SAMITEC INSTITUTE

Final Report # FP01/14

***In Vivo* Test**

Start of Experiment: December 23rd, 2013

End of Experiment: January 27th, 2014

COMPANY

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Additive tested: TOXFREE (MYCOAD AZ, COBIND AZ)

Animal species tested: fish (tilapia – *Oreochromis niloticus*)

Mycotoxin tested: Fumonisins

PARAMETERS TESTED

Performance data

Test performed in conformity with the recommendations of the Task Force of the Ministerio de Agricultura, Pecuaria e Abastec (MAPA) for the registration of Anti-Mycotoxins Additives. Trial conducted in conformity with the resolution no. 879 of February 15th, 2008 of CFMV for animal testing and animal welfare.

RESULTS

It contains the description of the evaluation of an Anti-Mycotoxins Additive (AMA) that complies with the requirements for approval.

EFFICACY *IN VIVO* OF THE ANTI-MYCOTOXINS ADDITIVE TOXFREE (MYCOAD AZ – COBIND AZ) ADDED TO A DIET CONTAMINATED WITH FUMONISINS.

An evaluation of the efficacy of the Anti-Mycotoxins Additive TOXFREE (MYCOAD AZ – COBIND AZ) – concentration of 0.10% - against a fumonisin challenge – 100ppm – in fish diets (tilapias – *Oreochromis* niloticus) was conducted in the SAMITEC Institute (Institute of Analytical, Microbiological and Technological Solutions) between December 23rd, 2013 and January 27th, 2014

FACILITIES

The study was performed in an experimental unit of SAMITEC. The experimental room, measuring 22 m^2, was maintained at an ideal temperature for the development of the animals of the age group used (25ºC). Negative pressure was maintained throughout the experiment. Fishes were allocated in 24 polypropylene cages with an individual capacity of 60 L each, for a total of 2500 L of a recirculating water system. Water recirculation was maintained at 3 L per minute, for 24h, with two biofilters built into the system.

Water temperature was controlled by an electric heater regulated by a thermostat. The daily water renovation rate was approximately 5%, considering the need for clearance of dejections and feed waste. Physic-chemical analysis of water was performed using a kit (Alfa Tecnoquimica) measuring ammonia nitrogen, dissolved oxygen, pH, water transparency, total alkalinity, total water hardness and temperature (Annex Figure 3).

ANIMALS AND FEEDING

180 fishes from the species *Oreochromis niloticus*, known as Tilapia of the Nile were used for the experiment. Average weight and total size at the start of the experiment (day 0) were 3.43 g and 5.54 cm, respectively. Handling of fishes was the same as used routinely for fish housed in cages: fishes were fed twice a day (8 and 18 hours), an amount equivalent to 5% of total biomass. Amount of diet fed was adjusted on days 0, 7 and 14 of the study, based on a sample of 100% of fishes included in the experiment. On weighing days, animals were fasted 6h before the measurement. The diet was isonutritious for the total duration of the experiment and its composition is represented on Table 1.

Table 1. Nutritional Levels of Fish Diets

Nutrients	Initial Diet (1-21days)
Crude Protein (%)	35.22
Metabolizable Energy (kcal/kg)	3,444
Calcium (%)	3.20
Available Phosphorus (%)	1.87
Crude Fiber (%)	2.54
Dry Matter (%)	90.73
Ashes (%)	10.65
Ether Extract (%)	11.16

MYCOTOXINS USED IN THE STUDY

Fumonisins (B_1, B_2) were obtained from a culture of a toxigenic strain of *Fusarium moniliforme*. Concentration of aflatoxins used int he study are shown in Table 2.

Table 2. Type and Concentration of Fumonisin used

Fumonisin Type	Concentration (%)
B_1	95.80
B_2	4.20

EXPERIMENTAL DESIGN

A Completely Randomized design was used, with the 180 fishes distributed in 3 treatments, with 5 repetitions for treatment, with 12 fishes in each repetition. Treatments were established as shown on Table 3.

Table 3 Treatments

Treatment	Number of fish	Fumonisin (ppm)	TOXFREE (MYCOAD AZ – COBIND AZ, %)
1	60	-	-
2	60	100	-
3	60	100	0.10

EXPERIMENTAL PARAMETERS

The following parameters were measured weekly (0, 7, 14 and 21 days of age)

- Live Weight of fishes obtained by individual measurement.
- Fish Size: Measured according to NAFO (Northwest Atlantic Fisheries Organization). It was measured in a straight line from the cranial end of the face to the end of the longest lobe of the tail fin when both lobes are depressed on the midline.

