RESEARCH ARTICLE

Carry-Over Rate of Aflatoxin B_1 from Feed to Milk and its Impact on Haemato-Biochemical Parameters in Nili-Ravi Buffaloes (*Bubalus bubalis*)

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Abstract

The present study was undertaken to establish the transmission/carry-over rate of aflatoxin B_1 (AFB_1) from contaminated feed to AFM_1 in milk of Nili-Ravi buffaloes. Twenty lactating buffaloes of almost similar weight (450-550 kg), lactation number (3-6) in early lactation (1-4 months) were divided into 5 treatments (T_1 to T_5), each having four replicates. The AFB_1 concentration in feed of T_1 to T_5 was 0, 28, 56, 84 and 113 ppb, respectively. The average milk yield (kg/d) in treatments T_1 to T_5 during the collection period of experiment was 9.31±0.04, 9.24±0.04, 9.35±0.06, 9.42±0.07 and 9.42±0.05; and the total AFM_1 excretion (µg/animal/5d) was 0.00±0.00, 19.20±0.04, 29.85±0.23, 39.00±0.14 and 47.74±0.14, respectively. The results revealed that the total AFM_1 excretion increased with increasing concentration of AFB_1 in feed. The average AFM_1 concentration in T_1 to T_5 was 0.00±0.00, 0.41±0.01, 0.63±0.01, 0.83±0.01 and 1.01±0.01 ppb, respectively. The carry-over (%) of aflatoxin, *i.e.,* transmission from feed to milk was 0.00, 0.76, 0.59, 0.51 and 0.48 in groups T_1 to T_5, respectively. The biochemical and haematological values in various treatment groups revealed that erythrocytes, PCV, haemoglobin and total protein content decreased ($p<0.05$); andGOT, GPT, GGT and ALP values increased ($p<0.05$) with increasing level of aflatoxin contamination in feed. The overall carry-over rate of aflatoxin in Nili-Ravi buffaloes was 0.59%, which might impose a health risk to the consumer. It was concluded that the daily intake of AFB_1 in Nili-Ravi buffalo should not exceed 500 µg or 28 ppb in the total dry matter intake to keep the AFM_1 content below permissible level.

Key words: Aflatoxin, Carry-over rate, Haemato-biochemical parameters, Milk safety, Nili-Ravi buffalo.

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Introduction

Aflatoxins are naturally occurring secondary metabolites produced primarily by toxigenic strains of *Aspergillus flavus* and *A. parasiticus* that colonize crops, including many staple foods and feed commodities. Contamination may occur either pre- or post-harvest and is more frequent in areas with a hot and humid climate (Singh, 2019[b]). Good agricultural practices and monitoring of feed aflatoxin level minimize but not eliminate the risk of milk contamination with AFM_1. AFM_1 is a metabolite of AFB_1 and was originally discovered in milk of humans and animals fed on mouldy grains containing AFB_1 (Singh, 2020). AFM_1, a somewhat less toxic metabolite of AFB_1, is a known carcinogen that contaminates the milk of animals fed AFB_1-contaminated feed. AFM_1 can be detected in milk within hours after administering an oral dose of AFB_1 to lactating dairy animals, indicating that at least part of its absorption occurs in the rumen (Moschini *et al.,* 2007).

AFB_1 present in feed gets transformed to 4-hydroxylated metabolite in liver and is excreted in milk as AFM_1. Aflatoxins are considered as one of the major causes of hepatocellular carcinomas. Synergistic effects of hepatitis B virus and aflatoxins have also been reported to cause hepatocellular carcinomas and risk is increased around 30-fold in the presence of both of them (Liu and Wu, 2010). The estimation of the carry-over rate of AFB_1 to AFM_1 in buffalo is of major importance in order to determine the acceptable AFB_1 level in feed.

The objective of the present investigation was to study the transmission/carry-over rate of AFB_1 from contaminated

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feed to AFM_1 in milk of Nili-Ravi buffalo in order to assess evidence-based recommendations for standards regarding a minimum recommended level (MRL) of AFB_1 in the feed of dairy animals.

Materials and Methods

Experimental Animals and Design

Twenty lactating Nili-Ravi buffaloes with similar body weights (450-550 kg), lactation numbers (3-6), and in early lactation stage (1-4 months) were selected from the herd of CIRB, Sub Campus, Nabha (Punjab, India) for the study. The animals were randomly allocated into five treatment groups (T_1 to T_5), each comprising of four replicates or animals.

