CONTROL OF AFLATOXIN IN COTTON SEED CAKE BASED DAIRY FEED FOR M1 FREE MILK PRODUCTION

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2021
In the name of Allah, the Most Beneficent, the Most Merciful
To

The Controller of Examinations,
University of Veterinary and Animal Sciences,
Lahore.

We, the Supervisory Committee, certify that the contents and form of the thesis, submitted by Mr. Murtaza Ali Tipu S/O Muhammad Iqbal Hussain (Regd. No. 2012-VA-394) have been found satisfactory and recommend that it be processed for further evaluation by the External Examiner(s) for award of the Degree.

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DEDICATION

This scientific work is dedicated to

My Parents

And

My caring wife and children
ACKNOWLEDGEMENTS

I am very thankful to Almighty Allah who is real force behind completion of this uphill, great and noble task. Innumerable thanks to Holy Prophet, Muhammad (SAWW) whose life is always a source of inspiration to do something for the welfare of mankind.

I would like to express my sincerest thanks to my supervisor Prof. Dr. Anjum Khalique, for the friendship, guidance, respect and patience that he has shown me over the years. I am fortunate to express my deep gratitude to for guiding me during difficult moments of my studies.

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(Murtaza Ali Tipu)
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ABSTRACT

The present Ph.D. research was designed to explore the detoxificant ability of chemical detoxificant in cotton seed cake (CSC) based diet and subsequent effect on milk yield, milk composition and aflatoxin M₁ in Nili Ravi buffaloes. For first experiment, four chemicals were selected i.e. copper sulphate, benzoic acid, calcium propionate and sodium bisulfide to treat CSC. Four levels of above four chemicals were selected (0.25%, 0.5%, 0.75%, 1.0%). These were treated against 100ppb CSC. As a result, calcium propionate (0.5%) level significantly (P<0.05) decreased the aflatoxin. In the second experiment three levels (0.25%, 0.5%, 0.75%) of calcium propionate treated CSC were used in dry buffaloes to see any adverse effect on the dry matter intake, blood chemistry, Body condition scoring and urine pH. One diet having no CSC and other without chemical treated CSC were also given to dry buffaloes. Calcium propionate 0.5% treated CSC has positive effect on body condition scoring and dry matter intake. In the third experiment three levels of calcium propionate i.e 0.25%,0.50% and 0.75% were used in cotton seed cake base diet infected with aflatoxin to see the effect on milk production, milk composition and aflatoxin M₁. Calcium propionate 0.5% significantly (P<0.05) reduced M₁. There was no change in milk yield and milk composition. In the fourth and last experiment best level of calcium propionate i.e. 0.5% was compared with a commercial toxin binder( sodium bentonite 1%) to see which chemical is better regarding reduction of M₁. Calcium propionate 0.5% performed well regarding reduction of M₁ as compared to sodium bentonite. No change in milk yield and milk composition was observed. As a conclusion, we may say that calcium propionate 0.5% is quite effective to minimize AFM₁ in milk.
GLOSSARY OF ABBREVIATIONS AND ACRONYMS

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ADG</td>
<td>Average daily (live weight) gain</td>
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<td>AFB&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Aflatoxin B&lt;sub&gt;1&lt;/sub&gt;</td>
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<td>AFB&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Aflatoxin B&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>AFM&lt;sub&gt;1&lt;/sub&gt;</td>
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<tr>
<td>BCS</td>
<td>Body condition score</td>
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<tr>
<td>BRI</td>
<td>Buffalo Research Institute</td>
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<tr>
<td>BW</td>
<td>Body weight</td>
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<tr>
<td>Ca&lt;sub&gt;H&lt;sub&gt;10&lt;/sub&gt;CaO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Calcium propionate</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>Ca(OH)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Calcium hydroxide</td>
</tr>
<tr>
<td>Cl</td>
<td>Chloride</td>
</tr>
<tr>
<td>CP</td>
<td>Crude protein</td>
</tr>
<tr>
<td>CSC</td>
<td>Cotton seed cake</td>
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<tr>
<td>CuSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Copper sulfate</td>
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<tr>
<td>DCAD</td>
<td>Dietary anion-cation difference</td>
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<tr>
<td>DM</td>
<td>Dry matter</td>
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<td>DMI</td>
<td>Dry matter intake</td>
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<td>HSCAS</td>
<td>Hydrated sodium calcium aluminosilicates</td>
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<tr>
<td>K</td>
<td>Potassium</td>
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<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>NaHS</td>
<td>Sodium bisulfide</td>
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<tr>
<td>NDF</td>
<td>Neutral detergent fiber</td>
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<tr>
<td>OTA</td>
<td>Ochratoxin A</td>
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<tr>
<td>SBM</td>
<td>Soybean meal</td>
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<td>UVAS</td>
<td>University of Veterinary and Animal Sciences</td>
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CHAPTER 1
INTRODUCTION

Pakistan is facing shortage of available feed resources for dairy production. A competition exists between increasing populations of human beings and livestock to grow crops or fodder. Meat production, milk yield and milk composition are affected most importantly by animal feed, in terms of both supply and quality of the feed. The biggest cost in livestock production is feed (Ribeiro et al. 2011).

Cotton is a leading cash crop in South Asia. In Pakistan in 2015-2016, cotton production was 9917 thousand bales (Ministry of Finance, 2017). Cotton seed cake (CSC) meal is a byproduct of the cotton seed industry and is commonly used in livestock feeds. The major problems with CSC include contamination of sand with usage of pesticides, availability in specific season, and adulteration with low quality material (Zahid et al. 2003). Cotton seed cake can also have a high incidence of aflatoxin contamination (Weidenner, 2012).

Recently, the quality of CSC has emerged as a big issue as most of its supply was thought to pose health problems in small as well as large ruminants. The inadequate processing and storage of CSC cause the toxin problem in this ingredient (Yunus et al. 2015). There is an increased awareness of human and animal health hazards due to the presence of fungi in human foods and livestock feeds (Huwig et al. 2001). Mycotoxins are secondary metabolites produced by various fungi species. Amongst all mycotoxins, aflatoxins are of importance in Pakistan due to the warm and humid weather. The most prevalent and actively potent toxin is aflatoxin B1 (AFB1). Improper storage can cause contamination of CSC by aflatoxins, ochratoxin and cyclopiazonic acid (Yunus et al. 2015). Animal food products such as milk and meat may contain aflatoxins; however, aflatoxins may also pose health issues to the animals themselves (Visconti, 1998). Many mycotoxins, in particular aflatoxins, are harmful to human beings as well as the animals consuming the contaminated feed, as aflatoxins are transmitted through infected milk. Aflatoxin M1 (AFM1) can contaminate cow milk due to the intake of feed contaminated with AFB1. After the intake of feed contaminated with AFB1, the mycotoxin is hydroxylated to AFM1, which is then transferred in to blood and through blood comes in to the milk, and when animals (calves in particular) and humans consume the milk they also consume AFM1 (Hashmi, 2016). The potential adverse effects on both human and animal health creates the demand for practical and cost-effective measures to reduce mycotoxin contamination of animal feeds. Although only a few have real practical application, many approaches have already been adopted to control this menace (Kolossova et al. 2009). Physical treatments are the first options used without involving any chemical like washing, polishing, mechanical separation, flotation and autoclaving (Jouany, 2007). Chemical treatments include calcium hydroxide (Ca(OH)2), ammoniation, copper sulfate (CuSO4), sodium bisulfide and various acids (Giovati et al. 2015). Structural changes can be done by chemical methods using chemicals like oxidizing agents, aldehydes, gases such as ammonia as well as bases (Oliveira and Corassin, 2014). Chemical detoxification can reduce the level of aflatoxin by up to 93-95% (Lee et al. 2015).

The previous studies are not clear about the exact concentration of various chemicals used to reduce aflatoxins in cattle feed. However, some levels are selected based on the earlier work to assess their efficacy to detoxify aflatoxins. The third option is the use of mycotoxin binders which are extensively used by animal feed industry but they adsorb some micronutrients (Kolossova et al. 2009).

In this study the effectiveness of calcium propionate and bentonite to decrease the concentration of AFM1 in milk. Moreover, the effect of these detoxificants on other parameters such an animal live weight, body condition score (BCS) and milk composition of buffaloes were also investigated.
CHAPTER 2
REVIEW OF LITERATURE

2.1 Use of Cotton Seed Cake in Livestock Feed

Cotton seed cake is produce from whole cotton seeds after extraction of oil from the seeds. It contains an average of 25% crude protein (CP) (Liadakis et al. 1993; Khan et al. 2009) and can be used in ruminant rations to supply both energy (due to its relatively high residual oil content) and protein. Of the protein contained in CSC, about 50% is protected from degradation in the rumen (Agrawal et al. 2003), and thus can be used as a protein supplement for growing and dairy animals (Gajera et al. 2013; Titi et al. 2013). It is the most common protein source used for ruminant production in Pakistan (Jabbar et al. 2006). It is also commonly used in India (Mudgal et al. 2003; Naik et al. 2013) and other cotton growing areas because of its availability and its high nutrient value (Broderick et al. 2013).

There are many reports of improvements in ruminant production in response to inclusion of CSC in the diet. Uddin et al. (2013) reported improvements in milk production, milk fat percentage, BCS and live weight when Holstein Friesian cross-bred cows were fed diets containing 35% CSC. Bunaji (Bos indicus) heifers supplemented with CSC had higher average daily gains and attained puberty at an earlier age than those supplemented with maize stover (Rekwot 2004). When CSC was substituted for soybean meal (SBM) in the diets of finishing lambs, it did not affect final body weight, daily weight gain and total weight gain of the lambs (Kandylis et al. 1992; Silva et al. 2016). de Assis et al. (2019) found CSC could totally replace SBM in diets for crossbred Boer goat kids in feedlot, with no alteration to the productive performance, intake and digestibility of nutritional components and nitrogen balance of the animals. When natural pasture hay was supplemented with CSC, the CP intake and daily body weight gain were increased and the carcass parameters of Sidama goats were also improved (Alemu et al. 2010).

2.2 Incidence of Mycotoxin in Cotton Seed Cake

Mycotoxins are secondary metabolites produced by various fungi species. Aflatoxins, ochratoxin A (OTA), deoxynivalenol, fumonisins, zearalenone, and patulin are among the most important mycotoxins. Human foods and animal feeds can become contaminated with mycotoxins through fungal growth prior to and during harvest, or as a result of inappropriate storage. Mycotoxins are mainly produced by Claviceps, Fusarium, Penicillium, Aspergillus and Alternaria (Huwig et al. 2001).

Under certain conditions, both crops and stored feeds can become contaminated by fungi and subsequently mycotoxins. These feeds can include CSC. In their study of Egyptian cotton seeds, cotton seed meal and CSC, Mazen et al. (1990) isolated 39 species and 16 fungal genera, with Aspergillus the most frequent genus. Cotton seeds and cotton seed products were naturally contaminated by AFB1 and aflatoxin B2 (AFB2). Other researchers have also reported the presence of aflatoxins in cotton seed products (Loosmore et al. 1964; Girgis et al. 1977; Coppock et al. 1987; Dhavan and Choudary 1995; Prasad et al. 1997; Kotinagu et al. 2015), including those utilised in Pakistan (Aman Ullah et al. 2016; Chohan et al. 2016; Iqbal et al. 2016). Recently, Shar et al. (2020) found that 88% of CSC samples assayed (n =110) from Pakistan were contaminated by aflatoxins, with over 73% of those samples with contamination concentrations higher than 20 μg/kg. In addition to aflatoxins, Yunus et al. (2015) found that, in Pakistan, CSC may also be contaminated with OTA, cyclopiazonic acid, equisetin, rubrofusarin, tenuazonic acid, 3-nitropropionic acid, and citrinin. In contrast, Mazen et al. (1990) found no citrinin, OTA, patulin, sterigmatocystin, diacetoxyscirpenol, T-2 toxin or zearalenone in the cotton seeds and cotton seed products assayed from Egypt.

