Association Between Aflatoxin M1 Excretion in Milk and Indicators of Rumen Fermentation in Bovines

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Research Article

Keywords: Aflatoxin M1, Bovines, ELISA, Milk, Rumen fermentation

Posted Date: July 16th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-695370/v1>

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Version of Record: A version of this preprint was published at Tropical Animal Health and Production on March 1st, 2022. See the published version at <https://doi.org/10.1007/s11250-022-03123-9>.

Abstract

Aflatoxins and its metabolites negatively impact the ruminant health and production. The present cross-sectional study was aimed to determine the effect of aflatoxins on rumen fermentation by deducing the correlation between the Aflatoxin M1 (AFM1) excretion in milk and indicators of rumen fermentation in bovines. The indicators of rumen fermentation were taken into account and correlated with AFM1 concentration in milk of respective 120 bovines [cattle (n = 82) and buffalo (n = 38)]. The AFM1 in milk samples (n = 120) was quantified by ELISA kit. The correlation analysis revealed that with increase in excretion of AFM1 in milk, the pH (r = 0.38), Methylene Blue Reduction Time (MBRT) (r = 0.43), Sedimentation Activity Time (SAT) (r = 0.31) and ammonia nitrogen content (r = 0.34) of rumen liquor increases; whereas, the Total Volatile Fatty Acids (TVFA's) content (r=-0.25), Total Bacterial Count (TBC) (r=-0.43) and Total Protozoal Count (TPC) (r=-0.14) of rumen liquor decreases. The results of the present study suggest that the presence of aflatoxins in rumen could have negative effect on the process of rumen fermentation. Therefore, the prevention of primary entry point(s) of AFB1 through the feed of bovines is important for the animal as well as public health.

Introduction

Aflatoxins are toxic fungal metabolites mainly produced by *Aspergillus flavus* and *A. parasiticus* (Okoth et al. 2018; Frisvad et al. 2019). Aflatoxins are mutagenic, genotoxic, teratogenic and carcinogenic (group 1) with aflatoxin B1 (AFB1) being most toxic (McCullough et al. 2019). The contaminated crops are the primary entry point of AFB1 in the food chain. The ingestion of AFB1 by dairy animals leads to the hydroxylation of the AFB1 toxin to a lesser toxic metabolite in the liver, i.e., Aflatoxin M1 (AFM1), which excretes through the milk and urine of the animals (Sharma et al. 2020). On ingestion, aflatoxins are first encountered by the ruminal fauna and flora which acts as the first line of defense against aflatoxins. Several bacterial and yeast strains isolated from rumen liquor of cattle have been shown to have some detoxifying effect on aflatoxins *in vitro* (Intanoo et al. 2018). In addition, adult ruminants are considered as more resilient to the adverse effects of aflatoxins than monogastric animals and young/immature ruminants (Mathur et al. 1976; Upadhaya et al. 2009; Khodabandehloo et al. 2019). After ingestion, AFB1 is degraded in rumen into aflatoxicol and the remaining is absorbed in the digestive tract and converted to AFM1 in liver, the same being excreted through milk (Nidhina et al. 2017). On the downside, the degraded aflatoxicol can almost entirely convert back into its more toxic parent compound AFB1 or AFM1 in liver (Benkerroum 2020). The AFB1 and AFB2 can also be produced by rumen microflora in ruminal environment, therefore, the rumen can also act as a source of AFM1 in milk of ruminants with ruminal pH also governing the production of aflatoxins (Nidhina et al. 2017).