Feeding and Housing

All buffaloes were housed individually under uniform management and environmental conditions. Each animal received 5 kg of concentrate mixture daily at the time of milking. The concentrate feed was formulated to contain 20% crude protein and 70% total digestible nutrients, using locally available feed ingredients. Additionally, 70 kg of freshly cut berseem clover (*Trifolium alexandrinum*) containing 18.01% dry matter was offered to each animal daily. Clean drinking water was made available round the clock.

Aflatoxin Treatment

The AFB_1 levels in the concentrate mixture were adjusted to deliver daily intakes of 0, 500, 1000, 1500, and 2000 µg per animal in treatments T_1 to T_5, respectively. The corresponding AFB_1 concentrations in the feed were 0, 28, 56, 84, and 113 ppb. The feeding experiment lasted for 17 days, comprising a 7-day adjustment period followed by a 10-day data and sample collection period.

Milk Sampling and Analysis

Milk yield was recorded twice daily at 4:00 AM and 4:00 PM throughout the 10-day collection period. A 100 mL composite milk sample was prepared daily for each animal by mixing equal volumes from the morning and evening milking. Samples were stored at -20°C until analysis. A F M $_1$ levels in milk were estimated using a Toxinometer (VICAM, USA), while AFB_1 content in feed was extracted following the method described by Pons *et al*. (1966) and quantified using UV-Spectrophotometry.

The carry-over rate of AFB_1 from feed to AFM_1 in milk was calculated using the formula:

$$\text{Carry-over (\%)} = \frac{\text{Total AFM}_1 \text{ in milk (ppb)}}{\text{Total AFB}_1 \text{ in feed (ppb)}} \times 100$$

Blood Sampling and Analysis

At the end of the experimental period, blood samples were collected from all animals, and analyzed for various haematological parameters.From half amount of the collected blood, serum was separated and analyzed for various biochemical parameters using Span Diagnostic kits as per manufacturer's protocols.

Statistical Analysis

The collected data were analyzed using Statistical Package for Social Sciences (SPSS) version 16.0. One-way analysis of variance was used to determine treatment effects, and Duncan's multiple range test was employed for *post hoc* comparison among means. A significance level of $p< 0.05$ was considered statistically significant.

Results and Discussion

Transmission of AFB_1 from Contaminated Feed to AFM_1 in Milk

The data pertaining to carry-over of AFB_1 from contaminated feed to AFM_1 in milk of Nili-Ravi buffaloes was statistically analyzed and the mean values are given in Table 1.

The average daily milk yield (kg/d) recorded during the sample collection period in treatment T_4 and T_5 was significantly higher ($p<0.05$) compared to T_2, while T_1 and T_3 remained intermediate. The total AFM_1 excretion (µg/ buffalo/5d) and the average concentration of AFM_1 in milk (ppb) increased significantly ($p<0.05$) and sequentially with AFB_1 dose from 0.00 ± 0.00 in T_1 to 47.74 ± 0.14 and 1.01 ± 0.01, respectively, in T_5. This demonstrates a clear dose-dependent linear increase in AFM_1 excretion with rising AFB_1 intake levels. Interestingly, the carry-over percentage of AFB_1 to AFM_1 in milk showed a decreasing trend with increased toxin intake. Statistically, T_2 had the highest carry-over rate, which was significantly different from T_3, T_4, and T_5. This may be

Table 1: Transmission of AFB_1 from feed to AFM_1 into buffalo milk (Mean ± SE, n=4)

Attributes	Treatments				
	T_1	T_2	T_3	T_4	T_5
Total AFB_1 intake (µg/d)	0.00±0.00	500^{a}	1000^{b}	1500^{c}	2000^{d}
Total milk yield (kg/5d)	46.58 ± 0.18^{ab}	46.22 ± 0.18^{b}	46.77 ± 0.30^{ab}	47.11 ± 0.36^{a}	47.14 ± 0.27^{a}
Average milk yield (kg/d)	9.31 ± 0.04^{ab}	9.24 ± 0.04^{b}	9.35 ± 0.06^{ab}	9.42 ± 0.07^{a}	9.42 ± 0.05^{a}
Total AFM_1 excreted (µg/ buffalo/5d)	0.00±0.00	19.20 ± 0.04^{a}	29.85 ± 0.23^{b}	39.00 ± 0.14^{c}	47.74 ± 0.14^{d}
Average AFM_1 excretion (ppb)	0.00±0.00	0.41 ± 0.01^{a}	0.63 ± 0.01^{b}	0.83 ± 0.01^{c}	1.01 ± 0.01^{d}
AFM_1 Carry-over (%) in milk	0.00±0.00	0.76 ± 0.00^{a}	0.59 ± 0.01^{b}	0.51 ± 0.00^{c}	0.48 ± 0.00^{d}

Means ± SE values with different superscripts (a–e) in a row denote significant differences ($p < 0.05$).

attributed to a possible saturation of metabolic or secretory pathways at higher toxin loads.