In hot and humid environments Aspergillus flavus is the dominate species responsible for the production of aflatoxins, particularly AFB1 and AFB2 (Herrera et al. 2019). Aflatoxins can have carcinogenic, teratogenic and mutagenic actions (Pier 1992). They can cause reduced weight gain, reduced feed efficiency and increased incidence of disease because of immunosuppression (Robens and Richard 1992; Yunus et al. 2015; Elgioushy et al. 2020). Aflatoxin B1 presents the highest degree of toxicity for animals, followed by the AFM1, aflatoxin G1, AFB2 and aflatoxin G2 (Gourama and Bullerman, 1995). Aflatoxin B1 is the most abundant aflatoxin in naturally contaminated dairy rations (Kaleibar and Helan, 2013), including those containing CSC (Chohan et al. 2016; Shad et al. 2020).
As previously reported, in Pakistan, CSC may also be contaminated with ochratoxins. In warmer climates, OTA is produced by *Aspergillus ochraceous* (Krogh 1987). In ruminants, OTA is subjected to microbial degradation in the rumen to nontoxic ochratoxin-alpha prior to absorption (Bruinink et al. 1998) and it is generally considered that animals with a developed rumen are largely unaffected by dietary OTA (Xiao et al. 1991). Recently, Hashimoto et al. (2015) confirmed that OTA is not carried over into (ruminant) milk or meat.

### 2.3 Aflatoxin M1

Aflatoxin M1 is the hydroxylated metabolite of AFB1. Approximately 0.3–6.2% of AFB1 is converted into AFM1 and excreted into milk, with the amount varying depending on the genetics of the animals, seasonal variation and environmental conditions (Unusan, 2006). Contamination of milk and dairy products by AFM1 has gained a worldwide importance (Giovati et al. 2015), being categorized by the International Agency for Research on Cancer as a class 2B toxin, a possible human carcinogen (Prandini et al. 2009).

Contamination of milk and dairy products is particularly of concern in many developing countries, particularly those located in tropical and subtropical environments (Iqbal et al. 2015). In a study of dairy cattle in Sudan, Amin et al. (2010) found AFM1 contamination in approximately 95% of samples analyses (n=44). Results from a survey in Southern Iran showed the presence of AFM1 in 55.56% of the milk samples assayed (Hashmi, 2016). In a study conducted in Pakistan, AFM1 contamination of buffalo, cow milk, goat milk, and sheep milk was found to be 34.5%, 37.5%, 20%, and 16.7%, respectively (Hussain et al. 2010). In another study conducted in Pakistan, Iqbal and Asi (2013) detected AFM1 in 61% of yogurt, 78% of white cheese, 59% of cheese cream and 45% of butter samples.

Aflatoxin M1 is very stable at high temperatures which is why it persists in dairy products (Govaris et al. 2002; Oruc et al. 2006). There are; however, many ways to decrease AFM1 contamination in milk and subsequently in dairy products. It can be done directly in milk by its treatment or indirectly by minimizing the level of toxins in feed being offered to dairy animals. There are many strategies before and after the harvest of feed crops to reduce toxin levels. The best approach to prevent the effect of mycotoxins is minimizing production of mycotoxins in the first place by harvesting grains at maturity and low moisture and then storing in cool and dry conditions (Cotty 1994; Rachaputi 2006). This, however, can be difficult to perform in countries with a warm and humid climate. In these environments, physical and chemical treatments are utilised to reduce the adverse effects of mycotoxins on both the animals and the products they produce.

### 2.4 Binders

The most applied method for protecting animals against mycotoxicosis is the utilization of adsorbents/binders mixed with the feed; however, their efficacy varies depending on the chemical structure of the adsorbents and the toxin.

#### 2.4.1 Activated charcoal

In aqueous solution, activated charcoal can adsorb most mycotoxins efficiently; however, different activated carbons have limited to no effects (Huwig et al. 2001). Galvano et al. (1996) showed reduced aflatoxin residues in milk of cows consuming different sources of charcoal. In contrast, Diaz et al. (2004) showed that low levels (45 g/cow daily) of activated carbon did not significantly reduce milk aflatoxin residues. Activated charcoal may; however, be more effective in binding zearalenone and/or deoxynivalenol (Döll et al. 2004; Bueno et al. 2005).

#### 2.4.2 Aluminosilicates

Aluminosilicates include zeolites, hydrated sodium calcium aluminosilicates (HSCAS) (Phillips et al. 1990) and aluminosilicate-containing clays such as bentonite, kaolinite, and montmorillonite (Elliott et al. 2020). Of these, HSCAS have been shown to have a high affinity for AFB1 (Phillips et al. 1990). In general, aluminosilicates have limited efficacy against zearalenone, ochratoxin and trichothecenes (Huwig et al. 2001). Recently; however, Vila-Donat et al. (2019) found a tri-octahedral bentonite was not only effective (*in vitro*) as a binder for AFB1 but also for OTA. EFSA(2013) investigated the efficacy of sepiolite (a clay containing a minimum of 60% sepiolite and a maximum of 30% smectite) and bentonite and found both were efficient in reducing the level of mycotoxins in feed.
REVIEW OF LITERATURE

Sulzberger et al. (2016) used clay (0.5%, 1 or 2%) orally to mitigate an aflatoxin challenge in Holstein cows. They found that the 2%/day of product reduced aflatoxin transfer from the rumen to the milk and feces. Milk yield and milk composition remained unaffected by the oral clay treatments. Feeding calcium montmorillonite clay at 0.58 to 1.17 % of dry matter intake (DMI) was an effective method to reduce the transfer and excretion of AFM₁ in milk with no negative effects on DMI, milk production, and milk composition. Gouda et al. (2019) found bentonite and montmorillonite decreased milk content of AFM₁ but had no effects on milk production in the goats. Gul et al. (2017) examined the effect of sodium bentonite as mycotoxin binder in poultry feed and its effects on production performance of laying hens. The addition of 2% sodium bentonite showed better feed and protein utilizations leading an increased egg production and reduced egg defects in layer hens.

2.4.3 Yeasts and yeast products

Yeast or yeast cell walls (YCW) can also be used as adsorbents for mycotoxins. In in vitro studies evaluating tested 30 commercially available YCW products and two different bentonites for binding aflatoxin and zearalenone, Fruhauf et al. (2011) and Sebastian et al. (2011) found all products were effective; however, there were some differences in their efficacy.

A limited number of studies involving these mycotoxin binders have been undertaken in ruminants. Pasha (2008) found that when Sahiwal cattle were subjected to either 500 ppb aflatoxin or 500 ppb aflatoxin plus 1% yeast sludge, the inclusion of the yeast sludge resulted in increased feed intake, milk production, and milk quality. Stanford et al. (2018) fed lambs diets containing ergot-contaminated barley screenings and found inclusion of the mycotoxin binder BiominII (contains diatomaceous earth, kaolin clay, yeast and plant extracts, and enzymes targeted for the degradation of zearalenones and trichothecenes) resulted in increased average daily gains and gain/feed ratios as well as increased N retention. Aazami et al. (2019) evaluated AFB₁ binding capacity of YCW components in dairy goats' diet and found that yeast (1→3)-β-d-glucan was more effective than YCW to bind AFB₁. Further, (1→3)-β-d-glucan was more effective than cell wall to reduce aflatoxin carryover from feed to milk.

In studies with broiler breeders, Manafi et al. (2012) compared the efficacy of bentonite and glucomannan (extracted from YCW) on aflatoxicosis, and found glucomannan was superior. Li et al. (2012) found addition of 2 g/kg of YCW enterosorbent partly neutralized the detrimental effects in broiler chickens fed naturally contaminated (Fusarium mycotoxins) feed. Mendieta et al. (2018) investigated the effect of Saccharomyces cerevisiae YCW in diets with low doses of AFB₁ and OTA, alone or in combination, on broiler performance and immune response. Weight gain, feed conversion and cellular immune response improved in the groups receiving YCW.

2.5 Chemical Detoxification

Calcium propionate is widely used as an anti-fungal agent in silage making (Dong et al. 2017). Bintvihok and Kositcharoenkul (2006) used different levels (0.25% and 0.50%) of calcium propionate and found it to be effective as an aflatoxin detoxificant agent for broiler chickens. Moreover Tipu et al.2020 compared calcium propionate (0.5%) with a commercial toxin binder (sodium bentonite 1%) to find best possible solution for aflatoxin. They concluded that both calcium propionate and sodium bentonite were quite effective at reducing AFM₁ in milk. Neither had harmful effects on the health of buffaloes or their milk production and composition.

There are a number of other chemical compounds that have been successful used as either anti-fungal agents. Arshad et al. (2012) found of copper sulfate, propionic acid and benzoic acid completely inhibited the growth of fungus. They also found the use of herbal compounds/spices garlic and onion (0.5%) was effective in inhibiting the growth of Aspergillus flavis and Aspergillus parasiticus.
2.6 Statement of the Problem

High levels of aflatoxin in cotton seed cake is a major concern for animal health. Various chemical methods and toxin binders are available to decrease the harmful effects of cotton seed cake feeding in dairy buffalo.

2.7 Research Objectives

The objectives of this research were as follows:

1- To evaluate the efficacy of various chemical methods to detoxify cotton seed cake.
2- To evaluate the palatability and intake of feeding the chemically treated cotton seed cake to buffalo.
3- To assess the outcome of feeding lactating buffaloes chemically-treated cotton seed cake on milk yield, composition and AFM1 concentration.
4- To determine the effects of toxin binders in comparison to chemically treated cottonseed cake.
2.8 References


European Food Safety Authority (EFSA) Panel on Additive and Products or Substances used in Animal Feed. 2013. Scientific opinion on the safety and efficacy of a preparation of bentonite and sepiolite (Toxfin® dry) as feed additive for all species. EFSA J. 11(4): 3179.


REVIEW OF LITERATURE


CHAPTER 3
EXPERIMENT NO. 1

3.1 Introduction

Feed shortage is a problem for livestock farming in Pakistan due to competition between meeting food needs of humans and animals. As a consequence livestock productivity is affected including meat and milk production. Feed is the biggest cost factor in dairy farming (Ribeiro et al. 2011).

Cotton is a leading cash crop in South Asia. Cotton production in Pakistan in 2015-2016 was 9917 thousand bales (Ministry of Finance, 2017). Cotton seed cake is a byproduct arising from the extraction of oil from the seed and is the most commonly used in feeding of dairy animals. However, there are some issues with its use including availability in specific seasons contamination with low quality materials and pesticides (Zahid et al. 2003) as well as a high incidence of aflatoxin (Weidenner, 2012).

In the recent past, the quality of CSC has emerged as a major issue as most of its supply was thought to pose health problems in small as well as large ruminants. Inadequate processing and storage of this product are the major causes of the mycotoxin contamination (Yunus et al. 2015).