Efforts to reduce human and animal exposure to aflatoxins have resulted in the establishment of regulatory limits for AFM1 in milk worldwide. The Food Safety and Standards Authority of India (FSSAI) and *Codex Alimentarius* of FAO/WHO have established the Maximum Permissible Limit (MPL) of 0.5 µg/L of AFM1 in bulk milk, while the European Union established the MPL at 0.05 µg/L (EC 2010, FSSAI 2011 and Codex Alimentarius Commission 2017). The available physical and chemical methods of

mycotoxin detoxification are restricted by problems concerning safety issues, possible losses in the nutritional quality, limited efficacy and cost implications (Intanoo et al. 2018), but recent studies suggest the use of gastro-intestinal microbials in detoxification process as a promising alternative (Kabak and Var 2008; Nguyen et al. 2020). However, limited information is available on the interactions between rumen microbes and aflatoxins, though the impact of rumen on degradation of aflatoxins has been reported (Kabak and Var 2008). In this context, the present study was designed to determine the possible effect aflatoxins have on rumen fermentation by finding the correlation between the AFM1 excretion in milk and indicators of rumen fermentation in bovines.

Materials And Methods

Sampling procedure

The present cross-sectional study was carried out across all the 22 districts of Punjab State of India during the period of July 2019 to February 2020. A total of 120 bovines [cattle (n = 82) and buffalo (n = 38)] were selected in the study (Table 1). The consent of the dairy farmers for enrollment in the study was obtained and the samples were taken in presence of regional veterinary doctors. The ethical permission for the collection of rumen liquor samples was taken from the Institutional Animal ethics Committee (IAEC) (IAEC/2019/188-221).

From each animal, 50 ml of milk and 15 ml of rumen liquor samples were collected aseptically under the supervision of field veterinarians. The milk collection was done in sterile collection tubes which were transported to the laboratory in ice-box and stored at -20°C until further analysis. The rumen liquor was collected in sterile collection tubes by needle puncture of rumen at the left paralumbar fossa using 20 ml syringe with 16-gauge needle under aseptic conditions. The collected samples were strained through double layer of muslin cloth as described by Lengemann and Allen (1955) and labeled properly as 'strained rumen liquor' (SLR) with unique identification number. The preservation of rumen liquor was done in saturated solution of mercuric chloride and in 8% formaldehyde solution separately. For analysis of rumen metabolites, 2-3 drops of mercuric chloride were added per 4-5 ml of rumen liquor sample and for rumen microflora count, 1 ml of formaldehyde was added per 1 ml of rumen liquor sample (Singh et al. 2018). The samples were immediately stored in ice box after collection and in -20°C upon reaching laboratory for further analysis. The district wise details of the collected samples are presented in Table 1.

AFM1 detection in milk samples by ELISA

All the collected milk samples (n=120) were subjected to commercial competitive Aflatoxin M1 ELISA (EuroProxima B.V., The Netherlands) having the limit of detection (LOD) of 5 pg/ml for milk samples. All the procedures were performed as per the instructions provided by the manufacturer. The milk samples were prepared for ELISA testing by carrying out centrifugation at 2000×g for 10 min at 4°C after being thawed to room temperature and the separated fat layer was removed by using spatula. The optical density (O.D.) values for each sample were recorded immediately at 450 nm by using microplate

spectrophotometer. The concentration of AFM1 in milk samples was calculated by using regression equation obtained from calibration curve built by plotting values of % maximal absorbance of standards versus the analyte equivalent concentration (pg/ml).

$$\% \text{ maximal absorbance} = \frac{\text{O. D. of standard or sample}}{\text{O. D. of zero standard}} \times 100$$

The O.D. of the standards and samples were averaged and corrected by subtracting the mean O.D. of blank wells before computation. The O.D. is inversely proportional to the AFM₁ concentration in the sample.

Analysis of rumen liquor

To study the correlation of aflatoxins on rumen fermentation, the rumen liquor parameters selected as indicators of rumen fermentation were: (1) Physico-chemical parameters [colour, odour and consistency, pH, Methylene Blue Reduction Time (MBRT), Sedimentation Activity Time (SAT)]; (2) Rumen metabolites [Total Volatile Fatty Acids (TVFA's) and ammonia nitrogen]; and (3) Rumen microflora count [Total Bacterial Count (TBC) and Total Protozoal Count (TPC)].

