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Administration of Suggested Formula to Improve the Reproductive Performance of Sheep and to Minimize the Negative Effects due to Ingesting Mycotoxin (S) - Contaminated Feed.

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ABSTRACT

This study is an attempt to prevent or minimize the negative probabilities due to ingesting feed contaminated with aflatoxins (AFs). A previous research studies for about 30 years leads to suggest this studied formula. An exposure study extended for 3 different stages was conducted using eighteen Egyptian male sheep at the growing ages. The 1st stage (pre-treatment) was extended for 2 weeks and suggested to compare the performance of animal groupings under the normal conditions before receiving any treatment, either level of contamination(s) or dosage(s) of additive. The 2nd stage (treatment) was extended for 4 weeks and the animals received different levels of aflatoxin(s) (10 mg/kilogram concentrated diet) and / or the studied formula at two levels (250 and 500 mg / head / day). The 3rd stage (post-treatment) was extended for 4 weeks and suggested to transfer treated animal groupings to receive sound diets free from any level of contamination. Dry matter intake (DM), apparent nutrient digestibilities, nutritive values, serum chemistry profiles and AFs concentrations in feed intake, orts, feces and urine; were evaluated. Data revealed that aflatoxins contaminated rations induced significant decrease in daily feed intake and the averages of body weight, body weight gain and feed conversion rates were dramatically affected during the exposure stage to aflatoxins. Additionally, serum constituents and ruminal measurements indicated impaired liver function and digestive disturbances in sheep fed aflatoxin. An addition of studied formula at doses between 250 and 500 mg / head / day, for exactelly 28 days, were able to modify rumen fermentation by changing protozoal activity and motility and could approximately normalized the adverse effects of aflatoxin contamination.

Keywords: Mycotoxin, aflatoxin(s), food contaminants, food additives

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INTRODUCTION

Mcotoxins constitute a significant problem for the animal feed industry and an ongoing risk to feed supply security [1, 2, 53]. Aflatoxins (AFs) are highly hazardous contaminants of common food and feed. Aflatoxin B1 in particular, the most predominant among aflatoxins, was thoroughly demonstrated to be highly toxic, mutagenic, teratogenic and carcinogenic in many animal species [22, 35, 53].

Both human and animal health has been dramatically affected in outbreaks of acute mycotoxicosis, but these tragic events may be only a part of the cost to society in terms of impaired health and productivity from the ingestion of sub-clinical levels of mycotoxins [1, 22, 46, 54]. Acute aflatoxicosis causes hepatitis, hemorrhage, and death. Reduced growth rate is the most sensitive clinical sign of chronic aflatoxicosis, and it may be the only readily detectable abnormality [16, 40].

The metabolism of aflatoxins is due mainly to sex and species differences besides the mitochondrial enzymatic reaction [5, 51]. The rate of metabolism, the repeativeness of exposure, the dosage and type of mycotoxin(s) were reported as limiting factors affect the type of toxic action of mycotoxicosis [1, 24, 46]**.**

In ruminants, the rumen is essentially a fermentation chamber in which the resident microbial population helps to digest the diet. Digestion of food in the rumen occurs by a combination of microbial fermentation and physical breakdown during regurgitation of the food by rumination. The rumen microbial population is very dense, containing 10^{10} bacteria/ml, 10^{6} protozoa/ml and 10^{3} fungi/ml. Ruminal ciliate protozoa play an important role in biodegradation of plant toxins and mycotoxins [57]. Moreover, Ruminal ciliates play an important role in regulation of ruminal condition such as pH. Ruminal ciliate protozoa eliminate certain pathogens from the digestive tract of ruminant, protecting them from disease and so improving the food safety of edible animal products [1, 32]**.**

More attention has focused on the alleviation or prevention of aflatoxicosis by manipulation of dietary nutrients [46] or the inclusion of chemiadsorptive compounds [30, 47,48]. Reports concerning aflatoxicosis in ruminants, in sheep in particular, vary. Some experiments showed that ruminants are more resistant to AFs poisoning than monogastric animals [2, 40]. However, the data in the literature on the extent of ruminal degradation of AF and its effects on rumen microbial activity are not consistent. A dose of 2.6 mg of AFB1 kg of diet significantly reduced feed intake and BW gain and increased serum enzyme activities indicative of liver damage [25, 29].

This work is an attempt to offer an appropriate formula to avoid or to minimize the harmful effects due to ingesting aflatoxin(s) contaminated feed and to evaluate the efficacy of the studied formula when ingested simultaneously with the contaminated feed by experimental animals, considering the animal(s) performance through: the digestibility of nutrients, growth performance, feed utilization, increasing the protozoal activities and its ability to minimize the adverse effects of aflatoxin-contaminated diet biochemical changes.

MATERIALS AND METHODS

The general layout of the study.

Firstly - The suggested additive formulation and application:

In our previous wark the suggested additive formula under investigation experimentally was applied on white rats and give +ve results in vitro so that we are going to examine its effect on farm animals in vivo. The ingredients and their quantities included in this appropriate formula were suggested considering the available our knowledge [46]**.** The combination (Table 1) is a group of food additives that legally used to convert the non antigenic structures to antigenic structures. The ingredients (Table 1) were obtained from the Egyptian Company for Chemicals and Pharmaceuticals, then the combination mixed well and packaged in the capsule form (250 mg) under sanitary conditions. The capsule form was given to animals every day 2 capsules /head.

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Table 1: The composition of the studied formula.

