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Examination of covariance and correlation in aflatoxin levels between feeds and dairy cattle milk in Northern Greece

D. Gourdouvelis,[1] V. Dotas,[1] D. Vouzaras,[2] I. Kaimakamis,[1] L. Hatzizisis,[3] M. Koidou,[4] V. Schina[1]

[1]Department of Animal Production, Faculty of Agriculture, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

[2]Department of Animal Sciences, University of Thessaly, GR-41500 Larisa, Greece

[3]Faculty of Agriculture, University of Ioannina, 47100 Arta, Greece

[4]Department of Agriculture, School of Agricultural Sciences, University of Western Macedonia, 53100 Florina, Greece

ABSTRACT: This study aimed to determine the concentration of aflatoxins and reveal the degree of correlation between feed and cow's milk. A total of 182 corn grain samples, 73 cotton seed samples, 183 maize silage samples, 183 total mixed ration samples, and 183 fresh cow milk samples were collected and analyzed using the ELISA method in 2021 and 2022 from farms in the Central Macedonia and Thessaly regions. The Pearson coefficient was employed to investigate the existence of correlations. The results indicated that the presence of aflatoxins in milk is significantly correlated with the level of aflatoxins in feed. The levels of aflatoxin concentrations in corn grain, cotton seed, and total mixed rations showed a presence of aflatoxins in cow's milk. Specifically, when the concentration of aflatoxins in either the corn grain or the total daily ration increases, the concentration of aflatoxins in the milk also rises, never exceeding the maximum permissible limit. The analysis is completed by capturing and examining scatter charts among the variables, which found strong correlations. In all cases, there is no evidence of non-linear relationships or strongly divergent values, and the dispersion of the points is small, indicating high correlation values ($r > 0.700$). Moreover, this study indicates that the feed used in dairy cattle must have zero or low levels of aflatoxins to produce aflatoxin-free milk. Careful observance of the rules and conditions in the production, harvesting, drying, storage, and maintenance of feed materials is of key importance in this regard.

Keyword: dairy cows; feeds; milk; aflatoxins; covariance and correlation analysis.

Correspondence author:
D. Gourdouvelis,
Department of Animal Production, Faculty of Agriculture, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
E-mail address: topidaias@gmail.com

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INTRODUCTION

The most dangerous mycotoxins are considered to be aflatoxins, which affect human and animal health through cirrhosis and acute liver damage, tumor induction, as well as causing immunosuppressive, mutagenic, teratogenic, and carcinogenic effects (Deshpande, 2002). They are mainly produced by *Aspergillus flavus* and *parasiticus* species and are highly stable in drying, storage, and processing of food and feed (Ding et al., 2012). The most important members of the aflatoxin family are aflatoxins B1, B2, G1, and G2. Among them, aflatoxin B1 (AFB1) is the most potentially teratogenic, mutagenic, and hepatocarcinogenic and has been recognized as a category 1 carcinogen by the International Agency for Research on Cancer (IARC) (2012), with serious impacts on health, agricultural and livestock productivity, and food safety (Benkerroum, 2020). High temperatures and humidity favor AFB1 distribution, though fungal growth and toxinogenesis can also occur during drought conditions (Ghadiri et al., 2019).

Moulds belonging to the gender *Aspergillus spp.* and *Penicillium spp*. They mainly develop in inappropriate storage conditions of cereals (e.g., when drying of cereals is not carried out, which therefore have a moisture content >14%) (Xu et al., 2007). A particular case could be *Aspergillus flavus*, because it can produce high concentrations of aflatoxins in seeds before their harvest and storage (Ting et al., 2020).

Regarding dairy milk, the European Union has set very low limits for AFM1 (0.050 μg/kg, lowered to 0.025 μg/kg in infant formulae) to protect consumers' health (EC Regulation 1881/2006). For feedstuffs, the corresponding maximum is 5 ppb according to Community Directive 100/2003. Aflatoxin (AF) B1is bioactivated by drug metabolizing enzymes to form the cytotoxic and carcinogenic metabolite AFM1, which is a dairy milk contaminant (Ghadiri et al., 2019). Aflatoxin (AFM1) is the hydroxylated form of AFB1, detected in milk and other dairy products obtained from animals that have consumed feed contaminated with AFB1 (Rahimi, 2010). A proportion of 0.3-6.2% of AFB1 ingested by ruminants is converted to AFM1 and goes into milk (Creppy, 2002).