Mycotoxins are produced by several fungal species of Claviceps, Fusarium, Penicillium, Aspergillus and Alternaria. It has been estimated that at least 300 of these fungal metabolites are highly toxic to animals and humans (Huwig et al. 2001). Improper storage can cause contamination by aflatoxin, ochratoxin and cyclopiazonic acid in CSC. There is an increased awareness for human and animal health hazards due to the presence of fungi and their toxic metabolites in feed and food (Huwig et al. 2001). As a consequence of feeding aflatoxin-contaminated feeds, animal food products like milk and meat may contain aflatoxins although other health issues to animals also prevail (Visconti, 1998). These mycotoxins are harmful to human beings as well, as aflatoxin B$_1$ (AFB$_1$) can be transmitted through infected milk. After the intake of feed contaminated with AFB$_1$, it is hydroxylated to aflatoxin M$_1$ (AFM$_1$), which is then transferred in to blood and through blood comes in to the milk, and when animals (calves in particular) and humans consume the milk they also consume AFM$_1$ (Hashmi, 2016). Because of the potential adverse effects on both animal and human health, there is need for practical and cost-effective measures to reduce contamination of feeds by mycotoxins. Although only a few have real practical application, a number of approaches have already been adopted to address this issue (Kolossova et al. 2009).

Physical treatment is first option to reduce mycotoxin contamination of feeds. This option involves washing, polishing, mechanical separation, flotation and autoclaving (Jouany, 2007). Chemical treatments include calcium hydroxide (Ca(OH)$_2$), ammoniation, copper sulfate (CuSO$_4$), sodium bisulfide (NaHS) and various acids (Giovatiet al., 2015).

The exact concentration of various chemicals to reduce aflatoxin contamination levels in cattle feed has not been clearly proven. Thus, the theme of this study was to assess the efficacy of various concentrations of copper sulfate, benzoic acid, calcium propionate (C$_6$H$_{10}$CaO$_4$) and sodium bisulfide in detoxifying AFB$_1$ and AFB$_2$.

3.2 Materials and Methods

This trial was carried out in Nutrition Laboratory at the Buffalo Research Institute (BRI), Pattoki, District Kasur. The concentrations of the chemical binders used were based on those reported in the literature. In the first step, about 80 kg of CSC with aflatoxin contamination levels of around 100 ppb was collected from different cotton expellers and the market. The level of natural AFB$_1$ and AFB$_2$ contamination of the CSC was determined using the Elisa Kit method (insert reference), as described in Section 3.3.3.

Four concentrations (0.25%, 0.5%, 0.75% and 1.0%) of copper sulphate, benzoic acid, calcium propionate and sodium bisulfide were used to treat aflatoxin-contaminated CSC as described below.

3.2.1 Treatment with copper sulphate, benzoic acid or calcium propionate

For each allocated chemical treatment (with three replicates) and a control, approximately 5 g (known weight) of the ground CSC was weighed in to a conical flask to which was added 9 mL of distilled water to bring the moisture level of the CSC to 180 g/kg. The flask were then autoclaved at 121°C for 15 min after which they were inoculated with 0.1 mm spore suspension (25×10$^7$ CFU/ml) of Aspergillus
parasiticus strain NRRL. The incubation of each was done at temperature of 27-30°C for 7 days in darkness. For spore counting 1 g of sample was taken, aflatoxin estimation of remaining sample at day 7 was done after drying and autoclaving. Benzoic acid was directly added to contaminated samples according to the respective doses. Every effort was ensured that treatment was equally distributed to every part of CSC

3.2.2 Treatment with sodium bisulfide

Sodium bisulfide was certified ACS grade. 1 kg portions of naturally contaminated CSC were treated by placing into 1 L flasks and chemical treatment was done according to their respective levels on the dry matter basis. Distilled water was also added to bring the moisture content of samples to approximately 20%. Flasks was sealed, thoroughly mixed by hand for a few minutes and stored (Samarajeewa et al. 1990)

3.2.3 Aflatoxin B1 and B2 concentrations.

Determination of AFB1 and AFB2 concentrations was done using ELISA kit method by using Aflatoxin Detection-8030 Veratox kit before and after the treatment. The number of blue - bordered dilution strips required for the samples and the standards was placed in a micro well strip holder. Equal numbers of antibody coated strip were also placed in a micro well strip holder. A multichannel pipette was used to deliver 100 mL of the conjugate into each of the blue bordered dilution wells. The multichannel was used to mix the sample by carefully pipetting it up and down three times. 100 mL of the mixture was immediately transferred into the antibody coated plate and incubated for 15 min at room temperature. The optical density reading for each micro well was recorded. A standard curve was generated using aflatoxin concentrations (for AFB1 and ABF2). The solutions covered thr range of 0.25-4 ng for AFB1 and 0.0625-1 ng for AFB2 (Leszczynska et al. 2001).

3.2.4 Proximate analysis

Samples of the untreated treated and untreated CSC were dried to constant weight at 55°C and then ground to a particle size of 2 mm using a Wiley mill. The samples (in duplicate) then underwent proximate analysis using the methods of AOAC (1990).

3.2.5 Statistical analysis

Data was analyzed by ANOVA technique through Proc-Mixed (SAS 9.1) (Steel et al. 1997).

3.3 Results

3.3.1 Aflatoxin concentrations

The effect of the chemical treatments on AFB1 and AFB2 concentrations in CSC is presented in Table 3.1. Only 0.5% calcium propionate, 0.25% benzoic acid and 0.25% copper sulfate were effective (P < 0.05) in reducing AFB1 concentrations. Calcium propionate 0.5% level decreased AFB1 from 100ug/kg to 16ug/kg followed by Benzoic acid 0.25% level which limited AFB1 to 23ug/kg. Copper sulfate (0.25%) level stood third by decreasing AFB1 to 25ug/kg. In the case of AFB2, Calcium propionate 0.5% level reduced the AFB2 from 10ug/kg to 1.5ug/kg (P < 0.05) followed by 2ug/kg which was shown by Calcium propionate 0.25% level, Copper sulphate 0.25% and Benzoic acid 0.25% level (Table 3.3). Treatment with sodium bisulfide had no effect (P > 0.05) in reducing the AFB1 and AFB2 concentrations in the CSC.
Table 3.1: Effect of chemical treatment on aflatoxin B₁ and B₂ concentrations in cotton seed cake.

<table>
<thead>
<tr>
<th>Chemical treatment</th>
<th>Concentration (%)</th>
<th>Aflatoxin concentration (µg/kg DM)</th>
<th>B₁</th>
<th>B₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>103⁺</td>
<td>10⁺</td>
</tr>
<tr>
<td>Copper sulfate</td>
<td>0.25</td>
<td>25ᵇ</td>
<td>2ᵇ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>50ᵃ</td>
<td>4ᵇ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>70ᵃ</td>
<td>7.5ᵃ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>88ᵃ</td>
<td>8ᵃ</td>
<td></td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>0.25</td>
<td>23.3ᵇ</td>
<td>2ᵇ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>39.9ᵇ</td>
<td>3ᵇ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>89.3ᵃ</td>
<td>8.5ᵃ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>98.4ᵃ</td>
<td>8ᵃ</td>
<td></td>
</tr>
<tr>
<td>Calcium propionate</td>
<td>0.25</td>
<td>27.5ᵇ</td>
<td>2ᵇ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>16ᵇ</td>
<td>1.5ᵇ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>49.1ᵃ</td>
<td>5ᵃ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>80ᵃ</td>
<td>8ᵃ</td>
<td></td>
</tr>
<tr>
<td>Sodium bisulfide</td>
<td>0.25</td>
<td>100ᵃ</td>
<td>9ᵃ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>95ᵃ</td>
<td>9ᵃ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>93ᵃ</td>
<td>9.5ᵃ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>90ᵃ</td>
<td>9.5ᵃ</td>
<td></td>
</tr>
</tbody>
</table>

Values within columns with varying superscripts differ significantly (P < 0.05)

3.3.2 Proximate analysis
Chemical treatment had no effect on the proximate analysis of the CSC (Table 3.2).

Table 3.2: Proximate analysis of cotton seed cake before and after chemical treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (%)</th>
<th>Crude protein</th>
<th>Fat</th>
<th>Fiber</th>
<th>Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>23.5</td>
<td>8.5</td>
<td>27.4</td>
<td>6.8</td>
</tr>
<tr>
<td>Copper sulfate</td>
<td>0.25</td>
<td>23.3</td>
<td>8.4</td>
<td>27.0</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>23.0</td>
<td>8.0</td>
<td>27.2</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>23.1</td>
<td>8.2</td>
<td>27.5</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>23.2</td>
<td>7.9</td>
<td>27.0</td>
<td>6.5</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>0.25</td>
<td>23.5</td>
<td>8.3</td>
<td>27.5</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>23.4</td>
<td>8.5</td>
<td>27.3</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>23.2</td>
<td>8.1</td>
<td>27.0</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>23.0</td>
<td>8.0</td>
<td>26.8</td>
<td>6.6</td>
</tr>
<tr>
<td>Calcium propionate</td>
<td>0.25</td>
<td>23.6</td>
<td>8.7</td>
<td>27.5</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>23.7</td>
<td>8.3</td>
<td>27.2</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>23.5</td>
<td>8.4</td>
<td>27.0</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>23.1</td>
<td>8.5</td>
<td>27.3</td>
<td>6.7</td>
</tr>
<tr>
<td>Sodium bisulfide</td>
<td>0.25</td>
<td>23.5</td>
<td>8.7</td>
<td>27.5</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>23.7</td>
<td>8.5</td>
<td>27.0</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>23.3</td>
<td>8.0</td>
<td>27.3</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>24.0</td>
<td>8.1</td>
<td>27.8</td>
<td>6.1</td>
</tr>
</tbody>
</table>

3.4 Discussion
Our results coincide with the Mukendi et al. (1991). They used different chemicals to detoxify aflatoxin. However, they used sodium sulfite, sodium hydroxide and hydrogen peroxide. The above-mentioned chemicals performed well. Our study is also in agreement with Bintvihok and Kositcharoenkul (2006). These scientists used different levels of calcium propionate in feed and 0.5% level was proved effective to control aflatoxin B₁. Moreover, our experiment is in partially live with Arshad et al. (2012),
Arshad et al. (2012) used chemicals in the culture of Aspergillus. The chemicals were only used for the Aspergillus species. Alam et al. (2014) also used calcium propionate to increase the storage time of feed. They used 0.5g level of calcium propionate for 1kg of finished feed. The level used in this study was lower as compared to our study. The difference of dose was due to different forms of feed. Alam et al. (2014) used broiler finisher feed, so less level was used. In our study, best results (P<0.05) were shown for 0.5 %level because cattle feed have normally higher level of aflatoxin (B1 &B2). Meanwhile, a study conducted by Ismail et al. (2018) concluded that reduction of aflatoxin (B1) is 85-90% through chemical detoxificants. In this study best reduction was 84% by calcium propionate.

Prevention of mycotoxin production begins by prevention of the growth of mycotoxin-producing fungi. Hussain and Ali (2012) reported completed inhibition of growth of Aspergillus parasiticus, by treatment with benzoic acid (0.2 - 0.5 %), copper sulphate (0.1 - 0.5 %) and propionic acid (0.2 - 0.5 %). Gowda et al. (2004) found that 0.2% benzoic acid completed inhibited aflatoxin production, while 0.08% copper sulfate resulted in a significant reduction in aflatoxin production. They also found sodium propionate (at 0.1, 0.2 and 0.5%) in feed eliminated aflatoxin production.

Most previous studies have involved the use of sodium bisulfite as opposed to sodium bisulfide used in this study. However, there are similarities in the findings. Hagler et al. (1982) found AFB2 was not susceptible to the action of sodium bisulfite, although there was reaction with AFB1, as also reported by Yagan et al. (1989). However, the form of sodium bisulfite used may impact results, with the powder form found to be ineffective while using 8% bisulfite solution reduced AFB1 in copra by 98% (Mercado et al. 1991).