STATISTICAL ANALYSIS

The statistical analysis was performed using descriptive statistical analysis (mean and coefficient of variation). Analysis of Variance (ANOVA) was performed using Bonferroni test ($P \leq 0.05$) for comparison of means. The analysis were done using Stagraphics Centurion XV version 15.1 software package.

RESULTS AND DISCUSSION

Following are the results of the trial and some comments.

1. AVERAGE WEIGHT OF TILAPIAS

Table 4 and Figure 1 show data on average weight of fishes from the start to d7, 14, 21, 28 and 35 of the experiment.

Table 4. Average Weight (g) of Tilapias of the Nile fed a diet containing fumonisin, with and without the addition of TOXFREE (MYCOAD AZ – COBIND AZ), during 35 days.

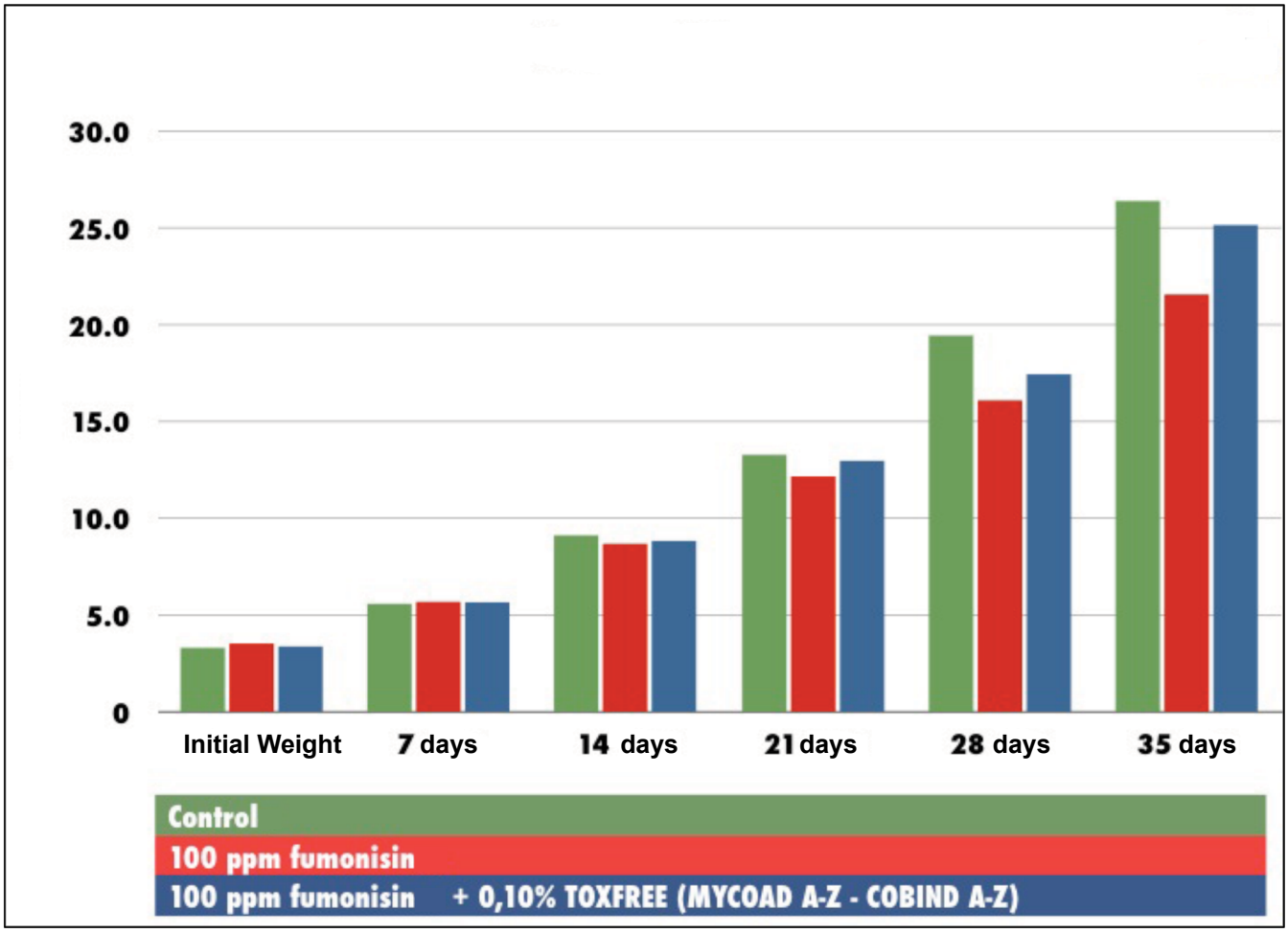
Treatment	d0		d7		d14		d21		d28		d35	
	ABW	CV	ABW	CV	ABW	CV	ABW	CV	ABW	CV	ABW	CV
Control	3.33[a]	21.4	5.60[a]	21.6	9.14[a]	26.9	13.30[a]	30.4	19.46[a]	23.7	26.41[a]	20.7
100ppm fumonisins	3.55[a]	20.2	5.70[a]	19.9	8.70[a]	24.	12.18[a]	27.5	16.11[b]	28.4	21.58[b]	29.7
100ppm fumonisins + Toxfree	3.40[a]	17.5	5.68[a]	20.6	8.85[a]	22.9	12.99[a]	27.6	17.46[ab]	28.9	25.17[a]	27.7
Mean	3.43	19.7	5.66	20.6	8.88	24.5	12.77	28.5	17.47	28.1	24.15	27.6

