. 1: pH profile of experimental solutions (neutral and acid pH) during the experimental conditions measured using an indwelling pH bolus. (n= 3 animals, error bar represent SD)

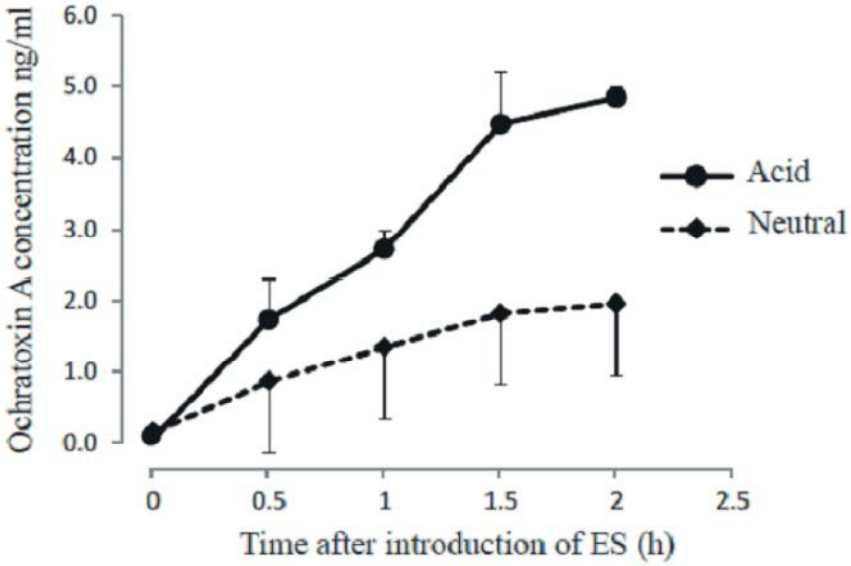

Fig. 2. Concentration of ochratoxin A in sheep plasma after introduction of a neutral or acid solution containing ochratoxin A in empty-washed rumens (n= 3 animals; error bars represent SD)

Disappearance of mycotoxins from the rumen is presented in Table 2. Aflatoxin B1 had the highest disappearance. The low pH solution increased significantly (*p=0.019*) AFB1 disappearance. Aflatoxins are lipophilic compounds and, although their pKa has not been reported, the increased disappearance observed at acid pH suggests a higher proportion of non-ionized molecules. Similarly, disappearance of OTA at low pH was two times higher than at neutral pH (*p=0.058*). This result is consistent with the estimated proportion of non-inonized OTA. Based on the pKa 7.5 of its phenolic group, the non-ionized fraction of OTA at pH 6.79 and 5.7 was 83.7% and 98.4%, respectively. The increased systemic availability of OTA in animals fed diets favouring a low rumen pH that was reported by other authors [5, 6] may be partially explained by this property. The high disappearance rate of OTA from ES acid solution was confirmed by the profile of concentration of OTA in plasma (Figure 2). Before exposure, OTA was not present in plasma but it was detected as early as 0.5 h after exposure. The concentration increased throughout the incubation time, particularly for the acid condition that

Table 2: Effect of pH on disappearance rates of mycotoxins in isolated rumen`s sheep model

	pH		
Mycotoxin	Neutral	Acid	*p-level*
Rate of disappearance (ng/h^{-1})			
Aflatoxin B1	1.42± 0.57	1.98 ± 0.52	*0.019*
Ochratoxin A	0.06 ± 0.03	0.17 ± 0.10	*0.058*
Fumonisin B1	0	0	*NS*

NS: non significant

reached up to 5 ng/ml 2 h after ES administration. For the neutral condition, the maximal concentration was 2 ng/ml. This correlates with the rapid disappearance rate of OTA in the rumen indicating that OTA was readily absorbed through the rumen wall and that the process was pH dependent. Chronic acidosis can cause parakeratosis and loss of integrity of the rumen epithelium [17]. This condition would additionally favour the systemic passage of mycotoxins in affected animals. In this work, however, the rumens were clinically healthy and the experimental time was too short to induce this type of lesions. In contrast to AFB1 and OTA, the concentration of FB1 in the rumen of sheep remained unchanged throughout the incubation (Table 2). FB1 has a pKa of 3.5 and, according to the Henderson-Hasselbalch equation, would be largely ionized at the pH used in this experiment, e.g. 99% with the acid ES and hence unsuitable for absorption. FB1 is soluble in water so that in the rumen is present in the full ionized form. FB1 has been shown to be poorly absorbed in the gastrointestinal tract and not metabolized by ruminants [18] and other animals [19, 20]. In conclusion, we show that AFB1 and OTA are absorbed in the rumen particularly at acid pH. Cereal-rich diets given to high producing animals might have a double negative effect by increasing both the dose and absorption of these mycotoxins, contributing potentially to an exacerbated toxic risk.