Several studies have reported the use of propionate salts to control fungus growth in stored feed products. Marin et al. (2000) reported a significant reduction in the total fungal viable count in maize with 0.05–1.0% propionate-based preservatives while 0.6% calcium propionate significantly depressed A. flavus count in poultry feed (Vanselow et al. 1985). Alam et al. (2014) found calcium propionate (5 g/kg DM) was an effective means of controlling aflatoxin production in poultry feed.

3.5 Conclusion

Contamination of Cotton Seed Cake with aflatoxin is one of the most important tasks which are being confronted by the feed manufacturing and common livestock owners. We used a variety of chemical detoxificants to mitigate this problem. In vitro Calcium propionate 0.5% gave good results to reduce AFB1&AFB2.

3.6 Acknowledgements

The author is especially thankful to Dr. Maqsood Akhtar, Chief Research Officer for BRI for funding for this study. The author is also grateful to Mr. Bilal Mansha and Mr. Umar Hayat, laboratory technicians at the Nutrition Laboratory at BRI for their assistance in sourcing of AF infected CSC and AF analysis.

3.7 References


4.1 Introduction

Aflatoxin is an emerging problem nowadays, originating from the intake of Aspergillus species infected feed or feed ingredients. The most commonly affected feeds are corn, peanuts and cotton seed, including cotton seed byproducts (Sulzberger et al. 2017).

Aflatoxin B₁ is the most toxic form of the aflatoxins, being immunosuppressive (Mohsenzadeh et al. 2016) and carcinogenic (McKean et al. 2006) in both animals and humans. Additional health impacts of aflatoxins include teratogenicity (Wangikar et al. 2005), genotoxicity (Gross-Steinmeyer and Eaton, 2012), hepatotoxicity (Lu et al. 2013) and cytotoxicity (Zhang et al. 2015; Ismail et al. 2018). Aflatoxins are strongly linked with growth impairment, including stunting and wasting in humans (Khangwiset et al. 2011; Magoha et al. 2014) and animals and poultry (Pimpukdee et al. 2004; Han et al. 2008). Clinical signs of chronic aflatoxin intoxication in cattle in particular include decreased appetite, weight loss, decreased feed efficiency, and decreased milk production (Queiroz et al. 2012).

Cotton seed cake (CSC) is a readily available protein source in Pakistan. However, in recent years, concerns have been raised over contamination by aflatoxins as a consequence of inadequate processing and storage (Yunus et al. 2015). The Commission of European Communities (2003) set a maximum accepted/residue levels for aflatoxin in animal feeds as 20 μg/kg ppb in all feed materials and in the most feedstuffs for cattle, sheep, goats, pigs and poultry, while it is 5 ppb in complete feeding stuffs for dairy animals and 10 μg/kg for complete feeding stuffs for calves and lambs. Results from various feed samples analyzed for AFB₁ at the Nutrition Division, Buffalo Research Institute (BRI) revealed AFB₁ concentrations above 100μg/kg, which may have significant impacts on animal health and production.

Calcium (Ca) propionate can be used to inhibit mycotoxin production (see Chapter 3) and has been shown to be effective in suppressing the germination, growth rate and aflatoxin production of Aspergillus flavus (Alam et al. 2010). The antimicrobial properties of Ca propionate involve the uncoupling of microbial substrate transport and oxidative phosphorylation from the electron transport system (Saftner et al. 2003). The aim of this study was to investigate the effectiveness of various concentrations of Ca propionate in mitigating any adverse effects of feeding aflatoxin-contaminated CSC to dry buffaloes. The parameters assessed included dry matter intake (DMI), average daily live weight gain (ADG), body condition score (BCS), urine pH and serum mineral concentrations.

4.2 Materials and Methods

All experimental methods were approved by the Animal Care and Ethical Committee (Approval number 1173) of the University of Veterinary and Animal Sciences, Lahore.

4.2.1 Animal housing and experimental design

The feeding trial was carried out at Buffalo Research Institute, Pattoki and was based on a random, complete blocks design. Twenty Nili Ravi dry buffaloes, weighing 600 ± 20 kg and of approximately 5 years of age, were selected. They were randomly assigned one to five dietary treatments, with each dietary treatment thus having four buffaloes. The animals were kept in covered sheds, grouped housed, however were individually fed. The feeding trial was conducted over a total period of 60 days, including 07 days dietary adaptation period.

4.2.2 Experimental diets

All of the buffaloes were fed at a level to meet maintenance requirements. They were all fed a concentrate (either “normal” or CSC) and their maintenance requirement was fulfilled with corn silage fed at the rate of 2.5% of BW (on DM basis) and wheat straw fed at the rate of 0.9% of BW (on DM basis). The CSC had an aflatoxin contamination level of at least 100ug/kg.

The dietary treatments involved variation to the concentrate ration fed and included: A) normal concentrate (Table 4.1); B) un-treated CSC; C) CSC treated with 0.25% Ca propionate; 4) CSC treated with 0.5% Ca propionate; and E) CSC treated with 0.75% Ca propionate. Treatment A represented a negative control, while Treatment B represented a positive control. The nutritive values of the normal concentrate, CSC, corn silage and wheat straw are shown in Table 4.2.
Table 4.1: Composition of the normal concentrate (without cotton seed cake).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Inclusion level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>15</td>
</tr>
<tr>
<td>Rape seed meal</td>
<td>10</td>
</tr>
<tr>
<td>Canola meal</td>
<td>6.5</td>
</tr>
<tr>
<td>Maize gluten meal (30%)</td>
<td>20</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>34</td>
</tr>
<tr>
<td>Molasses</td>
<td>12.5</td>
</tr>
<tr>
<td>Mineral mixture</td>
<td>0.5</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>1</td>
</tr>
<tr>
<td>Marble powder</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The daily feed allocations (for roughages and concentrate) were split into two equal portions, one fed at 0900, and the other fed at 1630. Feed refusals were recorded daily to enable determination of feed intakes (on a DM basis). The animals had *ad libitum* access to fresh, clean water at all times.

Table 4.2. Proximate analysis of diet ingredients. *Table 4.2*

<table>
<thead>
<tr>
<th>Feed ingredient</th>
<th>Dry matter (%)</th>
<th>Crude protein (%)</th>
<th>Fat (%)</th>
<th>Fibre (%)</th>
<th>Ash (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal concentrate</td>
<td>89.61</td>
<td>16.32</td>
<td>2.96</td>
<td>4.74</td>
<td>3.88</td>
</tr>
<tr>
<td>Cotton seed cake</td>
<td>91.9</td>
<td>23.44</td>
<td>8.8</td>
<td>27.40</td>
<td>6.80</td>
</tr>
<tr>
<td>Corn silage</td>
<td>72.5</td>
<td>8.5</td>
<td>1.8</td>
<td>27.1</td>
<td>9.8</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>90.5</td>
<td>3.0</td>
<td>0.1</td>
<td>41.8</td>
<td>10.9</td>
</tr>
</tbody>
</table>

4.2.3 Animal data

In addition to feed intake, data relating to live weight and BCS was also collected. The animals were weighed on a digital balance *YH-T6 Shanghai Yaohua* on fortnightly basis. The BCS of the animals was assessed every 2 weeks by two evaluators and scores were averaged (for each animal)

4.2.4 Collection and analysis of samples

During the last week of the feeding trial blood was taken from the coccygeal vein of each buffalo using a plain vacum tube (*Bio-Vac*) and 18 gauge needle. Serum samples were obtained by centrifugation of the tubes at 2,500 x g for 15 min at 4°C. After this samples were stored at -20°C till further analysis.

The serum samples were sent to Provincial Diagnostic Laboratory for analysis of Ca, potassium (K), sodium (Na) and chloride (Cl) concentrations. The urine was collected in midstream in a bucket and then transferred to a small beaker. The PH was measured using *MI 151 pH meter* (*Martini instruments*)

4.2.5 Calculations

Feed efficiency for each of the treatment groups was calculated by dividing total feed intake by the total live weight gain of the animals.

4.3 Results

4.3.1 Dry matter intake

The average DMI of the concentrate rations and roughages and total DMI are presented in Table 4.3. The buffaloes fed untreated CSC (Group B) had significantly higher total DMI compared with all other treatment groups. Those buffaloes fed either the “normal” concentrate (Group A) or CSC treated with 0.75% Ca propionate (Group E) had significantly lower (P < 0.05) total DMI compared with the other treatment groups. There was no difference (P > 0.05) in total DMI of those buffaloes fed CSC treated with either 0.25% (Group C) or 0.5% (Group D) Ca propionate.
Table 4.3 Effects of feeding cotton seed cake treated with different levels of calcium propionate on the dry matter intakes (kg/day) of dry buffaloes.

<table>
<thead>
<tr>
<th>Feed</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
<th>Group E</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentrate</td>
<td>4.00a</td>
<td>5.74a</td>
<td>5.22b</td>
<td>5.36b</td>
<td>4.2c</td>
<td>0.0001</td>
</tr>
<tr>
<td>Corn silage</td>
<td>7.84</td>
<td>7.67</td>
<td>7.90</td>
<td>7.84</td>
<td>7.55</td>
<td>0.37</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>4.52</td>
<td>4.31</td>
<td>4.22</td>
<td>4.25</td>
<td>4.33</td>
<td>0.55</td>
</tr>
<tr>
<td>Total</td>
<td>16.34± 3.02c</td>
<td>17.72± 1.31a</td>
<td>17.34± 1.18b</td>
<td>17.45± 1.10b</td>
<td>16.08± 1.15c</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation. Values within rows with varying superscripts differ significantly.

4.3.2 Live weight gain and body condition score

As shown in Table 4.4, neither the source of concentrate nor the level of feeding of Ca propionate had any effect (P > 0.05) on the ADG of the dry buffaloes.

Table 4.4. Effects of feeding cotton seed cake treated with different levels of calcium propionate on live weight and body condition score of dry buffaloes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
<th>Group E</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial live weight (kg)</td>
<td>583</td>
<td>612</td>
<td>605</td>
<td>595</td>
<td>615</td>
<td></td>
</tr>
<tr>
<td>Final live weight (kg)</td>
<td>613</td>
<td>654</td>
<td>644</td>
<td>649</td>
<td>646</td>
<td></td>
</tr>
<tr>
<td>Live weight gain (kg/d)</td>
<td>0.50±0.15</td>
<td>0.70±0.44a</td>
<td>0.65±0.25a</td>
<td>0.90±0.11a</td>
<td>0.51±0.34a</td>
<td>0.39</td>
</tr>
<tr>
<td>Initial BSC</td>
<td>3.0</td>
<td>2.9</td>
<td>3.1</td>
<td>3.0</td>
<td>3.15</td>
<td></td>
</tr>
<tr>
<td>Final BCS</td>
<td>3.2±0.24</td>
<td>3.25b±0.20</td>
<td>3.37c±0.14</td>
<td>3.63d±0.16</td>
<td>3.56e±0.23</td>
<td>0.0001</td>
</tr>
<tr>
<td>Feed efficiency</td>
<td>32.68±0.23</td>
<td>25.31b±0.15</td>
<td>26.67b±0.16</td>
<td>19.38c±0.20</td>
<td>31.52a±0.22</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation. Values within rows with varying superscripts differ significantly.