According to Sultana and Hanif (2009), ruminants are affected by mycotoxins to a lesser extent than other animal species. The rumen microflora can convert a range of mycotoxins into metabolites, which are less potent or even biologically inactive at common levels of exposure to mycotoxin action. However, this does not apply to all types of mycotoxins that contaminate feed materials. So around 10 μg/AFB1/ml concentrations completely stop many bacteria from growing. This suggests that the toxin could stop protozoan microorganisms from growing and metabolizing (Yiannikouris and Jouany, 2002).

Aflatoxin (AFM1) milk contamination shows substantial seasonal variation, with the highest concentrations observed during winter. This observation connects to the fact that, towards the end of summer, animals consume roughages more than concentrated feedstuffs, which they mainly consume during winter. In this season, concentrated feedstuffs contain higher levels of AFB1 (Çelik et al., 2005). In general, aflatoxins and their metabolites remain unchanged during milk processing (Harris and Staples, 1992). In the study by Kaniou-Grigoriadou et al. (2005), it is reported that after 4 days of milk storage at 0°C, 40% of the toxin is inactivated, while after 6 days, approximately 80% is inactivated.

The most widely used analytical techniques that have been applied for the determination of AFM1 in milk and its products are the enzyme immunoassay method (ELISA) and high-performance liquid chromatography (HPLC) coupled to a fluorescence detector (FLD) (Shephard, 2009; Turner et al., 2009). An essential aspect of the analysis process is that AFM1 is evenly distributed in milk and dairy products, which facilitates sampling. The ELISA method is the preferred choice because it is purely quantitative, reliable, simple to use, fast, low-cost, and has satisfactory sensitivity and specificity (Walker and Crowther, 2009).

In Greece, dairy cow farming is practiced as a business, particularly in Thessaly and Central Macedonia, where most of the cow's milk is produced. In these regions, dairy production systems heavily depend on locally produced forages, such as maize silage, and concentrated feedstuffs, including corn, cottonseed, and cottonseed cake, to meet the nutritional requirements of livestock. These feed components, when combined with protein-rich supplements like soybean meal and sunflower meal, cereal grains such as wheat and barley, and balanced mixtures of vitamins and trace elements, form the Total Mixed Ration (TMR), a standardized diet aimed at optimizing milk production and animal health (NRC, 2001).

Given the critical impact of aflatoxins on both human and animal health, as well as their implications for the safety of milk and dairy products, it is essential to investigate the interrelationship and covariance of aflatoxin contamination in locally sourced animal feeds and resulting milk. Such studies are crucial for developing effective mitigation strategies to ensure food safety and compliance with regulatory standards (EFSA CONTAM Panel, 2020).

MATERIAL AND METHODS

Samples collection

In collaboration with 20 selected producers (13 from Thessaly and 7 from Central Macedonia) a sampling program was implemented. This program involved collecting 4-5 milk samples, each 100 ml, after the morning or afternoon milking from each producer, randomly, once a quarter, during the years 2020-2021. The 5th sampling was conducted when deemed appropriate, mainly during particularly wet weather conditions. A total of 183 milk samples were collected, with 91 originating from 2020 and 92 originating from 2021.

At the same time, sampling was carried out for the four main categories of animal feed, specifically 182 corn grain samples, 183 maize silage samples, 183 TMR samples and 73 cotton seed samples, each weighing 100grams. The selection of the 20 farms included in the research was based on their use of these animal feeds from crops in the nearby area. The average annual milk production of the 20 farms was 9,137kg/cow, while the average number of animals per farm was 110 cows.

Determination of aflatoxins

The concentration of AFM1 and AFB1 aflatoxins in milk and feed samples was determined using the immunoenzymatic ELISA method, in an accredited laboratory with a 450nm spectrophotometer. Standard tests with standard solutions were used for the analyses, which were carried out in reduced light conditions since aflatoxins are sensitive to light.

The ELISA test is based on the specific binding of antigen (Ag) and antibody (Ab). The method's principle is that the antigen and the antibody bind in a particular bidirectional manner to form a complex that can be separated from the free molecules in the examined sample.

Document number 1: 2003 describes the standardized application of ELISA for determining aflatoxins in milk and animal feed. The specification for the method's sensitivity, specificity, and accuracy is also described.

