HOW RUMINAL ACIDOSIS CAN ALTER THE ABSORPTION OF MYCOTOXINS AND THEIR DETOXIFICATION?
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Bogor, February 2016

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RINGKASAN

DADIK PANTAYA. Bagaimana ruminal acidosis dapat merubah penyerapan mikotoksin dan detoksifikasinya? Dibimbing oleh SURYAHADI, KOMANG G WIRYAWAN dan DWIERRA EVVYERNIE


Tujuan dari disertasi ini mengkaji pengaruh ruminal acidosis pada penyerapan mikotoksin dan degradasinya di rumen. Untuk mencapai tujuan tersebut dilakukan 2 percobaan, percobaan pertama dengan judul : pH rendah meningkatkan penyerapan AFB1 dan OTA di rumen domba, dengan tujuan untuk mengetahui pengaruh pH terhadap penyerapan mikotoksin di rumen.

Kecepatan penyerapan diukur dengan menggunakan model temporally isolated rumen. Percobaan ini untuk mengevaluasi penyerapan mikotoksin di dalam rumen antara lain aflatoksin B1(AFB1), ochratoksin (OTA) and fumonis in B1 (FB1) menggunakan ternak domba dengan metode empty-washed rumen pada dua kondisi pH yang berbeda: asam dan netral. Hasil analisis menunjukkan bahwa kecepatan fraksional penyerapan (k) AFB1 dan OTA meningkat secara signifikan pada pH asam, sedangkan pada FB1 tetap tidak mengalami perubahan.

Pada percobaan ke dua dengan judul : Penyerapan dan detoksifikasi mikotoksin oleh mikrob di rumen berubah yang berkaitan dengan percobaan induksi SARA pada sapi perah non laktasi, dengan tujuan untuk mengkaji pengaruh pakan acidogenic dengan penambahan yeast terhadap penyerapan mikotoksin dan detoksifikasi mikotoksin oleh mikroba. Rancangan percobaan dengan double cross over, menggunakan 8 ekor sapi perah non laktasi dengan perlakuan pakan non acidogenic (NA) dan acidogenic (Ac) (15 vs 30.8% pati) dengan yeast dan tanpa yeast. Penambahan yeast dengan dosis 10^{10} CFU/hari (0.5 g/ekor/hari) Saccharomyces cerevisiae. Percobaan dilakukan selama 12 minggu dan percobaan dilakukan selama 4 periode dengan 14-hari per periode, yang diikuti pada akhir setiap periode seluruh ternak menerima single dose kontaminasi yang mengandung mikotoksin dengan dosis AFB1 (110 µg/kg), OTA (430 µg/kg), DON (450 µg/kg ) dan FB1 (3044 µg/kg) melalui canulla 4 jam setelah pemberian pakan pagi dan periode pengukuran sampel selama 3 hari.

Hasil penelitian menunjukkan bahwa pakan acidogenic (Ac) dapat menurunkan rataan pH rumen (6.46 vs 6.29) dan meningkatkan lama waktu pH di
bawah 5.6 (0.5 vs 2.77 jam/hari) dibanding NA, sehingga mendekati kriteria SARA. Karakterisasi produk fermentasi pakan Ac di rumen antara lain ditandai dengan meningkatnya konsentrasi total VFA (120-132 mM) dan proporsi propionat (14.5 vs 16.3%) serta menurunnya proporsi acetat (76.30 vs 74.34 %), sedangkan asam laktat masih dalam kisaran normal (2.68-3.09 mM). Penambahan yeast efektif mengurangi penurunan pH pada kondisi Ac (P<0.05) pada 4-10 jam setelah makan pagi. Terjadi pengaruh interaksi antara pakan dengan yeast terhadap konsentrasio isoacid (P<0.01), pada NA dan Ac penambahan yeast meningkatkan isoacid. Penurunan pH di rumen dapat meningkatkan penyerapan acidie mikotoksin, sedangkan pada percobaan ini penambahan yeast pada kondisi Ac belum efektif menurunkan penyerapan OTA dan AFB1, yang ditunjukkan dengan peningkatan ekstraksi OTA (P<0.05) mulai 24-72 jam dan ekstraksi aflatoxin M1 (AFM1) sebagai metabolit AFB1 (P<0.05) mulai 48-72 jam melalui urine. Penurunan microbial rumen Entodinium >100 mm dan holotrich protozoa (Isotricha dan Dasytricha) berkontribusi meningkatkan systemic bioavailability OTA dan DION pada pakan Ac yang terjadi pada awal kontaminasi. Penambahan yeast cenderung meningkatkan (P=0.07) konversi OTA ke OTα yang dikeluarkan melalui urine. Sementara itu pakan Ac dapat mempercepat eliminasi DON dan DOM1 melalui feces.

Kesimpulan, pH rendah dapat meningkatkan penyerapan AFB1 dan OTA. Pakan acidogenic dapat menurunkan pH rumen mendekati SARA, mempengaruhi karakteristik produk fermentasi di rumen dan memodifikasi profil toxikokinetik mikotoksin AFB1, OTA, DON dan FB1. Pada kondisi acidosis dapat meningkatkan penyerapan AFB1 dan OTA tetapi DON dan FB1 sedikit terserap di rumen. Ekstraksi DON tidak secara langsung dipengaruhi oleh pH, ekstraksinya berkorelasi dengan aliran keluar partikel pakan dari rumen. DON lebih cepat diekstraksi pada kondisi acidosis. Ekstraksi FB1 tidak dipengaruhi oleh rumen pH di rumen. Yeast mampu menghambat penurunan pH rumen setelah makan tetapi penambahan yeast belum menurunkan penyerapan AFB1 dan OTA. Yeast juga mampu menstimulasi detoksifikasi OTA di dalam rumen.

Kata kunci: penyerapan, mikotoksin, detoksifikasi, *sub-acute ruminal acidosis* (SARA), ruminansia
SUMMARY

DADIK PANTAYA. How ruminal acidosis can alter the absorption of mycotoxins and their detoxification? Supervised by SURYAHADI KOMANG G WIRYAWAN and DWIERRA EVYERNIE

Mycotoxins are toxic metabolites produced by some fungal species commonly found in foods and feeds, particularly in cereals. In intensive production systems, dairy and beef cattle are fed cereal-rich diets and, consequently, are more exposed to mycotoxins. Besides, this type of diet is associated with a higher risk of rumen acidosis that can also affect performance and animal health. In addition, the efficacy of microbial detoxification can be reduced during acidosis. For instance, some authors observed a decrease in the number of protozoa that are responsible of the degradation of some mycotoxins. Another consequence of acidosis is the potential modification of ruminal absorption of mycotoxins, which until now has received scarce attention. Yeast probiotic additives have been shown to reduce the post-feeding drop in rumen pH and to increase the number of rumen protozoa. This effect can be positive in reducing the absorption and toxicity of mycotoxins.

The aim of my dissertation is to evaluate the effect of ruminal acidosis on mycotoxins absorption and degradation in the rumen. To achieve this objective, two animal experiments were performed. In experiment 1, with the title: Low pH enhances rumen absorption of aflatoxin B1 and ochratoxin A in sheep. 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. The rumen absorption of three mycotoxins: aflatoxin B1(AFB1), ochratoxin (OTA) and fumonisin B1 (FB1) was evaluated in sheep using the empty-washed rumen method at two different pH: acid and neutral. The results showed that fractional absorption rate ($k$) of AFB1 and OTA increased significantly in pH acid while FB1 concentrations remained unchanged.

In experiment 2, in vivo experiment with the title: Ruminal absorption and Microbial Detoxification of Mycotoxins Changes Associated with Experimentally Induced Sub-Acute Acidosis (SARA) in Non-Lactating Dairy Cows. The aims of this study were to examine the effects of sub-acute ruminal acidosis (SARA), with or without yeast, on mycotoxin absorption and mycotoxin detoxification in the rumen. Eight non-lactating dairy cows (650 ± 115) kg fitted with rumen canulas were used in a double cross over design. Cattle were separated in 2 groups that received 30.8% starch for acidogenic (Ac) and 15.1% starch for non acidogenic diet (NA), diet with and without yeast supplementation (Saccharomyces cerevisiae, 0.5 g/d per animal corresponding to $10^{10}$ CFU). The whole trial lasted 12 weeks and the experiment was divided into 4 periods of 2 weeks, followed by a single administration of four mycotoxins-naturally contaminated feed containing aflatoxin B1(AFB1) (110 µg/kg DMI), ochratoxin A (OTA) (430 µg/kg DMI), deoxynivalenol (DON) (450 µg/kg DMI), and fumonisin B1 (FB1) (3044 µg/kg DMI) 4-h after feeding via cannula, and a 3-d measurement period.
Results demonstrated that Ac diet lowered mean ruminal pH (6.46 vs 6.29) and increased spent time of pH below 5.6 (0.5 vs 2.77 h/d) compared to NA, as the criteria of SARA. Ruminal fermentation in cows fed with Ac diet were characterized by increase total VFA (109 vs 126 mM) and molar proportion of propionate (14.5 vs 16.3%) and decrease molar proportion of acetate (76.30 vs 74.34 %) compared NA diet, while lactic acid was within normal range (2.68-3.09 mM). Yeast supplementation could effectively reduce decreasing pH in Ac (P<0.05) at 4-10 h after morning feeding. The interaction between diet and yeast treatments significantly affected proportion of acetate and isoacids proportion (P<0.01) before feeding. Low ruminal pH enhanced acidic mycotoxin absorption, while yeast was yet effective in reducing OTA and AFB1, shown from the increase of OTA excretion (P<0.05) at 24 to 72 h, and AFM1 excretion as AFB1 metabolite at 48-72 h through urine. Yeast supplementation tended to increase (P=0.07) OTA conversion to OTα through urinal excretion while Ac feed could accelerate fecal elimination of DON and DOM1.

In conclusion, low pH could increase absorption of AFB1 and OTA, acidogenic diet could lower rumen pH and close to SARA, affect the fermentation characteristics in the rumen and modified toxicokinetics profile of mycotoxins AFB1, OTA, DON and FB1. In acidosis condition AFB1 and OTA were rapidly absorbed but DON and FB1 low absorbed in the rumen. DON excretion is not directly affected by the rumen pH but its excretion correlated to feed passage rates from the rumen. DON was rapidly excreted in acidosis condition. FB1 excretion was not affected by rumen pH. *Saccharomyces cerevisiae* yeast could inhibit the decrease rumen pH after feeding but yeast supplementation did not affect in decreasing the absorption AFB1 and OTA. Yeast could stimulate mycotoxins OTA detoxification in the rumen.

Key words: absorption, mycotoxins, microbial detoxification, sub-acute ruminal acidosis (SARA), ruminant
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HOW RUMINAL ACIDOSIS CAN ALTER THE ABSORPTION OF MYCOTOXINS AND THEIR DETOXIFICATION?

DADIK PANTAYA

Dissertation
as part of requirement to fulfill PhD degree
at
Nutrition and Feed Science Study Program

THE GRADUATE SCHOOL
BOGOR AGRICULTURAL UNIVERSITY
BOGOR
2016
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Bogor, February 2016

Dadik Pantaya
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1. INTRODUCTION

Background

Mycotoxins

Mycotoxins was first discovered in 1960 at the time of massive poisoning in the United Kingdom related to 100,000 turkeys death, after consuming poultry feed containing groundnut cake contaminated with a group of compounds now called aflatoxin (Blount 1961). Another incidence of poisoning case was occurred in Cicadas Bogor Indonesia 1987-1988, in which 34 of pregnant sheep, 6 lambs and 9 male adult sheep died (Bahri et al., 1990). The investigation by Balitvet (Balai Penelitian Veteriner) in Bogor Indonesia has concluded that death was caused by a high concentration of DON in the concentrate feed. It is a fact that the relationship between the fungi–toxin-pathology with several cases of poisoning through animals.

Mycotoxin is a secondary metabolite product of fungi which can cause complex toxic effect. More than 300 secondary metabolites have been identified, while only 30 of those which are truly toxic (Streit et al., 2013). Consumption of a mycotoxin-contaminated diet may induce acute and long-term chronic effects in animals and humans, resulting in teratogenic, carcinogenic and oestrogenic or immune-suppressive effect. The AFB1 and its metabolites product AFM1 had carcinogenic effect in human and it is found in milk product. The chemical structures of mycotoxin are varies. Mycotoxins are organic compounds having low-molecular weight. Mycotoxin molecules are small and very stable and difficult to be eliminated and enter the food and feedstuff production chains while keeping their toxic properties.

Climatic condition in tropical countries like Indonesia with high humidity and temperature is ideal for the fungi growth. Among mycotoxin commonly found in the field such as aflatoxin B1 (AFB1), ochratoxin A (OTA), deoxynivalenol (DON) and fumonisins B1 (FB1) (Bryden 2012). Cattle’s exposure to mycotoxins affects cattle’s health and productivity and leads to economical loss. (CAST. 2003, Morgavi et al., 2003).

Consequences of High Cereals Diet to Mycotoxins

About 25% of world grains productions for food and feed have been contaminated with mycotoxins (CAST., 2003). Contamination is induced by environmental condition which is conducive for fungus growth such as high temperature and humidity in tropical region. Several types of Aspergillus sp, Fusarium are producing mycotoxins that frequently contaminates grains. Fungi are easily growing in grains during transportation and storage in environment with high temperature and humidity, accordingly contaminating feed. Mycotoxins rarely takes single contaminated form but multi contaminated instead (Binder et al., 2007, Fink-Gremmels 2008).

Some high energy feed ingredients easily contaminated by mycotoxins are derived from grains such as corn, rice bran and groundnuts meal. Moreover, contaminations in plantation and agriculture waste are copra meal, palm kernels, chiefly cassava, and cotton seed meal. Those feed ingredients are frequently used to compose concentrate for ruminant feed as energy source. Some analysis study
of aflatoxin (AFB1) contamination in Indonesia were reported 38 µg/kg and 49 µg/kg in copra cake and palm kernel cake, respectively (Pranowo et al., 2013), while in dairy cow farm and smallholder farm were 46.6 µg/kg and 54 µg/kg, respectively (Agus 2013). OTA contamination in corn was 10 µg/kg and 47-348 µg/kg (Setyabudi et al., 2012), DON contamination was 47-348 µg/kg (Setyabudi et al., 2012) and 324 µg/kg (Tangendjaja et al., 2008), and FB1 was 1193 µg/kg (Setyabudi et al., 2012).

Nuryono et al., (2005) reported that almost 100% fresh milk in Yogyakarta province was contaminated with AFM1 although none of contaminated samples exceeded the European Union (EU) limits for infant and adult consumption. Similarly, Agus (2013) revealed survey of AFM1 contamination in milk product of dairy farmers in Yogyakarta collected from individual farmer, business/farmer group, and cooling unit. The average of AFM1 concentration for all samples was 74.72 ng/L, whereas 95.6% exceeded EU limit of 5 ng/L.

Data showed that grains from agricultural product are highly prone to mycotoxins contamination which caused severe problem in quality and food security. From the perspective in dairy cattle, mycotoxins contaminated in feedstuffs are in dual concern. First, occurrence AFB1 in feed may affect the safety in milk and milk products, due to its carry over from feed to milk as AFM1. Other commonly occurring associated mycotoxins, including OTA, DON and FB1, are not considered in safety in milk due to the low carry over from fed to milk. The second concern is the detrimental effect that mycotoxins can have on animal health and productivity (CAST, 2003). For this reason, Indonesian government through National of Drugs and Food Controls therefore launched standard for mycotoxin maximum level mycotoxin contamination i.e., in corn were 20 µg/kg AFB1, 1000 µg /kg DON, and 2000 µg/kg FB1 and in milk was 5 ng/L. Whereas, European Commission released directive number 2002/32/CE for maximum level AFB1 in feed was 50 µg/kg and number 2006/575/EC for adult ruminants complete feed, among which were 100 µg/kg OTA, 2000 µg /kg DON and 50000 µg/kg for FB1.

The higher proportion of concentrate for feed increased mycotoxin exposure. The complexity of the materials feedstuffs for concentrate can enhance ruminant exposure to mycotoxins. Ruminant complete feed also included other ingredients based forage, feed supplement and some co-product can increase the possibility of multi mycotoxin exposure (Boudra and Morgavi 2006). Concentration of mycotoxin contaminated diet in ruminant feed depended on the composition and origin of ingredients in the rations.

**Consequences of High Cereals Diet to Acidosis**

The phenomenon is observed in Indonesian ruminant production system that can utilize concentrate feeds to supplement roughage based diet, either under drought condition since green feed is minimal, or to meet the higher nutrient requirements for animals in critical physiological states, such as lactating cows, or fattening beef. According to Wahyono and Hardianto (2004), for milk production or in fattening periods concentrate may form a high proportion in diets (over 40% and 75% respectively). Consumption of high-concentrate diet can cause rumen pH decrease called ruminal acidosis (Beauchemin et al., 2003, Lettat et al., 2012).
Acidosis can disturb ruminal metabolism affecting feed fermentation process in the rumen. Acidosis is divided into two, sub-acute ruminal acidosis (SARA) and acute acidosis. SARA is indicated by the decrease of rumen pH <5.6 for more than 3 hours per day. VFA level approximates 100-125 mM (Nagaraja and Titgemeyer 2007) lactic acid is within normal range or < 5 mM, and osmotic pressure is 250-280 mOsm/L, while acute acidosis experiences rumen pH decrease up to 5 -5.5, with total VFA 70-100mM, lactic acid >50mM, osmotic pressure 350-515 mOsm/L (Krause and Oetzel 2006, Owens et al., 1998).

Streptococcus bovis can convert pyruvate into lactate by Lactate dehydrogenase (LDH) or into acetyl CoA and formatted by pyruvate format-lyase (PFL) into acetic or ethanol. Lactic acid through methylmalonyl cycle CoA is converted into propionic acid. Lactate utilizing bacteria such as Megasphaera elsdenii and Selenomonas ruminantium can prevent the accumulating lactate. In normal condition, lactic acid synthesis is balanced with hydrolysis, but high concentrate supplementation causes imbalance synthesis rate with degradation, thereby accumulating lactic acid. Nagaraja and Titgemeyer (2007) stated that lactic acid is less absorbable than VFA because pKa of lactic acid is 3.7 while VFA (acetic, propionic and butyric) is 4.7-4.9. In acidic condition, VFA will undergo non-ionization that increases rumen absorbability.

Acidosis can also affect microflora population (such as protozoa) in the rumen which is responsible for degrading mycotoxin. The decrease of protozoa population can influence mycotoxin degradation (Galtier and Alvinerie 1976) and disturb mycotoxin metabolism before being absorbed in the rumen.