4.3.3 Feed efficiency

As shown in Table 4.4, feed efficiency for groups A,B,C,D and E was 32.68±0.23, 25.31±0.15,26.67±0.16,19.38±0.20 and 31.25±0.22.There was significant difference among the treatments (P<0.05)

4.3.4 Urine pH

There were not significant differences (P > 0.05) in urine pH, with the average (± standard deviation) of Groups A, B, C, D and E being 6.90 ± 0.61, 6.97 ± 0.41, 6.97 ± 0.63, 6.96 ± 0.14 and 6.97 ± 0.42, respectively.

4.3.5 Serum mineral concentrations

As shown in Table 4.5, there were no differences (P > 0.05) in any of the serum parameters assessed.

Table 4.5 Effects of feeding cotton seed cake treated with different levels of calcium propionate on serum calcium, potassium, sodium and chloride concentrations

<table>
<thead>
<tr>
<th>Serum</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
<th>Group E</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mg/dL)</td>
<td>10.27</td>
<td>10.15</td>
<td>9.72</td>
<td>10.42</td>
<td>10.65</td>
<td>0.36</td>
</tr>
<tr>
<td>Potassium (meq/L)</td>
<td>5.5</td>
<td>5.5</td>
<td>4.75</td>
<td>4.0</td>
<td>4.75</td>
<td>0.51</td>
</tr>
<tr>
<td>Sodium (meq/L)</td>
<td>77.25</td>
<td>87.25</td>
<td>82.5</td>
<td>71.5</td>
<td>83</td>
<td>0.75</td>
</tr>
<tr>
<td>Chloride (meq/L)</td>
<td>85.25</td>
<td>98.0</td>
<td>95.75</td>
<td>99.25</td>
<td>98.75</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation.
4.4 Discussion

4.4.1 Dry matter intake

With the exception of 0.75% Ca propionate treatment (Group E), the feeding of CSC resulted in a significant increase in total DMI compared with the feeding of the “normal” concentrate ration. Thus an aflatoxin contamination level of at least 100 ppb (as found in the CSC) had no adverse effects on DMI. Similar results were reported by Quiroz et al. (2012) for dairy cattle fed an AFB1-contaminated diet (75 ppb). In contrast, Choudary et al. (1998) reported significant reduction in DMI in a dose dependent manner in cattle dosed with 10 to 108.5 ppb of aflatoxin. The apparent resistance to any adverse effects of aflatoxin in the CSC may be due to the mature age of the buffaloes, with younger ruminant animals typically more susceptible to aflatoxin intoxication (Baines et al. 2013; Kaleibar and Helan 2013; Atherstone et al. 2016).

In comparing the groups fed CSC, treatment with any level of Ca propionate caused a significant (P < 0.05) reduction in total DMI (compared with the untreated CSC). Aside from its potential to detoxify aflatoxins, for ruminants, Ca propionate is effectively a gluconeogenic precursor and addition to the diet has improved animal performance in some situations (Overton and Waldron, 2004) McNamara and Valdez (2005) found Ca propionate supplementation (0.125 kg/d) increased DMI by 11% prepartum and by 13% postpartum in Holstein dairy cows. In contrast, Lee-Rangel et al. (2012) found supplementation of 10 g/kg DM of Ca propionate had no effect on feed intake of finishing Criollo lambs. Similarly, Zhang et al. (2017) found no differences in DMI in Jersey calves with the different feeding levels of Ca propionate. The reason(s) for the adverse effects of Ca propionate on total DMI of the dry buffaloes fed CSC is not readily apparent and warrants further research.

4.4.2 Live weight and body condition score

Despite differences in DMI, neither the type of concentrate ration fed nor treatment of CSC with Ca propionate impacted on ADG, of dry buffaloes, with all animals gaining weight over the feeding period. This is in agreement with other researchers who found that neither aflatoxin challenge nor the use of aflatoxin binders affected weight gain in Holstein cattle (Kutz et al. 2009; Queiroz et al. 2012; Sulzberger et al. 2017). However, Abdel-Latif et al. (2016) found supplementation of Ca propionate in primiparous buffalo cows during late gestation and early lactation significantly improved body weight. Zhang et al. (2017) found that although there were no differences in DMI, the addition of Ca propionate (10%) improved the growth performance of Jersey calves, likely as a result of increased supply of glucose precursors (Liu et al. 2010). The low level of Ca propionate supplementation used in this study would likely have made little difference to the supply of glucose precursors and account for the lack of differences in ADG between the treatment groups.

4.4.3 Feed efficiency

As shown in Table 4.4, feed efficiency for groups A,B,C,D and E was 32.68±0.23, 25.31±0.15,26.67±0.16,19.38±0.20 and 31.25±0.22. There was significant difference among the treatments (P<0.05). The feed efficiency was better in group consuming 0.5% treated cotton seed cake as compared to other groups. The feed efficiency was higher in group A and then C. The best results were taken in 0.5%-treated cotton seed cake. Lee-Rangel et al. (2012) found supplementation of 10 g/kg DM of Ca propionate had no effect on the feed conversion ratio of finishing Criollo lambs. In contrast, in studies using broiler chickens, Bintvihok and Kositcharoenkul (2006) found supplementing Ca propionate in an aflatoxin-contaminated diet resulted in improved feed conversion ratio. Broiler chickens are much more susceptible to aflatoxins that ruminants and the detoxifying effects of the Ca propionate would account for the improvement in the feed conversion ratio.

4.4.4 Urine pH

Urine pH is a useful method for predicting urine Ca concentrations, particularly in periparturient dairy cattle (Constable et al. 2019) and combined with no changes to in serum Ca concentrations indicates that the feeding of Ca propionate had no effect on Ca homeostasis of the dry buffaloes. Urine pH also indicates any changes in the dietary cation-anion difference (DCAD). When DCAD decreases, urine pH decreases (Block, 1994). Treatment of CSC with Ca propionate had no effect on DCAD.
4.4.5 Serum mineral concentrations
Neither the source of concentrate nor the treatment of CSC with Ca propionate had any effect on serum Ca, K, Na and Cl concentrations, and supports that there were also no changes in DCAD. Calcium propionate can be absorbed by across the rumen and increase the ionized Ca level in the blood (Zhang et al. 2020). However, Stokes and Goff (2001) found no change in plasma Ca concentrations of dairy cows administered Ca propionate at calving.

4.5 Conclusion
The feeding of CSC contaminated by aflatoxins had no adverse effects on DMI or ADG of dry buffaloes. Treatment of the CSC with Ca propionate (to detoxify the aflatoxins) resulted in decreased DMI but with no effects on ADG, indicating an increase in feed efficiency.

4.6 Acknowledgements
The author is thankful to the staff at the Livestock Experiment Station, Bhunikey, Pattoki (a sub centre of BRI Pattoki) for selection of buffaloes, conducting of the feeding trial and sample collection.

4.7 References


CHAPTER 5
EXPERIMENT NO. 3

5.1 Introduction

Aflatoxins are secondary metabolites produced by Aspergillus species (Maki et al. 2016). The most common kinds of aflatoxin are aflatoxin B1 (AFB1) and aflatoxin B2 (AFB2). These metabolites are immunosuppressive and carcinogenic in humans as well as animals (Peers et al. 1987). Feed is commonly contaminated with AFB1 and AFB2 both pre- and post-harvest, with the latter due to inadequate processing and storage (Yunus et al. 2015).

Cotton seed cake (CSC) is the most extensively used protein source for ruminant nutrition in Pakistan. Cotton seed cake is often contaminated with aflatoxins (Mazen et al. 1990; Dhaven and Chudary, 1995; Rustom, 1997; Weidenner, 2012), including in Pakistan (Ashiq, 2015; Chohan et al. 2016; Akbar et al.2020; Shar et al. 2020). Contamination of feeds with AFB1 and AFB2 are major concerns for both the general livestock feed and dairy industries of Pakistan.

When rations contaminated with AFB1 are fed to lactating ewes (Battacone et al. 2003), cattle (Diaz et al. 2004; Xiong et al. 215; Gonclaves et al. 2017) or buffaloes (De Roma et al. 2017; Aslam et al. 2016; Naveed et al. 2018; Guo et al. 2019), the toxin is metabolized in liver into the hydroxylated derivative aflatoxin M1 (AFM1) which is then transported to mammary gland via blood and excreted in milk (Kuilman et al.1998). Aflatoxin M1 will come in the milk following 2–3 days of ingestion of aflatoxin-contaminated feed. When a feed without aflatoxins is subsequently fed, the AFM1 concentration reduce to zero in 2–3 days (Veldman et al. 1992).

As reported in Chapter 3, treatment of CSC with Ca propionate is one means of detoxifying CSC. The objective of this experiment was to examine the effectiveness of several concentrations of Ca propionate in mitigating any adverse effects, including AFM1 concentrations in milk, of feeding aflatoxin-contaminated CSC to lactating buffaloes.

5.2 Materials and Methods

5.2.1 Animal care and housing

All experimental methods were approved by the Animal Care and Ethical Committee (Approval number 1173) of the University of Veterinary and Animal Sciences (UVAS), Lahore. The experiment commenced on 12.11.2018 and concluded ended on 30.01.2019 and was conducted at the Buffalo Research Institute, Pattoki District Kasur.

The experiment consisted of four 20-d periods and involved milking at 400 and 1600, and individual feeding at 08:00 hour. The buffaloes were individually tethered.

5.2.2 Animals, experimental design and treatments

Sixteen Nili Ravi, multiparous buffaloes of about 600 ± 50 kg of BW were used in a 4 ×4 Latin square design feeding trial. For this body weight, it was estimated that buffaloes would consume 15-16 kg DM. The naturally contaminated (100 ppb of AFB1) was sourced from the local market, and the AFB1 contamination level confirmed by ELISA analysis at the World Trade Organization laboratory, UVAS, Lahore.

The CSC (RDH Company) was treated with three levels of Ca propionate (0.25%, 0.5% and 0.75%). In each of 20-d period, the buffaloes were randomly assigned to one of four dietary treatments, thus by the end of the trial tenure all of the buffaloes had been part of each dietary treatment. All animals were fed the same base diet (corn silage, wheat straw and CSC) ad libitum (approximately 3% of BW).

The dietary treatments were based on the treatment of CSC with varying levels of Ca propionate and included: (1) untreated CSC control (CON) (2) low dose Ca propionate (CaP 0.25%); (3) medium dose Ca propionate (CaP 0.5%); and (4) high dose calcium propionate (CaP 0.75%).

5.2.3 Feed and faeces collection and analysis.

Samples of the CSC, wheat straw and fodder were taken on Day 18 of each period and subsequently pooled by period. To enable determination of DMI, the feed samples were oven-dried at 65°C at Nutrition Division, BRI, Pattoki District Kasur, Pakistan.
During the last 3 d of each period, all faeces were collected (from individual animals) to enable determination of DM digestibility CP digestibility and neutral detergent fiber (NDF) digestibility.

Duplicate feed samples and faeces were analysed for DM DM (method 930.15; AOAC International, 2000), N (method 990.03; Leco FP-528 Nitrogen Combustion Analyzer, LecoCorp. St. Joseph, MI), and NDF (Van Soest et al. 1991) contents.

5.2.4 Milk data collection

Individual milk production was measured and recorded daily. Measurements from the first 15 d of each period were used to evaluate milk production. Milk samples were taken during the morning and night milking of Days 14 and 15 were analyzed for fat, lactose, SNF, milk density and protein (AOAC International, 2000) at the Nutrition Laboratory, UVAS, Ravi Campus, Pattoki using Milkotroniclactoscan S 60.

For determination of AFM1 concentrations, the milk samples were processed according to official AOAC International methods. Briefly, samples were warmed to 37°C, centrifuged for 20 min at 2,000 x g, and defatted.