Statistical analysis

A linear regression model was developed to explore the quantitative relationship between aflatoxin levels in animal feed and the resultant contamination in milk. The dependent variable ("milk") represented the concentration of aflatoxin M1 (AFM1) in cow's milk (expressed in parts per trillion, ppt). In contrast, the independent variables included aflatoxin concentrations in the main feed components: total mixed ration (TMR), measured in parts per billion (ppb). These variables were selected based on their established contribution to dietary intake and contamination pathways observed in prior studies.

The dataset consisted of 183 paired observations collected across dairy farms in Thessaly and Central Macedonia during 2020-2021. Sampling was conducted quarterly, with an additional round when weather conditions warranted. Feed and milk samples were analyzed using the ELISA method to ensure precise and consistent quantification of aflatoxins.

The statistical analysis was done using IBM SPSS Statistics (version 29). Initially, descriptive statistics were computed to summarize critical characteristics of the dataset, including means, standard deviations, and ranges for all variables. This step was crucial for identifying variability and ensuring data quality.

Pearson correlation coefficients were calculated to determine the strength and significance of relationships between feed components and milk aflatoxin levels. These coefficients ranged between -1 and +1, with positive values indicating a direct relationship. Strong correlations ($r > 0.7$, $p < 0.05$) were observed between:

- Corn grain and TMR aflatoxin concentrations.
- TMR and milk aflatoxin levels.
- Corn grain and milk aflatoxin levels.

These findings justified including the selected independent variables in the regression model, as they demonstrated a strong and statistically significant linear association with the dependent variable.

Following the correlation analysis, a linear regression model was constructed to quantify the predictive impact of each feed component on milk aflatoxin levels. The regression equation was structured as follows:

$$\text{AFM1 in milk (ppt)} = \beta0+ \beta1\{\text{TMR}\}+ \varepsilon$$

Where:

- β0: Intercept (baseline milk aflatoxin level when all predictors are zero).

- β1: Regression coefficients representing the rate of change in milk aflatoxin levels for a unit change in each predictor.
- ε: Error term.

The model provided insights into how each feed component contributes to milk contamination, informing risk management strategies in dairy farming.

Scatterplots of residuals and diagnostic tests confirmed the model's validity. They showed no evidence of multicollinearity or heteroscedasticity and supported the assumption of linear relationships. The regression analysis was a powerful tool for bridging the gap between feed quality and milk safety.

RESULTS

Effect of year and quarter of sampling

Table 1 presents the mean values, the standard deviation and minimum and maximum value of the determined aflatoxin expressed in ppb and in ppt for the studied feed and milk respectively per calendar year. In addition, a statistically significant difference between the averages is investigated using the ANOVA variance analysis method at a significance level of p≤0.05.

There are no statistically significant differences in the averages for the presence of aflatoxins in corn grain, maize silage, and total mix ration during the periods under study. In contrast, in cotton seed and cow's milk, it was found that the concentrations of aflatoxins in 2020 were higher compared to 2021. These values reflect statistically significant differences (p=0.000 and p=0.006) determined by the analysis of variance (ANOVA). Figures 1 and 2 highlight the mean values (ppb) of the aflatoxin presence in feed by quarter and by year 2020 and 2021, respectively.

According to Figure 1, the highest value of aflatoxin occurs in corn seed samples during the third quarter of the year. A high level of aflatoxin (1.32 ppb) also appears in total ration samples, for the same period, while maize silage shows the lowest level of 0.15 ppb. For the year 2021 (Figure 2) the highest aflatoxin levels were observed in the last quarter. The presence of aflatoxin M1 expressed in ppt, in the cow's milk samples is highlighted in Figure 3 for the years 2020 and 2021.

Figure 3 shows that the highest value (27.63ppt) occurs in the samples from the 3rd quarter of 2020, while the lowest value is from the 2nd quarter of 2021 (12.24ppt). The first quarters of 2020, show a higher concentration of aflatoxin compared to 2021.

Correlations between variables

Pearson's correlation coefficient was used to investigate the existence of a correlation between the sets of values of aflatoxin concentrations in feed and cow's milk samples. Coefficient values ranged from -1.00 to +1.00 with values approaching 1.00 indicating a statistically significant association. Table 2 presents the degree of correlation between the values of the variables used.

Table 1. Mean values, standard deviation, maximum and minimum of sample aflatoxins.