**Mycotoxin Absorption in the Rumen**

Mycotoxins enter the cattle’s body through mycotoxin contaminated feed, which later go through metabolism process in the digestive tract. Mycotoxins absorption in the digestive tract usually occur by passive diffusion (Yiannikouris and Jouany 2002). Varied molecular structure of mycotoxins can cause different physical and chemical characteristics thus affecting mycotoxin absorption in the rumen. Most mycotoxins have low-molecular weight. Mycotoxin absorption in the digestive tract occurs when the molecule is in its non-ionized form so that permeable through cell membrane in the rumen.

Molecular ionization of xenobiotic compounds in the digestive tract is influenced by acid dissociation constant (pKa), pH and solubility of the molecule (David and William 2007). Values pKa = -log (K_a), based to Henderson–Hasselbalch equation, if pH-pKa = 0, then molecule 50% is ionized and 50% is non ionized, if pH-pKa > 0.5, then molecule is 75% ionized and 25% non ionized or 75% non ionized and 25% ionized. Degree of ionization of a weak acid and base dependent on the pH of medium (Deshpande 2002).

For example, mycotoxin OTA is a weak acid that in acidic condition can be quickly absorbed, while a weak base compound will be slowly absorbed. According to Boudra and Morgavi (2006), mycotoxins OTA and AFB1 are quickly absorbed in the rumen. Therefore, it is suggested that acidosis can increase absorption of several mycotoxins in the rumen, which in turn can increase the mycotoxin exposure to the cattle. Increasing ruminal pH in the rumen...
is expected to overcome acidosis and to decrease mycotoxin absorption in the rumen.

**Mycotoxin Detoxification**

Mycotoxin detoxification process is an effort to decrease mycotoxin toxicity. Several strategies can be employed to reduce mycotoxin contamination. Preventive effort in farm with good agricultural practices, i.e. proper storage of commodities can minimize mycotoxin level, but climatic condition and insect intervention cannot fully guarantee the decrease of mycotoxins in agricultural product. Feed is likely contaminated in some different concentration according to the proportion of the contaminated feed materials in complete feed, therefore decontamination or detoxification can be used to remove and reduce mycotoxin contamination.

Some physical and chemical detoxification methods have been conducted to reduce mycotoxin-contaminated in grains, but study of detoxification by organic agents are extensively researched in recent years. The organic agents such as yeast can degrade mycotoxin into less toxic or inactive metabolite product to bind mycotoxin *in vitro* (Boudra and Morgavi 2006).

**Hypothesis and Alternative Solution**

The diversity of materials feed ingredients for concentrate can potentially increase ruminant to mycotoxins exposure. Using a variety of feed ingredient increases the probability of multiple-mycotoxin contamination. On the other hand, ruminal acidosis due to consuming feed readily fermentable carbohydrates from grains is assumed to affect mycotoxin absorption in rumen. Acidosis in rumen can modify microbial population in rumen that lowers protozoa population that serves in mycotoxin detoxification (Galtier and Alvinerie 1976). The decreasing protozoa population is assumed to affect degradation and mycotoxin metabolism before being absorbed in rumen.

Several methods have been used to overcome acidosis in cattle farming, such as bicarbonate buffer, ionophores antibiotic (monensin), and virginiamycin. However, there are several problems in using antibiotic due to the resistant characteristic of antibiotic towards diseases and cattle product residue. Yeast is used as a technological alternative to overcome acidosis. Supplementation of *Saccharomyces cerevisiae* in feed increased pH as previously reported by several authors (Chaucheyras-Durand *et al.*, 2008, Lascano and Heinrichs 2009) but until now, there is no study about capacity of live yeast in reduced mycotoxin absorption in the rumen, particularly in acidosis condition. On the other hand acidosis can reduce protozoa population, and modified microbe population in the rumen, consequently, detoxification of mycotoxins is reduced.

Mycotoxin detoxification process is an effort to decrease mycotoxin toxicity, which can be conducted by reducing mycotoxin absorption and degrading mycotoxin into less toxic metabolite. One approach to reduce mycotoxin absorption in the rumen could be done by regulating rumen pH. The extent to which compound is in its ionized and non ionized form is an indicator of its potential absorption across ruminal epithel membranes. Yeast can play a double role by increasing pH and reducing rumen pH decrease after feeding (post
feeding) in the rumen (Chaucheyras-Durand et al., 2008), also as nutrient supply to increase protozoa population.

Based on those studies, ruminal pH regulation has important influence in mycotoxin absorption in the rumen. Changes of pH in the rumen during acidosis condition may influence mycotoxin absorption, therefore, in order to know the effect of rumen pH on mechanism mycotoxin absorption and efficacy yeast to reduce mycotoxin absorption in acidosis and non acidosis, we did two experimental studies in ruminants. In the first study, the mycotoxin absorption was carried out using Empty Washed Rumen (EWR) technique with sheep as model. In this experimental model, we evaluated disappearance rate of AFB1, OTA and FB1 in rumen sheep. The second study was in vivo study on eight non-lactating dairy cows used double cross-over design given acidogenic and non-acidogenic diet supplemented with probiotic yeast to investigate their effect on mycotoxin absorption and mycotoxin detoxification by rumen microbes.

Mycotoxin contamination was conducted naturally on feedstuff using the four mycotoxins (multiple-contamination) with the single-dose mycotoxins administration. The analysis determination of detoxification agents was based on absorption, distribution, metabolism, and excretion (ADME) technique developed by the European Food Safety Authority (EFSA).

Objectives

1. To investigate the effect of ruminal pH on mycotoxin absorption.
2. To investigate the effect of acidogenic diet on ruminal pH, fermentation characteristic and mycotoxin absorption in the rumen.
3. To investigate the capability of yeast S. cerevisiae in stabilizing ruminal pH and mycotoxin detoxification by rumen microbes

Benefit of The Study

To contribute knowledge on mechanism of mycotoxin absorption related to acidogenic diet, so that the diet can be formulated as alternative solution for detoxification in ruminant cattle farming.
2. LOW pH ENHANCES RUMEN ABSORPTION OF AFLATOXIN B1 AND OCHRATOXIN A IN SHEEP

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 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 low pH. The fractional disappearance rates at acid and neutral pH for AFB1 were, respectively, 1.98±0.52 and 1.42±0.57 %/h (p<0.019) and for OTA were 0.16±0.10 and 0.06±0.03 %/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, low pH in the rumen increases the absorption of AFB1 and OTA, potentially contributing to an exacerbated toxic risk.

Key words: low pH, 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 (IARC 2002). 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 (Martin et al., 2006). 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 pH (Martin et al., 2006). 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 (Galtier and Alvinerie 1976) usually decrease in numbers under this dietary conditions (Lettat et al., 2012, Martin et al., 2006) and in animals fed high concentrate diets an increase of the systemic availability of OTA has been described (Xiao et al., 1991).
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 2006) 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 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 FB1 from 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 (Doreau et al., 1997). Experimental solutions at pH 5 or 7 were based on citrate and phosphate buffer, and contained 60 mM acetate, 15 mM 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 CO₂ gas for at least 2 h before used.

Table 2.1 Composition of experimental solution (1 L)

<table>
<thead>
<tr>
<th>Item</th>
<th>pH 5</th>
<th>pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid (0.1 M) (ml)</td>
<td>490</td>
<td>190</td>
</tr>
<tr>
<td>Sodium Phosphat dibasic (0.2 M) (ml)</td>
<td>510</td>
<td>810</td>
</tr>
<tr>
<td>VFA mixture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate (mM)</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Propionate (mM)</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Butyrate (mM)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Co-EDTA (mg)</td>
<td>3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

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 (Truckess and Pohland 2001).
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 (French Ministry of Agriculture 1995) experimental protocol was approved by the Regional Ethics Committee of Animal Experimentation (No CE 31-11).

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 block 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 (Doreau et al., 1997). 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 CO₂ 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., (2010). 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

Experimental solution 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 α-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 C₁₈ 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 desolvation 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.

Table 2.2 Transition reactions monitored by LC-ESI-MS/MS, cone and collision voltages

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>MW</th>
<th>Precursor ion</th>
<th>Daughter ion</th>
<th>Cone voltage (V)</th>
<th>Collision energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B1</td>
<td>312</td>
<td>313.0</td>
<td>128</td>
<td>45</td>
<td>60</td>
</tr>
<tr>
<td>Fumonisin B1</td>
<td>721</td>
<td>722.0</td>
<td>81</td>
<td>50</td>
<td>57</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>403</td>
<td>404.1</td>
<td>239.19</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>α-Zearalenol (IS)</td>
<td>322</td>
<td>323.0</td>
<td>123</td>
<td>15</td>
<td>21</td>
</tr>
</tbody>
</table>

Transition in bold is used for quantification

1Internal standard
MW = Molecule weight

The multiple reaction monitoring (MRM) transitions and the applied cone voltages and collision energies are summarised in Table 2.1. 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 (Boudra and Morgavi 2006). Samples for VFA were analysed by gas chromatography according to (Morgavi et al., 2003). The concentration of Cr-EDTA was determined by atomic absorption spectrometry using a Perkin-Elmer Model 2380 spectrophotometer as described by Uden et al. (1980).

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, \( \mu \) 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 \( (p > 0.05) \), together with the disappearance rates of VFA that were in accord with previous reports \( (Bergman, 1990, Dijkstra et al., 1993, Storm et al., 2012) \) 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 \( (1.07 \pm 0.3\% /h) \) than in neutral pH \( (0.58 \pm 0.3\% /h) \) \( (p = 0.1) \). 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 2.1). 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 \( \text{CO}_2 \) in the gas phase that combines with water to form \( \text{HCO}_3^- \) \( (Aschenbach et al., 2011) \) 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 \( (Dijkstra et al., 1993) \).

Disappearance of mycotoxins from the rumen is presented in Table 2.2. Aflatoxin B1 had the highest disappearance. The low pH solution increased significantly \( (P=0.019) \) AFBI disappearance. Aflatoxins are lipophilic compounds and, although their \( \text{pKa} \) has not been reported, the increased disappearance observed at low 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-ionized OTA. Based on the \( \text{pKa} \) 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 \( (Blank and Wolffram 2009, Xiao et al., 1991) \) 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 2.2). Before exposure, OTA was not present in plasma but it was detected as early as 30 min after exposure. The concentration increased throughout the incubation time, particularly for the acid condition that reached up
Table 2.3. Effect of pH on fractional disappearance rates of mycotoxins in isolated rumen’s sheep model

<table>
<thead>
<tr>
<th>Mycotoxin</th>
<th>Rate of disappearance (%/h)</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neutral</td>
<td>Acid</td>
</tr>
<tr>
<td>Aflatoxin B1</td>
<td>1.42 ± 0.57</td>
<td>1.98 ± 0.52</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>0.06 ± 0.03</td>
<td>0.17 ± 0.10</td>
</tr>
<tr>
<td>Fumonisin B1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

NS : non significant

Figure 2.1. pH profile of experimental solutions (neutral and low pH) during the experimental conditions measured using an indwelling pH bolus. (n=3 animals, error bar represent SD)
Figure 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)

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. 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 (Prelusky et al., 1995) and other animals (Cavret and Lecoeur 2006, Gbore et al., 2010). The transfer of organic compounds such as mycotoxins through the epithelium depends on many factors; among them the degree of ionization plays a significant role in the absorption process (Blank et al., 2003, Xiao et al., 1991).

CONCLUSION

We show that AFB1 and OTA were absorbed in the rumen particularly at low pH. AFB1 and OTA disappearance from the rumen was followed by a concomitant increase of OTA concentration in plasma. Low rumen pH might have a double negative effect by increasing both the dose and absorption of these mycotoxins, contributing potentially to an exacerbated toxic risk. Thus, the solution to this problem was how to maintain rumen pH so that it did not stimulate acidosis condition.
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3. RUMINAL ABSORPTION AND MICROBIAL DETOXIFICATION OF MYCOTOXINS CHANGES ASSOCIATED WITH EXPERIMENTALLY INDUCED SUB-ACUTE RUMINAL ACIDOSIS (SARA) IN NON-LACTATING DAIRY COWS

ABSTRACT

The aims of this study were to examine the effects of sub-acute ruminal acidosis (SARA), with or without yeast, on mycotoxin absorption and mycotoxin detoxification in the rumen. Eight non-lactating dairy cows (650 ± 115) kg fitted with rumen canulas were used in a double cross over design. Cattle were separated in 2 groups that received 30.8% starch for acidogenic (Ac) and 15.1% starch for non acidogenic diet (NA), diet with and without yeast supplementation (Saccharomyces cerevisiae, 0.5 g/d per animal corresponding to 10^{10} CFU). The whole trial lasted 12 weeks and the experiment was divided into 4 periods of 2 weeks, followed by a single administration of four mycotoxins-naturally contaminated feed containing aflatoxin B1 (AFB1) (110 µg/kg DMI), ochratoxin A (OTA) (430 µg/kg DMI), deoxynivalenol (DON) (450 µg/kg DMI), and fumonisin B1 (FB1) (3044 µg/kg DMI). Results demonstrated that Ac diet lowered mean ruminal pH (6.46 vs 6.29) and increased spent time of pH below 5.6 (0.5 vs 2.77 h/d) compared to NA, as the criteria of SARA. Ruminal fermentation in cows fed with Ac diet were characterized by increase total VFA (109 vs 126 mM) and molar proportion of propionate (14.5 vs 16.3%) and decrease molar proportion of acetate (76.30 vs 74.34 %) compared NA diet, while lactic acid was within normal range (2.68-3.09 mM). Yeast supplementation could effectively reduce decreasing pH in Ac (P<0.05) at 4-10-h after morning feeding. The interaction between diet and yeast treatments significantly affected proportion of acetate and isoacids proportion (P=0.01) before feeding. Low ruminal pH enhanced acidic mycotoxin absorption, while yeast was yet effective in reducing OTA and AFB1, shown from the increase of OTA excretion (P<0.05) at 24 to 72 h, and AFM1 excretion as AFB1 metabolite at 48-72 h through urine. Declining Entodinium>100mm and holotrich protozoa (Isotricha and Dasytricha) contributed to increase systemic bioavailability of OTA and DON in Ac feed only at the initial contamination (3-h post contamination). Yeast supplementation tended to increase (P=0.07) OTA conversion to OT α through urinal excretion, while Ac diet could accelerate fecal elimination of DON and DOM1. In conclusion, results showed that induced sub-acute ruminal acidosis (SARA) varied their ability to modify toxicokinetics pattern on AFB1, OTA, DON and FB1 in non lactating dairy cows. When yeast was supplemented in the diets, it could improve microbial detoxification of OTA and there was positive effect in the ruminal fermentation profile.

Keywords: mycotoxins, absorption, microbial detoxification, sub-acute ruminal acidosis (SARA)
INTRODUCTION

Mycotoxins are toxic metabolites produced by some fungi species that are commonly found in foods and feeds, particularly in cereals (Binder et al., 2007, Scudamore and Livesey 1998). They induce a variety of toxic responses in animals, including neurological, hepatotoxic and immunotoxic effects (decrease of the functioning immune system) (Morgavi and Riley 2007) intensive dairy and beef production systems, high concentrate diets, often used to maximize performances, may induce sub-acute ruminal acidosis (SARA). As for mycotoxins, acidosis can have a negative impact on the economy of the farm. The losses can range from a simple decrease in production (reduced intake and performances) up to more serious health problems (Martin et al., 2006).

Resistance of ruminants against some of mycotoxins is well known. A common accepted explanation for this natural recalcitrancy is the capacity of the rumen microbial ecosystem to degrade some mycotoxins (Binder et al., 1997, Kiessling et al., 1984, King et al., 1984, Mobashar et al., 2012, Ozpinar et al., 1999).

Acidosis affects the biodiversity of the rumen microbiota that can have a negative effect on digestibility and the efficacy of microbial detoxification. For instance, some authors observed a decrease in the number of protozoa (Martin et al., 2006) that are responsible to the degradation of some mycotoxins (Galtier and Alvinerie 1976). Another consequence of acidosis is the potential modification of ruminal absorption of mycotoxins. The systemic availability of ochratoxin A (OTA) increased when animals were fed high concentrate diets (Blank et al., 2003, Xiao et al., 1991). Up to now, there are no studies on the interaction between ruminal acidosis and exposure to mycotoxins in ruminants.

Several approaches have been investigated to prevent SARA. Among them, the use of probiotics that could stabilize ruminal pH and improve animal production (Chaucheyras-Durand et al., 2008, Lettat et al., 2012). These latter effects can be positive in reducing mycotoxins’ absorption and toxicity. The aim of this study was to evaluate the effect of ruminal acidosis with and without yeast probiotic on both ruminal absorption of mycotoxins and the efficacy of the microbiota to degrade them.

MATERIALS AND METHODS

Preparation of Mycotoxin-Contaminated Feeds

Aflatoxins (AFB1) and ochratoxins (OTA) were produced via fermentation of wheat by Aspergillus flavus and Aspergillus ochraceus as described by Boudra et al., (2013). Corn naturally contaminated with Fumonisins (FB1) or deoxynivalenol (DON) were locally obtained from an experimental field (Limagrain, Clermont-Ferrand, France). The four contaminated feeds were then ground to a powder to pass a 1-mm screen. The levels of mycotoxins in the homogenized contaminated-feed were analyzed in quadruplicate, and the concentration was 30 ± 0.1, 237 ± 16, 30 ± 0.3, and 237± 0.3 μg/g (Mean ± SD, n=3) for AFB1, OTA, FB1, and DON, respectively. The four mycotoxin-contaminated feeds were then hand-mixed in order to homogenize them.
Animals and Experimental Procedures

The experiment was conducted at the experimental animal facilities the INRA’s Herbivores Research Unit (Saint-Genès Champanelle, France). Procedures with animals were conducted in accordance with the guidelines for animal research of the French Ministry of Agriculture and applicable European guidelines and regulations for experimentation with animals (French Ministry of Agriculture 1995). The experimental protocol was approved by the Regional Ethics Committee on animal experimentation (CE n° 22612). Mycotoxins are dangerous and were manipulated following appropriate safety precautions.