No	Component(s)	Concentration (gm/kg)	The percentage
1	Active silica	50 gm	5 %
2	Reduced glutathione	15 gm	1.5 %
3	Zinc sulphate	15 gm	1.5 %
4	Carbo-diimide	0.1 gm	0.001 %
5	Ascorbic acid	12 gm	1.2 %
6	Choline chloride	100 gm	10 %
7	Lactose	Up to kg	80.8 %

Secondly - *Animals and rations*.

Eighteen apparently healthy Egyptian male Baladi sheep with a mean body weight of 25 - 35 ± 2.34 kg, and age from 6 to 8 months; were selected from a local private farm of Giza province, Egypt, passed through three main stages, which lasted ten weeks as follows:

a- The first pre-treatment stage extended for two weeks to make the animal will be adapted for the tested diet, and to avoid any differences in both type and number of ruminal microflora before treatment. During this stage, all experimental animals were fed sound rations (without aflatoxins or studied formula), checked and observed for any abnormalities. The daily ration contained 70 % concentrated diet (60 % wheat, 9 % soy meal and 1 % mineral/vitamin mixture) and 30 % hay as roughage.

Table 2: Ingredient and Chemical composition of the experimental diets (% D.M.) offered to male sheep during the treatment stage of experiment (4 weeks).

Item	Groups, DM composition %:					
	Group1	Group2	Group 3	Group4	Group 5	Group6
Ingredient %						
Wheat (Aflatoxin free)	60	60	60	57.30	57.30	57.30
Soy meal	9	9	9	9	9	9
[a]AFs- contaminated material (wheat)	0.0	0.0	0.0	2.7	2.7	2.7
Berseem hay	30	30	30	30	30	30
[b]Minerals/vitamin mixture	1	1	1	1	1	1
[c]The studied formula (as capsule)	0.0	1	2	0	1	2
Chemical compositiond						
Crude protein (CP)	11.98					
Crude fiber (CF)	26.88					
Ether Extract (EE)	2.98					
Nitrogen Free Extract (NFE)	58.16					
Organic matter (OM)	94.97					
Ash	5.03					
DE, K cal/kg	2.93					
Moisture	8.55					

N.B. a- 2.70 % AFs- contaminated material equal to 10 mg AFs or 6.48 mg AFB1/kg concentrated feed on dry matter bases. All ingredients except AFB1-cont.wheat were AFB1- free

b- Other ingridients: 1.2% limestone, .24% calcium phosphate, .38% KCl, .4% Co-I salt, .5% trace mineral mix, and 20% vitamin mix. The trace mineral mix was formulated to provide 22 mg of &SO,, .ll mg of Se, 28 mg of ZnO, 28.5 mg of MnO, 750 mg of MgO, 2.0 g of KC1, and 1.6 g of Co-I NaCl per kilogram of mixed diet. The vitamin mix was formulated to provide 7,000 IU of vitamin A, 3,000 IU of vitamin D3, and 6 IU of vitamin E per kilogram of mixed diet.

c- Each one capsule contains 250 mg the studied formula.

d- DM, OM, CP, CF, EE and NFE; are means of dry matter, organic matter, crude protein, crude fiber, ether extract, and nitrogen free extract, respectively.

*DE = 4.36-0.049×NDF = 28.924 + 0.657 (CF %) according to Cheeke [18].

b- The second treatment stage, was extended for further four weeks and the tested animals divided randomly into six experimental groups in equal numbers of animals after approximately similar weight and age, and the first three groups served as control groups and fed on sound rations (free from ochratoxins). The 1st one fed on non polluted ration and had no additive to act as "negative control". The 2nd and 3rd groups acted as "positive control", in which the 2nd group fed on non polluted ration + 1 capsule (250 mg studied formula) from examined studied formula / head /day; but the 3rd group fed on non polluted ration + 2 capsules (500 mg studied formula) / head /day. On the other hand, the 4th group fed on AFs-polluted ration only without any additive formula, but 5th group fed on polluted ration + 1 capsule (250 mg studied formula) from examined formula / head /day. 6th group fed on polluted ration + 2 capsules (500 mg studied formula) /head /day.

c- The 3rd stage (post treatment period) was extended for 4 weeks and the animals received normal ration to study the reversible and irreversible effects.

Feeds were offered in two equal portions at 0700 a.m and 1800 p.m. to meet NRC nutritional requirements [38]. Berseem hay (BH) was offered once daily at 2100 p.m. Fresh water was freely available to animals. Animals were biweekly weighed in the morning before offering any feed or water. Live body weight changes and feed intakes were recorded at biweekly intervals. Chemical analysis and aflatoxins estimation were conducted at Laboratory of Food toxins and contaminants, National Research Centre, Egypt. The protozoal count was conducted at Regional Center for Food and Feed (RCFF), Agricultural Research Center, Ministry of Agriculture, Egypt. The selected dose of AFB1, the selected dose of studied formula and duration period of AFs-exporure, were literature based [11, 15, 17, 46]**.** Studied formula was offered in encapsulated form to prevent palatability problems reported in previous studies [14, 15].