Year		Corn grain (ppb)	Cotton seed (ppb)	Maize silage (ppb)	Total mix ration (ppb)	Milk (ppt)
2020	Average	1.42	0.48	0.30	0.95	20.97
	S.D.	1.26	0.70	0.42	0.87	12.50
	Min	0.00	0.00	0.00	0.00	3.00
	Max	4.60	4.00	2.70	4.00	53.00
2021	Average	1.50	0.03	0.21	1.03	16.47
	S.D.	1.36	0.17	0.36	0.91	9.28
	Min	0.00	0.00	0.00	0.00	4.00
	Max	4.00	1.30	2.50	4.00	38.00
2020-2021	Average	1.46	0.25	0.25	0.99	18.73
	S.D.	1.31	0.55	0.40	0.88	11.21
	Min	0.00	0.00	0.00	0.00	3.00
	Max	4.60	4.00	2.70	4.00	53.00
Significance (p≤0.050)		0.668	0.000	0.102	0.502	0.006

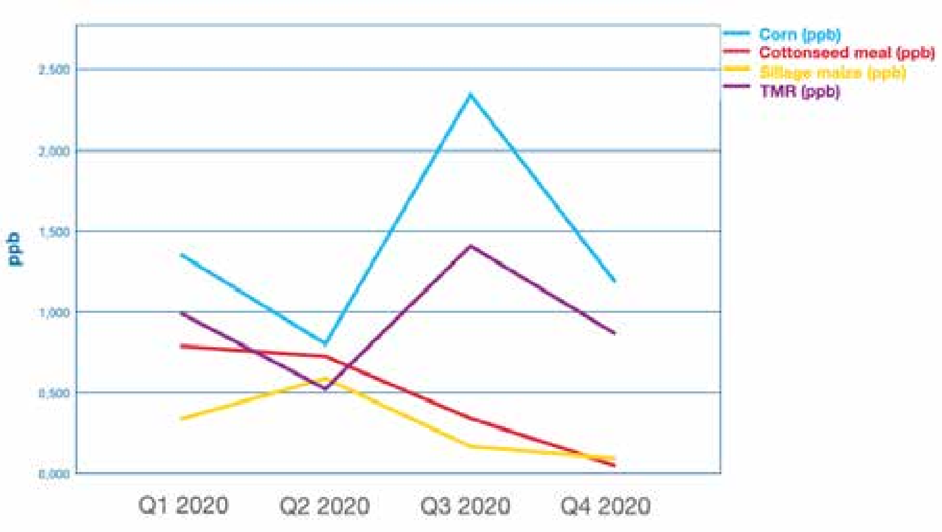

Figure 1. Mean value of aflatoxin B1 (ppb) in feed by quarter of the year 2020.

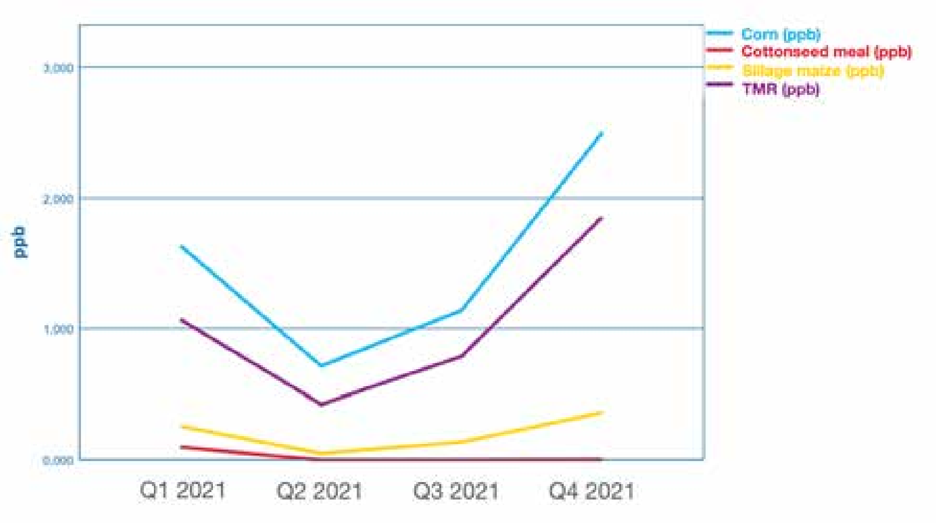

Figure 2. Mean value of aflatoxin B1 (ppb) in feed by quarter of the year 2021.

Figure 3. Aflatoxin M1 (ppt) values in cow's milk samples by quarter for the years 2020 - 2021.

Table 2. Correlation of the variables used (years 2020-2021).