Physico-chemical parameters:

1. Colour, odour and consistency

After the collection of rumen liquor, the colour, odour and consistency of rumen liquor samples were immediately recorded as described by Garry (2002). The observations were grouped into 2 categories: normal and abnormal (Supplementary Table 1). The normal group included, greenish-yellow to yellowish-brown colour, aromatic odour and slightly viscous consistency. The observations recorded other than normal were grouped under abnormal category.

2. pH of rumen liquor

The pH of rumen liquor was determined immediately after collection by digital pH meter.

3. Methylene blue reduction test (MBRT)

Methylene blue reduction test was performed as per the method described by Dirksen (1979). A total of 0.25 ml of 0.03% methylene blue solution was added in 5 ml of freshly collected rumen liquor in a test tube and incubated in water bath at room temperature. The time taken for the reduction of methylene blue by the microbial constituents of rumen liquor, *i.e.*, for discoloration of sample, was recorded by using a plain rumen fluid as a basis for comparison.

4. Sedimentation activity test (SAT)

Sedimentation activity test was performed according to the method described by Dirksen (1979). The freshly collected rumen liquor was observed in glass test tubes kept in water bath at 39°C. The time required for sedimentation and floatation was referred to as sedimentation activity time.

Rumen metabolites:

1. Total volatile fatty acids (TVFA)

Total volatile fatty acids concentration in rumen fluid was estimated by the method of Barnett and Reid (1957). A total of 1 ml of SRL was transferred into Markham's micro-Kjeldahl distillation apparatus and 1 ml of scaribrick buffer (10% potassium oxalate and 5% oxalic acid in equal volumes) was added to it. The cup was made air tight with stop cork and by adding some water in it. The steam distillation was carried and approximately 75 ml distillate was collected. To the distillate, few drops of phenolphthalein indicator were added and titrated against standard 0.01N NaOH solution.

$$\text{TVFA's (mEq/L)} = \text{Amount of standard base used (ml)} \times 10$$

2. Ammonia nitrogen (NH₃-N)

The Conway micro diffusion technique (Conway 1957) was used to estimate NH₃-N in SRL. In the inner chamber of Conway cell, 1 ml of 2% boric acid solution containing mixed indicator was taken and 1 ml of clear SRL was pipetted into the outer compartment. Further, 1 ml of 50% potassium carbonate solution was added slowly into the outer compartment opposite to SRL. After covering the micro-diffusion cell, it was gently rotated clockwise and anticlockwise at a horizontal plain to mix contents of outer chamber followed by incubation for 1 hour. Thereafter, contents of the inner chamber were titrated against standard 0.01N H₂SO₄ solution. Simultaneously, a blank of 1 ml distilled water was also titrated against standard acid.

$$\text{Ammonia nitrogen } \left(\frac{\text{mg}}{\text{dl}} \right) = \frac{\text{Amount of standard acid used} \times 0.14}{\text{Volume of sample taken (ml)}} \times 100$$

Rumen microflora count:

1. Total bacterial count

Total bacterial count was determined by using Nigrosin slide technique as per the method described by Gall et al. (1949). In brief, the preserved sample of SRL was thawed and shaken vigorously in order to separate microbes from feed particles and to break microbial clumps. The thawed rumen liquor sample was centrifuged at 3000 rpm for 5 min and the supernatant was serially diluted in 1:10000 ratio with distilled water. The diluted bacterial suspension was mixed well and 0.01 ml of diluted

suspension was taken onto a clean grease free glass slide. A loopful of saturated Nigrosin stain was added to 0.01 ml of diluted suspension. The sample was mixed and uniformly spread over 2×2 cm area of glass slide with the platinum loop. The smear was dried immediately over a preheated (about 60°C) hot plate. The bacterial counting was done in total 30 microscopic fields from 2x2 cm area of stained smear under 100X objective of microscope. The total bacterial count per ml of rumen liquor was calculated by the formula given below:

$$\text{TBC per ml of sample} = \text{Number of bacteria per field} \times \text{Number of microscopic field (1000)} \times \text{Dilution factor (10}^6\text{)}$$