Digestibility trial

At the end of 3rd week of the treatment period, all animals from each group were taken to determine the digestibility and nutritive value of expermintal diets. Samples of rations, refusal, feces and urine were taken daily, for seven days. Refusals were collected daily at 07.30h, weighed, sampled, and then stored. Total daily fecal output for each animal was also collected, weighed, homogenised and 10% of feces samples were dried at 70º C for 24 hr, then blended and kept for aflatoxins analysis according to the AOAC methods [7]**.** Different parameters of digestibility of the different six feeding treatments were measured as: dry matter intake, digestible crude protein intake, percentage of total digestible nutrients (TDN, %), total digestible nutrient intake (TDNI) and percentage digestible crude protein (DCP, %). Nutrient consumed during the digesitibility trail were calculated according to feeds consumed and their chemical composition (AOAC, 1980) as follows:

TDN % = % DCP + % DCF + % DNFE + 2, 25 x % DCEE
With DCP: digestible crude protein
DCF: digestible crude fibre
DNFE: digestible nitrogen-free energy
DCEE: digestible crude ether extract TDNI = TDN% x DMI
With DMI: Dry Matter Intake (DM eaten) Digestibility (D) % = (DMI – DMO/DMI) x 100% With DMO: Dry Matter Output (DM in faeces) DCP % = (DCP/DMI) x 100%**.**

Sampling

Blood samples

Blood samples were taken weekly from the jugular vein prior to the morning feeding, at the following times: 0, 6, 13, 20 and 27 days of treatment period. Blood samples were placed on ice, allowed to clot and after centrifugation; serum was separated and frozen at -20 °C until it was analyzed for several metabolic variables (AFB 1, aflatoxicol, glutamate –oxalacetate transaminase (GOT), glutamate-pyruvate transaminase (GPT), urea and creatinine.

Sampling of rumen liquor

Rumen fluid samples were collected from all animals groups using a rubber stomach tube at 3 hrs post feeding and handled [36]. For experiment, approximately 250 ml of ruminal contents was collected from sheep by stomach tube, separated into two portions; the first was used for immediate determination of pH using digital pH-meter, while the 2nd portoin was transported to the laboratory at 39°C in anaerobic condition to be used for rumen protozoal count, identification and activity estimation. Tubes and equipments were rinsed with 5% formalin and then saline following each use to prevent artificial inoculation of viable protozoa among animals.

Sampling of feces and urine

During the 3rd wk of the experiment, total feces and urine of animals were collected twice daily over a 7-days period. Urine was collected from a sealed brown color bottle, which was also placed 1-day before the toxin administration. Feces were collected in fecal bags. Following the collection period, total samples of urine and feces from each animal were homogenized, and aliquot samples were stored at -20°C until further analysis.

Analytical methods

Preparation of aflatoxin(s) - artificially contaminated ingredient

Aflatoxins (AFs) which were used in this study were produced by a culture of Aspergillus parasiticus NRRL 2999 (obtained from the Mycotoxin Lab., N.R.C., Dokki, Giza, Egypt.) on wheat which was used as a basal material [49]. The infected medium was incubated for 17 days at 28 °C. Qualitative and quantitative assay for the presence of aflatoxins in the contaminated substrate has been carried out using HPLC (Agilent Technologies, Waldbronn, Germany) as recommended by the AOAC methods [7]**.** The obtained data exhibited that each kg of the contaminated wheat material was proved to include 240 mg B1, 30 mg B2, 85 mg G1 and 15 mg G2 /kg contaminated material. The AFs within the wheat material consisted of the wheat meal was incorporated into the basal diet in the ratio 2.7. % of the daily ration, to provide the desired level of 10 mg of total AFs or 6.48 mg AFB1/Kg diet. The diet containing AFs was analyzed and the presence of parent aflatoxins was confirmed by HPLC.

Aflatoxins analysis

All standards of aflatoxins were purchased from sigma company, USA. All Chemicals and solvents used were of ACS grade. Thin layer TLC aluminum plates recoated with 0.25 mm silicagel 60 (Merk). Aflatoxin(s) in feed and feces samples were extracted by B.F. method as described in the AOAC methods [6]. Extracts were dissolved in soul chloform and vortex, 20µl aliquot and 10µ of the standards were stopped on TLC plates and developed in dark room with chloroform: actone (90:10). After drying the spots were examined with U.V at A wave length of 365 nm. The AFB1 and its metabolites B2α and aflatoxicol were analyzed in the feces and urine samples according to method of Richarda & Lyona [44]**.**

The chemical composition of the diet and feces

The chemical analysis of feeds and feces were carried out according to the AOAC methods [6]. Feed samples were collected on days: 0, 14, and 28; and composited. Samples of each group diet were analyzed for determination of dry matter (DM), organic matter (OM), percentage of crude protein (CP), ether extract (EE), crude fibers (CF), and nitrogen-free extract (NFE), [7]. Concentrates, hay, and feces were successively ground in mills with 3- and 1-mm screens. Nitrogen was determined using the standard Kjeldahl procedure with K2SO4 and CuSO4 as catalysts. The OM was determined by ashing at 550°C overnight. The NDF (cell wall), ADF, and 72% H2SO4 lignin were analyzed as described by Van Soest *et al.* [52], except that NaSO3 was not used in the NDF preparation.

Rumen protozoal count in rumen content

The total protozoal count was conducted according to Abou El-Naga [4]. Two 5 ml duplicate liquors of rumen content were separately taken and diluted five times by addition of 15 ml saline solution and 5ml of lugol's iodine solution. Immediately after gentle shaking, one ml liquor was taken up in a one ml wide mouthed graduated pipette. As quickly as possible, exactly 0.1 ml was pour on a dry clean slide which was then carefully covered by a dry clean cover slide with a dimensions of 23 × 51 mm (total area of 1173 mm2). Counting was carried out using the low power; 30 fields in each slide were counted and chosen as representative to the whole area. The average count in 30 fields, which represents the protozoal count per one square mm area of the field, was multiplied by 1173 (the area of the cover slide) to give the protozoal count in 0.1 ml of the diluted sample, which represents 0.02 ml of original sample. Therefore, the total protozoal count /1 ml rumen content = average count in 30 field ×1173×50. Each of the two diluted duplicate was counted and average was calculated.