The present study reported that AFM_1 was detected in the milk from first milking after ingestion of the AFB_1 contaminated feed, *i.e.,* the AFM_1 can be detected in the milk of Nili-Ravi buffaloes within 12 h of ingestion of AFB_1 contaminated feed. This finding was in agreement with those of earlier reports, wherein, the presence of AFM_1 in milk was detectable from the first milking after the AFB_1 ingestion by the animal (Diaz *et al.,* 2004). This result confirmed the rapid excretion of AFB_1 metabolite in milk as reported by Diaz *et al*. (2004) and Masoero *et al*. (2007). Aflatoxins are rapidly absorbed through membranes by a passive mechanism due to its low molecular weight, namely 312.27 and 328.27 Dalton for AFB_1 and AFM_1, respectively (Yiannikouris and Jouany, 2002). The AFM_1 in blood plasma was detected in 15 min after AFB_1 intake (Moschini *et al.,* 2008). Moschini *et al*. (2006) reported that lactating cows fed a 5mg AFB_1 bolus had detectable blood plasma AFM_1 and AFB_1 concentrations as soon as 15 min after treatment, indicating both a rapid absorption of AFB_1 through the rumen wall and metabolism into AFM_1. The authors in this study used the retinol palmitate plasma level as a marker for intestinal adsorption (Bertoni *et al*., 2001), which indicated a probable AFB_1 absorption at the rumen level and an intestinal contribution to the AFM_1 plasma level 120 min after drenching.

In the present study, the total aflatoxin excretion per animal showed an increasing trend with increasing concentration of AFB_1 in feed. The AFB_1 concentration in feeds of T_1 to T_5 was 0, 28, 56, 84 and 113 ppb, respectively. The various dietary treatments were prepared keeping in view the detectable level of AFM_1 in milk. Similarly, recent findings by Xiong *et al.* (2023) confirmed that dairy cows fed with diets containing low levels of AFB_1 (as low as 20 ppb) exhibited detectable concentrations of AFM_1 in milk, while cows on diets below this threshold often showed no measurable AFM_1 excretion.

In the present study, the carry-over rate (%) of aflatoxin from feed to milk ranged between 0.48 in T_5 to 0.76 in T_2. Similarly, carry-over in dairy cows milked twice daily was usually 1-2% of the ingested AFB_1 for low-yielding cows (<30 kg milk yield/day) and up to ~6% for high-yielding cows (>30 kg milk yield/day) (Diaz *et al.,* 2004; Masoera *et al.,* 2007). Bantaokul and Ruangwises (2010) reported a transfer of 2.35% in Holstein Friesian cows with AFM_1 within the range of 0.035-11 µg/kg, which was comparable to the milk carry-over observed in the present study. Masoero *et al.* (2007) reported a transfer of 1.29 and 2.70% in low (21.2 kg/day) and high (41.8 kg/day) producing Holstein cows, which is higher to the carry-over rate reported in the present study. Also, Britzi *et al*. (2013) suggested that milk production is the main factor affecting the carry-over rate, with an average of 2.5% for low production cows (< 35 l/day) and 5.4% for high production cows (>35 l/day). In the present study, the overall carry-over rate of aflatoxin in Nili-Ravi buffaloes was 0.59%, which might impose a health risk to the consumer.

The present study reported 0.41 (T_2) to 1.01 (T_5) ppb AFM_1 in the milk of Nili-Ravi buffaloes. These levels of milk contamination were, however, higher than 0.037 ppb AFM_1 reported by Michlig *et al*. (2016). Alonso *et al*. (2010) reported 0.028 ppb AFM_1 at dairy farms at Villa Maria (Argentina). Therefore, several factors affect the aflatoxin carry-over in ruminant as the differences between species (Battacone *et al.,* 2003); individual variability of lactating animals, rumen degradation activity, hepatic and rumen biotransformation to aflatoxicol and other metabolites other than AFM_1 (Auerbach *et al.,* 1998), differences in term of induction of the enzymatic AFB_1 oxidation system and in the mammary gland permeability.