Table 3.1 Composition of experimental diets

<table>
<thead>
<tr>
<th>Item</th>
<th>NA</th>
<th>Ac</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM, %</td>
<td>87</td>
<td>87</td>
</tr>
<tr>
<td>Ingredient (% of DM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Prairie hay</td>
<td>76.2</td>
<td>55.0</td>
</tr>
<tr>
<td>- Wheat</td>
<td>21.6</td>
<td>44.1</td>
</tr>
<tr>
<td>- Ca, Mineral (1), Vitamin</td>
<td>2.16</td>
<td>2.16</td>
</tr>
<tr>
<td>Chemical composition, (% DM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- NDF</td>
<td>53.5</td>
<td>41.1</td>
</tr>
<tr>
<td>- ADF</td>
<td>31.9</td>
<td>24.5</td>
</tr>
<tr>
<td>- CP (crude protein)</td>
<td>8.5</td>
<td>10.3</td>
</tr>
<tr>
<td>- Starch</td>
<td>15.1</td>
<td>30.8</td>
</tr>
<tr>
<td>Feed value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- PDIE (2) g/kg DM</td>
<td>734</td>
<td>841</td>
</tr>
<tr>
<td>- PDIN (3) g/kg DM</td>
<td>517</td>
<td>621</td>
</tr>
<tr>
<td>- UFL (4)</td>
<td>7.30</td>
<td>8.9</td>
</tr>
</tbody>
</table>

1) Mineral Composition (kg): Contents (per kg of premix, DM basis): 25 g, Mg 45 g, Na 30 g, Ca 1.300 mg, Zinc 6.000 mg, Mg 3.500 mg, I 90 mg, Co 36 mg, Se 20 mg, Vitamin A 400.000 U/kg, Vitamin D3 120.000 U/kg, Vitamin E 1.000 U/kg. CMV : calcium, mineral Vitamin.

2) PDIE (Protéines Digestibles dans l’intestin permise par Energy (E)): g of protein that is synthesized in the rumen from breakdown of dietary energy and is absorbed in the small intestine;

3) PDIN (Protéines Digestibles dans l’intestin permise par Azote (N) : g of protein that is synthesized in the rumen from breakdown of dietary nitrogen and is absorbed in the small intestine

4) UFL : Unité Fourragère Lait : energy value of ruminant feed

(1 UFL is equivalent of 1kg of air dried barley = 1.7 Mcal) (INRA 1989)

NA: non acidogenic; Ac: Acidogenic diet

Cows were housed in a tie-stall barn and had ad libitum access to water and minerals salts blocks. Eight non-lactating Holstein cows (650 ± 115 kg) fitted with rumen cannula were allotted to 2 diets: non acidogenic (NA) and Acidogenic diet (Ac) in a double cross over design with four 14-d periods. In addition two experimental diets based on starch proportion (15 vs 30.8%) with and without yeast supplementation. The yeast supplement (Saccharomyces cerevisiae, CNCM I-1077) was provided from Lallemand SA (Toulouse, France), and also administered at 0.5 g /d per animal (1 x 10^{10} CFU) before morning feeding via
rumen cannula to ensure the entire consumption by cows. Cows fed twice daily at 10.7 kg DM/day/head. Hay was fed twice daily at 08:00 (60%) and 14:00 (40%). Concentrate diet was fed at 07:00-hour. The chemical composition of the experimental diets used in the basal diet and feed challenges for acidosis induction is presented in Table 3.1. Feeds were analyzed and the tested mycotoxins OTA, AFB1, DON and FB1 were not detected.

Each animal received a constant dose of contaminated feed that was divided into 4 equivalent portions and administered via the cannula in different places of the rumen that ensures a better homogenization of mycotoxins in the rumen and complete ingestion. The contaminated-feed was administered 4h after the morning feed that corresponding to the pH drop. The design of experiment is shown in Figure 3.1. The whole trial lasted for 12 weeks and the experiment was divided into 4 periods of 2 weeks and 4 weeks washout periods. Each period consisted of a 14-day without mycotoxins followed by a single administration and 3 days of sample collection. Body weights and intake were monitored throughout the experiment.

Sample Collection

Samples of rumen content, urine and feces were collected the day before mycotoxins administration and for 3 days thereafter. Urine and feces excreted over 24-h periods were collected for 3 d and weighed. Urine was collected in a clean container containing sodium azide (50 ml/L of urine). Aliquots of 4 mL were transferred into clean tubes and stored at -20°C until analysis. Feces were manually homogenized and a representative sample (~100 g) was dried (50°C, 72 h), ground to pass a 1-mm screen and stored 20 °C until analysis.

Rumen content samples (~500 g) for fermentation, microbiological parameters and mycotoxins analysis were taken through the cannulae in different parts of the rumen before 0-h and 4-h after morning feeding and at the following time after mycotoxins administration. Samples for mycotoxins analysis were collected at 0 (before administration), 1.5, 3, 6, 12, 24, 48 and 72 h after administration and stored at -20 °C. For mycotoxins analysis, aliquot of 200 g of the whole rumen content was added to 100 ml of PBS buffer and homogenized for 1-min cycles with Laboratory Blender (Georgia, USA).

Approximately 5 g were transferred to 15-ml PP tubes and immediately frozen to stop any microbial activities. Another portion of the rumen sample (~100g) was strained through a polyester monofilament fabric (250 μm mesh aperture) and samples of the liquid filtrate were taken for fermentation parameters analysis: protozoa, volatile fatty acid (VFA), lactic acid and ammonia. For VFA, 0.8 mL of ruminal filtrate was mixed with 0.5 ml of a 0.5 N HCl solution containing 0.2% metaphosphoric acid and 0.4% crotonic acid and frozen at -20 °C. Two ml of filtrate were transferred to PP tubes for lactic acid and 1 ml of filtrate transferred to PP tubes 2 ml containing 0.1 ml 5% acid ortho-phosphoric acid for ammonia determination and stored at -20°C until analysis.

For protozoa counting 2 ml filtrate to 10 ml PP tube containing 2 ml MFS (methyl green formaldehyde solution) and stored in the room temperature in the
dark until used for protozoa counting. Protozoa were enumerated in a Dolfuss cell (Elvetec Services, Clermont-Ferrand, France), using a photonic microscope.

Figure 3.1 Diagram showing rumen juice, urine and feces sampling after introduction contaminated feed. Arrows show contaminating time at the end each periods. P: periods, C: introduction of contaminated mycotoxins, S: sampling times for rumen juice, urine and feces

Ruminal pH was continuously measured in the rumen started at the second week (7-d to 14-d) of each period using e-Cow bolus as described by Phillips et al., (2010). The bolus identity number for each animal was entered into the computer software. Ruminal pH readings were taken every 15 min and stored by the data logger. The daily ruminal pH data were summarized as average pH, min pH and duration below pH 5.6 for 24-hours period. The amounts of dry matter intake (DMI) were recorded for each cow daily. Diets were sampled weekly. Pooled samples were oven dried at 105°C for 24-h (AOAC. 2005).

Parameter Fermentation
Volatiles fatty acids was determined by gas chromatography (CP 9002 Gas Chromatograph, Chrompack, Middelburg, Germany) (Morgavi et al., 2003). Lactate concentration was determined by an enzymatic method (Enzyplus EZA 891+, D/L-Lactic Acid, Raisio Diagnostics, Rome, Italy) as described in Lettat et al., (2010). For NH₃-N, thawed samples were centrifuged at 10,000 g for 10 min and NH₃-N concentration was determined in the supernatant using the Berthelot reaction. The reaction was carried out in duplicate in 96-well plates and read using the Nano-quant Infinite M200 spectrophotometer (Tecan Austria GmbH, Grödig, Austria).

Mycotoxins Analysis

Extraction Procedure
Mycotoxins in different matrices were extracted and analyzed. For urine, extraction with CHCl₃-isopropanol (SI-1): Urine samples (5 ml) were diluted with 3 ml distilled water/methanol. After addition of 3 ml of chloroform-isopropanol (97:3, v/v), the mixture was extracted on a horizontal shaker for 20 min. The mixture was centrifuged at 5000 rpm for 5 minutes, and the top aqueous phase was removed by aspiration. An aliquot of the extract (2 ml) organic layer was
transferred into clean tubes and evaporated to dryness at 45 °C under nitrogen. The dried residues were reconstituted in 200 µl of 0.1% of acetic acid in methanol, vortex-mixed for 20 seconds, and 10 µl were injected onto the LC-MS system.

For rumen juice and feces, mycotoxins were analyzed according to (Lattanzio et al., 2007) with a slight modification (SI-2). Briefly, the polar mycotoxins fumonisin B1, deoxynivalenol and deepoxy-deoxynivalenol were extracted with 25 ml of phosphate buffer saline (PBS) in a horizontal shaker (Peters et al., 2013) for 15 min at 60 rpm, and then centrifuged at 5000 rpm for 5 minutes. Total supernatant will be filtered through a Whatman GF/A glass, and 10 ml will be transferred into 15-ml falcon tubes = Extract A. The apolar mycotoxins (aflatoxin B1, ochratoxin A aflatoxin M1 and ochratoxin α) were extracted from the solid residue of the previous extraction with 20 ml methanol and centrifuged at 5000 rpm / 5 min. An aliquot of supernatant (10 ml) is transferred into a clean PP -tube and reduced to ~ 6 ml. The methanol extract was diluted with PBS (20 ml) and filtered through a Whatman GF/A glass = Extract B.

Purification Procedure

Twenty five milliliters of the extract A was applied to the immunoaffinity columns (IAC) (myco 6 in 1) at a flow rate of 1-2 drop/second. The IAC was washed with 15 ml of PBS at a flow rate of 4-5 drop/sec. Ten milliliters extract B was applied to IAC at flow rate of 1-2 drop/second. The IAC was washed with 10 ml distilled water (DW) at a flow rate of 4-5 drops/second until air comes through the column and then the IAC was eluted using 3 ml of methanol at flow 1 drop/sec until air comes through the column. The elute was evaporated at dryness /N2 at 50°C (duration 2 hours) and redissolved with 0.2 ml of mobile phase (30% of B).

LC-MS/MS Analysis

The chromatographic system was an Alliance 2695 module (Waters Corporation, St-Quentin-en-Yvelines, France). Separation was performed at room temperature on a Kinetex C18 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 desolvation 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 summarized in Table 2.1. The most intense transition reaction was used for quantification purpose while the
others were employed for analyte confirmation. Data were collected in centroid mode with an acquisition rate of 0.2 s, a constant dwell time of 10 ms and an interscan delay of 0.02 s.

Statistical Analysis

Data of DMI, nutrient intake, DM intake, pH, VFA, lactic acid, ammonia, protozoa, mycotoxins and its metabolite excretion were analyzed using the MIXED procedure of SAS. Least square means and standard errors were determined using the LSMEANS for nutrient content of treatments, and differences of least square means were determined using the PDIFF statement with the following mixed linear model:

\[ y_{ijk} = \mu + T_i + Y_j + (T \times Y)_{ij} + A(i)k + \epsilon_{ijk} \]

Where \( y \) is the dependent variable, \( \mu \) is the overall mean, \( T \) is the fixed effect of the treatment, \( Y \) is the fixed effect of yeast supplementation, \( T \times Y \) is the treatment \( \times \) yeast interaction, \( A \) is the random effect of animal tested within treatment, and \( \epsilon \) is the random residual. All data were analyzed using SAS version 9.1 (SAS Institute Inc., Cary, NC). All statements of statistical significance are based on a probability of \( P < 0.05 \). Trends are discussed at a statistical significance of \( P < 0.10 \).

RESULTS AND DISCUSSION

General Conditions of the Experimental Cows

The experiment was conducted according to the protocol designed previously. All the experimental animals were in normal conditions without any health disturbance and insults during the four periods of experiment consisted of diet adaptation period and sampling period.

Feed and Nutrients Consumptions and Dry Matter Digestibility

Averages of diet and nutrient consumptions and dry matter digestibility of the experimental cows during the experiment are presented in Table 3.2. Based on the statistical analysis, it was found that there was no interaction effect of diet or ration type and yeast supplementation on dry matter, protein, NDF, ADF, and starch intakes, and dry matter digestibility. The type of diet or ration had a significant effect on nutrient consumption, except on dry matter intake. As compared to cows fed with NA ration, cows fed with Ac ration had lower NDF (5.82 vs. 5.53 kg/d) and ADF (3.48 vs. 2.70 kg/d) consumptions and higher protein (0.93 vs. 1.13 kg/d) and starch (1.64 vs. 3.39 kg/d) consumptions. Based on the diet composition (Table 3.1), the increased starch and protein consumptions in the cows fed with Ac ration were probably associated with the higher proportion of grain in the Ac diet as compared to NA diet. The use of Ac diet potentially causes SARA disturbance.
In this study, yeast supplementation increased dry matter digestibility (P<0.02). It was found that cows fed with ration or diet supplemented with yeast had higher dry matter digestibility (64.35%) as compared to those fed with diet without yeast supplementation (61.56%). Dry matter digestibility had higher (P<0.03) for cows fed with Ac than NA (64.2 vs. 61.3%). The digestibility of DM increased with increasing starch intake, caused by higher amount of soluble carbohydrates. (Table 3.2). The result indicated that yeast could stimulate the process of rumen fermentation by increasing bacterial activities in the rumen. Yeast supplementation was reported to increase dry matter digestibility (Marden et al., 2008), stimulate the activities of cellulolytic bacteria and increase fiber digestibility (Harrison et al., 1988). However, it was reported that yeast supplementation could reduce redox potential (Eh) that caused the rumen environment became more anaerobic that stimulated and increased bacterial growth in the rumen (Chaucheyras-Durand et al., 2008).

**Rumen pH**

The Effects of Ration Type and Yeast Supplementation on Rumen pH

The averages of rumen pH in this experiment are presented in Table 3.3. There was no interaction effect of diet type and yeast supplementation on average pH, minimum pH, and the duration of rumen pH below 5.6. However, cows fed with Ac diet had lower (P<0.05) mean rumen pH (pH=6.29) and minimum rumen pH (5.24) as compared to those fed with NA diet (rumen pH and minimum rumen pH were 6.46 and 6.08, respectively). The fluctuation of pH values during 24-hours observations are presented in Figure 3.2. The duration of rumen pH lower than 5.6 was an indicator of SARA. When the duration of rumen pH lower than 5.6 was 2.77 hours/day while in the cows fed with NA diet the duration of rumen pH below 5.6 was very short i.e., only 0.5 hour/day, more than 3 hours per day it was an indication of acidosis (Gozho et al., 2005b, Oetzel et al., 1999). During the acidosis condition, the duration of rumen pH below 5.6.

In this study, the decreased rumen pH in the cows fed with Ac diet was caused by the increased starch consumption (3.39 kg/d) and the decreased fiber consumption as compared to those fed with NA diet (Table 3.2). Starch supplementation by 3.88 kg/day could reduce average rumen pH to 5.56 in steer (Beauchemin et al., 2003) and concentrate supplementation in high proportion could decrease rumen pH to 5.1 (Ghorbani et al., 2002). The low fiber content of the diet could reduce saliva secretion and production that decreased buffer capacity that eventually decreased rumen pH (Yang 2002). Therefore, simulation in this experiment successfully decreased and reduced rumen pH similar to that in SARA condition.

Yeast significantly affected rumen pH. Yeast supplementation in the diet increased rumen pH (6.45) and minimum rumen pH value as compared to control without yeast supplementation (6.27). The duration of rumen pH below 5.6 was shorter (1.61 hours) in cows supplemented with yeast as compared to control.
Table 3.2 Dry matter and nutrient intake (kg/d) and dry matter digestibility (%)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NA Without Yeast</th>
<th>Yeast Addition</th>
<th>Ac Without Yeast</th>
<th>Yeast Addition</th>
<th>SEM D</th>
<th>SEM Y</th>
<th>SEM D x Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMI, kg/d</td>
<td>10.88</td>
<td>10.95</td>
<td>11.02</td>
<td>11.01</td>
<td>0.18</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Nutrient intake</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein, kg/d</td>
<td>0.92</td>
<td>0.93</td>
<td>1.13</td>
<td>1.13</td>
<td>0.03</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>NDF, kg/d</td>
<td>5.82</td>
<td>5.86</td>
<td>4.53</td>
<td>4.52</td>
<td>0.15</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>ADF, kg/d</td>
<td>3.47</td>
<td>3.49</td>
<td>2.70</td>
<td>2.70</td>
<td>0.09</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Starch, kg/d</td>
<td>1.64</td>
<td>1.65</td>
<td>3.39</td>
<td>3.39</td>
<td>0.16</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>DM Digestibility</td>
<td>60.45</td>
<td>63.00</td>
<td>62.59</td>
<td>65.70</td>
<td>0.18</td>
<td>0.03</td>
<td>0.02</td>
</tr>
</tbody>
</table>

DMI: dry matter intake; NS: non significant; SEM: standard error mean
D: diet effect; Y: yeast supplementation effect
NA: non acidogenic
Ac: acidogenic

Diarang mengutip sebagian atau seluruh karya tulis ini tanpa mengutip tanpa menunjukkan sumber, baik buku, jurnal, atau lainnya, dapat dikenal sebagai plagiasi.
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Table 3.3 Ruminal pH profile in non-lactating dairy cows fed a non acidogenic (NA) and acidogenic (Ac) diets (n=8) with and without yeast supplementation

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Diet (D)</th>
<th>Yeast (Y)</th>
<th>SEM</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NA</td>
<td>Ac</td>
<td>Without Yeast</td>
<td>Yeast Addition</td>
</tr>
<tr>
<td>Mean pH</td>
<td>6.46</td>
<td>6.29</td>
<td>6.27</td>
<td>6.45</td>
</tr>
<tr>
<td>Min Ruminal pH</td>
<td>6.08</td>
<td>5.24</td>
<td>5.51</td>
<td>5.68</td>
</tr>
<tr>
<td>Time spent pH &lt; 5.6 (h/24h)</td>
<td>0.50</td>
<td>2.77</td>
<td>1.98</td>
<td>1.61</td>
</tr>
</tbody>
</table>

NS: non significant, D, diet effect; Y, yeast supplementation effect NA: non acidogenic Ac: acidogenic; SEM: standard error mean

NA: non acidogenic; Ac: acidogenic

Figure 3.2 The diurnal pattern, (24-h) ruminal pH on non acidogenic and acidogenic diet with and without yeast supplementation. Arrows show sampling time rumen juice and administration mycotoxin contaminated diet (4-h after morning feeding at 11:00 a.m)
without yeast supplementation (1.98 hours). Based on these results, acidogenic condition with feeding the experimental cows with ration contained 30.8% starch could cause the SARA condition

In this experiment, it was clear that yeast supplementation could inhibit (P<0.05) the decrease in rumen pH 4 to 10-h after morning feeding in the cows fed with Ac diet. This condition was related to the ability of yeast to stimulate the growth of lactate-utilizing bacteria as was reported by Callaway and Martin (1997) that Saccharomyces cerevisiae yeast could stimulate the growth of lactate bacteria such as Megasphaera elsdenii and Selenomonas ruminantium, so that the rumen pH did not decrease. The use of Saccharomyces cerevisiae yeast was also reported to increase rumen pH by 0.5 unit as compared control without yeast supplementation Bach et al., (2007). However, Erasmus et al., (2005) reported that yeast supplementation did not affect rumen pH. The different results could probably due to the difference in diet composition, i.e., protein content of the ration was high (18.1%) with low fiber content (NDF was 31.2% and ADF was 19.2%) while in our experiment, protein content was 10.3%, NDF was 41.1% and ADF was 24.5%. Yeast supplementation has a chance to reduce the incidence of SARA condition by maintaining the rumen pH relatively high and shorten the duration of rumen pH below 5.6. The Ac diet or ration decreased the rumen pH similar to SARA condition. The effect of yeast in preventing acidosis is also depends upon the type of diet used. This hypothesis needs to be tested by using various types of diets.