Evaluation of protozoal activity and motility

Survival rate was evaluated according to Nasbimana *et al.* [36]. The survival rate was estimated by counting the proportion of motile ciliate under a microscope. Motility of ciliates was examined and counts were repeated 5 times per sample and the mean was calculated to be considered as individual reading.

Determination of ruminal pH

The pH values of the collected rumen juice samples were estimated by means of an electric pH–meter (Wissens Chaftlish tehcnisch werkstatten D 8/20 weitheim Ph 40) according to Nassar [37].

Determination of several metabolic variables

The profiles of several metabolic variables were measured calorimetrically in the serum samples of each animal using spectrophotometer (Instruction Manual UV-1201, Shimadzu) and commercial kits. Urea and creatinine [28], GOT and GPT [43]; were purchased from (Stanbio Laboratory, North Main, Boerne, TX USA).

Statistical analysis

The differences in feed intake, average daily gain, nutritive values and apparent digestibility coeffecients, were examined using F- Test through the analysis of variance (ANOVA) according to Snedecor & Cochran [50]. The differences among periods were tested using Duncan's multiple range test [21]. All data were represented by means ± standard error (SE). All differences were considered statistically significant at ($P < 0.05$).

RESULTS AND DISCUSSION

Feed efficiency entire the 28-days feeding period

It is interest to note that nutrient consumed during feeding period was calculated according to feeds consumed and their chemical composition. Sheep data for the body weight gains, dry matter intake, and nutritive values of all experimental groups are presented in table 3 & figure 1.

When examined during the entire 28-d feeding period, sheep fed aflatoxins-contaminated rations with or without studied formula had significantly lower ($P< 0.05$) daily feed intake, TDN, DCP and body weight gain, the greatest decrease was seen in sheep fed AFs- contaminated diet only. However, Studied formula treatment significantly ($P< 0.05$) imoproved the recorded numbers of those parameters especially at high dose of studied formula (table 3 & figure1). These results are supported by [3,27,46].

In regard to nutritive values, the nutritive value as TDN for the six experimental groups showed comparable results and ranged between 53.38 and 29.96 %. Exept for the two groups 3 & 4, there were no significat differences between the four other groups (groups; 1, 2, 5, 6). The low DCP (11.52 %) for the aflatoxin-treated group tended to be significant ($P<0.05$) the lowest digestible crude protein (DCP), while those

of negative control group, and the suggested formula supplemental groups were 14.8, 14.87, 15.08, 13.7 and 13.92 %; for groups; 1, 2, 3, 5 and 6, respectively. The lower nutrient digestibilities of groups fed aflatoxin–contaminated diets resulted in lower TDN% and DCP % than that of other groups, and this may be due to a tendency for reduced digestibility of nutrients.

The use of studied formula, as feed supplement to sheep diet in this study, had no adverse effect on DM intake or average daily gain where aflatoxins may disrupt growth and function of ruminal microorganisms and together with changes in volatile fetty acids (VFA) production may be responsible, in part, for the decreased growth and performance seen in ruminants fed aflatoxins [33].

Feed conversion expressed as kg DM /kg body weight gain were 10.71, 12.14, 7.54 and 11.34% for groups 2, 3, 5 and 6; respectively. This indicated that addition of studied formula to sheep diets improved DM conversion for these groups, copmared to the aflatoxin-treated group. We suggest that studied formula may increase rumen microbial fermentation in the direction which total VFA and ammonia-N concentrations increased. The results of Molero, *et al.* [34] may support this hypothesis.

Our results were not in agreement with those obtained in vitro by Castillejos *et al.* [17], who found that thymol at higher doses (500 mg/L) decreased total VFA and ammonia-N concentrations, and increased the acetate to propionate ratio. Differences in those results than our results may attribute to other factors (Oil ingredients and dosing level, composition of the diet and /or others).

In this study, feeding AFs markedly decreased feed intake and daily gain remained lower in sheep fed AFs, particularly in sheep fed dietary aflatoxins alone. The exact mechanism by which AFs impairs growth is unknown, but it is probably multifactorial, involving disturbances in carbohydrate, lipid, and protein metabolism, nutrient interactions, and disturbances in hormonal metabolism [2, 41]**.** Additional signs of poisoning include poor appetite, reduced feed intake, and immunosuppression common in chronic aflatoxicosis, may account (at least partially) for reduced performance [40, 41].

Table 3: The efficacy of studied formula and / or aflatoxin(s)-contaminated diet on sheep performance (Nutrients intake, nutritive values, body weight gain and feed conversion) during the treatment period.