The columns were washed twice with 10 mL of double distilled, deionized water (MilliQ 18.2 M cm), and eluted with 4 mL of acetonitrile. The column temperature and injection volume were 40°C and 10uL, respectively. The AF standards were purchased from Sigma Chemical Co. (St. Louis, MO). All solvents were obtained from Fisher Scientific (Pittsburgh, PA). The AF levels were quantified with the instrument software (Empower 2, Waters Corporation, Milford, MA).

5.2.5 Calculations

5.2.5.1 Milk AFM1 transfer

Milk AFM1 transfer variables were calculated as follows:
Excretion in milk (□ g/d) = concentration of AFM1 in milk (µg/L) x milk yield (L/d)

Transfer to milk (%) = excretion of AFM1 in milk (□ g/d) / AFB1 consumption (□ g/d) x 100

5.2.5.2 Apparent nutrient digestibility

Digestibility% = \frac{\text{Feed Intake–Faeces go out}}{\text{Feed Intake}} \times 100

5.3.6 Statistical analysis

Data were analyzed as replicated 4 x 4 Latin Squares by SAS (Version 9.3, SAS Institute Dnc. Cary, NC). Statistical significance for all treatments effects was declared at P < 0.05.

5.3 Results

5.3.1 Nutritive value and aflatoxin contamination of the diet

The nutritive value and aflatoxin contamination of the diet fed to the lactating buffaloes is presented in Table 5.1.

5.3.2 Dry matter intake and milk production

As shown in Table 5.2, treatment of CSC with Ca propionate had no significant result (P > 0.05) on either total DMI or milk production. However, addition of Ca propionate resulted in a significant increase in the intake of CSC intake.

5.3.3 Milk composition

Treatment of CSC with Ca propionate had no significant result (P > 0.05) on milk fat, protein and lactose contents and milk density (Table 5.3). Overall, milk composition averaged 8.22 ± 1.71% for fat and 3.38 ± 0.35 for protein.

5.3.4 Aflatoxin in milk (AFM1)

Dietary inclusion of Ca propionate showed decreases (P < 0.001) in the transfer of aflatoxins to milk and thus concentrations of AFM1 in milk, as shown in Table 5.3. The most effective level of inclusion of Ca propionate to minimise AFM1 concentration was 0.5%, followed by 0.75% and then 0.25% (P < 0.001).

5.3.5 Apparent digestibility of nutrients

As shown in Table 5.4, treatment of CSC with Ca propionate had no effect (P > 0.05) on the apparent digestibility of DM, CP and NDF.
Table 5.1 Ingredient, chemical composition and predicted nutritive value of the diet fed to lactating Nili Ravi buffaloes.

<table>
<thead>
<tr>
<th>Ingredient/parameter</th>
<th>Dry matter content (%)</th>
<th>As fed basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn silage</td>
<td>54.47</td>
<td>75.95</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>23.86</td>
<td>12.66</td>
</tr>
<tr>
<td>Cotton seed cake</td>
<td>19.09</td>
<td>10.13</td>
</tr>
<tr>
<td>Mineral premix</td>
<td>2.58</td>
<td>1.27</td>
</tr>
</tbody>
</table>

**Nutrient content (as analysed)**

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>% DM</th>
<th>As fed basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>9.55</td>
<td>4.66</td>
</tr>
<tr>
<td>Acid detergent fiber</td>
<td>31.97</td>
<td>15.6</td>
</tr>
<tr>
<td>Neutral detergent fibre</td>
<td>46.87</td>
<td>22.87</td>
</tr>
<tr>
<td>Ether extract</td>
<td>4.09</td>
<td>1.99</td>
</tr>
<tr>
<td>Ash</td>
<td>7.92</td>
<td>3.86</td>
</tr>
<tr>
<td>Non-fibrous carbohydrate</td>
<td>33.3</td>
<td>16.25</td>
</tr>
</tbody>
</table>

**Nutrient content as predicted using CNCPS* system**

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>% CP</th>
<th>% DM</th>
<th>As fed basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rumen undegradable protein (%) CP</td>
<td>37.53</td>
<td></td>
<td>37.53</td>
</tr>
<tr>
<td>Rumen degradable protein (%) CP</td>
<td>62.47</td>
<td></td>
<td>62.47</td>
</tr>
<tr>
<td>Metabolisable protein (%) DM</td>
<td>5.96</td>
<td></td>
<td>2.91</td>
</tr>
<tr>
<td>Metabolisable energy (mCal/kg DM)</td>
<td>2.12</td>
<td></td>
<td>1.03</td>
</tr>
<tr>
<td>Net energy for lactation (mCal/kg DM)</td>
<td>1.36</td>
<td></td>
<td>0.67</td>
</tr>
<tr>
<td>Salicylic acids (%) DM</td>
<td>3.58</td>
<td></td>
<td>1.75</td>
</tr>
<tr>
<td>Sugar (%) DM</td>
<td>4.05</td>
<td></td>
<td>1.98</td>
</tr>
<tr>
<td>Starch (%) DM</td>
<td>20.47</td>
<td></td>
<td>9.99</td>
</tr>
</tbody>
</table>

* Cornell Net Carbohydrate and Protein
### Table 5.2 Effect of dietary addition of calcium propionate on dry matter intake and milk production of Nili Ravi buffaloes consuming an aflatoxin contaminated diet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Level of inclusion of calcium propionate (%)</th>
<th>SEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (Control) 0.25 0.5 0.75</td>
<td></td>
<td>Treatment Linear Quadratic</td>
</tr>
<tr>
<td>Corn silage</td>
<td>7.30 7.5 7.89 8.10 0.03 0.4 0.20 0.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat straw</td>
<td>3.94 3.7 3.80 3.88 0.03 0.6 0.14 0.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotton seed cake</td>
<td>3.80 4.27 4.00 4.05 0.02 0.4 0.30 0.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Dry Matter intake(Kg/d)</td>
<td>15.04 ± 0.16 15.47 ± 0.20 15.69 ± 0.20 16.03 ± 0.20 0.02 0.50 0.20 0.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk yield (L/d)</td>
<td>10.46 ± 2.60 10.16 ± 2.52 10.18 ± 2.44 10.23 ± 2.38 0.20 0.46 0.48 0.26</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation

### Table 5.3 Effect of dietary addition of calcium propionate on the milk composition and aflatoxin M1 concentration of milk produced by Nili Ravi buffaloes consuming an aflatoxin contaminated diet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Level of inclusion of calcium propionate (%)</th>
<th>SEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (Control) 0.25 0.5 0.75</td>
<td></td>
<td>Treatment Linear Quadratic</td>
</tr>
<tr>
<td>Milk fat (%)</td>
<td>8.23 ± 1.64 8.00 ± 1.85 8.50 ± 1.65 8.19 ± 1.76 0.42 0.27 0.58 0.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk protein (%)</td>
<td>3.40 ± 0.32 3.40 ± 0.33 3.38 ± 0.33 3.42 ± 0.40 0.08 0.81 0.96 0.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solids non fat (%)</td>
<td>7.50 ± 0.47 7.77 ± 0.46 7.66 ± 0.53 7.61 ± 0.60 0.13 0.37 0.67 0.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk density (%)</td>
<td>25.38 ± 4.07 25.53 ± 3.87 25.20 ± 4.07 25.18 ± 4.26 1.00 0.92 0.62 0.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactose (%)</td>
<td>4.36 ± 0.29 4.31 ± 0.32 4.22 ± 0.24 4.26 ± 0.24 0.06 0.50 0.20 0.53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFM1 (ug/kg)</td>
<td>1.69 ± 0.02 0.84 ± 0.03 0.30 ± 0.03 0.45 ± 0.02 0.01 0.0001 0.0001 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFM1 transfer (%)</td>
<td>1.69a 0.84b 0.30 0.45c 0.01 0.0001 0.0001 0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation. Values within rows with varying superscripts differ significantly (P < 0.05).

### Table 5.4 Effect of dietary addition of calcium propionate on apparent nutrient digestibility of lactating Nili Ravi buffaloes consuming an aflatoxin contaminated diet

<table>
<thead>
<tr>
<th>Apparent digestibility</th>
<th>Level of inclusion of calcium propionate (%)</th>
<th>SEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (Control) 0.25 0.5 0.75</td>
<td></td>
<td>Treatment Linear Quadratic</td>
</tr>
<tr>
<td>Dry matter</td>
<td>63.43 ± 3.30 61.75 ± 2.97 62.68 ± 3.53 64.00 ± 2.55 0.207</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude protein</td>
<td>64.37 ± 3.30 63.87 ± 3.18 65.93 ± 2.90 65.87 ± 3.13 0.158</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutral detergent fiber</td>
<td>36.62 ± 2.02 36.56 ± 2.52 37.00 ± 1.96 36.37 ± 2.47 0.886</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

25
5.4 Discussion

5.4.1 Dry matter intake and milk production

Compared with fattening cattle, dairy cows (and therefore potentially dairy buffaloes) are considered more sensitive to aflatoxins (Applebaume et al. 1982). However, in this study the feeding of aflatoxin-contaminated (100 ppb) CSC did not have any adverse effects on the lactating buffaloes, or conversely there was no benefit of detoxification using Ca propionate. Dietary inclusion of varying levels (0.25, 0.5 and 0.75%) Ca propionate had no effect on total DMI, when contaminated CSC was included in the diet fed to dry buffaloes. Similar results were reported by Quiroz et al. (2012) and Maki et al. (2016) for dairy cattle fed AFB1-contaminated diets. However, addition of Ca propionate affected the intakes of the CSC. Same results were observed by Tipu et al.(2020) where addition of Ca propionate did not affect DMI in lactating buffaloes

Applebaume et al. (1982) reported significant decreases in milk production in cows receiving 13 mg of impure AFB1 per day, and thus it would be assumed that inclusion of detoxification agents would result in increased milk production. However, dietary inclusion of Ca propionate had no effect on milk production, which was not unexpected given that it had no effect on total DMI. Similarly, Kutz et al. (2009), Xiong et al. (2015) and Sulzberger et al. (2016) found no differences in milk yield, in dairy cows fed AFB1-contaminated feed and a detoxifying agent(s). Similar trend was also noted by Tipu et al. (2020) while using Ca propionate in lactating buffaloes.

5.4.2 Milk composition

As found in other studies involving dairy cows (Kutz et al. 2009; Xiong et al. 2015) goats (Smith et al. 1994) and ewes (Firmin et al. 2011; Battacone et al. 2012), addition of a detoxification agent (in this case Ca propionate) to an aflatoxin-contaminated diet had no effect on milk composition. Similar observations were observed by Tipu et al. (2020) while using Ca propionate as detoxificant agent in lactating buffaloes. In contrast; however, Queiroz et al. (2012) found that while the presence of aflatoxin had no effect on most milk components, it decreased milk protein concentration and milk fat yield.

5.4.3 Aflatoxin in milk (AFM1): The carryover of dietary aflatoxin into milk was 1.69% (control) which was within the range of 1 and 6% reported by the European Food Safety Authority (EFSA, 2004), with high-producing cows having greater carryover than cows with moderate-to-low milk production (Frobish et al. 1986). Secretion and transfer of aflatoxin into the milk were lower in the buffaloes supplemented with Ca propionate, supporting its usefulness as a means of partially detoxifying AFB1 and thereby reducing the amount converted to AFM1 and subsequently excreted in milk. There was not; however, a linear decrease in milk AFM1 concentrates in response to increasing inclusion levels of Ca propionate, with the greatest reduction achieved with 0.5%, with 0.75% being intermediate between 0.5% and 0.25% Ca propionate. This was also supported by Tipu et al.(2020)where they observed that Ca propionate 0.5% significantly(P<0.05) lowered M1in lactating buffaloes

5.4.4 Digestibility of nutrients

The use of Ca propionate for the detoxification of aflatoxins found in CSC had no effect on the digestibility of either DM, CP or NDF. In contrast, Ewuola et al. (2013) found aflatoxins impaired nutrient digestibility in goats. Kiyothong et al. (2012) found improved digestibility in cattle with the use of a mycotoxin deactivator and Gouda et al. (2019) reported enhanced nutrient digestibility when clay minerals were used as sorbents for mycotoxins in the diets of lactating goats.