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REFERENCES

1. IARC. World health organization international agency for research on cancer. Aflatoxins: B1, B2, G1, G2, M1. In: Some traditional herbal medicines, some mycotoxins napthalene and styrene. Lyon, France. . IARC Monographs, 2002, 82: 171-5.
2. Martin, C., L. Brossard and M. Doreau, 2006. Mechanisms of appearance of ruminal acidosis and consequences on physiopathology and performances. Prod Anim, 19(2): 93-107.
3. Lettat, A., P. Noziere, M. Silberberg, D. Morgavi, C. Berger and C. Martin, 2010. Experimental feed induction of ruminal lactic, propionic, or butyric acidosis in sheep. J Anim Sci., 88(9): 3041-6.
4. Galtier, P. and M. Alvinerie, 1976. *In vitro* transformation of ochratoxin A by animal microbial floras. Ann Rech Vet., (7):1.
5. Blank, R. and S. Wolffram, 2009. Effects of live yeast cell supplementation to high concentrate diets on the toxicokinetics of ochratoxin A in sheep. Food Addit Contam, 26(1):119-26. doi: 10.1080/ 02652030802320600.
6. 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 bioavailability of ochratoxin A in the rumen of sheep. J. Anim Sci., 69: 3715-23.
7. Boudra, H. and D.P. Morgavi, 2006. Development and validation of a HPLC method for the quantitation of ochratoxins in plasma and raw milk J. Chromatogr B., 843(1-2):295-301. doi: 10.1016/j.jchromb.2006.06. 018.
8. Doreau, M., E. Ferchal and Y. Beckers, 1997. Effects of level of intake and of available volatile fatty acids on the absorptive capacity of sheep rumen. Small Ruminant Research, 25(2):99-105. doi: 10.1016/s0921-4488(96)00982-0.
9. Trucksess, M.W. and A.E. Pohland, 2001. Mycotoxins protocols. Totowa, New Jersey: Humana Press; 2001.
10. Phillips, N., T. Mottram, D. Poppi, D. Mayer and M.R. McGowan, 2009. Continuous monitoring of ruminal pH using wireless telemetry. Animal Production Science, 50(1):72-7. doi: http://dx.doi.org/ 10.1071/AN09027.
11. Morgavi, D.P., H. Boudra, J.P. Jouany and D. Graviou, 2003. Prevention of patulin toxicity on rumen microbial fermentation by SH-containing reducing agents. J. Agric Food Chem., 51(23): 6906-10. doi: 10. 1021/jf034505v.

12. Uden, P., P.E. Colucci and P.J. Vansoest, 1980. Investigation of chromium, cerium and cobalt as markers in digesta-rate of passage studies. J Sci Food Agric, 31(7): 625-32. doi: 10.1002/jsfa.2740310702.
13. Bergman, E.N., 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiological Reviews, 70(2): 567-90.
14. Dijkstra, J., H. Boer, J. Vanbruchem, M. Bruining and S. Tamminga, 1993. Absorption of volatile fatty-acids from the rumen of lactating dairy-cows as influenced by volatile fatty-acid concentration, pH and rumen liquid volume. Br J. Nutr., 69(2): 385-96. doi: 10.1079/bjn19930041.
15. Storm, A.C., N.B. Kristensen and M.D. Hanigan, 2012. A model of ruminal volatile fatty acid absorption kinetics and rumen epithelial blood flow in lactating Holstein cows. J. Dairy Sci., 95(6): 2919-34.
16. Aschenbach, J.R., G.B. Penner, F. Stumpff and G. Gäbel, 2011. Role of fermentation acid absorption in the regulation of ruminal pH. J. Anim Sci., 89(4): 1092-107. doi: 10.2527/jas.2010-3301.
17. Krause, K.M. and G.R. Oetzel, 2006. Understanding and preventing subacute ruminal acidosis in dairy herds: A review. Animal Feed Science and Technology, 126(3): 215-36.
18. Prelusky, D.B., M.E. Savard and H.L. Trenholm, 1995. Pilot study on the plasma pharmacokinetics of fumonisin B1 in cows following a single dose by oral gavage or intravenous administration. Natural Toxins, 3(5): 389-94. doi: 10.1002/nt.2620030511.
19. Cavret, S. and S. Lecoeur, 2006. Fusariotoxin transfer in animal. Food Chem Toxicol, 44(3): 444-53. doi: 10.1016/j.fct.2005.08.021.
20. Gbore, F.A., R.I. Yinusa and B. Salleh, 2010. Evaluation of subchronic dietary fumonisin B-1 on nutrient digestibility and growth performance of rats. Afr J. Biotechnol, 9(38): 6442-7.