	Expermental groups (Means ±SE)						**LSD (P<0.05)**
	Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**	
Daily DM intake, gm	1216±23.2a	1307± 31.5 b	1176 ± 18.6 a	1085± 26.5 c	1182 ±34.1 a	11168 ±22.6a	85.5
Body weight							
Initial B.W.	34.3±0.48a	36.7 ±0.69 b	31 ±1.12 c	29.8±1.23 c	32.3 ±0.62 d	30 ±0.93 c	1.74
Final B.W.	39.2±0.86a	41.5±0.89b	35.8±0.63 b	31.5±0.92d	35.3 ±1.23e	34.2 ±2.03e	1.56
Total B.W. gain (kg)	4.9 ±0.23 a	4.8 ±0.34 a	4.8±0.19 a	1.7±0.09b	3 ±0.19c	4.2 ±0.66 d	0.48
B.W. gain (gm/day)	175a	171.4a	171.4a	60.7b	107.1c	150d	18.3
Nutritive values:							
TDN intake (gm/day)	649±2.13b	684 ±3.6b	623.77±1.65c	324.8±1.14 a	504.4 ±3.11b	568 ±2.25b	18.40
DCP intake (gm/day)	96±3.7d	101.7±8.4d	94±4.8c	37.4±2.5a	69.1±5.48b	79±6.1c	23.15
TDN%	53.38	52.33	53.01	29.96	42.67	48.63	12.59
DCP%	14.8	14.87	15.08	11.52	13.7	13.92	-
% Feed conversion ,as							
B.W. gain / DMI, kg	11.66±0.13b	10.71±0.09b	12.14±0.17c	4.73±0.14a	7.54±0.12d	11.34±0.10d	0.83

a,b,c,d, e Means in the same row having different superscripts are significantly different at (p<0.05)

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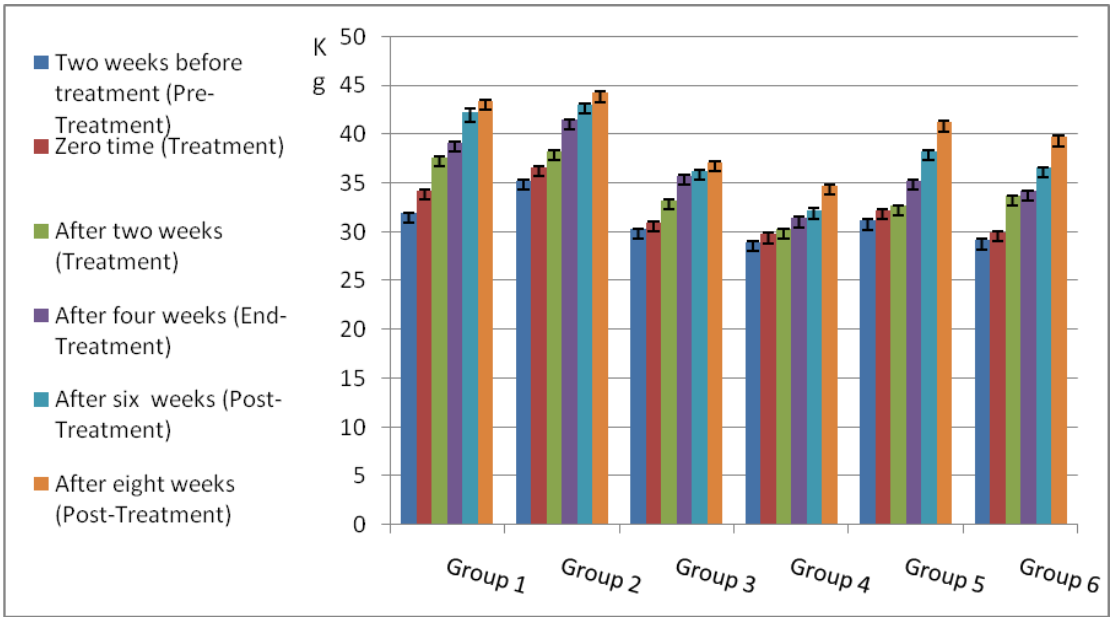

Figure 1: Body weight of sheep as affected by the studied formula and / or aflatoxin(s)-contaminated diet during experiment.

The efficacy of studied formula and / or aflatoxin(s)-contaminated diet on some rumen liquor parameters

Results of pH values, protozoal count, motility and activity of rumen protozoa are presented in tables 4 & 5 and figure 2. Aflatoxin(s)-treated groups recorded higher pH values, decreased protozoal count and reduced protozoal motility and activity at three hours post feeding; compared with the control groups.

Adding studied formula, improved the negative effects of aflatoxin treatment, and these effects of the suggested formula increased as their level increased (tables 4 & 5 and figure 2). These results are in agreement with those found by Mahmoud and Evans & Martin [23, 31]**.** These differences in pH values (table 4) may be related to fermentation process of both non-structural and structural carbohydrates and production of volatile fatty acids (VFAs) as which affected the pH to same limit until they were proportionally and relatively absorbed from the rumen wall. This assumption is in agreement with the conclusion of Reddy and Reddy [42]**,** who stated that the pH values were inversely related to VFAs. The present results and those of the previous studies indicate that diet may influence the pH and probably the type of microflora that developed in the rumen.

Diets which promote rapid fermentation in the rumen result in a rapid production of VFA, which is usually associated not only with a reduction in pH of the rumen, but also with a change in the microbial population, particularly the ruminal protozoa [29, 55]**.** In vitro, the main studied formula componants, Carvacrol and thymol at higher doses (300 mg/L); increased pH values and butyrate proportion, and decreased acetate and propionate proportions, and total VFA concentration [13]. Feeding essential oils to dairy cattle increased ruminal pH and ADF digestion, but had no effects on protozoal counts or animal performance [10, 11]. No effects of the addition of CRINA (A blend of essential oils containing thymol, eugenol, vanillin and limonene, among other compounds; patent EP 0646321 B1 [9]; on DM intake and growth rate of beef cattle. The differences in results may attributed to differences in the experimental conditions (Feeding and animal type, treatment period, the tested material, etc) [45]. Ruminal motility may be affected by single acutely toxic doses of AFs [20] ; however, effects of lower, more environmentally prevalent concentrations have not been determined. Such effects on ruminal motility would potentially contribute to the decreased gain seen in chronic intoxication (table 5 & figure 2).