The limited duration of the digestibility study (3 d) may have impacted results, as typically digestibility studies are conducted over 7 d (Ribeiro et al. 2011; Johnson et al. 2019) although other researchers have utilised only 3 d (Dermauw et al. 2013). In recent studies investigating apparent nutrient digestibility in buffaloes, 6 d (Paraw et al. 2019), 7 d (Farghaly et al. 2017; Wanapat et al. 2018; Chaji et al. 2020) and 10 d (Sharma et al. 2017) faecal collection periods have been utilised

5.5 Conclusion

Feed contamination with mycotoxin and especially with AF is a major challenge which is being faced by the dairy and feed industry of Pakistan. The hot and humid weather of Pakistan favours this problem. This AF problem is very common in cotton seed cake. This AF is transferred to M1 in the milk
of dairy animals and is carcinogenic in nature. Calcium propionate at 0.5% level is safe and a quite effective method to reduce AFM1 concentrations in milk, having no detrimental effects on milk yield and milk composition.

5.6 References


Gouda GA, Khattab HM, Abdel-Wahhab MA, El-Nor SA, El-Sayed HM, Kholif SM. 2019. Clay minerals as sorbents for mycotoxins in lactating goat’s diets: Intake, digestibility, blood


6.1 Introduction

Aflatoxins are secondary metabolites produced by *Aspergillus* species (Maki *et al.* 2016). The two main types of aflatoxin are aflatoxin B1 (AFB1) and aflatoxins B2 (AFB2). These metabolites are immunosuppressive and carcinogenic in humans as well as animals (Peers *et al.* 1987). Feed may be contaminated with AFB1 and AFB2 both pre- and post-harvest. Cotton seed cake (CSC) is readily available across Pakistan. The problem of contamination of CSC with AFB1 and AFB2 is a major issue in both the feed and livestock industries in Pakistan. CSC have high incidence of AF (Weidenner, 2012). The post harvesting contamination is more in CSC which is due to inadequate process and storage (Yunus *et al.* 2015).

When aflatoxin-contaminated diets are given to lactating cattle or buffaloes, the toxin is metabolized in liver to a hydroxylated derivative called AFM1 (Kuilman *et al.* 1998), transported to mammary gland via blood and excreted to milk. As reported in Chapter 5, Ca propionate is one means of reducing AFM1 concentrations in the milk of buffaloes fed diets containing aflatoxin-contaminated CSC.

Bentonites are smectite clays and organic substances can be adsorbed in the clay, (Dakovic *et al.* 2008). Effective feed additive must reduce the bioavailability of AF with no effects on animals’ performance nor the nutritional content of animal products. The objective of this experiment was to reduce AFM1 concentration in milk and to determine responses on milk composition by intruding calcium propionate in AF contaminated CSC.

6.2 Materials and Methods

6.2.1 Animal care and housing

All experimental methods were approved by the Animal Care and Ethical Committee of University of Veterinary and Animal Sciences, Lahore (Approval number 1173). The experiment was started on 01.02.2019 and ended on 01.04.2019 at Buffalo Research Institute, Pattoki District Kasur. The buffaloes were tied individually to note daily feed offered and refusal. The experiment consisted of four 20-d periods daily care involved milking at 400 and 1600, individual feeding at 08:00 hour for approximately 3% body weight dry matter intake, and refusals were collected, weighted, and noted individually.

6.2.2 Animals, experimental design and treatments

Twelve Nili Ravi multiparous buffaloes of about 600 ± 50kg of BW were used in replicated 4 × 4 Latin squares. The test products were calcium propionate (0.5 %) of RDH Company and sodium bentonite (Alltech Company). It was thought that buffaloes would consume 15-16 kg of DM. The Cotton Seed Cake (CSC) of 100 ug/kg AFB1 was searched at local market. The AF content was initially determined by ELISA analysis. The AF concentration was verified by WTO lab, University of Veterinary & Animal Sciences Lahore. CSC was treated with calcium propionate (0.5%) and sodium bentonite (1.0%). In each 20-d cows within a square were named to 1 of 3 dietary treatments (1) control (CON), comprising of untreated Cotton Seed Cake with wheat straw and adlib fodder (2) Medium dose Ca propionate (CaP 0.5%) (3) Sodium bentonite (NaB 1.0 %).

6.2.3 Sampling and data collection

Feed sampling, samples of Cotton Seed Cake, Wheat Straw and fodder were Collected on d 18 of each period and subsequently pooled by period. The feed samples were dried at 65°C in an oven to determine DM at Nutrition Division, Buffalo, Research Institute., Pattoki District Kasur, Pakistan.

At the last 3 days of each period digestibility trial was carried out to know the DM digestibility CP digestibility and NDF digestibility. The total collection of faeces was adopted. Analysis included DM (method 930.1; AOAC international, 2000) N (method 99.03; Leco). Analyses included DM (method 930.15; AOAC International, 2000), N (method 990.03; Leco FP-528 Nitrogen Combustion Analyzer, LecoCorp., St. Joseph, MI), NDF (Van Soest *et al.* 1991).

6.2.4 Milk data collection:

Individual milk production was measured and recorded daily; measurements from the first 15 d of each period were used to evaluate milk production; additionally, milk samples were collected during
the morning and night milking of d 14 and 15. These samples were analyzed for fat, lactose, SNF, milk density and protein (AOAC International, 2000) by Nutrition Laboratory, UVAS, Ravi Campus, Pattoki using milkotroniclactoscan S 60

For AF determination, milk samples were processed according to official AOAC International methods. Briefly, samples were warmed to 37°C, centrifuged for 20 min at 2,000 x g, and defatted.

The columns were washed two times with 10 mL of double distilled, deionized water (MilliQ 18.2 M cm), and eluted with 4 mL of acetonitrile. Samples were evaporated to dryness under constant nitrogen. All solvents were obtained from Fisher Scientific (Pittsburgh, PA). The AF levels were quantified with the instrument software (Empower 2, Waters Corporation, Milford, MA).

6.2.5 Statistical analysis
Data were analyzed as replicated 3 x 3 Latin Squares using the SAS (Version 9.3, SAS Institute Dnc., Cary, NC). Statistical significance for treatments effects was presented at P<0.05.

6.3 Results
6.3.1 Dry matter intake
The dry matter intake (DMI) of different treatments was same (P>0.05). DMI of control, Cap 0.50%, and NaB was 15.67kg, 15.67kg and 15.66 kg respectively (Table 6.2).

6.3.2 Cotton seed cake intake
The Cotton Seed Cake intake posed a similar trend ((P>0.05) across the treatments. Cotton Seed Cake intake is less (4.57 ± 0.78) kg in treatment having 100ug/kg AFB₁. The Cotton Seed Cake intake improved with addition of calcium propionate (CaP) to 4.65 ± 0.71kg and (4.64 ± 0.71) kg for NaB

6.3.3 Milk production
Milk production was same (P=0.42) across all the groups with mean of 10.25 kg/day.

6.3.4 Milk composition
This study demonstrated similar values of fat (P=0.49), protein (P=0.69), SNF (P=0.69), milk density (P=0.97) and lactose (P=0.23) across all the levels (Table 2). Overall milk composition averaged 8.22 ± 1.71% fat and 2.78 ± 0.35.

6.3.5 Aflatoxin in milk (AFM₁):
The AFM₁ contents are given in table 6.3. The AFM₁ across all the treatments were different (P<0.05). The AFM₁ was significantly lower in the CSC treated with CaP 0.5% (0.29ug/kg) followed by NaB (1.0%) 0.30ug/kg. Specifically, transfer rate was reduced from 1.70% (Control) to 0.29% (CaP 0.5%)

6.3.6 Digestibility of nutrients
The digestibility of nutrients was the same (P>0.05) i.e., dry matter (P=0.966), Crude protein (P=0.0018) and Neutral Detergent Fiber (0.314). The AF Challenge and detoxificants did not affect the digestibility of nutrients.
Table 6.1: Ingredient and chemical composition and predicted nutritive value

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>% DM</th>
<th>AF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn silage</td>
<td>54.47</td>
<td>75.95</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>23.86</td>
<td>12.66</td>
</tr>
<tr>
<td>Cottonseed Cake</td>
<td>19.09</td>
<td>10.13</td>
</tr>
<tr>
<td>Mineral Premix</td>
<td>2.58</td>
<td>1.27</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
<td></td>
</tr>
</tbody>
</table>

**Nutrient, Analyzed Content**

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Control</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry Matter (%)</td>
<td>100</td>
<td>48.8</td>
</tr>
<tr>
<td>Forage (%)</td>
<td>78.33</td>
<td>88.61</td>
</tr>
<tr>
<td>Crude Protein (%)</td>
<td>9.55</td>
<td>4.66</td>
</tr>
<tr>
<td>ADF (%)</td>
<td>31.97</td>
<td>15.6</td>
</tr>
<tr>
<td>NDF (%)</td>
<td>46.87</td>
<td>22.87</td>
</tr>
<tr>
<td>EE Total (%)</td>
<td>4.09</td>
<td>1.99</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>7.92</td>
<td>3.86</td>
</tr>
<tr>
<td>NFC (%)</td>
<td>33.3</td>
<td>16.25</td>
</tr>
</tbody>
</table>

**Predicted Using CNCPS System**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RUP (%CP)</td>
<td>37.53</td>
<td>37.53</td>
</tr>
<tr>
<td>RDP (%CP)</td>
<td>62.47</td>
<td>62.47</td>
</tr>
<tr>
<td>RDP (%)</td>
<td>5.96</td>
<td>2.91</td>
</tr>
<tr>
<td>ME (mCal/kg)</td>
<td>2.12</td>
<td>1.03</td>
</tr>
<tr>
<td>NEI (mCal/kg)</td>
<td>1.36</td>
<td>0.67</td>
</tr>
<tr>
<td>Salicylic Acids (%)</td>
<td>3.58</td>
<td>1.75</td>
</tr>
<tr>
<td>Sugar (%)</td>
<td>4.05</td>
<td>1.98</td>
</tr>
<tr>
<td>Starch (%)</td>
<td>20.47</td>
<td>9.99</td>
</tr>
</tbody>
</table>

*RUP: Rumen undegradable protein predicted using CNCPS evaluation
RDP: Rumen degradable protein predicted using CNCPS evaluation
MP: Metabolizable protein predicted using CNCPS evaluation
NEI: Net energy for lactation predicted using CNCPS evaluation

Table 6.2: Effect of dietary addition of calcium propionate and sodium bentonite on the cotton seed cake intake, dry matter intake and milk production of Nili Ravi buffaloes consuming an aflatoxin contaminated diet