[a– b] Means in the same column with different superscripts differ by Bonferroni test ($P \leq 0.05$)

ABW = Average Body Weight (g)

CV = Coefficient of Variance (%)

Figure 1. Average Weight (g) of Tilapia fed a diet containing fumonisin, with and without the addition of TOXFREE (MYCOAD AZ – COBIND AZ) during 35 days.



1.1 Discussion

Table 4 and Figure 1show the average weight of Tilapia at time of allocating (3.43 g). The absence of a significant difference ($P \leq 0.05$) in the weight of fishes across treatments, indicates the same body condition among fishes allocated to the different treatments at the start of the trial.

At day 35 , the presence of 100ppm of fumonisins in the diet of Tilapia, resulted in a lower body weight (-18.28%) when compared to control treatment. On the other hand, fishes receiving 100ppm fumonisins + 0.10% TOXFREE (MYCOAD AZ -COBIND AZ) showed a significant higher average body weight (+16.63%) than fishes receiving 100ppm fumonisins in the diet.

2. AVERAGE TOTAL SIZE OF TILAPIAS

Table 5 and Figure 2 show the values for average total size of tilapias during the experimental period.

Table 5. Average Total Size (cm) of Tilapias of the Nile fed a diet containing fumonisin, with and without the addition of TOXFREE (MYCOAD AZ – COBIND AZ), during 35 days.