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Table 4: Mean pH values after three hours post feeding during the study.

Groups	Stages of experiment					
	Two weeks before treatment	Ten weeks treatment stage				
		Zero time	After two weeks	After four weeks	After six weeks	After Ten weeks
Group 1	6.28±.001Aa	5.41±.012Aa	4.99±.003Ba	5.25±.009Aa	6.89	5.98
Group 2	6.23±.006Ab	5.35±.017Ab	5.12±.028Aa	5.23±.035Aa	5.33	5.49
Group 3	6.61±.053Ab	6.01±.007Ab	5.39±.016Aa	5.37±.017Aa	6.84	6.08
Group 4	6.22±.003Aa	5.79±.008Aa	5.20±.031Bb	5.23±.012Bb	5.22	5.11
Group 5	5.96±.008Ab	5.90±.016Ab	5.19±.016Aa	5.15±.015Aa	5.48	5.51
Group 6	5.96±.006Ab	5.88±.043Ab	5.47±.004Aa	5.13±.026Aa	5.34	5.17
LSD	0.61					

N.B: Different capital letters in columns between means denote significant difference between treatment in the same period at ($p<0.05$) and vice versa. But means in the same row having different small superscripts denote significant change between periods in the same treatment and vice versa

Table 5: Evaluation of protozoal activity and motility.

The semi-quantitative determination of both motility and activity of rumen protozoa classified the obtained results into four degrees with certain symbols.

Groups	Stages of experiment					
	Two weeks before treatment	Ten weeks treatment stage				
		Zero time	After two weeks	After four weeks	After six weeks	After Ten weeks
Group 1	++++	++++	++++	++++	++++	++++
Group 2	++++	++++	++++	+++	++++	++++
Group 3	++++	++++	+++	+++	+++	+++
Group 4	++++	++++	+++	++	+++	+++
Group 5	++++	++++	+++	++	++	++++
Group 6	++++	++++	+++	++	++	+++

The 1st (+), 2nd (++), 3rd (+++) and 4th (++++) degrees refer to weak, moderate, good and extremely of protozoal motility and activity, respectively.

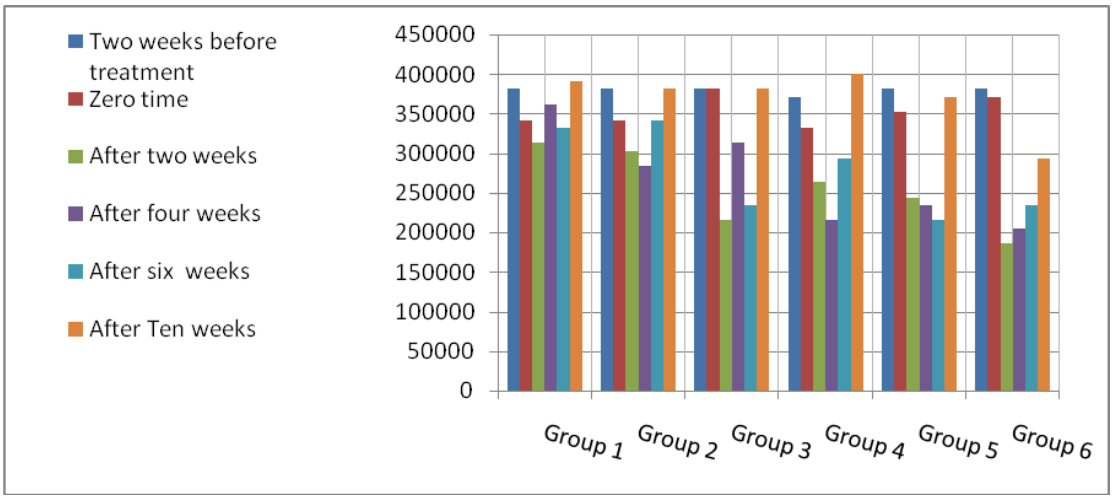

Figure 2: Mean protozoal count (cell / mL rumen liquer) after three hours post feeding dietary aflatoxins and / or studied formula during the study.

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The biochemical changes in different studied groups as affected by the studied formula and / or aflatoxin(s)-contaminated diet during experiment period.

Data for selected serum constituents are presented, for the three different stages of this study, in tables 6 & 7. During the pre-treatment stage the average(s) of the transaminases (GOT and GPT) level showed the normal picture with no differences between groups. During the treatment stage and compared with those fed sound rations with or without studied formula, serum activities of GOT and GPT were significantly ($p \leq 0.05$) elevated in sheep fed AFs-contaminated diets (table, 6).

Similarly, in table 7, urea and creatinine concentrations were higher ($p \leq 0.05$) at the end of the treatment period for group which fed AFs-contaminated diet only (group 4). However, no significant differences were noted for groups fed AFs-contaminated diets plus studied formula either at low or high levels (groups, 5 and 6). Except for the group 4 which fed aflatoxin-contaminated diet only, the post-treatment stage showed gradual decrease back to normal to reach almostly the same values of control group after 4 weeks from stopping exposure to aflatoxin(s). Such response was reported by some investigators [1, 16] when exposed mice to contaminated diet containing aflatoxin B_1.

Table 6: The biochemical change in serum GOT & GPT activities in different studied groups as affected by the studied formula and / or aflatoxin(s)-contaminated diet during experiment period.