Kinetic profiles of rumen pH

The pH kinetic values during 24-h observation are presented in Figure 3.3. The figure showed slopes (time x pH ) during 0 to 4-h after feeding on the cow fed NA diet had lower of slope (slope = 0.10 unit/h) as compared to those fed with Ac diet (slope=0.25 unit/h). In contrast, minimum pH had higher for cows fed NA than Ac (6.2 vs 5.5) at 4-h after morning feeding.

Increased slope pH values in the cows fed Ac diet was caused by increased starch intake and the decreased fiber intake as compared fed with NA diet. Ruminal acidosis results for consumption of readily fermentable carbohydrates (Lettat et al., 2012). Acidosis mechanism is showed in Figure 3.4. According Gozho et al., (2005b) starch supplementation of high proportion could decrease of rumen pH under 5.6 until 187 min/d. Where as high fiber content of the diet NA could increase saliva secretion and production that increased buffer capacity so that min pH was increased, as similar previously reported by Owens et al., (1998).

In slope pH during 4-24-hour after feeding on the cow fed Ac diet slope profile higher (0.06 unit/h) than that fed NA diet (0.02 unit/h). The higher slope profile in Ac diet due to decreased pH in Ac diet was higher than NA diet. Time to recovery to increased pH in unit/h faster in Ac than NA. The increased rumen pH in Ac more high that NA due to absorption rate VFA. According to Dijkstra et al., (2012) that in low pH rate of absorption VFA faster than neutral pH. In NA diet rumen pH was relative konstan in neutral pH condition during 4-24-hours after feeding. Therefore from this result showed different of pH profile among diet type as expected and it was viable for SARA simulation.
Figure 3.3 pH kinetic values, relationship between time and rumen pH at 0-4-h (A) and 4-24-h (B) after feeding in non acidogenic and acidogenic diet.

Figure 3.4 Step by step microbial of ruminal acidosis. Adapted from (Nocek 1997)
Rumen Fermentation

VFA Concentration

The effects of ration or diet type and yeast supplementation on rumen metabolites (VFA, isoacid, lactic acid, valerate, and ammonia) production were measured prior to feeding (0-h) and 4-h post-feeding are presented in Table 3.4. Based on statistical analysis, there was no interaction effect of diet type and yeast supplementation on total and individual VFA (acetate, propionate, and butyrate) production and C2:C3 ratio. Averages total VFA concentrations found in this experiment ranged from 70 to 132 mM that were in normal ranges. Ac ration or diet significantly affected total and individual VFA concentrations. Total and individual VFA concentrations were higher (P<0.05) in cows fed with Ac ration or diet as compared to those fed with NA diet, especially prior to feeding (0-h).

The increased total VFA concentration in cows fed with Ac diet caused a decrease in rumen pH. VFA is a product of carbohydrate metabolism by microbes in the rumen that normal concentration is around 80-160 mM. Concentrations of total VFA in cows fed with Ac ration or diet ranged from 121.70 to 132.0 mM. The increased total VFA concentration indicated that the condition was very close to SARA criteria i.e., around 150 mM (Nagaraja and Titgemeyer 2007). Total VFA concentrations in cows fed with diet supplemented with yeast were higher than in those fed without yeast supplementation. The increased total VFA concentration was associated with the increased dry matter digestibility when the diet was supplemented with yeast. The increase in total VFA concentration in cows fed with Ac diet could also be associated with the conversion of lactic acid to propionate and acetate (Wiryawan and Brooker 1995). One of SARA criteria in addition to rumen pH is total VFA concentration, since low rumen pH is caused by the increased VFA production.

Averages molar proportion of fermentation products found in this experiment are presented in Table 3.5. There was no interaction effect of diet type and yeast supplementation on butyrate and valerate-caproate percentage, except in acetate percentage. However, there was an interaction effect of diet type and yeast supplementation on molar proportion of acetate (P=0.01). Cows fed with NA diet supplemented with yeast had higher acetate proportion (79.31%) while those fed with Ac diet supplemented with yeast had lower acetate proportion (74.34 %). Therefore, yeast and diet type affected the activities of cellulolytic bacteria in the rumen that produced acetate. The results were associated with the tendency (P=0.06) of higher proportion of molar concentration of propionate by 16.3% in cows fed Ac diet as compared to those fed with NA diet (14.5%).

The decreased acetate production in cows fed with Ac diet was caused by the increased proportion of propionate. Yeast supplementation in cows fed with Ac diet could positively decrease acetate proportion since yeast was assumed to increase lactate conversion to propionate due to the lower production of acetate. It was reported that yeast was able to stimulate the growth of lactic-utilizing bacteria that converted lactate to propionate (Martin et al., 2006). In cows fed with Ac diet, the effect of yeast supplementation was more significant as compared to those fed...
Table 3.4  Effect of yeast probiotic supplementation on ruminal fermentation characteristics in non-lactating dairy cows fed a non- acidogenic (NA) and acidogenic (Ac) diets (n=8)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Time (h)(1)</th>
<th>NA Without Yeast</th>
<th>Ac Without Yeast</th>
<th>Ac Yeast Addition</th>
<th>SEM</th>
<th>D</th>
<th>Y</th>
<th>D x Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total VFA (mM)</td>
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<td>115.72</td>
<td>93.24</td>
<td>7.19</td>
<td>0.02</td>
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</tr>
<tr>
<td></td>
<td>4</td>
<td>100.68</td>
<td>118.90</td>
<td>121.70</td>
<td>132.48</td>
<td>7.25</td>
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<td></td>
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<tr>
<td>Acetate</td>
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</tr>
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<td>88.74</td>
<td>96.02</td>
<td>5.01</td>
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<td></td>
</tr>
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<td>1.31</td>
<td>0.003</td>
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<tr>
<td></td>
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<td>21.53</td>
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<td>0.96</td>
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</tr>
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<td></td>
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<td>11.89</td>
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<tr>
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<td>0.79b</td>
<td>0.38ab</td>
<td>0.41a</td>
<td>0.05</td>
<td>0.03</td>
<td>0.004 0.02</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.56</td>
<td>0.85</td>
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<td>0.05</td>
<td>0.003</td>
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</tr>
<tr>
<td>Valerate</td>
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<td>0.31</td>
<td>0.72</td>
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<td>0.14</td>
<td>0.06</td>
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<td></td>
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<td>0.63b</td>
<td>0.68b</td>
<td>0.66b</td>
<td>0.05</td>
<td>0.006 0.03</td>
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</tr>
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<td></td>
</tr>
<tr>
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<td>0.21</td>
<td>0.19</td>
<td>0.48</td>
<td>0.07</td>
<td>NS NS NS</td>
<td></td>
</tr>
<tr>
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<td>1.41b</td>
<td>1.06ab</td>
<td>1.08ab</td>
<td>0.08</td>
<td>NS 0.0008 0.003</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>5.31</td>
<td>4.76</td>
<td>4.84</td>
<td>0.21</td>
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<td></td>
</tr>
<tr>
<td>C2 : C3</td>
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<td>5.00</td>
<td>4.64</td>
<td>4.98</td>
<td>0.21</td>
<td>NS NS NS</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>2.16</td>
<td>2.5</td>
<td>1.73</td>
<td>1.35</td>
<td>0.23</td>
<td>NS NS NS</td>
<td></td>
</tr>
</tbody>
</table>

Mean within the same group (diet and yeast) with the different superscripts differ (P<0.01), SEM : standard error mean

(1) From time feeding; D, diet effect; Y, yeast supplementation effect; NA: non acidogenic; Ac: acidogenic

a, b, Different letters indicate differences (P<0.01)
Table 3.5  Effect of yeast probiotic supplementation on ruminal fermentation characteristics in non-lactating dairy cows fed a non-acidogenic (NA) and acidogenic (Ac) diets (n=8)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Time (h)(^{(1)})</th>
<th>NA Without yeast</th>
<th>NA Yeast addition</th>
<th>Ac Without yeast</th>
<th>Ac Yeast addition</th>
<th>SEM</th>
<th>D</th>
<th>Y</th>
<th>D x Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate (%)</td>
<td>0</td>
<td>79.31(^{a})</td>
<td>76.30(^{b})</td>
<td>75.35(^{b})</td>
<td>74.34(^{b})</td>
<td>0.67</td>
<td>0.03</td>
<td>0.008</td>
<td>0.01</td>
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<tr>
<td></td>
<td>4</td>
<td>72.80</td>
<td>74.65</td>
<td>73.44</td>
<td>73.49</td>
<td>0.52</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Propionate (%)</td>
<td>0</td>
<td>14.56</td>
<td>14.50</td>
<td>16.21</td>
<td>16.51</td>
<td>0.48</td>
<td>0.06</td>
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<tr>
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<td>16.68</td>
<td>14.98</td>
<td>16.16</td>
<td>16.17</td>
<td>0.46</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Butyrate (%)</td>
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<td>4.90</td>
<td>6.64</td>
<td>6.84</td>
<td>6.89</td>
<td>0.35</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8.15</td>
<td>8.17</td>
<td>8.38</td>
<td>8.15</td>
<td>0.39</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Isoacids (^{(2)}) (%)</td>
<td>0</td>
<td>0.88(^{b})</td>
<td>1.96(^{b})</td>
<td>0.92(^{b})</td>
<td>1.23(^{b})</td>
<td>0.10</td>
<td>0.04</td>
<td>0.006</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.37</td>
<td>1.39</td>
<td>1.06</td>
<td>1.02</td>
<td>0.08</td>
<td>0.009</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Valerate and Caproate</td>
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<td>0.28</td>
<td>0.43</td>
<td>0.58</td>
<td>0.79</td>
<td>0.07</td>
<td>0.03</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.80</td>
<td>0.63</td>
<td>0.84</td>
<td>0.88</td>
<td>0.07</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(^{a,b}\) Mean within the same group (diet and yeast) with the different superscripts differ (P<0.01)
\(^{(2)}\) Isoacid (isobutyrate + isovalerate)
(1), (2) From time feeding: D, diet effect; Y, yeast supplementation effect
SEM: standard error mean; NA: non acidogenic; Ac: acidogenic
with NA diet. In cows fed with Ac diet, diet type would stimulate *Streptococcus bovis*, which is an efficient starch-utilizing and lactate-producing bacteria with vary rapid growth, becomes dominant in high-starch diets. According Russell and Hino (1985) that the decreased in pH and the accumulation of glycolytic intermediet (i.e. piruvat, fructose-1.6-diphosphat) inhibits pyruvate formate-lyase (PFL) and the subsequent formation format and acetate to the benefit of lactate dehydrogenase (LDH) (activities that increased lactate production that were further converted to propionate through methylmalonil CoA and propionil CoA cycles that eventually produced propionate (Jouany 2006) (Figure 3 5). In this process, yeast plays a significant role.

The increased propionate (C3) production in cows fed with Ac diet decreased the ratio of C2:C3 (acetate:propionate) (P<0.01) by 4.75 as compared to cows fed with NA diet (5.4). This value was higher than that reported by Kowalik et al., (2012) that found the ratio of C2:C3 was around 4.1 in high fiber diet (diet contained mead cow hay 87.5%, and NDF 55%). The difference could be related to the different NDF content of the ration. According to Weimer et al., (1999), the decrease in C2:C3 ratio in acidogenic diet is caused by the decreased cellulolytic activities at the acidic pH and the increase in amylolytic activities. The effect of Ac diet on the ratio of C2:C3 indicated the increased activities of rumen amylolytic microbes.

In this experiment, there was not significant effect of diet type and sampling time 4-h after morning feeding on total VFA and individual VFA (propionate and acetate). This sampling time is representative of maximum fermentation and formation of metabolite products in the rumen, as previously reported by (Franzolin and Dehority 2010, Lascano and Heinrichs 2009). Non significant effect of diet treatment at 4-h on total VFA , acetate and propionate due to the conversion of lactic acid by lactic acid-utilizing bacteria in Ac diet was

![Figure 3.5 Regulation of lactate production by *Streptococcus bovis*](image-url)

LDH = *Lactate dehydrogenase*, PFL = *Pyruvate formate-lyase*

Adapted from (Russell and Hino 1985)
not yet optimum, thus variation of propionate and acetate production between diet type was not achieved. According to Ghorbani et al., (2002) the effect of treatment diet occurred 12 hours after morning feeding on VFA, while Silberberg et al., (2013) reported feeding history of individual animal caused ruminal biochemical and microbial parameters change. Therefore, non different among concentration total VFA and individual VFA (acetate, propionate, and butyrate) at 4-h are probably due to feed composition.

Isoacid

Isoacid concentrations indicating the total concentration of isovalerate and isobutyrate ranged from 0.67 to 1.41 mM. There was an interaction effect of diet type and yeast supplementation on isoacid concentration (P=0.01) at 0-hour feeding (prior to feeding). In cows fed with NA diet, yeast supplementation increased isoacid concentration while in those fed with NA diet, the effect of yeast supplementation was depend on time.

The percentage of isoacid was affected by the interaction of diet type and yeast supplementation (P=0.03). Yeast supplementation both in Ac and NA diets increased the proportion of isoacid. This result indicated the effect of yeast supplementation on the fermentation process of branch-chain amino acids in relation to the stimulation of proteolytic enzyme activities.

Yeast supplementation could also increase isoacid proportion (P<0.025) even though in a very small proportion (ranged from 1.23 to 1.96%) when compared to those reported by Ghorbani et al., (2002) (3.9%). However, the result found in the present experiment was similar to that reported by Lettat et al., (2010) i.e., 1.5%. Isoacid is produced by fermentation of branch-chain amino acids. In the present experiment, however, it was assumed that the yeast preparation also contained amino acid (isoacid) that could increase isoacid concentration in the rumen. The increased isoacid concentration was assumed to be directly correlated with the increased proteolytic activities of rumen bacteria, as was reported by Oeztuerk (2009). Isoacid production and concentration could be used as a parameter index of amino acid production and amino acid deamination in the rumen (Jouany et al., 1998). Yeast supplementation could stimulate the activity of proteolytic enzymes that finally increased the production of isoacid.

Lactic Acid

Lactic acid concentration in this experiment was affected by the diet composition. In cows fed with Ac diet, lactic acid concentration (2.9 mM) was higher (P<0.01) as compared to that in cows fed with NA diet (0.99 mM), especially 4-hours after feeding. Average lactic acid concentration in cows fed with Ac diet ranged from 2.68 to 3.09 mM and these values were still in the normal range (<5 mM) and was one of the criteria of SARA condition. The normal concentration and condition of lactic acid is an indication that lactic acid is not accumulated in the rumen and the rate of lactic acid synthesis is balance with its rate of degradation (Owens et al., 1998).

The increase in starch content with almost twice in the Ac diet produced a higher concentration of lactic acid in the rumen. It was reported that concentrate with high starch content decreased rumen pH due to the increased production of
lactic acid in the rumen (Beauchemin et al., 2003). In the present experiment, the induction of SARA condition was conducted by using wheat grains that could decrease rumen pH rapidly. It was reported that wheat grains are sources of starch that are rapidly degraded (Sauvant et al., 2006). In the rumen, starch is rapidly fermented by rumen microbes that produced lactic acid. The normal concentrations of lactic acid indicated that lactic acid produced is directly converted to propionate that finally contributed to the lower rumen pH.

**NH₃-N (Ammonia)**

In this experiment, NH₃-N concentrations in the rumen ranged from 3.44 to 4.52 mM. There was no interaction effect of diet type and yeast supplementation on ammonia concentration. Ammonia is a product of protein hydrolysis. In the present experiment, protein metabolism did not significantly affect ammonia production and concentrations in the rumen.

**Protozoa Population**

Averages rumen protozoa numbers are presented in Table 3.6. There was no interaction effect of diet type and yeast supplementation on total protozoa population in the rumen (Entodinium < 100 mm, Entodinium > 100 mm, Dasytricha, except for Isotricha). There was an interaction effect of diet type and yeast supplementation on Isotricha (P=0.01) population, especially 4-hours after feeding. In cows fed with Ac diet, yeast supplementation decreased Isotricha population. However, in cows fed with NA diet, yeast supplementation increased Isotricha population. NA diet had higher fiber content as compared to Ac diet. The increased Isotricha population in cows fed with NA diet was due to the tendency of these protozoa to stick and attach to plant particle (Williams 1986). Probably ini NA condition with yeast to the benefit for Isotricha population.