	Corn grain	Cotton seed	Maize silage	TMR	Milk
Corn grain	1				
Cotton seed	0.078	1			
Maize silage	0.143	0.435**	1		
TMR	0.725**	0.178*	0.310**	1	
Milk	0.705**	0.361**	0.394**	0.800**	1

** Significance at p≤0.05 level*
*** Significance at p≤0.01 level*
Source: Calculation on SPSS

From the analysis results, it appears that the null hypothesis was rejected (i.e., the correlation coefficient between the variables is zero). In all cases of the variables studied, positive correlations are calculated. A strong degree of correlation (r>0.700, p≤0.01) was shown between the variables: i) corn grain - TMR (r= 0.725), ii) corn grain - milk (r= 0.705), and iii) TMR - milk (r= 0.800).

Control of dispersion of variable values

Figures (scatterplots) 4-6 for strongly correlated variables did not show a non-linear relationship or significantly divergent values. The dispersion slopes display straight lines, indicating a clear linear relationship rather than a curvilinear one. This reinforces the analysis and demonstrates the degree of correlation among the variables that emerged it.

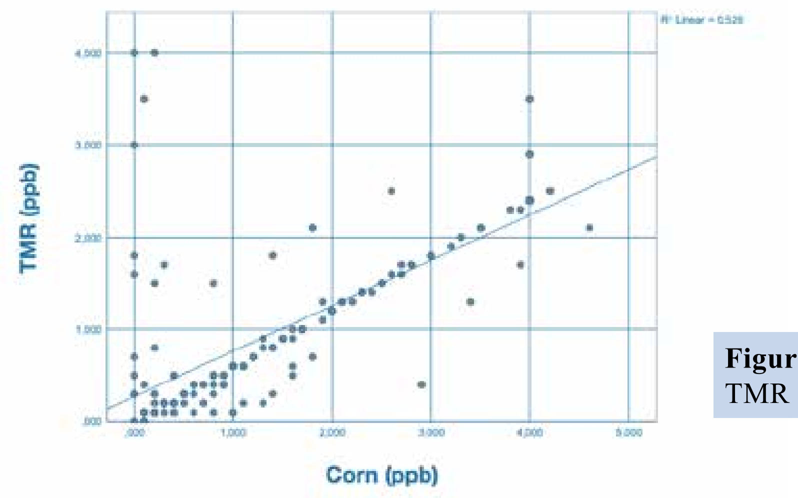

Figure 4. Scatter plot of variable values TMR - Corn (years 2020-2021).

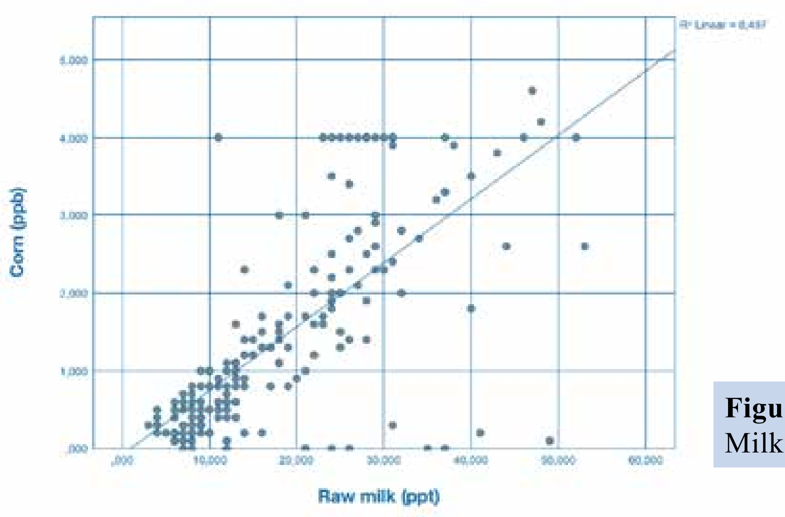

Figure 5. Scatter plot of variable values Milk - Corn (years 2020-2021).