Groups	Serum levels (IU / L) of GOT & GPT during 3 different stages of the study					
	SGOT			SGPT		
	Two weeks before treatment	Four weeks treatment	Four weeks post treatment	Two weeks before	Four weeks treatment	Four weeks post
1	44.31±6.3Aa	49.22±5.6Aa	39.49±5.9Aa	18.30±2.6Aa	16.54±2.4Aa	20.17±2.4Aa
2	39.86±5.5Aa	38.99±4.1Aa	44.29±4.6Aa	15.38±1.9Aa	13.1±1.1 Aa	19.55±1.8Aa
3	36.40±3.9Aa	39.36±6.5Aa	29.42±3.5Aa	14.12±1.8Aa	8.67±0.5 Aa	10.92±0.8Ba
4	47.28±7.5Aa	85.44±8.3Cc	60.95±6.4Bb	15.04±2.5Aa	58.50±4.3Cc	38.97±4.7Cb
5	46.88±4.1Aa	61.63±6.1Bb	45.80±5.3Aa	11.08±1.3Aa	27.58±2.2Bb	20.69±3.2Ab
6	35.77±4.9Aa	48.36±4.2Aa	42.02±7.5Aa	13.40±1.4Aa	20.44±2.5Bb	11.28±1.1Aa
LSD	11.73			7.85		

N.B: Different capital letters in columns between means denote significant difference between treatment in the same period at ($p<0.05$) and vice versa. But means in the same row having different small superscripts denote significant change between periods in the same treatment and vice versa.

Table 7: The biochemical change in serum urea & creatinine concentrations in different studied groups as affected by the studied formula and / or aflatoxin(s)-contaminated diet during experiment period.

Groups	Serum concentrations (mg / 100 ml) of urea & creatinine during 3 different stages of the study					
	Urea			Creatinine		
	Two weeks before treatment	Four weeks treatment	Four weeks post treatment	Two weeks before	Four weeks treatment	Four weeks post
1	26±3.4 Aa	28±4.2 Aa	29±3.8 Aa	49.14±2.17Aa	50.4±4.3 Aa	52.75±2.7 Aa
2	21±5.5 Aa	23±5.3 Aa	19±5.1 Ba	45.53±5.03Aa	46.25±2.8 Aa	46.12±1.8 Aa
3	28±2.4 Aa	26±1.3 Aa	25±2.9 Ba	50.64±2.13Aa	50.18±3.2 Aa	50.5±4.15 Aa
4	25.5±2.3Aa	56±3.7 Ca	48±6.2 Ca	47.75±4.2 Aa	110.32±5.7Cc	93.17±6.7 Bb
5	23±4.5 Aa	39±2.8 Ba	28±3.8 Aa	43.24±3.56Aa	69.03±6.5 Bb	50.5±5.6 Aa
6	22±3.9 Aa	35±4.6 Ba	26±1.7 Aa	46.68±2.81Aa	53.2±2.8 Aa	47.13±3.36Aa
LSD	6.71			7.53		

N.B: Different capital letters in columns between means denote significant difference between treatment in the same period at ($p<0.05$) and vice versa. But means in the same row having different small superscripts denote significant change between periods in the same treatment and vice versa.

As mentioned before by many invistigators, the effects of AFs on liver function can vary with the amount, duration of intoxication and the serum levels of GOT and GPT increased after liver damage because of

increased membrane permeability or because of liver cell necrosis and cytosol leakage into the serum [1, 39, 46].

Our results were similar with those observed by Harvey *et al.,* [25], who reported that a dose of 2.6 mg of AF/kg of diet significantly increased serum enzyme activities as indicative of liver damage. Similar findings have been reported in sheep [25], goats [1, 19], cattle [26], and in rats [1, 46]. In dairy sheep, the daily intake of pure AFB1 at level ranged between 32 and 128 µg / d for an exposure period of 1 wk did not alter liver enzymatic activity [8].

Our results may lead us to suggest that 10 mg of Afs / kg of diet was sufficient to impair performance and cause liver damage and kidney dysfunction in male sheep, but studied formula could protect sheep from hepato-toxicities and kidney dysfunction which induced by AFs-contaminated diet when used as a sole diet for sheep.

Proportional urinary and fecal excretion of aflatoxin B1, and its corresponding metabolite aflatoxicol, for sheep fed AFs-contaminated diets.

The proportional excretions of AFB1 and its metabolite aflatoxicol via feces and urine as well as the % recovery of thes toxins are shown in table 8 & figure 3. The concentrations of AFB1 and aflatoxicol in feces and urine varied according to dietary treatment. Prior to initial exposure to aflatoxin (on day 0 of the experiment), there was no detectable AFs in the urine or feces of the tested sheep. After four weeks of exposure, Afs as ng / mL of urine and Afs as ng / gm of feces were observed in all aflatoxin - treated groups and the most of the excreted AFB1 and its metabolite aflatoxicol were found in the urine for all groups fed aflatoxin-contaminated diets with or without the suggesting formula. (table 8). These results indicate that some of the AFs were not degraded by the ruminal microflora, and it was absorbed and distributed in the animal tissues. Also the major excretory route was found to be the urine (accounting for 23.9% to 68.8% of the tatal AFs-excretion forms, whereas less than 20% of these forms were excreted in the feces.

Table 8: Proportional urinary and fecal excretions (mg/head/day) of aflatoxin B1, and its corresponding metabolite (aflatoxicol) of sheep fed aflatoxin(s)-contaminated diets for 28 days .