In order to reduce human exposure of aflatoxin, various countries have formulated regulations for setting maximum acceptable limits for aflatoxin. In India, the maximum permissible limit of AFM_1 in milk is 0.5 ppb (FSSAI, 2020), which is comparable to that of T_2 group in the present investigation. The AFM_1 contents in group T_3, T_4 and T_5 were higher than that of 0.5 ppb permissible limit. Based on the present study, it has been inferred that the daily intake of AFB_1 in Nili-Ravi buffaloes per animal should not exceed 500 µg or 28 ppb in the total dry matter intake.

However, the field conditions present a different scenario wherein high level of AFB_1 contamination has been reported (Singh, 2019[a,b]), due to high level of mycotoxin contamination in animal feed and feed ingredients. The situation is alarming as far as carry-over of mycotoxins from feed to milk is concerned. The toxicity of AFM_1 is 2-10% less than that of AFB_1 (Creppy, 2002). AFM_1 was initially classified

Table 2: Effect of varying levels of AFB1 on haemato-biochemical parameters of buffalo

Treatment	RBCs (10^6/µL)	WBCs (10^3/µL)	Hb (g/dL)	PCV (%)	Glucose (mg/dL)	Total protein (g/dL)	Cholesterol (mg/dL)
T_1	5.73±0.04^{d}	6.96±0.03^{ab}	8.56±0.03^{e}	29.52±0.04^{c}	65.39±0.04^{c}	6.91±0.04^{d}	112.19±1.08^{c}
T_2	5.65±0.03^{c}	6.99±0.01^{b}	8.39±0.04^{d}	29.49±0.02^{c}	65.41±0.32^{c}	6.55±0.02^{c}	116.10±1.61^{a}
T_3	5.59±0.04^{c}	6.93±0.03^{ab}	8.20±0.02^{c}	29.31±0.01^{b}	63.88±0.38^{b}	6.41±0.04^{b}	110.17±2.04^{e}
T_4	5.31±0.03^{b}	6.89±0.04^{a}	7.95±0.04^{b}	28.05±0.03^{a}	63.32±0.39^{ab}	6.31±0.04^{b}	113.41±3.16^{b}
T_5	5.12±0.03^{a}	6.91±0.03^{ab}	7.69±0.02^{a}	27.98±0.02^{a}	62.41±0.35^{a}	5.96±0.02^{a}	111.91±1.22^{d}

Mean ± SE values bearing different superscripts within the column differ significantly ($p<0.05$).

by the International Agency for Research on Cancer (IARC) as a group 2B that is possible carcinogen for humans and is now known to have genotoxic and cytotoxic potential. It has now been placed in the group 1 of definite human carcinogens along with AFB_1 (IARC, 2002).

Effect of Varying Levels of AFB_1 on Haemato-Biochemical Parameters

The data pertaining to haemato-biochemical parameters was statistically analyzed and the mean values are presented in Table 2.

The RBCs and Hb values decreased ($p<0.05$) with increasing levels of aflatoxin intake. The values in T_1 (control) were significantly ($p<0.05$) higher compared to other treatments (T_2 to T_5), though RBCs did not differ ($p>0.05$) between T_2 and T_3. The WBCs in T_1 was statistically similar to those of other treatments. The WBCs in T_2 was higher ($p<0.05$) than that of T_4.The PCV of T_1 was higher ($p<0.05$) than those of T_3, T_4 and T_5.The PCV in T_1 was statistically similar to that of T_2. The glucose content in T_1 was higher ($p<0.05$) than those of T_3, T_4 and T_5treatment groups. The glucose content in T_1 did not differ with that of T_2. The total protein in T_1 was higher ($p<0.05$) than those of other treatment groups (T_2 to T_5). The total protein in T_2 was higher ($p<0.05$) than those of T_3, T_4 and T_5, but the content between T_3 and T_4 was statistically similar. The total protein significantly decreased, which might be due to the decrease in both of albumin and globulin levels. In the present study, the cholesterol content did not differ ($p>0.05$) among various treatments. Similar results were observed in dairy ewes, dairy goats, and dairy cows (Huang *et al.,* 2018). The failure to detect difference in cholesterol content could be attributed to the low level of AFB_1 in feed.