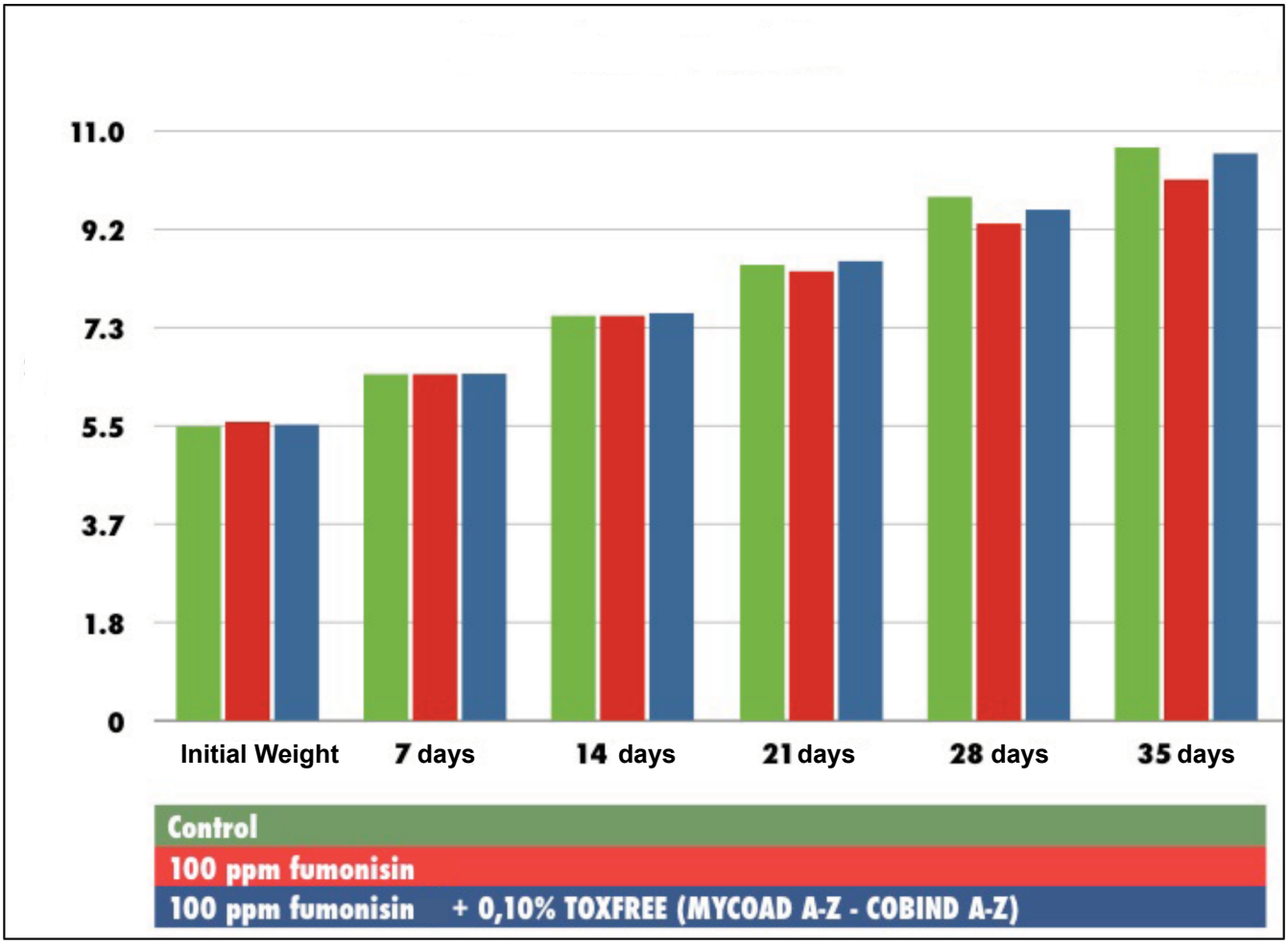
Treatment	d0		d7		d14		d21		d28		d35	
	ATS	CV	ATS	CV	ATS	CV	ATS	CV	ATS	CV	ATS	CV
Control	5.50[a]	6.7	6.47[a]	8.2	7.56[a]	10.7	8.51[a]	13.2	9.78[a]	9.1	10.70[a]	7.5
100ppm fumonisins	5.58[a]	6.	6.47[a]	7.2	7.56[a]	8.4	8.40[a]	9.6	9.28[b]	9.6	10.10[b]	10.
100ppm fumonisins + Toxfree	5.53[a]	5.6	6.48[a]	6.9	7.61[a]	8.6	8.58[a]	10.5	9.54[ab]	11.2	10.60[a]	9.7
Mean	5.54	6.3	6.48	7.3	7.58	9.1	8.49	11.0	9.51	10.2	10.44	9.6

[a– b]Means in the same column with different superscripts differ by Bonferroni test ($P \leq 0.05$)

ATS = Average Total Size (cm)

CV = Coefficient of Variance (%)

Figure 2. Average Total Size (cms) of Tilapia fed a diet containing fumonisin, with and without the addition of TOXFREE (MYCOAD AZ – COBIND AZ) during 35 days.



2.1 Discussion

During the experimental period (35 days) , the presence of 100ppm of fumonisins in the diet of Tilapia, signifcantly reduced the average total size (- 5.60%) when compared to the control treatment. On the other hand, fishes receiving 100ppm fumonisins + 0.10% TOXFREE (MYCOAD AZ -COBIND AZ) showed a significantly higher average total size (+4.95%) than fishes receiving 100ppm fumonisins in the diet.

CONCLUSSIONS

1. The harmful effects of the inclusion of fumonisins at 100ppm concentration in the diet of Tilapia of the Nile (*Oreochromis niloticus*) are clearly shown during a period of 35 days.

2. According to the parameters evaluated in this study, TOXFREE (MYCOAD AZ – COBIND AZ), an Anti-Mycotoxins Additive, showed a significant efficacy at the inclusion rate of 0.10% against a challenge of 100 ppm of fumonisins in the diet of Tilapia of the Nile (*Oreochromis niloticus*) during 35 days.

The results obtained in this experiment refer to the product provided by SPECIAL NUTRIENTS INC, and are specific for the sample provided. Any future modification in the product, regarding chemical or physical characteristics should result on a re-evaluation.

Santa Maria, March 20th, 2014

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Figure 3 - Picture of the facility used in the study

Figure 4 - Picture of the body and Liver development of Fish (T1 = control; T2 = 100 ppm Fumonisin; T3 = 100 ppm Fumonisin + 10 % Toxfree (Mycoad AZ - Cobind AZ))