Items	Animal grouping					
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
1- Average daily intake (mg/head)						
AFB1	0	0	0	8.31±0.14	9.2±0.09	8.574±0.05
2- Average daily excreted in feces (mg/head) and % Recovery,						
AFB1 excreted	0	0	0	.05929 ±0.04	0.9887±0.06	0.9442±0.008
Aflatoxicol excreted	0	0	0	0.1301±0.06	0.5933±0.02	0.7273±0.03
AFB1 recovery,%	100	100	100	0.71	10.75	11.02
Aflatoxicolrecovery,%	100	100	100	1.57	6.45	8.48
3- Average daily excreted in urine (mg/head) and % Recovery,						
AFB1 excreted	0	0	0	1.5193±0.001	2.82 ± 0.08	2.948 ± 0.04
Aflatoxicol excreted	0	0	0	0.4667±0.05	1.642±0.04	2.522 ±0.03
AFB1 recovery,%	100	100	100	18.28	30.65	34.38
Aflatoxicolrecovery,%	100	100	100	5.62	17.85	29.42
Total recovery,%	100	100	100	32.6	65.7	83.3

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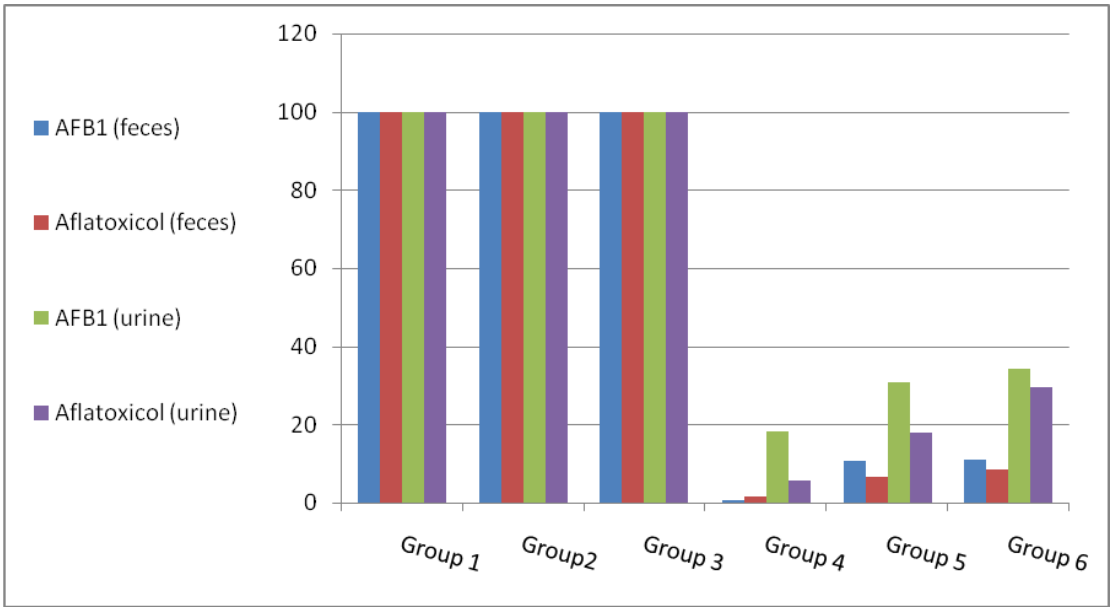

Figure 3: % recovery of fecal excretions of fecal and urinary excretions of aflatoxin B1, and its corresponding metabolite (aflatoxicol) of sheep fed aflatoxin(s)-contaminated diets for 28 days.

Studied formula-treatment significantly increased fecal and urinary excretion of aflatoxicol; this might be explained by a more pronounced renal elimination, which in turn might result in higher biliary secretion of aflatoxicol in these groups. The lower toxicities of AFB1 and aflatoxicol in mammals are mainly as a result of a faster rate of clearance via urine and feces compared with that of AFB1 [12], these results were in the same trend with. Therefore, processes that enhance the conversion of AFB1 to aflatoxicol will tend to reduce the general toxicity of AFB1 in the animal itself or the toxic potential of AF-contaminated feed (e.g., when dietary aflatoxin is administered to ruminants rather than to non ruminants). This implies that the amount of AFS in the rumen greatly affected the amount of AFB1 in the blood in our study. Also, these results demonstrated that the rate of disappeaxance of AFB1 from the rumen was much higher in sheep fed aflatoxin-contaminated diets with the additive formula than in sheep fed aflatoxin- contaminated diet only. This may be due to that diet influences the pH and probably the type of microflora that developed in the rumen, and as a result, the rate of hydrolysis of AFB1 was reduced in sheep fed aflatoxin-contaminated diet only compared with those fed aflatoxin-contaminated diet with the additive formula [29, 55].

CONCLUSIONS

In conclusion, all treated animals were responding to contaminated diets and to the additive as well. Aflatoxins contaminated rations induced significant decrease in daily feed intake, TDN, DCP and body weight gain. Additionally, serum constituents and ruminal measurements indicated impaired liver function and digestive disturbances in sheep fed aflatoxin. An addition of studied formula at doses between 250 and 500 mg / head / day, for exactelly 28 days, were able to modify rumen fermentation by changing protozoal activity and motility and could approximately normalized the adverse effects of aflatoxin, perhaps attributed to its effect on ruminal pH and improving digestibility and animal performance. The exact mode of antitoxic action may need further clarification and there is an urgent need to conduct further in vivo studies with studied formula, which may provide a useful tool to improve efficiency of nutrient utilization in the rumen and could be recommended to sheep diet with possibility of aflatoxin contamination.

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