2. Total Protozoal count (TPC)

The rumen protozoal count was done as per the method described earlier by Naga and El-Shazly (1969). A total of 5 ml of sample was taken through wide bore (3.5 mm) pipette into a test tube. Then 15 ml of normal saline solution (0.85%) was transferred and thereafter 5 ml Lugol's iodine was added. The solution was mixed gently and 0.1 ml of sample was transferred swiftly to a dry clean slide and spread under a glass cover of known area (24×60 mm). A total of 30 fields were counted per slide both for ease and accuracy and total protozoal count per ml of rumen liquor was calculated by the formula given below:

$$\text{TPC per ml of sample} = \text{Number of protozoa per field} \times \text{Number of microscopic field (1000)} \times \text{Dilution factor (100)}$$

Statistical analysis

All the data entry and the computation of mean, standard deviation, maximum and minimum concentrations were carried out using Microsoft® Excel 2010. The correlation analysis was taken as the measure for the degree of association between AFM1 excretion in milk and indicators of rumen fermentation computed as Pearson Correlation Coefficient (r) by using Microsoft® Excel 2010. The correlation coefficient was categorized into weak (0-0.25), fair (0.25-0.5), good (0.5-0.75) and excellent (0.75-1) correlation based on r value as described by Cohen (2013).

Results

Occurrence of AFM₁ in bovine milk samples from Punjab

Covering all the districts of Punjab (n = 22), a total of 120 milk samples were collected and analyzed for the presence of AFM1. Out of the tested samples, 63.3% (76/120) of the samples exceeded the maximum permissible limit (MPL) of the European Union (EU) set by the European Commission (EC) (i.e., 0.05 µg/L); whereas, 10.83% (13/120) of samples were above the MPL set by Food Safety and

Standards Authority of India (FSSAI) (i.e., 0.05 µg/L). On analysis, the overall mean concentration $\pm$ standard deviation (SD) of AFM₁ concentration in milk samples was found to be 0.22 $\pm$ 0.36 µg/L.

Correlation between AFM1 excretion in milk and indicators of rumen fermentation in bovines

The Pearson Correlation Coefficient (r) was calculated between the AFM1 concentrations in milk of bovines and their respective rumen liquor parameters. The details of data on AFM1 in milk and rumen liquor parameters are presented in Supplementary Table 1.

Of the physico-chemical parameters analyzed, the data on colour, odour and consistency of the rumen fluid samples was found non suitable to deduct any significant association with AFM1 excretion in milk. The pH of the rumen liquor samples was found to be positively correlated with AFM1 excretion in milk having the correlation coefficient (r) of 0.384 (category: fair correlation) with p-value of 0.000015 (statistically significant). The methylene blue reduction time of rumen liquor significantly increased (p-value of <0.00001) with increase in excretion of AFM1 in milk (r= 0.429; fair correlation). Another indicator of rumen microbial activity, SAT also significantly increased (p-value of 0.0005) with increase in excretion of AFM1 in milk (r= 0.312; fair correlation).

The analysis of rumen metabolites revealed TVFA's content of rumen liquor to be negatively correlated (fair correlation) while ammonia nitrogen content to be positively correlated (fair correlation) with correlation coefficient of -0.249 and 0.337 respectively. Both the associations were found to be statistically significant (p-value- TVFA's: 0.006; p-value- ammonia nitrogen: 0.00017).

For determining the effects of aflatoxins on rumen microflora, the bacterial and protozoal counts were taken into account. The analysis showed that as the AFM1 excretion in milk increased, both the TBC (r =-0.429) and TPC (r =-0.137) of rumen liquor decreased. The TBC was found to have fair correlation with the AFM1 excretion in milk with p-value of < 0.00001 (statistically significant) however, the TPC of rumen liquor had a weak correlation which was also statistically non-significant (p-value=0.136). The correlation coefficient of various indicators of rumen fermentation has been summarized in Table 2.