The increase in protozoa population is depend on the availability of soluble carbohydrate produced and available that can be used as an energy source and carbon source. It was reported that the decreased Isotricha population in the rumen of cows supplemented with yeast was assumed to be caused by the negative effects of nutrients and compounds produced by the yeast on Isotricha growth and development (Kowalik et al., 2011). In contrast, Isotricha number increased at 4-h on NA diet. The population of Entodinium > 100 mm and Dasytricha in cows fed with Ac diet was lower (P<0.01) as compared to those fed with NA diet, especially 0 and 4-h after feeding. The decreased population of these two protozoa could be caused by the sensitivity of these two protozoa to the decreased pH during acidosis. It was reported that protozoa is sensitive to the decrease in pH (Franzolin and Dehority 2010). In the present experiment, population of protozoa was 95% dominated by genera Entodinium < 100 mm and only 5% was contributed by Entodinium >100 mm, Isotricha, and Dasytricha. SARA condition potentially decrease population of genera Entodinium >100 and holotrics protozoa.
Table 3.6 Effect of yeast probiotic supplementation on rumen protozoa numbers in non-lactating dairy cows fed a non-acidogenic (NA) and acidogenic (Ac) diets (n=8)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Time (h)</th>
<th>NA Without Yeast</th>
<th>NA Yeast Addition</th>
<th>Ac Without Yeast</th>
<th>Ac Yeast Addition</th>
<th>SEM</th>
<th>P-values</th>
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<td></td>
<td>(h)(1)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>D</td>
</tr>
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<td>5.49</td>
<td>5.42</td>
<td>5.55</td>
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<td>4</td>
<td>5.46</td>
<td>5.48</td>
<td>5.29</td>
<td>5.32</td>
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<td>Entodiniomorphs&lt;100 µm</td>
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<td>5.48</td>
<td>5.41</td>
<td>5.54</td>
<td>0.05</td>
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<td>5.44</td>
<td>5.47</td>
<td>5.28</td>
<td>5.32</td>
<td>0.07</td>
<td>NS</td>
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<td>3.31</td>
<td>3.10</td>
<td>2.69</td>
<td>0.14</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.18</td>
<td>0.06</td>
</tr>
<tr>
<td>Dasytrichae</td>
<td>0</td>
<td>3.43</td>
<td>3.56</td>
<td>2.66</td>
<td>2.43</td>
<td>0.15</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.07</td>
<td>3.10</td>
<td>1.61</td>
<td>1.36</td>
<td>0.24</td>
<td>0.007</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Mean within the same group (diet and yeast) with the different superscripts differ (P<0.01)

1. From time feeding:
2. D, diet effect; Y, yeast supplementation effect
3. SEM: standard error mean; NA: non acidogenic; Ac: acidogenic
1. Dilihat mempublikasikan secara seluruh koran, lihat ini compon masyarakat kampung 

2. Dilihat mempublikasikan secara seluruh koran, lihat ini compon masyarakat kampung

3. Dilihat mempublikasikan secara seluruh koran, lihat ini compon masyarakat kampung

4. Hak cipta bagi dilihat dengan Lindung-Lindung

Bogor Agricultural Institute

Hak cipta Milik IPB (institut Pertanian Bogor)
Acidogenic diet could cause the rumen condition similar to SARA condition that eventually affected diet metabolism and rumen protozoa population. The effect of SARA on multi-mycotoxin contamination would be discussed in the following section.

**Toxicokinetics of Mycotoxins in the Rumen and Its Balance Excretion**

The experimental cows were in healthy conditions and there was no health disturbance found after multi mycotoxin contamination. Before mycotoxins contamination was conducted, there was no detectable mycotoxins and their metabolites in the rumen. Based on statistical analysis, there was no interaction effect of diet type and yeast supplementation on the concentrations of AFB1, OTα, DON, DOM1 and FB1 in the rumen both at the sampling times of 3 and 6 hours after contaminations, except on AFM1 and OTA, there was an interaction effect at the time of 6 h sampling.

The excretion of mycotoxins in urine and feces are presented in Table 3.7-3.10. There was no interaction effects of diet type and yeast supplementation on the excretions of AFB1, AFM1, OTA, DON, DOM 1 and FB1 that was detected in feces, and AFM1, OTα and OTA in the urine at the collection time of 24, 48, and 72 hours after multi-mycotoxin contaminations. Average total excretion of each mycotoxin is presented in Table 3.11. There was no interaction effect of diet type and yeast supplementation on the excretion of OTA, DON and FB1, except AFB1.

**Aflatoxin B1 (AFB1)**

Average concentrations and excretions of AFB1 and AFM1 (as a metabolite of AFB1) are presented in Table 3.7. Based on time of sampling, concentration of AFB1 at the 3 hours sampling was higher (0.85 ng/g) as compared to that at the time of 6 hours sampling (0.53 ng/g), even though statistically not significant. This non-significant effect could be related to the high variability among the experimental cows that was reflected in the high SEM. The trend of decrease in concentration 6 hours after contamination indicated that there was an increased absorption of AFB1 with the increase in incubation time in the rumen. The same results were reported by Yiannikouris and Jouany (2002) that AFB1 was absorbed rapidly by passive diffusion in the rumen. However, Moschini *et al.* (2006) reported that AFB1 was detected in blood plasma only in 20 minutes after contamination in the rumen. AFB1 has low molecular weight (312.27 MW) and is easily soluble in the lipid (lipophilic) so that it easily crossed the rumen cell membrane (Fernández *et al.*, 1997). Therefore, the decrease in AFB1 concentration is caused by the rapid absorption of the molecule in the rumen.

At the average concentration of AFB1 based on diet type, it was clear that in the Ac diet, portion of the AFB1 absorbed was higher (0.53 ng/g) as compared to NA diet (0.11 ng/g), after 6 hours after contamination. This decrease trend of AFB1 indicated that AFB1 in cows fed with Ac diet was absorbed rapidly in the rumen. This results is similar to that reported by Pantaya. *et al.* (2014) that the rate of disappearance of AFB1 in low pH is higher than in neutral pH in the rumen of sheep. The increase in AFB1 absorption is assumed to be caused by the
increase in the percentage of unionized molecules of AFB1 in acid environment. However, Fink-Gremels (2008) assumed that acidosis condition will increase the absorption. This decrease trend of AFB1 indicated that AFB1 in cows fed with Ac diet was absorbed rapidly in the rumen. The increase in AFB1 absorption is assumed to be caused by the increase in the percentage of unionized molecules of AFB1 in acid environment. From this fact, the absorption of AFB1 increased in the condition of acidosis.

Table 3.7 Daily excretion of AFB1 and AFM1 in feces urine and concentration on rumen juice (RJ) (ng/g) in dairy cows fed contaminated diet alone or with yeast

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>Time (h)(1)</th>
<th>NA Without yeast</th>
<th>Yeast addition</th>
<th>Ac Without yeast</th>
<th>Yeast addition</th>
<th>SEM</th>
<th>D</th>
<th>Y</th>
<th>D x Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1</td>
<td>3</td>
<td>0.59</td>
<td>0.69</td>
<td>1.29</td>
<td>0.83</td>
<td>0.17</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.52</td>
<td>0.55</td>
<td>0.54</td>
<td>0.52</td>
<td>0.05</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AFM1</td>
<td>3</td>
<td>0.08</td>
<td>0.09</td>
<td>0.06</td>
<td>0.07</td>
<td>0.02</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.06a</td>
<td>0.12a</td>
<td>0.04a</td>
<td>0.04a</td>
<td>0.01</td>
<td>0.01</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>AFM1</td>
<td>24</td>
<td>12.2</td>
<td>10.7</td>
<td>6.7</td>
<td>9.8</td>
<td>1.5</td>
<td>0.07</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>23.4</td>
<td>23.4</td>
<td>18.0</td>
<td>18.1</td>
<td>1.4</td>
<td>0.03</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AFM1</td>
<td>24</td>
<td>39.4</td>
<td>32.9</td>
<td>44.1</td>
<td>54.8</td>
<td>5.3</td>
<td>0.08</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>47.4</td>
<td>37.0</td>
<td>49.6</td>
<td>49.6</td>
<td>4.5</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AFM1</td>
<td>24</td>
<td>25.6</td>
<td>8.2</td>
<td>18.4</td>
<td>26.2</td>
<td>5.3</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td></td>
<td>48</td>
<td>7.7</td>
<td>6.3</td>
<td>16.9</td>
<td>19.4</td>
<td>2.2</td>
<td>0.01</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AFM1</td>
<td>72</td>
<td>3.1</td>
<td>4.6</td>
<td>6.8</td>
<td>10.7</td>
<td>1.5</td>
<td>0.06</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

(1) From time feeding: D, diet effect; Y, yeast supplementation effect, SEM : standart error mean
AFB1 (aflatoxin B1), AFM1 (aflatoxin M1); NA: non acidogenic; Ac: acidogenic

AFM1 was detected in the rumen at the time of samplings 3 and 6 h after contamination. There was an interaction effect of diet type and yeast supplementation on the concentration of AFM1 (P=0.02), especially 6 h after contamination. The highest concentration was found in cows fed NA diet supplemented with yeast i.e., 0.12 ng/g, and the lowest was in the cows fed with Ac diet i.e., 0.04 ng/g either supplemented or non-supplemented with yeast. This result indicated that NA diet supplemented with yeast could increase AFM1, however, Ac diet supplemented with yeast decreased AFM1 concentration. The increase in AFM1 concentration in NA diet supplemented with yeast probably caused by the binding of cell wall of yeast with the AFM1 at the glucomannan or β-1,3-glucan groups so that AFM1 concentration increased (Corassin et al., 2013), while the decrease in AFM1 concentration in Ac diet was assumed to be due to the increased rate of diet passage so that AFM1 disappeared rapidly from rumen since acidosis condition will increase diet passage in the rumen so that the decrease in AFM1 concentration was due to the rapid elimination through feces (Gressley et al., 2011).
AFM1 detected in the rumen was assumed to be the results of back diffusion of AFM1 from enterocytes cell and then bound to yeast cell wall. According to Gratz et al., (2007), AFM1 was synthesized and formed in enterocytes cells and diffuse back to the lumen in the rats that was then bound to sequestering compound such as yeast cell and lactic acid bacteria (LAB). Further study is needed to study the origin of AFM1 in the rumen.

Diet type and yeast supplementation did not significantly affect the urine excretion of AFM1 24-hours after mycotoxin contamination. This non-significant result is assumed to be due to a high individual variation. Battacone et al., (2009) reported that higher variation of AFM1 excretion pattern was positively correlated with the variation of rumen activities that affect the gradual release of toxins from tissue to circulation that eventually affect the rate of bioconversion of AFB1 to AFM1 in the liver and the process of excretion through feces and urine.

Further, the average of AFM1 excretion in the cows fed with Ac diet was higher (13.45 µg) (P<0.01) as compared to those fed with NA diet (5.43 µg) 48 and 72 hours after mycotoxins contamination. Similar to that result, average excretion of AFB1 through feces in cows fed with Ac diet was lower (18.1 µg) than in those fed with NA diet (23.4 µg) (P<0.03) 48 hours after mycotoxin contamination. This trend indicated that there was a correlation between the increased AFM1 excretions through urine with the decrease in AFB1 excretion through feces. The number of AFB1 absorbed is then excreted as AFM1 through urine, while AFB1 that has not been converted to AFM1 is secreted through feces through biliary excretion. AFM1 excreted through urine at the time of 48 and 72 hours after mycotoxins contamination was assumed to be from enterohepatic circulation. Previous study by Battacone et al., (2009) reported that excretion patterns of AFM1 through milk are occurred through enterohepatic circulation after 24-hours of mycotoxins contamination in dairy goat that contaminated with a single dosage of AFB1. The same pattern was occurred in the excretion of AFM1, i.e., the excretion increased after 24-hours (the second day) through urine.

The excretion of AFM1 through feces tended to be higher (P=0.08) in the cows fed with Ac diet (49.34 µg) as compared to those fed with NA diet (36.15 µg) 24-hours after contamination. This result indicates the correlation between the decreased AFM1 concentrations in the rumen with the increased AFM1 excretion through feces in the cows fed with Ac diet. In the cows fed with Ac diet, AFM1 was assumed to be excreted rapidly with the diet particles. The elimination of AFB1 and AFM1 through feces is come from the biodegradation of unabsorbed AFB1 while biliary excretion comes from the absorbed AFB1 metabolite (Mykkänen et al., 2005).

In the present experiment, yeast supplementation did not significantly effective in decreasing the absorption in the cows fed with Ac diet that was thought to be caused by, 1) the ability of the yeast to increase pH in the limited acidosis due to the low doses, or 2) the ability to adsorb AFB1 is limited and is not optimum. The absorption of AFB1 in the rumen of dairy cows is occurred very rapidly and reached the peak concentration in the plasma 20 minutes after the experimental cows consumed diet contaminated with AFB1 (Gallo et al., 2008). Therefore, the difference in pH gradient caused the difference in absorption. In
the lower pH, the absorption is increase. The limited ability of yeast cell to bind was assumed to affect the absorption of AFB1 in the cows fed with the Ac diet.

Ochratoxin A (OTA)

Acidogenic diet significantly affect the concentration of the OTA in the rumen (Table 3.8). The average concentration of OTA based on time after contamination showed that the concentration was higher 3 h after contamination (4.1 ng/g) as compared to 6 h after contamination (1.5 ng/g), even though statistically was not significant. The individual variations of the OTA concentrations were high as indicated by the high SEM. The trend of this decreasing concentration indicated that the occurrence of OTA absorption or hydrolysis of OTA to OTα. Previous research by Blank and Wolffram (2009) reported that OTA concentration after contamination showed a trend of decreasing concentration and the very rapid absorption from the rumen until 12 hours after contamination, that was followed by the increase in OTα production. This result indicated that the longer the incubation, the hydrolysis of OTA to OTα was increase in addition to the increase in OTA absorption.

Table 3.8. Daily excretion of OTA and OTα (µg) in feces and urine and concentration on rumen juice (RJ) (ng/g) in dairy cows fed contaminated diet alone or with yeast

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>Time (h)(1)</th>
<th>NA</th>
<th>Yeast Addition</th>
<th>D</th>
<th>Y</th>
<th>D x Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTA</td>
<td>Without Yeast</td>
<td>Yeast</td>
<td>Without Yeast</td>
<td>Yeast</td>
<td>SEM</td>
<td>D</td>
</tr>
<tr>
<td>RJ Concentration (ng/g)</td>
<td>3</td>
<td>1.04</td>
<td>2.31</td>
<td>7.74</td>
<td>5.44</td>
<td>1.27</td>
</tr>
<tr>
<td>6</td>
<td>0.31b</td>
<td>1.289b</td>
<td>2.56a</td>
<td>1.25a</td>
<td>0.40</td>
<td>NS</td>
</tr>
<tr>
<td>OTα</td>
<td>3</td>
<td>0.19</td>
<td>0.14</td>
<td>0.16</td>
<td>0.3</td>
<td>0.04</td>
</tr>
<tr>
<td>6</td>
<td>0.18</td>
<td>0.25</td>
<td>0.27</td>
<td>0.3</td>
<td>0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Feces Excretion (µg)</td>
<td>24</td>
<td>11.0</td>
<td>11.0</td>
<td>3.1</td>
<td>4.3</td>
<td>1.3</td>
</tr>
<tr>
<td>48</td>
<td>5.8</td>
<td>9.8</td>
<td>5.4</td>
<td>6.3</td>
<td>1.1</td>
<td>NS</td>
</tr>
<tr>
<td>Urine Excretion (µg)</td>
<td>24</td>
<td>0.31</td>
<td>0.19</td>
<td>1.30</td>
<td>1.09</td>
<td>0.2</td>
</tr>
<tr>
<td>48</td>
<td>0.00</td>
<td>0.01</td>
<td>0.14</td>
<td>0.09</td>
<td>0.03</td>
<td>0.1</td>
</tr>
<tr>
<td>72</td>
<td>212.9</td>
<td>213.4</td>
<td>199.0</td>
<td>257.2</td>
<td>18.5</td>
<td>NS</td>
</tr>
</tbody>
</table>

(1). From time feeding; SEM: standart error mean
D, diet effect; Y, yeast supplementation effect; NA: non acidogenic; Ac: acidogenic
OTA (ochratoxin A), OTα (ochratoxin α)

The average concentration of OTA based on the diet type indicated or showed that in the cows fed with Ac diet, the OTA concentration (5.69 ng/g) was higher (P<0.05) as compared to those fed with NA diet (1.68 ng/g), especially 3 hours after contamination. This result showed that the degradation of OTA in the cows fed with Ac diet was decrease. The bioavailability of the OTA in the rumen was increase in the cows fed with Ac diet. This result was assumed to correlate with the decrease in the degradation of the OTA. Systemic bioavailability of OTA in the rumen increased in the cows fed with Ac diet associated with the decrease...
in population of protozoa *Entodinium* *>100* mm and protozoa holotrics (Tabel 3.6). Previous study by Xiao *et al.*, (1991) reported that there was an increase in the systemic bioavailability of OTA in the experimental animals when fed with ration with high concentrate content. This result indicated that the protozoa play a role in hydrolysis of OTA.

From the statistical analyses showed that there was an interaction effect of diet type and yeast supplementation on the OTA concentration in the rumen 6 hours after contamination. The highest OTA concentration was found in the cows fed with Ac diet without yeast supplementation (2.56 ng/g), while the lowest concentration was found in the cows fed with NA diet without yeast supplementation (0.30 ng/g). This result showed that Ac diet supplemented with yeast effectively increased the hydrolysis of OTA as compared to NA diet. The opposite result was found in Ac diet without yeast supplementation that decreased OTA degradation. The decrease in pH in the cows fed with Ac diet was assumed to affect the protozoa capacity to degrade OTA. The degradation of OTA is dominantly conducted by the protozoa (Kiessling *et al.*, 1984). However, the other study reported that the degradation of OTA was dominantly conducted by the bacteria as compared to protozoa (Mobashar *et al.*, 2012). These different results could be caused by the composition of the ration used.

The increase in systemic bioavailability of OTA was assumed to be caused by several possibilities i.e., 1) the effect of decreasing population of protozoa of the holotrich type, 2) modification of bacterial population that affect the degradation of OTA in the cows fed with Ac diet in the long term (3 days), even though population and the type of this bacteria was not analyzed in this experiment. The difference in bioconversion tended to be affected by the yeast supplementation. It was assumed that yeast could supply the nutrients for the growth and development of microbes degrading OTA and this effect was observed in the rumen at the time of sampling 6 hours after contamination.

The Ac diet significantly affect the average of OTA excretion through urine. The OTA excretion in the cows fed with Ac diet (1.9 µg) was higher (P<0.01) as compared to those fed with NA diet (0.35 µg) at the time of 24, 48 and 72 hours after contamination. This result indicated the increase in absorption OTA in the cows fed with Ac diet. OTA is a weak acid and has pKa 7.5 (Xiao *et al.*, 1991), and its absorption will increase at the low pH in the rumen. Yeast supplementation did not significantly decrease the absorption of OTA. This non-significant effect was assumed to be due to the limited capacity of the yeast in increasing the pH in the cows fed with Ac diet. The difference in the pH of the rumen of the cows fed with Ac and NA diet was around 0.4 unit so that it will significantly increase the percentage of non-ionized OTA molecules that finally increased the OTA absorption in the cows fed with Ac diet.

OTA was still detected in urine and feces. This indicated that OTA was not completely hydrolyzed in the rumen. The same result was reported that OTA was found in the milk and urine (Boudra *et al.*, 2013). In this present experiment, OTA was hydrolyzed about 78% and this result was similar to that reported by Blank *et al.*, (2003) that in sheep rumen OTA could be hydrolyzed up to 78%. The other study reported the higher hydrolysis of OTA up to 90% (Miller, 2008) while Blank and Wolffram (2009) found 88% hydrolysis in sheep. The different in degradation ability was probably due to the difference in diet composition and...
animals used. It was found that diet composition affect the rumen microbes that play an important role in the hydrolysis of OTA.