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Parameters</th>
<th>Control</th>
<th>Calcium Propionate (0.5%)</th>
<th>Sodium Bentonite</th>
<th>P-Value</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cotton Seed Intake(kg)</td>
<td>4.57 ± 0.78</td>
<td>4.65 ± 0.71</td>
<td>4.64 ± 0.71</td>
<td>0.45</td>
<td>0.10</td>
</tr>
<tr>
<td>2.</td>
<td>Dry Matter Intake(DMI)(kg)</td>
<td>15.67 ± 0.56</td>
<td>15.67 ± 0.57</td>
<td>15.66 ± 0.57</td>
<td>0.98</td>
<td>0.08</td>
</tr>
<tr>
<td>3.</td>
<td>Milk Production(kg)</td>
<td>7.72 ± 2.62</td>
<td>7.46 ± 2.69</td>
<td>7.25 ± 2.68</td>
<td>0.15</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Means with different superscripts differ statistically (p<0.05)
Table 6.3: Effect of dietary addition of calcium propionate and sodium bentonite on the milk composition and aflatoxinM1 concentration of milk produced by Nili Ravi lactating buffaloes consuming an aflatoxin contaminated diet

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Parameters</th>
<th>Control</th>
<th>Calcium Propionate (0.5%)</th>
<th>Sodium Bentonite</th>
<th>P-Value</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Milk Fat (%)</td>
<td>7.98 ± 1.90</td>
<td>7.64 ± 1.65</td>
<td>7.627 ± 0.96</td>
<td>0.49</td>
<td>0.23</td>
</tr>
<tr>
<td>2.</td>
<td>Milk Protein (%)</td>
<td>3.35 ± 0.48</td>
<td>3.43 ± 0.55</td>
<td>3.452 ± 0.40</td>
<td>0.69</td>
<td>0.07</td>
</tr>
<tr>
<td>3.</td>
<td>SNF (%)</td>
<td>8.02 ± 0.70</td>
<td>8.17 ± 0.52</td>
<td>7.848 ± 0.52</td>
<td>0.69</td>
<td>0.07</td>
</tr>
<tr>
<td>4.</td>
<td>Milk Density (%)</td>
<td>23.25 ± 4.47</td>
<td>23.05 ± 4.04</td>
<td>25.066 ± 4.41</td>
<td>0.97</td>
<td>0.57</td>
</tr>
<tr>
<td>5.</td>
<td>Lactose (%)</td>
<td>4.38 ± 0.36</td>
<td>4.378 ± 0.34</td>
<td>4.528 ± 0.33</td>
<td>0.23</td>
<td>0.04</td>
</tr>
<tr>
<td>6.</td>
<td>Afla Toxin in Milk (M1) ug/kg</td>
<td>1.70 ± 0.03^a</td>
<td>0.29 ± 0.04^b</td>
<td>0.30 ± 0.03^b</td>
<td>0.0001</td>
<td>0.006</td>
</tr>
<tr>
<td>7.</td>
<td>AFM1 Transfer (%)</td>
<td>1.70^a</td>
<td>0.29^b</td>
<td>0.30^b</td>
<td>0.0001</td>
<td>----</td>
</tr>
</tbody>
</table>

Means with different superscripts differ statistically (p<0.05).

Table: 6.4: Effect of dietary addition of calcium propionate and sodium bentonite on apparent digestibility of Nili Ravi lactating buffaloes consuming an aflatoxin contaminated diet

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Parameters</th>
<th>Control</th>
<th>Calcium Propionate (0.5%)</th>
<th>Sodium Bentonite</th>
<th>P-Value</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>DM Digestibility</td>
<td>62.50 ± 2.96</td>
<td>62.50 ± 2.81</td>
<td>62.25 ± 2.22</td>
<td>0.96</td>
<td>0.40</td>
</tr>
<tr>
<td>2.</td>
<td>CP Digestibility</td>
<td>65.50 ± 2.31^a</td>
<td>67.750 ± 2.59^a</td>
<td>63.16 ± 1.99^b</td>
<td>0.00</td>
<td>0.28</td>
</tr>
<tr>
<td>3.</td>
<td>NDF Digestibility</td>
<td>35.46 ± 2.31</td>
<td>35.666 ± 1.61</td>
<td>36.587 ± 1.83</td>
<td>0.31</td>
<td>0.25</td>
</tr>
</tbody>
</table>

6.4 Discussion

6.4.1 Dry matter intake and milk production

Compared with fattening cattle, dairy cows (and therefore potentially dairy buffaloes) are considered more sensitive to aflatoxins (Applebaum et al. 1982). However, in this study the feeding of aflatoxin-contaminated (100 ppb) CSC did not have any adverse effects on the lactating buffaloes, or conversely there was no benefit of detoxification using Ca propionate 0.5% and Na bentonite 1%. Ca propionate and Na bentonite had no effect on total DMI, which is the same as the results reported in Chapter 4, when contaminated CSC was included in the diet fed to dry buffaloes. Similar results were reported by Quiroz et al. (2012) and Maki et al. (2016) for dairy cattle fed AFB1-contaminated diets.

The CSC intake posed a non-significant difference (P>0.05) among the treatment. The results of this experiment are in line with the findings of Jones and Ewart (1979) who stated that an AFB1 contaminated diet (0.02mg/kg) decreased feed intake in Friesian cattle. Similarly, our results are in accordance with the findings of Pasha (2008) describing decrease in feed intake of Sahiwal dairy cross by feeding a 500 g/kg AFB1 contaminated feed.

Applebaum et al. (1982) reported significant decreases in milk production in cows receiving 13 mg of impure AFB1 per day, and thus it would be assumed that inclusion of detoxification agents would result in increased milk production. However, dietary inclusion of Ca propionate and Na bentonite had no effect on milk production, which was not unexpected given that it had no effect on total DMI as it was supported by Tipu et al (2020) who reported no effect on DMI and milk production in Ca propionate,Na...
bentonite and control groups in lactating buffaloes. Meanwhile, Kutz et al. (2009), Xiong et al. (2015) and Sulzberger et al. (2016) found no differences in milk yield, in dairy cows fed AFB1-contaminated feed and a detoxifying agent(s).

6.4.2 Milk composition
As found in other studies involving dairy cows (Kutz et al. 2009; Xiong et al. 2015) goats (Smith et al. 1994) and ewes (Firmin et al. 2011; Battacone et al. 2012), addition of a detoxification agent (in this case Ca propionate and Na bentonite) to an aflatoxin-contaminated diet had no effect on milk composition. This was also stated by Tipu et al. (2020) who used same these two chemical detoxificants in lactating buffaloes and no change in milk composition was observed. In contrast; however, Queiroz et al. (2012) found that while the presence of aflatoxin showed no effect on most milk components, it decreased milk protein concentration and milk fat yield.

6.4.3 Aflatoxin in milk (AFM1):
The transfer of dietary aflatoxin into milk was 1.70 % (control) which was within the range of 1 and 6% reported by the European Food Safety Authority (EFSA, 2004), with high-yielding cows having greater carryover than cows with moderate-to-low milk yield (Froshish et al. 1986). Secretion and transfer of aflatoxin into the milk were lesser in the buffaloes supplemented with Ca propionate and Na bentonite supporting their usefulness as a means of partially detoxifying AFB1 and thereby reducing the amount converted to AFM1 and subsequently excreted in milk. The efficacy of Ca propionate was slightly better than Na bentonite. This was also stated by Tipu et al. (2020) who reported that both these chemical detoxificants significantly (P<0.05) reduced AFM1 in lactating buffaloes. Similar transmit rates have been described for dairy cows getting toxin affected feeds (Harvey et al.; 1991; X long et al. 2015).

6.4.4 Digestibility of nutrients
The use of Ca propionate and Na bentonite for the detoxification of aflatoxins found in CSC had no effect on the digestibility of either DM, CP or NDF. The same was observed by Tipu et al.(2020) who reported no difference in digestibility of nutrients while using Ca propionate and Na bentonite in buffaloes. In contrast, Ewuola et al. (2013) found aflatoxins impaired nutrient digestibility in goats. Kiyothong et al. (2012) found improved digestibility in cattle with the use of a mycotoxin deactivator and Gouda et al. (2019) reported enhanced nutrient digestibility when clay minerals were used as sorbents for mycotoxins in the diets of lactating goats.

The limited duration of the digestibility study (3 d) may have impacted results, as typically digestibility studies are conducted over 7 d (Ribeiro et al. 2011; Johnson et al. 2019) although other researchers have utilised only 3 d (Dermauw et al. 2013). In recent studies investigating apparent nutrient digestibility in buffaloes, 6 d (Paraw et al. 2019), 7 d (Farghaly et al. 2017; Wanapat et al. 2018; Chaji et al. 2020) and 10 d (Sharma et al. 2017) faecal collection periods have been utilized. Meanwhile our experiment results are also different from Stanford et al. 2018 in which they also mentioned improved digestibility of nutrients in lambs fed with Biomin treated diet. The reason behind it might be species difference and managerial difference.

6.5 Conclusion
Cotton seed cake is the most frequently used protein ingredient in Pakistan. But the incidence of AF in this ingredient limits its usage in livestock feed. Different strategies are in progress to mitigate this problem. We in this study compared calcium propionate (0.5%) with a commercial toxin binder (sodium bentonite) to see best possible solution to this menace. In our case both calcium propionate and sodium bentonite are quite effective to reduce AFM1 in milk. Both had no harmful effects on the health of buffaloes as well as their milk production and composition.

6.6 References


Ribeiro SS, Vasconcelos JT, Morais MG, Ítavo CBCF, Franco GL. 2011. Effects of ruminal infusion of a slow-release polymer-coated urea or conventional urea on apparent nutrient digestibility, in situ
CHAPTER 7
SUMMARY

The research was carried out to explore the detoxificant ability of chemical ability of different chemical agents against cotton seed cake (CSC). The first target was to pick the best chemical detoxificant among four chemical detoxificants i.e. calcium propionate, copper sulphate, sodium bisulfide and benzoic acid on the basis of invitro examination. After it was established that Ca propionate 0.5% performed well as compared to other agents invitro examination. This treatment did not affect the nutrient profile of CSC. This best level of chemical treatment was used in animal trial.

In the second experiment, different doses (0.25%,0.5%,0.75%) of Ca propionate were used in cotton seed cake based diets. Ca propionate except 0.75% decreased the DMI of dry buffaloes as compared to untreated group. This was contradictory to Overton & Waldon 2004. Actually this experiment was conducted on dry buffaloes. There was not improvement in ADG in above mentioned doses. Literature tells that high dose rate (10%) improved ADG in exotic cattle but that high dose level does not seem to be economical in Pakistan circumstances. The blood parameters, BCS and feed efficiency were also unchanged across all the groups of second experiment.

The results were somewhat different in lactating buffaloes in experiment no.3. DMI was unchanged in treated and untreated groups. The positive effect of calcium propionate 0.5% was the reduction of AFM1 in milk.

In fourth experiment, Ca propionate 0.5% was compared with 1% Na bentonite was evaluated in a 3×3 Latin Square Design. There was no major difference in both agents in reduction of AFM1. Both reduced AFM1 concentration in milk. DMI, Milk yield and milk composition was same across all the treatments of trial no.4 (Smith et al. 2014, Firmin et al. 2011). To sum up, there was no apparent benefit was observed in dry buffaloes of use of calcium propionate. It did not increase DMI, ADG and BCS of non-lactating buffaloes. However calcium propionate was beneficial in reducing AFM1 concentration in milk. Calcium propionate performed well as compared to sodium bentonite, a commercial toxin binder. Neither had harmful effects on the health of buffaloes or their milk production and composition. More studies are required to explore the use of chemical detoxificants and commercial toxin binders in other feed ingredients which have high levels of aflatoxin, such as 30% maize gluten meal. The interaction of blood metabolites with AFM1 and the effects in buffaloes and other species are also important topics for future studies.