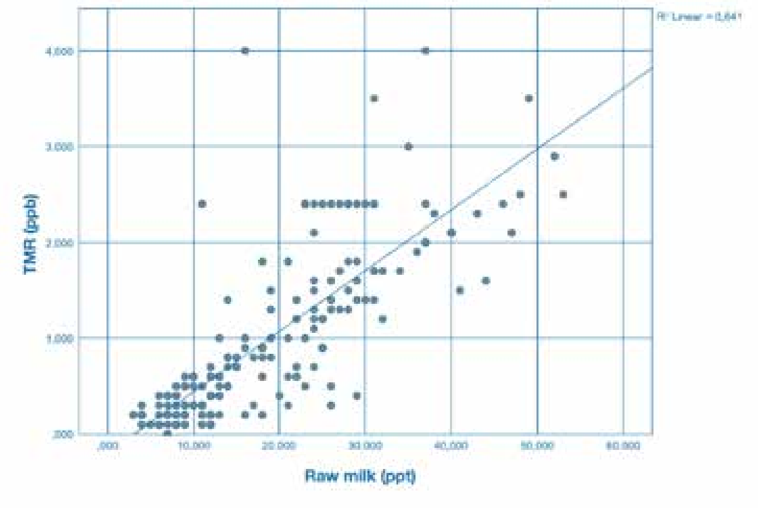

Figure 6. Scatter plot of variable values Milk - TMR (years 2020- 2021).

The regression modelling

The regression model demonstrated a strong overall fit, with an R-squared value of 0.936. This indicates that the predictors could explain approximately 93.6% of the variation in milk aflatoxin levels.

Among independent variable, the following were statistically significant ($p \leq 0.05$):

- TMR: A key predictor with a regression coefficient of 12.53 suggests that a unit increase in aflatoxin levels in TMR results in a proportional rise in milk aflatoxin levels by 12.53 ppt. More clearly, milk aflatoxin (AFM1) increases by 12.53 ppt for every ppb increase in TMR aflatoxin.

Table 3. Regression model summary.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	0.968[a]	0.936	0.920	9.196898

a. Predictors: (Constant), TMR
Source: Calculation on SPSS

DISCUSSION

The average aflatoxin content of cotton pie and milk were significantly ($p < 0.05$) higher in 2020 compared to 2021. The differences are likely due to the varying weather conditions that prevailed during the harvesting of cotton nuts, the ginning process and the storage of the cotton seed (Xu et al., 2007; Tola & Kebede 2016).

In contrast, no statistically significant differences existed between the years for corn grain, maize silage and the TMR. The lack of differences may be attributed to the relatively small percentage of cottonseed participation in the final TMR (2kg per 20kg dry matter).

Many researchers (Kaniou-Grigoriadou et al., 2005; Magan et al. 2011; Damianidis et al., 2018) have reported that weather conditions during the harvest, storage and general management of fodder, likely influenced aflatoxin content by either promoting or inhibiting the growth of *Aspergillus flavus* in corn grain. Moreover, since corn grain represents a significant portion of the TMR, its impact on milk aflatoxin content is noteworthy. This likely explains

Table 4. Regression model analysis.

Coefficients [a]

Model		Unstandardized Coefficients		Standardized Coefficients			Collinearity Statistics	
		B	Std. Error	Beta	t	Sig	Tolerance	VIF
1	(Constant)	23.348	5.960		3.917	.017		
	TMR	15.530	1.637	0.968	7.656	.002	1.000	1.000

a. Dependent Variable: milk (ppt)
Source: Calculation on SPSS

the significantly lower levels of aflatoxins in cow's milk in 2021, compared to 2020.

Strong (r>0.7) and statistically significant (p<0.01) correlations between the variables of feed and cow's milk align with findings from other studies (Battilani et al., 2012, Xu et al., 2021), indicating that aflatoxin levels in milk are significantly influenced and closely correlated with those in animal feed, particularly in the TMR, which is also highly susceptible to attacks by *Aspergillus flavus*.

These findings highlight the critical role of feed management in ensuring milk safety. The high significance of TMR aflatoxin levels emphasizes the need for stringent quality controls during feed production, storage, and handling. The results align with previous studies (Xu et al., 2021) demonstrating the vulnerability of maize-based components to fungal contamination.

CONCLUSION

The regression model provides a predictive tool for estimating milk contamination levels based on feed analysis, enabling proactive measures to minimize public health risks. Future research could enhance the model by incorporating additional variables, such as climatic conditions or feed storage practices, to account for unmeasured confounders. The linear regression analysis underscores the interconnectedness of feed and milk safety. By identifying critical contributors to milk aflatoxin levels, the study offers actionable insights for dairy farm management, advancing toward sustainable and knowledge-based production systems.

It has practical value in line with "knowledge-based raw milk production". This should move us closer to sustainable animal production systems and intelligent livestock that improve raw milk safety and quality.

CONFLICT OF INTEREST

None declared

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