Several biochemical and haematological parameters are affected by aflatoxin exposure.In the present study, decrease in erythrocytes, PCV, haemoglobin and total protein recorded was in agreement with earlier report (Huff *et al.*,1986). Decreased haemoglobin content due to feeding aflatoxin contaminated feed was also reported by Naseer *et al.* (2017). Aflatoxin decreases total serum proteins, alpha, beta and gamma globulins, with IgG being more sensitive than IgM. The serum total protein is an indicator of protein synthesis. The present study revealed that AFB_1 has a dose-dependent effect on biochemical and haematological parameters in Nili-Ravi buffalo.

Influence of Varying Levels of AFB_1 on Enzyme Activity

The data pertaining to various enzymes (GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; GGT, γ-glutamyl transpeptidase; and ALP, alkaline phosphatase) activity are presented in Table 3.

The GOT and GPT activities in control (T_1) were lower ($p<0.05$) than those of other treatment groups (T_2 to T_5).The values in T_2were lower ($p<0.05$) than those of T_3, $T_4$and T_5.The GOT and GPT values in T_5 were lower ($p<0.05$) than those of T_1, T_2, T_3 and T_4.The GOT and GPT value in T_3 did not differ ($p>0.05$) from that of T_4. The GGT activity in T_1 was higher ($p<0.05$) than those of T_3, T_4 and T_5, but the values between T_1 and T_2 did not differ.The GGT value in T_5 was lower ($p<0.05$) compared to other treatment groups barring T_3, wherein it was statistically similar. The ALP activity in T_1 was lower ($p<0.05$) than those of other treatment groups (T_2 to T_5). The ALP activity increased ($p<0.05$) with increasing levels of aflatoxin intake.

Aflatoxicosis is also reported to degenerate the hepatocytes leading to leakage of hepatic enzymes into the circulation resulting in the elevated levels of these enzymes (Singh *et al.,* 2013). The increased activities of GOT, GPT, and ALP are sensitive indicators of both hepatic tissues and biliary system impairment (Singh *et al.,* 2013). In the present study, an increase in the serum GOT, GPT, and ALP has been reported. Increase in the activity of serum ALP, AST and ALT was observed from the onset of animal exposure to the aflatoxin (Luna-Lopez *et al.,* 2025). The significant increase in the serum GOT and GPT was also reported in aflatoxin intoxicated animals in earlier studies (Bingol *et al.,* 2007). Earlier researchers also reported an increased serum activity of these enzymes (Silambarasan *et al*., 2016; Singh, 2019[c]). A reported increase in the serum aspartate transaminase was observed 24 to 48 h after AF intoxication in steers, where these changes were dose-related. The AFB_1 has been reported to provoke the liver function through inducing apoptosis plus disturbing the cellular enzymatic activities (Mughal *et al*., 2017). Some researchers reported no significant changes in total protein and alkaline phosphatase (ALP) following aflatoxin intoxication, suggesting that serum glycocholic acid is a more sensitive indicator of biliary impairment or cholestasis than ALP or bilirubin (Yu *et al.,* 2024).

Table 3: Effect of varying levels of AFB_1 on enzymatic activity of Nili-Ravi buffalo

Treatment	GOT (U/L)	GPT (U/L)	GGT (U/L)	ALP (U/L)
T_1	90.65±1.08^a	44.61±0.32^a	122.45±0.91^{ab}	78.51±0.70^a
T_2	96.65±1.05^b	50.97±0.70^b	121.97±1.06^a	81.57±0.63^b
T_3	99.78±0.67^c	55.86±0.39^c	126.47±1.04^{cd}	85.66±0.52^c
T_4	102.39±1.51^c	57.17±0.87^c	125.43±1.32^{bc}	88.36±0.48^d
T_5	110.47±0.55^d	61.21±0.52^d	129.30±1.09^d	90.08±0.45^e

Mean ± SE values bearing different superscripts within the column differ significantly ($p<0.05$).

Conclusion

It was concluded from the study that the daily intake of AFB_1 in Nili-Ravi buffalo should not exceed 500 µg or 28 ppb in the total dry matter intake to keep the AFM_1 content below permissible level. Erythrocytes, packed-cell volume, haemoglobin and total protein content decreased,while the GOT, GPT, GGT and ALP activity increased with increasing level of aflatoxin intake in Nili-Ravi buffaloes suggesting hepatic and renal damage.

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