The effect of yeast on the OT\(\alpha\) concentration in the urine was detected at the time of daily collection even though statistically it was not significant. The measurement of OT\(\alpha\) excretion in urine for 72 hours showed that yeast supplementation tended to increase OT\(\alpha\) excretion in the urine (\(P=0.07\)) as indicated by the higher OT\(\alpha\) urine concentration (2512 \(\mu\)g) in the cows supplemented with yeast as compared to cows without yeast supplementation (2182 \(\mu\)g). This result indicated that yeast could increase the conversion of OTA to OT\(\alpha\).

Yeast had a significant role in production of OT\(\alpha\) by degrading OTA to OT\(\alpha\). This result was similar to results reported by Péteri et al., (2007) and Oeztuerk (2009). This result indicated that yeast had a positive effect on decreasing the toxicity of OTA. Yeast could stimulate the growth of bacteria producing carboxypeptidase A enzyme (EC.3.4.17.1) that converts OTA to OT\(\alpha\) by degrading the amida bond in OTA. This result was assumed to be related to the increase in isoacid percentage and concentration in the rumen due to the activities of proteolytic enzymes (Table 3.4). However, this results is different from the previous result Blank and Wolfram (2009) reporting that yeast supplementation had no effect increase hydrolysis of OTA to OT\(\alpha\) in the diet contain 70% concentrate. This difference is probably due to the different diet composition since the higher the quality of ration the lower the role of yeast in detoxification of OTA.

**Deoxynivalenol (DON)**

The averages of DON concentrations in the rumen are presented in Table 3.9. Based on the time after contamination, the concentration of DON 3 hours after contamination (3.38 ng/g) was higher than that at 6 hours after contamination (1.62 ng/g). The trend of decreasing concentration of DON in the rumen could be caused by three possibilities i.e., the absorption, the conversion of DON to DOM1 and the outflow of the DON from the rumen along with the diet particles. From this result, it was assumed that the decrease in DON was supposed to be due to the conversion of DON to de-epoxy deoxynivalenol (DOM1). The rumen microbes have the abilities to convert and degrade DON to DOM1.

The same result was observed in different diet types. The concentration of DON tended to be higher (\(P<0.1\)) (4.81 ng/g) in cows fed with Ac diet as compared to those fed with NA diet (1.91 ng/g), especially 3 h after contamination. The bioavailability of DON in the rumen was increase in cows fed with Ac diet and it was assumed to be correlated with the decrease in degradation of DON. The rumen microbes are able to degrade DON to de-epoxy DON (DOM1) by the de-epoxydation reaction (Côté et al., 1986, Karlovsky 2011). In the present experiment, the decrease in population of protozoa in the cows fed with Ac diet was assumed to be correlated with the decrease in degradation of DON, even though was only detected mainly at 3 hours after contamination. This result was different from that reported by Kiessling et al., (1984) that DON incubated in the rumen fluid in the anaerobic condition did not change and was not degraded.
In addition, the other study stated that *Eubacterium BBSH 797* bacteria obtained from bovine have the ability to degrade DON (Fuchs et al., 2002, Schatzmayr et al., 2006). Based on this fact, it was clear that diet type could affect protozoa population in the rumen that finally affect DON concentration, even though in the present experiment the effect was observed in very short period for 3 h after contamination.

Table 3.9  Daily excretion of DON and DOM 1 in feces (µg) and concentration on rumen juice (RJ) (ng/g) in dairy cows fed contaminated diet alone or with yeast

<table>
<thead>
<tr>
<th>Mycotoxin</th>
<th>Time (h)</th>
<th>NA Without Yeast</th>
<th>NA Yeast Addition</th>
<th>Ac Without Yeast</th>
<th>Ac Yeast Addition</th>
<th>SEM</th>
<th>D</th>
<th>Y</th>
<th>D x Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>DON</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RJ</td>
<td>3</td>
<td>1.32</td>
<td>2.5</td>
<td>6.71</td>
<td>2.98</td>
<td>0.88</td>
<td>0.08</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.17</td>
<td>5.3</td>
<td>1.02</td>
<td>0.00</td>
<td>1.21</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>DOM1</td>
<td>3</td>
<td>0.54</td>
<td>0.43</td>
<td>0.57</td>
<td>1.03</td>
<td>0.12</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td></td>
<td>6</td>
<td>0.79</td>
<td>0.72</td>
<td>0.84</td>
<td>1.06</td>
<td>0.13</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>DON</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feces</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>470.7</td>
<td>451.3</td>
<td>529.5</td>
<td>530.6</td>
<td>92.2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>108.6</td>
<td>96.9</td>
<td>246.8</td>
<td>425.1</td>
<td>51.7</td>
<td>0.01</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>DOM1</td>
<td>24</td>
<td>304.6</td>
<td>289.3</td>
<td>384.8</td>
<td>436.0</td>
<td>48.9</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>181.2</td>
<td>85.7</td>
<td>347.5</td>
<td>380.4</td>
<td>57.4</td>
<td>0.04</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

(1) From time feeding; SEM: standart error mean
D, diet effect; Y, yeast supplementation effect; NA: non acidogenic; Ac: acidogenic
DON (deoxynivalenol), DOM 1 (de-epoxy nivalenol)

Based on time after contamination, the concentration of DOM1 3 hours after contamination was lower (0.64 ng/g) as compared to 6 hours after contamination (0.85 ng/g). The trend of increase in DOM1 in this present experiment indicated the increase in DON hydrolysis with the increase the duration of incubation in the rumen. DOM1 in this experiment was detected in the rumen 3 hours after introduction of mycotoxin. Swanson et al., (1987) reported that the formation of DOM1 was detected in the rumen of dairy cows in *in vitro* experiment after incubation time of more than 12 hours. Acidogenic diet significantly affect the excretion of DON and DOM1, especially 48 hours after contamination. The excretion of DON in feces was higher (530 µg) (P<0.01) in the cows fed with Ac diet as compared to those fed with NA diet (461 µg). The trend of increasing DON excretion in the cows fed with Ac diet was caused by the difference in diet type that affect the rate of diet passage in the gastrointestinal tract (Dehory and Orpin 1997) so that DON was excreted rapidly at the acidosis condition. The same condition was observed in DOM1 concentration that was higher (246 µg) (P<0.04) as compared to cows fed with NA diet (133 µg.). DON and DOM1 were excreted faster when the rumen condition was acidogenic that was assumed to be correlated with the low fiber content of the ration. The lower fiber content of the ration would increase the passage of diet in the rumen so that increase the rate of elimination through the feces.
In the present experiment, DON was not detected in the form of DOM1 in the urine during 72 hours collection after contamination. DON and DOM1 were only detected in feces 24-hours after contamination both in the cows fed with Ac and NA diets. This result indicated that most of DON and DOM1 were excreted along with the diet particles. According to Dänicke and Brezina (2013), there are two models of compartments in the excretion systems of DON and DOM1 (Figure 3.6) i.e., 1) they were absorbed in the form of DON and DOM1 to form conjugate and they were excreted into urine and feces, 2) they were excreted along with the diet particles to feces.

Figure 3.6. Metabolism DON in cattle (Pestka 2007)

The absorbed DON and DOM1 were then could be conjugated to form glucuronic-DON that are excreted in urine and feces through enterohepatic circulation. In the other pathway, the unabsorbed DON and DOM1 were then excreted through feces or in the unconjugated form. According to Côté et al., (1986) the contamination of DON conducted for 5 days, the DOM1 was detected in a significant concentration (>30 ppb) in urine, feces and milk only after more than 72 hours after contamination of cows fed with 30% concentrate and hay. Further, it was stated that the recovery of DON after 20% contamination, the most proportion was in the unconjugated DOM 1 (96%) and DON (4%). The composition of DON and DOM1 excretions are affected by the type diet used.

**Fumonisins B1 (FB1)**

The average of FB1 concentration 3 hours after contamination was higher (87.69 ng/g) as compared to 6 hours after contamination (72.8 ng/g). The concentrations of FB1 were constant and only slightly decreased from 3 to 6 hours after contamination. This result showed that the absorption of FB1 in the rumen is very low so that most of it was excreted along with the diet particles through the feces. FB1 is highly polar and very easily dissolved in water (very water soluble) and easily ionized. Some previous studies reported by (Prelusky et al., 1995, Riley and Voss (2006)) the same results that FB1 was poorly absorbed in the
gastrointestinal tract. However, Voss et al., (2007) added that the absorption in the rumen was varied by 1-2%. The FBI was minimally absorbed in the rumen and it was not detected in the blood plasma of the cattle. FB1 has the hydrophilic and water soluble properties at the pKₐ 3.5, when the FB1 will be completely ionized in the rumen (Pantaya. et al., 2014).

Table 3.10 Daily excretion of FB1 (µg) in feces and concentration on rumen juice (RJ) (ng/g) in dairy cows fed contaminated diet alone or with yeast

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>Time (h)(1)</th>
<th>NA Without Yeast Concentration (ng/g)</th>
<th>Ac Yeast Addition Concentration (ng/g)</th>
<th>SEM</th>
<th>D</th>
<th>Y</th>
<th>D x Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB1 RJ</td>
<td></td>
<td>3</td>
<td>75.0</td>
<td>59.1</td>
<td>128.5</td>
<td>88.2</td>
<td>12.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>55.1</td>
<td>125.4</td>
<td>55.1</td>
<td>55.9</td>
<td>37.60</td>
</tr>
<tr>
<td>Feces</td>
<td></td>
<td>24</td>
<td>6,050</td>
<td>6,420</td>
<td>4,894</td>
<td>4,145</td>
<td>699</td>
</tr>
<tr>
<td>FB1</td>
<td></td>
<td>48</td>
<td>1,241</td>
<td>1,542</td>
<td>2,617</td>
<td>3323</td>
<td>285</td>
</tr>
</tbody>
</table>

(1), From time feeding; SEM: standart error mean
D, diet effect; Y, yeast supplementation effect; NA: non acidogenic; Ac: acidogenic

FB1 (fumonisin B1)

In the cow fed with Ac diet, the concentration of FB1 tended to be higher (108.4 ng/g) (P<0.1) as compared to those fed with NA diet (67.04 ng/g), especially 3 hours after contamination. This result indicated that the concentrations of FB1 were fluctuated and Ac diet affect the FB1 concentrations 3 hours after contamination in the rumen. The increase in the concentration of FB1 in the cows fed with Ac diet was indicated that there was the binding of FB1 to BAL bacteria that finally slowed and inhibited the excretion.

In the cows fed with Ac diet, the excretion of FB1 through feces (4519 µg) were tended to be lower (P<0.1) 24-hours after contamination as compared to those fed with NA diet (6235 µg), while the excretion of FB1 was increase in the cows fed with Ac diet (2970 µg) (P<0.05) as compared to those fed with NA diet (1391 µg) 48 jam (2 days) after contamination. This result showed that cows fed with Ac diet had slower FB1 excretion and had correlation with the increase in FB1 concentration in the rumen. In the cows fed with Ac fed, the excretion of FB1 tended to be lower as compared to that in the cows fed with NA diet 24-hours after contamination. These results were assumed that there was a modification of microbial population during the SARA condition in the rumen and gastrointestinal tract that stimulated the growth and development of LAB bacteria such as S.bovis and Lactobacilli. The lower FB1 excretion has direct correlation with the population of Lactic acid bacteria (LAB) that was increase and bind to FB1, even though the effect of this inhibition or deceleration only effective 24-hours. The same results were reported by Niderkorn et al., (2009) that LAB could bind to FB1 at the site of peptidoglycant groups. The concept of FB1 binding by using LAB bacteria and propionic bacteria was developed in the previous research by Niderkorn et al., (2006). The slow elimination rate of FB1 in the cows fed with Ac diet confirmed the previous research that there is a binding of FB1 by bacteria in the acidic condition. The variation of FB1 binding that cause the inhibition and
slowed down the excretion of FB1 was assumed to be due to the difference in composition of cell wall among bacteria.

**Balance Excretion**

**Aflatoxin B1 (AFB1)**

The average of total excretion of AFB1 is presented in Table 3.11. Based on the statistical analysis, there was an interaction effect of diet type and yeast supplementation (P=0.07) on the total excretion of AFB1, with the highest total excretion was found in cows fed with Ac diet supplemented with yeast (183.1 µg) and the lowest was found in the cows fed with NA diet supplemented with yeast (120 µg). This result indicated that in Ac diet, yeast supplementation could increase AFB1 excretion while in NA diet, yeast supplementation decrease AFB1 excretion. The dosage of AFB1 contamination was 1.200 µg, while the amount of AFB1 excreted were around 120 - 183.1 µg or around 10-15%, so that the amount of retained in the body was almost 85-90%, that is possibly retained in liver, kidney, rumen, and blood plasma. In the previous study, the total AFB1 that was recovered in the urine and feces after single dose of contamination was around 9-10% in cattle (Allcroft et al., 1968). AFB1 and its metabolite were found in the liver and kidney tissues after the sheep consumed diet contaminated with AFB1 (Fernández et al., 1997) and most part of it was found in the rumen (Trucksess et al., 1983).

The highest excretion in the cows fed with Ac diet supplemented with yeast could probably due to the increase in the binding of the AFB1 by the yeast as was associated with the increase in fecal excretion in the cows fed with Ac diet. Yeast has the ability to bind AFB1 in the cell wall of the yeast at the glucomannans or β-1,3-glucans residues that reduce and inhibited the absorption of AFB1 and increased the excretion (Huwig et al., 2001). Therefore, yeast could accelerate the elimination of AFB1 from the body of the animal during acidosis condition even though in this case, yeast had not been able to reduce the absorption of AFB1.

**Ochratoxin A (OTA)**

Yeast supplementation significantly affects OTA excretion. Total OTA excretion tended to be higher (P=0.07) (3971 µg) in cows supplemented with yeast as compared to non-supplemented cows (3448 µg). This result showed that yeast supplementation contributed to the increase in degradation of OTA to OTα. The dosage of OTA at the time of contamination was 4.740 µg so that total recovery of the OTA excretion was 79-95%. This result is almost similar to previous study (83.9%) in sheep (Blank and Wolffram 2009). Yeast plays a role in increasing total OTA excretion due to its effect on stimulating the capacity and growth of microbes degrading OTA. In a single dosage experiment Blank and Wolffram (2009) by oral administration of 2 mg/kg OTA, the recovery of OTA was 90.1%, with the conversion to OTα by 90%. Therefore, yeast supplementation could increase OTA excretion both in cows fed with NA and Ac diets.
Deoxynivalenol (DON)

In the cows fed with Ac diet, the excretion of DON was higher (1684 µg) (P<0.03) as compared to those fed with NA diet (1018 µg). The Ac diet could accelerate DON elimination. Based on the intake and their concentrations in feces and urine, the higher rate of passage in the Ac diet was assumed to accelerate the DON excretion. The dosage of DON at the time of contamination was 6.000 µg so that the excretion recovery was 22.45% of the initial concentration at the time of contamination. This result indicated that most of DON contaminated was retained in the body. Dänicke and Brezina (2013) stated that almost 90% of consumed DON was disappeared when they pass through gastrointestinal tract. DON is rapidly detected in the plasma 5 minutes to 24-hours after contamination and DON was detected in blood plasma, bile, liver, and kidney even though its transmission rate was low (Sobrova et al., 2010). However, Keese et al., (2008) reported that the DON content of milk is very low. Therefore, the cause of low recovery was probably that DON is retained in the body.

Fumonisins B1 (FB1)

There was no a significant effect of diet type and yeast supplementation on total excretion of FB1. The diet type also did not affect the total excretion of FB1. The dosage of FB1 that was contaminated was 33180 µg, so that the total excretion recovery was 22.58%. According to Prelusky et al., (1995) cumulative excretion recovery in urine and feces on the second day was 20%. The low recovery was assumed to be due to the retention of FB1 in the body and organs. Even though the absorption of FB1 is very low, it is very rapidly distributed in the body and it was detected in liver and kidney (Voss et al., 2007). The other study by Caloni et al., (2000) reported that it was assumed that FB1 was deaminated in the rumen so that it was not detected in the rumen, while according to Smith and Thakur (1996) there was a 12.5% decrease in FB1 concentration 9 hours after incubation in on the mode the buffer system. Therefore, in general, the SARA condition did not affect the total excretion of FB1 so that it does not affect the balance excretion of FBI even though it is needed a further study of excretion of FB1.
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   b. Pengutipan tidak merugikan kepentingan yang wajar IPB.
2. Dilarang mengumumkan dan memperbanyak sebagian atau seluruh karya tulis ini dalam bentuk apapun tanpa izin dari Institusi.
Table 3.11 Cumulative excretion of AFB1, OTA, DON and FB1 (µg) in non lactating dairy cow fed non acidogenic and acidogenic diet

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>Contaminated diet (µg)</th>
<th>Matrixs</th>
<th>NA</th>
<th>Yeast</th>
<th>Yeast Addition</th>
<th>SEM</th>
<th>P-values</th>
<th>Excretion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFBM1</td>
<td></td>
<td>Feces</td>
<td>86.8</td>
<td>68.8</td>
<td>93.7</td>
<td>8.9</td>
<td>0.12</td>
<td>NS NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>36.4</td>
<td>19.2</td>
<td>41.9</td>
<td>7.5</td>
<td>0.08</td>
<td>NS NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>1200</td>
<td>154.5</td>
<td>120.0</td>
<td>183.1</td>
<td>8.6</td>
<td>0.06 NS 0.07 10-15</td>
</tr>
<tr>
<td>OTA</td>
<td></td>
<td>Feces</td>
<td>16.8</td>
<td>20.7</td>
<td>8.5</td>
<td>10.7</td>
<td>2.1</td>
<td>0.03 NS NS</td>
</tr>
<tr>
<td>OTA</td>
<td></td>
<td>Urine</td>
<td>0.3</td>
<td>0.4</td>
<td>2.1</td>
<td>1.7</td>
<td>0.3</td>
<td>0.01 NS NS</td>
</tr>
<tr>
<td>OTA</td>
<td></td>
<td>Total</td>
<td>4740</td>
<td>3484.7</td>
<td>3852.7*</td>
<td>3397.9</td>
<td>4071.6*</td>
<td>169.0 NS 0.07 NS 79-95</td>
</tr>
<tr>
<td>DON</td>
<td></td>
<td>Feces</td>
<td>485.8</td>
<td>375.0</td>
<td>732.3</td>
<td>816.4</td>
<td>101.1</td>
<td>0.07 NS NS</td>
</tr>
<tr>
<td>DON</td>
<td></td>
<td>Feces</td>
<td>579.3</td>
<td>548.2</td>
<td>776.3</td>
<td>955.7</td>
<td>130.8</td>
<td>0.02 NS NS</td>
</tr>
<tr>
<td>DON</td>
<td></td>
<td>Total</td>
<td>6000</td>
<td>1089.3</td>
<td>942.0</td>
<td>1545.2</td>
<td>1812.8</td>
<td>219.0 NS NS 22.45</td>
</tr>
<tr>
<td>FB1</td>
<td></td>
<td>Feces</td>
<td>7292.3</td>
<td>7693.2</td>
<td>7512.5</td>
<td>7468.4</td>
<td>812.0</td>
<td>NS NS NS 22.58</td>
</tr>
</tbody>
</table>

1Expressed in term AFB1 equivalent according to MW AFB1/MW AFM1(312.2/323.3)*µg AFM1
2Expressed in term OTA equivalent according to MW OTA1/MW OT-α (403.8/256.6)*µg OT-α
3Expressed in term DON equivalent according to MW DON/MW DOM1(296.3/280.3)*µg DOM1

P<0.1 between treatment; NA: non acidogenic; Ac: acidogenic; SEM: standart error mean.
CONCLUSION

The acidogenic diet could reduce pH of the rumen and the duration of rumen pH below 5.6 similar to the criteria of SARA condition. Sub-acute ruminal acidosis (SARA) could affect the profile of mycotoxin toxicokinetics of AFB1, OTA, DON and FB1 in ruminants. The total AFM1 and OTA excreted through the urine increased in the condition of SARA. The use and supplementation of yeast could increase the rumen pH, and it has positive effects on the rumen fermentation and shortened the duration of SARA condition, and affect the ability and capacity of rumen microbe to detoxify mycotoxin OTA.