Discussion

The excretion of Aflatoxin M1 in milk presents a continuous threat to food safety. The present study reported the AFM1 prevalence in milk from Punjab to be 10.83% with respect to the national standards, i.e., FSSAI. The epidemiological studies from other parts of India also showed the high presence of AFM1 in milk with 20.6% prevalence in Haryana (Sharma et al. 2020), 38% in Karnataka and 17.3% in Tamil Nadu (Siddappa et al. 2012), 75% in Goa (Kanungo and Bhand. 2014) and 9% in Uttar Pradesh (Rastogi et al. 2004).

The present study provides an insight on the possible alteration in rumen environment by the ingestion of aflatoxins by animals. The negative 'fair correlation' of -0.429 between the TBC of rumen liquor and AFM1 excretion in milk showed that the presence of aflatoxins has negative impact on the bacterial population

in rumen. The results of the present study were in agreement with Mathur et al. (1976) who reported that AFB1 affected some rumen micro-organisms morphologically and physiologically and also inhibited their growth. Fink-Gremmels (2008) and Jiang et al. (2012) have also reported AFB1 of having negative impact on rumen microflora. However, no previous data was available on the effect aflatoxins have on rumen protozoal population.

The inhibitory effect of aflatoxins on the microbial growth and activity can be further supported by the increasing MBRT and SAT of rumen liquor with increasing AFM1 excretion in milk (Supplementary Table 1). The present study revealed that the TVFA's content of rumen liquor and AFM1 excretion in milk were negatively correlated. The findings of our study are in concordance with previous studies of Mathur et al. (1976), Jiang et al. (2012), Khodabandehloo et al. (2019) and Jiang et al. (2020) also reported decrease in TVFA's concentration in rumen liquor on increasing AFB1 intake in diet. While Edrington et al. (1994) found no differences in ruminal VFA's concentrations in growing lambs fed 2.5 mg AFB1 per kg diet; and Helferich et al. (1986) also reported that AFB1 at 60–600 mg/kg did not influence the production of VFA in cattle. The decrease of VFA's production in rumen can be attributed to impaired fermentation of rumen due to decrease in population and activity of rumen microflora as a result of high aflatoxin concentration in rumen (Fink-Gremmels 2008; Jiang et al. 2012).

In present study, the ammonia nitrogen concentration in rumen significantly increased with increase in excretion of AFM1 in milk which is in concordance with Khodabandehloo et al. (2019), Jiang et al. (2020) which reported an increase in ammonia nitrogen concentration of rumen liquor on increasing AFB1 in diet of ruminants. On the contrary, Jiang et al. (2012) reported ammonia nitrogen concentration declined with the increase in AFB1 dosage. The increase in ammonia nitrogen concentration in rumen with increase in AFM1 excretion through milk of bovines could be due to the negative impact aflatoxin have on growth and activity of rumen microflora as reported in previous (Fink-Gremmels 2008; Jiang et al. 2012) and in the present study which reduces the utilization of ammonia nitrogen by rumen microbes for bacterial protein synthesis in turn leading to rise in the ammonia nitrogen concentration of rumen. The increased ammonia nitrogen content raises the pH of rumen which further leads to increased denaturation of proteins and peptides into ammonia nitrogen.

Regarding the effect of aflatoxins on ruminal pH, the analysis in present study revealed pH of rumen to be increasing with increase in excretion of AFM1 in milk. However, the previous studies show conflicting results with the Khodabandehloo et al. (2019) reporting an increase in pH of rumen liquor of rams by increasing the concentration of AFB1 in feed. In contrary to the results by Nidhina et al. (2017) who reported that low ruminal pH favors aflatoxin production in-vitro and vice-versa. The increase in pH of rumen with increasing AFM1 concentration in milk could be due to decrease in TVFA's production and increasing ammonia nitrogen concentration as a result of negative impact on the growth of rumen microflora (Supplementary Table 1) and disruption of rumen fermentation (Fink-Gremmels 2008; Jiang et al. 2012) caused by increased aflatoxin concentration in rumen. Therefore, ingestion of aflatoxins certainly impacts ruminal digestion and in turn the overall health of the animal.