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Morgavi DP, Riley RT. 2007. An historical overview of field disease outbreaks known or suspected to be caused by consumption of feeds contaminated with Fusarium toxins. *Anim Feed Sci Tech* 137(3–4):201-212.


4. GENERAL DISCUSSION

Raw materials for concentrate are commonly consist of variety of feed ingredients that potentially exacerbated mycotoxin exposure. Using a variety of feed ingredient increases the probability of multiple-mycotoxin contamination. Mycotoxins contaminated feed may exert antinutritional effect reduce in animal performance. Some authors reported that cattle’s exposure to mycotoxins affects cattle’s health and productivity and leads to economical loss. (CAST. 2003, Morgavi et al., 2003).

Mycotoxins has various molecular structures causing differences on physical and chemical characteristics (Table 4.1), such as polarity and solubility, which can affect the absorption mechanism in the rumen when ingested. In addition, ruminal microorganism play an important role in modulating nutrient absorption and mycotoxins degradation. The absorption process in the digestive tract is affected by pH and pKa like in the other xenobiotic molecules (David and William 2007). Low pH condition in the rumen (ruminal acidosis) can modify the mycotoxin absorption and increase the risk mycotoxin exposure.

Dairy cows with high genetic quality need energy and protein to increase milk production. High energy consumption from concentrate can increase propionic acid which later be converted into lactose influencing milk production. However, excessive concentrate consumption can decrease ruminal pH, reduce ruminal fiber digestibility and increase ruminal acidosis. The sub-acute ruminal acidosis (SARA) is very prevalent in commercial dairy cow farm which disturb production performance and animals health.

Ruminal acidosis will affect the microorganism population in the rumen, such as instability in the rumen microbial population, feed fermentation disorder and decrease microbes capability to detoxify mycotoxins. Low pH in the rumen can decrease protozoa population responsible for OTA mycotoxin degradation (Kiessling et al., 1984).

Based on this information, the first study (Chapter 2) was aimed to study the effect of rumen pH on mycotoxin absorption. The study was conducted using empty washed rumen (EWR) technique with sheep as model. EWR is a technique to isolate the rumen by closing the reticulo omasum duct at the orifice by using balloon chateter, then collecting saliva oesophagus use balloon chateter to prevent the saliva entering the rumen, hence, isolating the rumen.

The EWR method is developed by Doreau et al., (1997) and Dijkstra et al., (1993) in studying VFA (Volatile Fatty Acid) absorption in the rumen. The advantages of this technique can be used to measure the absorption rate in the rumen in real condition, this technique is easy to adopt in incubation within experimental solution, and by using this technique, the sampling can be conducted at point time with certain time interval.

In the recent study, we found that the rate of disappearance of AFB1 and OTA enhances in low pH. Mechanism of absorption is correlated to ionization and non-ionization state in the solution. The ionized form is often unable penetrate the membranes due to it low lipid-solubility whereas the non ionized form is lipid-soluble to diffuse across the cell membranes. In recent studies, mycotoxin absorption rate AFB1 and OTA was increases in low rumen pH due
to degree of non-ionized in low pH was higher than neutral pH. Based to Henderson–Hasselbalch equation as shown in Figure 4.1

Table 4.1 Structure of molecule mycotoxins (Yiannikouris and Jouany 2002)

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>Structure of molecule</th>
<th>Scientific name</th>
<th>Chemical and physical properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B1</td>
<td><img src="image" alt="Aflatoxin B1 structure" /></td>
<td>N-[[((3R)-5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl]carbonyl]-L-phenylalanine</td>
<td>C₂₀H₁₈O₆NCl, MW: 312.3, CAS 303-47-9</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td><img src="image" alt="Ochratoxin A structure" /></td>
<td>2,3,6a,9a-tetrahydro-4-methoxy cyclopenta[c]furo[3',2':4,5]furo[2,3-h][1]benzopyran-1,11-dione</td>
<td>C₁₇H₁₂O₆, MW: 408.8, CAS 1162-65-8</td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td><img src="image" alt="Deoxynivalenol structure" /></td>
<td>12,13-epoxy-3α,7α,15-trihydroxy trichothec-9-en-8-one</td>
<td>C₁₅H₂₀O₆, MW: 296.3, CAS 51481-10-8</td>
</tr>
<tr>
<td>Fumonisin B1</td>
<td><img src="image" alt="Fumonisin B1 structure" /></td>
<td>1,2,3-propanetricarboxylic acid, 1,1'-[1-(12-amino-4,9,11-trihydroxy-2-methyltridecyl)-2-(1-methylpentyl)-1,2-ethanediyl] ester</td>
<td>C₃₄H₅₉NO₁₅, MW: 721.8, CAS 116355-83-0</td>
</tr>
</tbody>
</table>
As the pH decreased more the acid molecules become non ionized. The converse is true is pH increase. If pH-pKa =0 the molecules 50 %b is ionized and 50% is non ionized, if pH-pKa>0.5 the molecules 75 % is ionized and 25% is non ionized, whereas as if pH-pKa > 1 the molecules 99 % ionized or 99 % is in non-ionized. The degree of non ionized is showed in Table 4.2.

The simulation of degree ionized and non-ionized mycotoxins in the rumen is calculated as follows:

- **OTA**
  In low pH pH-pKa = 5.6 – 7.5 = - 1.9, whereas in neutral pH pH-pKa = 6.7- 7.5 = - 0.8. OTA in low pH was higher percentage non-ionized molecules compared to percentage non-ionized in neutral pH. Therefore, OTA was faster absorbed in low pH compared to in neutral pH.

- **FB1**
  In low pH pH-pKa, 5.6 – 3.5= 2.1, whereas in neutral pH pH-pKa, 6.7- 3.5 = - 3.2 Therefore FB1 in low pH and neutral molecules 99.5% is ionized or non absorbed in the rumen.

Figure 4.1  Variation of the % formation AH (non-ionized), and A⁻(ionized) with the difference between the pH and pKa of the acid based to Handerson-Hasselbalch equation

For a weak organic acid, a decrease of one pH unit results in tenfold increase in the concentration of the non-ionized form. Conversely, an increase of one pH unit results in a tenfold increase in the concentration of ionized form (Kevin et al., 2008).

<table>
<thead>
<tr>
<th>Micotoxins</th>
<th>Rumen pH</th>
<th>pKa</th>
<th>Acid (5.6)</th>
<th>Neutral (6.7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTA</td>
<td></td>
<td>7.5</td>
<td>98.2</td>
<td>86.3</td>
</tr>
<tr>
<td>FB1</td>
<td></td>
<td>3.5</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>AFB1</td>
<td>n.a</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

n.a = non available, pKa= acid dissociation constant
In the range pH this experimental was confirmed mycotoxin is based on its solubility in fat, hence the order was AFB1>OTA>FB1, where AFB1 with very lipophilic characteristic had higher rate of disappearance compared with OTA, while FB1 with hydrophilic could not be absorbed in the rumen. The low pH solution increased significantly AFB1 disappearance. Aflatoxins are lipophilic compounds and, although their pKa has not been reported, the increased disappearance observed at low pH suggests a higher proportion of non-ionized molecules. Therefore the degree of ionization in of weak acid or base is dependent of pH medium in the rumen.

For experimental validation, this study used VFA as absorption marker. VFA consists of acetate, propionate, butyrate which is a product organic matter fermentation process in the rumen. This study showed that fractional absorption rates of the VFA with an initial pH of pH 5.6 were, in order: butyrate >propionate >acetate with correspond ionized percentage: 84.75%, 84.21%, and 86.25%, respectively. While in neutral pH of 6.7, the ionized percentage: 98.7%, 98.5% and 98.9%, respectively, according to Handerson-Hasselbalch. This study result reported that the fractional of disappearance rate in low pH higher than neutral pH, similar with previously study by Aschenbach et al., (2011).

Based on the solubility of VFA in fat (lipophilicity and highly permeable), the butyrate acid was 14 times more lipophilic than acetic acid. VFA absorption process in the rumen is by passive diffusion and only permeable non-ionized molecule can pass through bilayers membranes like ruminal epithelium. This concept is developed in previous study done by Gabel et al., (2002). Polarity and solubility characteristic of molecule could effected absorption rate in the digestive tract such as rumen. Therefore, mycotoxin absorption occurs through passive diffusion across the ruminal epithel is similar to VFA absorption mechanism that in this study used as marker absorption.

As described on conclusion of the first study, pH acid in the rumen can increase absorption rate of acidic mycotoxins (i.e: AFB1and OTA). Low pH not only can cause metabolism disorder in the rumen but also potentially increase mycotoxin absorption. It would be interesting to simulate the acidosis in in-vivo experiment with the acidogenic diet and yeast for pH stabilizing used Saccharomyces cerevisiae. For this purpose, the second study used acidogenic diet and yeast on mycotoxin absorption and mycotoxin detoxification by ruminal microbes using non-lactating dairy cow.

Effect of acidogenic diet with yeast on rumen pH and ruminal fermentation product as was described in Chapter 3, such as individual VFA, lactic acid and ammonia concentration. Acidogenic diet can modify fermentation product responsible for decreasing pH close to SARA condition and decreasing protozoa population. Several interesting finds from this study related to yeast addition to feed were as follows:

**Subacute Ruminal Acidosis (SARA) Characteristic**

SARA was defined as condition of time spent rumen pH under 5.6 for more than 3 h/d, according to several authors (AlZahal et al., 2008, Gozho et al., 2005a). In this study, Ac diet reduced rumen pH but not as expected. Cattle characterized by spent time pH <5.6 were slightly shorter (2.77 h/d) than expected
might be due to yeast supplementation so that inhibited decreased rumen pH after feeding. Although the time under pH 5.6 was shorter than that of SARA, metabolite products in the rumen showed SARA criteria, e.g. total VFA increased (132 mM), acetate proportion decreased, and propionate proportion increased, compared to 150 mM on total VFA by (Nagaraja and Titgemeyer 2007).

Lactic acid concentration in normal condition (0 to 5 mM) was similar to that previously reported (Ghorbani et al., 2002). In contrast, Wiryawan and Brooker (1995) reported acute acidosis ruminal lactate exceeded 100 mM in sheep. Normal concentration of lactic acid indicates that lactic acid is not accumulated in the rumen and the concentration influences rate of lactic acid synthesis and its degradation (Owens et al., 1998). Therefore, although not all parameters in this research corresponded to the criteria of SARA, the formation of metabolite rumen products were similar to SARA condition, thereby viable for SARA simulation.

Stabilization of Rumen pH with Yeast

Probiotic yeast are currently well accepted and widely used in animal feeding, especially since some of them have been officially authorized as feed additives in Europe (Directive 96/51 EC). It is now generally accepted, yeast removes harmful oxygen from rumen environment to be more anaerobic (Chaucheyras-Durand et al., 2008) and provides various growth factor and some micronutrient in the rumen. Yeast utilization of this study is effective in increasing pH during acidosis. Yeast has aerob facultative characteristic and can not survive in anaerobic environment such as in the rumen, therefore, yeast needs to be added every day. The amount of yeast in the rumen should be sustained a minimum of 10^5 CFU/g rumen contents (Jouany 2003).

In this research used Saccharomyces cerevisiae yeast, CNC strain CNCM 1-1077, which is commercial product of probiotic feed. Yeast has been long used to improve livestock production and can increase the average of pH in the rumen (Chaucheyras-Durand et al., 2008). In this study, yeast addition with a dose of 0.5 g/head of livestock/day (equivalent 10^10 CFU/day). This dose was similar to that used by (Bach et al., 2007, Blank and Wolffram 2009). According to Thrune et al., (2009) yeast affected the improvement of rumen pH from 6.32 to 6.53.

Yeast could increase the average of rumen pH, minimum pH and reduced the decreasing rumen pH for 4-10-hours after morning feeding so it will reduce SARA condition. Consequently, rumen pH was maintained at values compatible with an efficient rumen function. In Figure 4.1 showed, mode of action according Chaucheyras-Durand et al., (2008) that one strain S. cerevisiae was able to outcompete S. bovis for utilization of sugars, the reduction in quantity of fermentable sugars available for bacteria consequently limited the amount of lactate produced. The role of yeast in rumen is suspected able to stimulate lactic acid-utilizing bacteria like Megasphaera elsdenii and Selenomonas ruminantium so that able to increase the conversion of lactic acid to propionate, in addition yeast increases cellulolytic and amylolytic bacteria fermentation process thus increasing total VFA as energy resource. They produce ethanol, glycerol, peptides and amino acids as end-products which are essencal for the lactate fermenting bacteria (Chaucheyras-Durand et al., 2008).
Therefore, yeast addition can reduce pH decrease in acidogenic diet. Yeast effect towards the increase of rumen pH was expected to modify mycotoxins AFB1 and OTA absorption.

Other results showed the relation between acidogenic diet and mycotoxin metabolism in the rumen. Acidosis can modify microbial population in the rumen, like protozoa. In this study there was microbe effect towards mycotoxin detoxification.

**Ruminal Microbes Effects against Mycotoxin Detoxification**

Acidosis is able to affect microbial mycotoxin detoxification ability. In this experiment the effect is clearly visible. In OTA analysis, 3 hours after contamination in rumen, in Ac feed, there was an increase in OTA systemic bioavailability, which together with the decreasing of protozoa population genera *Entodinium* > 100 mm, *Isotricha* and *Dasytricha*. Increasing of OTA bioavailability indicates decreasing of OTA degradation become OTα. However, the amount of degradation at the beginning of this contamination is not linear with the excretion pattern of OTα produced through urine. It is suspected that Ac feed effect only at the beginning of contamination. Role of other microbes such as bacteria also allegedly involved in OTA detoxification (Mobashar et al., 2012).

OTA can be biodegraded through the hydrolysis of the amide bond that links the L-β-phenylalanine molecule to the OTα moiety (Figure 4.3).
Based on the chemical structure of the molecule of OTA, one speculates about the kind of proteolytic enzymes (exopeptidase) developing the highest degrading activity. Carboxypeptidase could be considered to be particularly effective. The first reported protease able to hydrolyze OTA was carboxypeptidase A (EC 3.4.1.7.1) from bovine pancreas. Ruminant are able to biodegrade OTA following the pathway that yields phenylalanine and OTα (Kiessling et al., 1984) with rumen protozoa. In this result was found indicate yeast has the a important role in OTA biotransformation.

The role of yeast in OTA mycotoxin detoxification was visible benefit to protozoa on the increasing or degrading of OTα metabolites product both in non-acidosis condition or acidosis. The addition of yeast was able to increase OTA biocconversion became OTα, this is caused by yeast can supply useful nutrient for the growth of proteolytic bacteria that can contribute to amide bond hydrolyze in OTA. Some of fermentation parameters were seen to contribute to this process i.e. 1) yeast addition was able to stimulate related to the increasing of proteolytic enzyme's activity was produced by bacteria (exopeptidase), 2) yeast was able to increase pH in rumen more suitable environment for protozoa in the rumen. 3) yeast produced carboxypeptidase enzymes (Abrunhosa et al., 2010) (Figure 4.4).

Whereas, previously reported by Abrunhosa et al., (2010) found that carboxypeptidase Y (CPY) (EC 3.4.16.1) from S. cerevisiae is also able to
hydrolyze OTA with optimal activity at temperature 37°C and pH 5.7. From those facts, yeast was able to contribute in supplying nutrition for bacterial growth which is directly related with production of proteolytic enzyme that stimulate OTA hydrolisis to OTα and phenylalanine.

The Role of Yeast in the Dynamics of Mycotoxin Concentration in The Rumen

Another interesting finding was the dynamics of the concentration in the rumen. The addition of yeast was suspected to have a role in binding mycotoxin. In the concentration analysis in the rumen, AFB1 and OTA concentration based on time of 3 - 6 h, showed concentration trends looked decreased in all treatments, this indicated that AFB1 and OTA experienced rapid absorption. This mycotoxin was suspected largely been absorbed in the rumen at 9 hours. Thus, these results showed OTA and AFB1 are absorbed rapidly accros to membranes cell in the rumen.

In contrast, AFM1 concentration in NA diet treatment with yeast that showed the highest increase. These results indicated yeast was able to bind AFM1. The ability of the yeast cell wall in binding AFM 1 toxin has been explained in a study conducted by Corassin et al., (2013). In addition, the ability of yeast in binding the AFB1 has been explained in a study conducted by (El-Nezami et al., 1998). Increasing yeast to bind AFM1 in NA caused slowly eliminated through feces. In contrast on Ac diet treatment, decreasing concentration AFM1 in rumen correlated to rapid elimination in feces. Therefore, these results demonstrated this yeast and diet treatments will be affected the excretion pattern of AFM1 through feces.

DON and FB1 are polar mycotoxins that slightly absorbed in the rumen, so that only slight absorption in the rumen wall. DON and FB1 concentration in the rumen showed a declining trend except for NA treatment with yeast addition showed an increase trend. A decrease in the DON and FB1 concentration is thought to be caused by mycotoxin outflow from rumen along with feed particle. In contrast with NA treatment with yeast occurred the pattern of concentration increasing from 3 - 6 h. This increased might be caused by, 1) the occurrence of DON and FB1 binding by yeast which is then stay longer in the rumen, or 2) redeglutisi process, which feed particles re-enter the rumen so the concentration has increased in 6 hours. Yeast in the rumen was expected to have a role in the DON and FB1 binding so the concentration will be increased. In contrast with Ac diet which caused the feed rate increased so all mycotoxin outflow will be increased from rumen.

The role of yeast can bind mycotoxin in the first 10-hours and within 24-hours of collection both in urine and feces did not provide significant effect in treatment based on the type of diet. From this fact can be used as a reference of mycotoxin in the rumen was very dynamic, the movement of rumen contents outflow could affect the concentration of mycotoxin sample analysis, this is caused by rumen contents always moving during the digestion process.
The Effect of Ration Type and Yeast Supplementation in Excretion of Mycotoxins through Feces and Urine

The rate of absorption of AFB1 and OTA differed significantly between diets. Their absorption rate was especially influenced by acidogenic diet leading to a greater rate of absorption than that of non-acidogenic diet. In acidogenic diet, fecal excretion of OTA with and without yeast supplementation decreased by more than three folds 24-h after mycotoxins administration. This decrease in fecal excretion was correlated with a greater urinary excretion of OTA after 24-h and 48h. Similarly, acidogenic diet reduced fecal excretion of AFB1 and increased elimination of its major metabolite AFM1 in urine. In contrast, DON and FB1 were not affected by the acidogenic diet. These results show that lower ruminal pH induced by high-grain diets increase AFB1 and OTA bio availability. The effect of diet on the bioavailability of OTA was reported by Xiao et al., (1991) who observed a four fold increase in OTA absorption in sheep fed high proportions of cereals.

To evaluate the effect of ruminal acidosis on mycotoxins’ bioavailability, we tested in a previous study, the disappearance of AFB1, OTA and FB1 in a temporarily isolated rumen sheep model at acid (pH 5) and neutral pH (pH 7). In this isolated rumen model without ruminal contents and hence, reduced load and activity of ruminal microbes disappearance of OTA and AFB1 was greater in acidic conditions, while FB1 concentration remained unchanged. In this experiment, we also showed that OTA is absorbed through ruminal wall because its disappearance from the rumen was confirmed by its Gaussian profile in plasma. In acidic conditions, the increased disappearance of OTA and AFB1 suggests the presence of greater proportions of non-ionized molecules.

The transfer of organic compounds such as mycotoxins through the epithelium depends on many factors; among them the degree of ionization plays a significant role in the absorption process (Blank et al., 2003). Other factors also playing a role are lipophilicity and the size of molecules. Ionization is mainly influenced by the pH of the medium and only non-ionized compounds are absorbed. At pH 5, OTA (pKa=7.5) and AFB1 are partially ionized.

In our study, the increase in OTA bioavailability is probably due to a passive diffusion rather than a decrease in OTA degradation by ruminal microbiota as reported by Blank et al., (2003) This is in agreement with results obtained by Xiao et al., (1991) who showed that the disappearance of OTA from the rumen and the corresponding apparition of its metabolite OTα was much faster for hay-fed than for grain-fed sheep. Therefore, AFB1 and OTA were rapidly absorbed due to its molecules more non-ionized forms in acidogenic diet. Yeast supplementation was increased rumen pH but yeast did not affect in decreasing the absorption AFB1 and OTA in the acidogenic diet.

From this experimental data showed that acidogenic diet influences DON and DOM1 excretion. DON and DOM1 are faster to be eliminated at the acidosis condition. The increasing DON and DOM 1 in cows fed with Ac diet were caused by different in the diet type that affect the rate of feed passage in the gastrointestinal tract so that DON was excreted rapidly at the acidosis condition. DON and DOM1 were faster excreted through feces that assumed to be correlated in the low fiber content of the diet. The low fiber content on the ration would increase the passage of diet in the rumen (Dehority and Orpin 1997).
DON and DOM 1 excretion were delayed through feces due to they more absorbed in the rumen. In the studies, in non acidogenic diet total excretion of DON and DOM1 was decreased about 10% compared to acidogenic diet. This is in agreement to Dänicke and Brezina (2013) was reported that different feeding generally result in a slower ingesta transit through the gastro-intestinal tract possibly a longer contac time to the absorptive surface, so that DON and DOM 1 more absorbed. Therefore, DON excretion is not directly affected by the rumen pH but its excretion correlated to feed passage rates from the rumen. In this study, DON and DOM1 was rapidly excreted in acidosis condition.

The absorption of FB1 in the rumen is very low so that most of it was excreted along with the diet particles through the feces. FB1 is highly polar and very easily dissolved in water (very water soluble) and easily ionized. Some previous studies reported by Prelusky et al., (1995) the same results that FB1 was poorly absorbed in the gastrointestinal tract. The FB1 was minimally absorbed in the rumen and it was not detected in the blood plasma of the cattle. FB1 has the hydrophilic and water soluble properties at the pKa 3.5, when the FB1 will be completely ionized in the rumen (Pantaya. et al., 2014).

The total of excretion between acidogenic diet and NA indicate over 2 days the elimination of FB1 kinetic was similar. This showed that both acidosis condition and non-acidosis has no effect on FB1 excretion.

It is well established that ruminants are more resistant to OTA and DON exposure because these mycotoxins are extensively degraded by ruminal microbes into less toxic OTα (Ringot et al., 2006) and DOM1 (Karlovsky 2011, Ringot et al., 2006), the two other tested mycotoxins (AFB1 and FB1) remain unchanged in the rumen (Prelusky et al., 1995, Voss et al., 2007). As ruminal acidosis is associated with significant changes in microbiota composition (Fernando et al., 2010), in this work we also evaluated the ruminal capability to degrade OTA and DON. There were no differences in OTα and DOM1 between the 2 diets. This is explained by the high individual variability observed in rumen content samples, probably due to the heterogeneity of the matrix and the difficulty to take representative samples from whole rumen contents of large animals.

**Excretion Mechanism through Biliary Excretion**

The experimental result was described excretion of AFB1, OTA, DON and FB1 and their metabolite product though urine for AFM1 and OTα , DOM 1 through feces in Chapter 3. Following ingestion AFB1 was quickly absorbed in the rumen, then AFB1 entered the liver through hepatic portal blood supply (Wilson et al., 1985). Metabolism of xenobiotics including AFB1 can divided into three phases, bioactivation (phase I), conjugation (phase II) and deconjugation (Phase III). On the biotransformation phase are mainly of oxidation of AFB1 to metabolites such as AFM1 (Figure 4.5). The oxidation-reduction reaction involving Cytochrome 450 enzyme (CYP450) in enterocytes cell and liver (Kuilman et al., 2000), AFM1 formed was more easily soluble in water (hydrophilic) and was excreted through urine.
The next process on the detoxification phase II (conjugation), AFB1 reacted with GSH (glutathione) formed AFB1-GSH with the help of glutathione-S-transferase (GST) catalisator, meanwhile for AFM1 reacted with glucuronic acid formed AFM1-glucuronic conjugate which is then through biliary excretion pathway excreted through the fecal, produced the product so that easily excreted through fecal. Conjugate of epoxide and hydroxylated AFB1 metabolites are readily excreted via the bile into the intestinal tract, where they might be subject to bacterial deconjugation as phase III reaction.

Afterward, the conjugate product had a deconjugation reaction (phase III reaction), hydrolysis reaction with beta glucuronidase enzyme catalyst which is formed by intestinal microflora (gut flora). Product from those reactions is free AFM1 returned to have lipophilic character then through the hepatic portal vein to liver called enterohepatic circulation. Deconjugation model is illustrated in Figure 4.7.

On this study AFB1 excreted as AFM1 with the proportion of 25% through urine (urine pathway), while 75% through fecal as AFM1 and AFB1. The percentages of AFM1 excretion obtained in the present study are in similar with those reported by Firmin et al., (2011). Most of the AFB1 excretion pathway through bile (biliary pathway) and then eliminated through fecal. Mycotoxin with molecule weight less than 500 DA toxin tend excreted through urine, while more than 500 DA (dalton) excreted through fecal via biliary excretion (i.e: AFB1-gluthatione MW 619 g/mol).

Biotransformation of OTA is dependent on the cytochrome P450 enzymes. OTA can undergo hydroxylation, glucurono and sulphate-conjugation and glutathione conjugation (Ringot et al., 2006). However, the major metabolic pathway of OTA is represented by its hydrolysis to OTα, by cleavage of the peptide bond, which can occur enzymatically (by carboxipeptidases, trypsin and chemotripsin) in the presence of the large intestine (Mobashar et al., 2012). With regard to fecal excretion, OTA have been detected in feces. However, it is difficult to distinguish whether or not the levels of OTA detected in feces are due to non-absorbed OTA, to the intestinal OTA secretion or to biliary excretion (Table 4.6).

DON in rumen have de-epoxydation reaction resulted de epoxy deoxynivalenol (DOM 1) and deacetilated product by ruminal microbes (ruminal microorganism) (Swanson et al., 1987) (Figure 4.6). Then DON have conjugation reaction with glucuronic acid formed DON-glucoronic conjugation (MW 490...
g/mol) (Table 4.3) which then through biliary excretion to intestine then have enterohepatic circulation. Biliary excretion pathway can slow down toxin elimination from the body.

deoxynivalenol (DON)  de-epoxy deoxynivalenol (DOM 1)

Figure 4.6 Structure of molecules DON and DOM 1

Figure 4.7 Principal deconjugation reaction mycotoxins with glucuronides in intestine
### Table 4.3 Chemical structure of mycotoxins conjugate with glucuronide

<table>
<thead>
<tr>
<th>Conjugation mycotoxins</th>
<th>Molecules structure and molecules weight (MW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTA- glucuronide</td>
<td><img src="image1" alt="OTA glucuronide structure" /></td>
</tr>
<tr>
<td></td>
<td>MW 597.9 g/mol</td>
</tr>
<tr>
<td>OTα-glucuronide</td>
<td><img src="image2" alt="OTα glucuronide structure" /></td>
</tr>
<tr>
<td></td>
<td>MW 450.74 g/mol</td>
</tr>
<tr>
<td>(Han et al., 2013)</td>
<td></td>
</tr>
<tr>
<td>DON-3-glucuronide</td>
<td><img src="image3" alt="DON-3 glucuronide structure" /></td>
</tr>
<tr>
<td></td>
<td>MW 490.17 g/mol</td>
</tr>
<tr>
<td>DON-15-glucuronide</td>
<td><img src="image4" alt="DON-15 glucuronide structure" /></td>
</tr>
<tr>
<td></td>
<td>MW 490.17 g/mol</td>
</tr>
</tbody>
</table>

MW: Molecular weight
5. CONCLUSION AND PERSPECTIVES

CONCLUSION

From several experimental series conducted, the conclusions derived were as follows:
1. Low pH could increase the absorption of AFB1 and OTA. In contrast, FB1 was not absorbed in the rumen.
2. Acidogenic diet could lower rumen pH and close to SARA, affect the fermentation characteristics in the rumen and modified toxicokinetics profile of mycotoxins AFB1, OTA, DON and FB1. In acidosis condition AFB1 and OTA were rapidly absorbed but DON and FB1 were low absorbed in the rumen. DON excretion did not directly affect by the rumen pH but its excretion correlated to feed passage rates from the rumen. DON was rapidly excreted in acidosis condition. FB1 excretion was not affected by rumen pH.
3. Saccharomyces cerevisiae yeast could inhibit the decrease rumen pH after feeding but yeast supplementation did not affect in decreasing the absorption of AFB1 and OTA. Yeast could stimulate mycotoxins OTA detoxification in the rumen.

PERSPECTIVES

Since mycotoxin contamination can not be avoided in current agricultural practices, detoxification strategies are very much needed. Microbial detoxification should be a promising choice since it can be a specific, effective and environmental friendly strategy for reducing and/or eliminating possible contaminations of mycotoxins in food and in feed.

Acidogenic diet can cause rumen pH decrease called ruminal acidosis. Acidosis could increase absorption AFB1 and OTA, modified microbe population, i.e., decreasing protozoa population. Biological, chemical, or physical treatments can reduce ruminal acidosis in ruminants. The acidic status of rumen content can be controlled by good management practices in the farm, i.e., slow adaptation concentrate feeding. One approach to reduce acidosis pressure is the use of probiotic yeast. The use of yeast is a technological alternative being explored to overcome the problem decrease rumen pH during acidosis. In this recent studies, yeast could inhibit decrease rumen pH after feeding but yeast supplementation did not affect in decreasing the absorption AFB1 and OTA.

The ability of the yeast in reducing the mycotoxin absorption is limited due to possibility a low doses. In this study, animals received the yeast with dose 0.5 g/heads/day (equivalent 10^{10} CFU/day) that administrated at morning feeding. The result showed that supplementation with yeast can increase mean rumen pH for 10-h, but it did not significantly effect in decreasing the absorption of AFB1 and OTA, so that needed extention of time in stabilizing rumen pH. Therefore, further work need to be done under controlled mycotoxin contaminated with
yeast was given every 10-12 h to achieve the optimum result in stabilizing rumen pH.

The future use of yeast has a good prospect to conform mycotoxins exposure especially for ruminants. This knowledge is important as a basis for choosing a new generation of yeast. The future studies can develop the role of yeast in the detoxification of mycotoxins.
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Morgavi DP, Riley RT. 2007. An historical overview of field disease outbreaks known or suspected to be caused by consumption of feeds contaminated with Fusarium toxins. *Anim Feed Sci Tech* 137(3-4):201-212.


## APPENDICES

### Appendix 1  Fungi produced mycotoxins (Yiannikouris and Jouany 2002)

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Mycotoxins</th>
<th>Structure of molecule</th>
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</thead>
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<tr>
<td><em>Aspergillus flavus</em></td>
<td>Aflatoxin B1</td>
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<tr>
<td><em>A. parasiticus</em></td>
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<td><em>A. nomius</em></td>
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<td><em>Penicillium vertucosium</em></td>
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<td><em>Aspergillus clavatus</em></td>
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<td><em>F. sporotrichioides</em></td>
<td>Deoxynivalenol</td>
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<td><em>F. graminearum</em></td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
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</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>Fusarium moniliforme</em></td>
<td>Fumonis B1</td>
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<tr>
<td><em>F. proliferatum</em></td>
<td></td>
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</table>
Appendix 2  Xenobiotic metabolism

Appendix 3  Major routes of mycotoxin bioconversions in biological systems
(Yiannikouris and Jouany 2002)

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Oxidation</th>
<th>Reduction</th>
<th>Hydrolysis</th>
<th>Conjugation</th>
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<tbody>
<tr>
<td></td>
<td>CYP450</td>
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<td>AFB1</td>
<td>Epoxide</td>
<td>Aflatoxicol</td>
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<td>AFQ1</td>
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<tr>
<td>OTA</td>
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<td>OTα</td>
<td></td>
</tr>
<tr>
<td>DON</td>
<td></td>
<td></td>
<td></td>
<td>DOM1</td>
</tr>
<tr>
<td>FB1</td>
<td></td>
<td></td>
<td></td>
<td>Aminopentol</td>
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## Appendix 4 Principal reaction of biotransformation

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<th>Reaction</th>
<th>Enzymes</th>
<th>Localisations</th>
<th>Substrats</th>
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<td>Oxidation</td>
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<td>Flavine mono oxigenase</td>
<td>Microsome</td>
<td>Amine tersier</td>
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<td>Cythrome P450</td>
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<td>Reduction</td>
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<td>Hydrolase</td>
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<td>Cytosol</td>
<td>Ester</td>
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<td></td>
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<td>Microsome</td>
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<td>Peptidase</td>
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<td>Microsome</td>
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<tr>
<td>Epoxide hydrolase</td>
<td>Microsome</td>
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<td>Phenol, thiol</td>
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<td>Cytosol</td>
<td>Amines, acids</td>
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<td>Microsome</td>
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### Appendix 5 Body weight (kg) of cows used in the research

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### Appendix 6 Setting of lot of cows used during in research

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CUD : Coefficient d’utilisation digestive
Appendix 7 Water intake of cows used in the research

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**Appendix 8. Feed nutrient calculation**

- **Item**: [List of items]
- **Value**: [List of values]
Appendix 9  Mycotoxins metabolism (Fauzet-Marquis, 2005)

Appendix 10  Unconjugation in terminal Ileum
Appendix 11  Lactic acid conversion to propionate via Methylmalonyl CoA and Propionyl CoA
Appendix 12 Documentation of activity during \textit{in vivo} experiment

1. Experimental non lactating dairy cow

2. Installing properties for urines collection

3. Falcon tube for collecting feces

4. pH meter and blender

5. Rumen juice samples in tube PP 10 ml

6. Purification using column 6 in 1
Appendix 13 Cannulated sheep in empty washed rumen method
BIOGRAPHY

Author was born on October 17th, 1967 in Sleman, Yogyakarta, Indonesia. Author was graduated from Faculty of Animal Husbandry at UGM in 1986, then got his Master degree from Animal Science at IPB, Bogor 2003. Author lectured at Animal Science Departemen of State Polytechnic Jember. Author had enrolled degree at Nutrition and Feed Science at Bogor Agricultural University in 2010 in Double Degree Indonesia Perancis (DDIP) and Cotutelle program with École doctorale des Sciences de la Vie Santé.

During study, author presented an article Do ruminal acidosis alter the absorption of mycotoxins and their detoxification? Oral Communications in Conseil Scientifique 2012, in INRA Hebivores 1213, Theix and Poster Presentation in JED (Journée Ecole Doctoral) 2013, with title: Low pH enhances rumen absorption of aflatoxin B1 and ochratoxin A in sheep, in Université Blaise Pascal as part student academic activity in France. Furthermore presented oral presenter in Program for “IUMS Outreach Program on Food Safety and International Conference on Mycotoxin” at Gadjah Mada University in Yogyakarta on December 2014. As a research team, together with Dr Ir Suryahadi DEA, Prof Dr Ir Komang G Wiryawan and Dr Ir Dwiera Evvyernie A MS MSc, Author was granted grant of Hibah IJR (International Joint Research) with title Probiotic Treatment as Agent of Mycotoxin Detoxification in Ruminants with contract No 509/SP2H/PL/ Dit.Libtabmas/VII/2011.