The present study has the limitation of not considering physiological, nutritional or other toxin effects on rumen flora of studied animals; therefore, we acknowledge that further *in vitro* and/or *in vivo* experimental studies need to be conducted in order to prove the causative effect(s) of aflatoxin on rumen microbes.

In conclusion, the present study reported that the excretion of AFM1 in milk of bovine is positively correlated with the pH, MBRT, SAT and ammonia nitrogen content of rumen liquor increases, while negatively correlated with TVFA's content, TBC and TPC of rumen liquor. The above-mentioned results suggest that the presence of aflatoxins in rumen have negative impact on ruminal fermentation. However, further *in-vitro* and *in-vivo* studies are required to get detailed knowledge on which type of microbial population in rumen is inhibited by presence of aflatoxins and the associated inhibitory factors responsible for it.

Declarations

Acknowledgment:

Authors are thankful to the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana for providing necessary help to write the manuscript. The financial support received from the projects of 'Rashtriya Krishi Vikas Yojana (RKVY), India' is thankfully acknowledged. The authors also thank the farmers and veterinary doctors of Punjab who assisted with sample collection during the study.

Funding: The financial support received from the projects of 'Rashtriya Krishi Vikas Yojana (RKVY), India' is thankfully acknowledged.

Conflict of Interest Statement: Authors declare no competing interests

Data availability statement: All relevant data are within the paper and its supporting information files.

Code availability: Not applicable

Authors' contributions: PD and JSB conceived and designed research. HT and PD conducted sample collection and experiments. PD, JSB, RS and GS analyzed data and supervised the research. HT and PD wrote the manuscript. JSB, RS and GS reviewed and edited the manuscript. All authors read and approved the manuscript.

Statement of informed consent: Farmer informed consent was asked during fieldwork. All applicable international, national, and/or institutional guideline for the care and use of animals were followed.

Ethics approval: The ethical permission for the collection of rumen liquor samples was taken from the Institutional Animal ethics Committee (IAEC) (IAEC/2019/188-221).

Consent to participate: The consent of the dairy farmers for enrollment in the study was obtained and the samples were taken in presence of regional veterinary doctors.

Consent for publication: All persons included as co-authors on this manuscript have provided their consent to be herein included.

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Tables

Table 1: District-wise distribution of milk samples and rumen liquor of bovines

Sl.No.	Districts	Milk samples	Rumen liquor samples
1.	Amritsar	8	8
2.	Barnala	4	4
3.	Bathinda	5	5
4.	Faridkot	4	4
5.	Fathegarh Sahib	4	4
6.	Ferozepur	5	5
7.	Fazilka	5	5
8.	Gurdaspur	4	4
9.	Pathankot	4	4
10.	Hoshiarpur	6	6
11.	Jalandhar	6	6
12.	Kapurthala	4	4
13.	Ludhiana	10	10
14.	Mansa	5	5
15.	Moga	6	6
16.	Mohali	4	4
17.	Muktsar	5	5
18.	Navanshahr	3	3
19.	Patiala	7	7
20.	Rupnagar	4	4
21.	Sangrur	9	9
22.	Tarn Taran	8	8
Total		120	120

Table 2: Correlation between AFM1 excretion in milk and indicators of rumen fermentation

	Parameters	Correlation coefficient (r)	p-value
Physico-chemical parameters	pH	0.384 (positive fair correlation)	0.000015*
	MBRT	0.429 (positive fair correlation)	0.00001*
	SAT	0.312 (positive fair correlation)	0.0005*
Rumen metabolites	TVFA's	-0.249 (negative fair correlation)	0.006*
	Ammonia nitrogen	0.337 (positive fair correlation)	0.00017*
Rumen microflora	TBC	-0.429 (negative fair correlation)	0.00001*
	TPC	-0.137 (negative weak correlation)	0.136

*Statistically significant (i.e., p-value <0.05)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarytable1.docx](#)