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Use of novel microbial and phyto-biotic feed additives in mycotoxins degradation *in vitro* and their potential *in vivo* application in fish diet

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ABSTRACT

Aims: This study focused on new fish feed additives that could supply a nutritional value and inhibit or eliminate mycotoxins. Four novel feed additives, including *Albizia lebbeck* (L.), *Leucaena leucocephala* leaf extracts, *Serendipita indica* and *Bacillus megaterium* were applied to contaminated fish feed; besides investigating the toxicity of these new fish feed additives.
Methodology and results: Our data exhibited that the different tested feed additives were not toxic for brine shrimp larvae or fish. *Albizia lebbeck* extract at a concentration 0.5% was highly effective in detoxifying mycotoxins with efficacy ratios of 88.01%, 93.89% and 92.89% for aflaB$_1$, aflaG$_1$ and CPA, respectively and *L. leucocephala* at 0.5% had efficacy ratios of 93.52% and 100% for aflaG$_1$ and CPA, respectively. The addition of *S. indica* with a concentration of 0.75% was highly effective for the usage of good feed approximately free of mycotoxins, with efficacy ratios of 85.65%, 90.81% and 100% for aflaB$_1$, aflaG$_1$ and CPA, respectively. Moreover, *B. megaterium*, with a concentration of 0.75% was recommended for detoxification.
Conclusion, significance and impact of study: Studied new feed additives as feed additives in fish diets to eliminate mycotoxin with the potential of providing antioxidant activity. Results suggest that mycotoxins degradation can happen *in vitro* and *in vivo* by applying new fish feed additives in the fish diet.

Keywords: Bacillus megaterium, bioassay, mycotoxin degradation, plant leaves extracts, *Serendipita indica*, toxicity

INTRODUCTION

The international demand for fish and fish products worldwide is increasing due to the growing population, higher incomes and greater consideration of fish as part of a healthy diet (FAO, 2020). This increase in aquaculture production is associated with increasing feed manufacturing sectors (Goda *et al.*, 2019). Many mycotoxins, such as aflatoxins, fumonisins, deoxynivalenol, zearalenone and moniliformine, are commonly and consistently present in the ingredients used to make fish feed (Pietsch, 2020). *Aspergillus flavus*, *A. parasiticus* and many other Aspergilla and Penicillia species produce four types of aflatoxins: B$_1$, B$_2$, G$_1$ and G$_2$. *Aspergillus flavus* can produce AFB$_1$, AFG$_1$ and cyclopiazonic acid (CPA), *A. parasiticus* produces AFG$_1$ and AFG$_2$, in addition to AFB$_1$ and AFB$_2$ (Yu *et al.*, 2004), which causes growth inhibition, disease infestation with high mortality rates and accumulation of mycotoxins in edible fish parts, which in turn increases the danger of coming into contact with both humans and animals (CAST, 2003; Deng *et al.*, 2010; Alasmari and Sakran, 2020).

Aflatoxins (AFs) usually contaminate fish feeds made of corn, which is essential as a feed ingredient. Maize plants are often contaminated with Aspergilli producing aflatoxins in the field, which can be developed during grain storage and consequently contaminate fish feeds (Njobeh *et al.*, 2009; Levic *et al.*, 2013). Aflatoxin B$_1$ (AFB$_1$) is the most toxic to humans as well as animals, including non-human primates, birds, fish and rodents (Yu, 2012). AFB$_1$ is mutagenic, carcinogenic, teratogenic and immunosuppressive (Bbosa *et al.*, 2013b). All these may interfere with the normal process of protein synthesis as well as inhibit several metabolic systems. Hence, it causes damage to various organs, especially the liver, kidney and heart, which causes liver tumors in fish, animals and humans (Pietsch, 2020). Acute aflatoxicosis

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in fish, as in other animals, occurs when moderate to high doses of aflatoxin are ingested (Bbosa *et al*., 2013a). Signs of acute aflatoxicosis in rainbow trout include anemia, pale gills, reduced hematocrit values, edema, frequent haemorrhage, alteration in nutrient metabolism and liver damage (Santacroce *et al*., 2008). Several approaches have been applied to detoxify mycotoxins during crop harvesting, postharvest and storage (Spadaro and Garibaldi, 2017), such as physical, chemical and biological methods (Siciliano *et al*., 2016). The disadvantage of using chemical and physical methods is that they have a common drawback (Fashandi *et al*., 2018). The biological methods used for detoxification include the involvement of microorganisms and phyto-feed additives such as plant extracts (Kolosova and Stroka, 2011; Mansour *et al*., 2011).

Microorganisms (including fungi and bacteria) and specific enzymes isolated from microbial systems can convert mycotoxins with varying efficiency to non-toxic or less-toxic products (Ahad *et al*., 2017). Several microbes are associated with aflatoxin decontamination, which may be used to produce aflatoxin-free food or feed (Kim *et al*., 2017). Biological detoxification was used to prevent aflatoxicosis in fish (Nayak *et al*., 2007; Hegazi, 2013), Ji *et al.* (2016) and Ahad *et al.* (2017) demonstrated that the biological degradation of mycotoxins has shown promising results because it works under mild, environmentally friendly conditions. Some strains of lactic acid bacteria, such as the order Lactobacilli including *Lactobacillus* and some strains of fungi, including *A. parasiticus*, *Trichoderma viride*, *Mucor ambiguus* and other fungi, were able to degrade AFB_1 with different degrees of success (Verheecke *et al*., 2016). In addition, earthly bacteria as antagonistic microorganisms are able to inhibit toxigenic fungus growth and AFs production (Siahmoshteh *et al*., 2016). The detoxification of mycotoxins by *Lactobacillus* sp in food products is the physical absorption of mycotoxins by *Lactobacillus* strains (Lili *et al*., 2018). Moreover, some of the antifungal metabolites released by bacteria or fungi can reduce and inactivate toxins, as investigated by (Abdel-Shafi *et al*., 2018), who reported new and safe microorganisms such as *Bacillus cereus*, *Brevibacillus* sp1. and *Brevibacillus* sp2. able to degrade AFs and inhabit *Aspergillus flavus* NRRL 3145 production. The strain *B. megaterium* bacteria has bioremediation activity and is considered a good source of industrial proteins because it is both a desirable cloning host and produces a large variety of enzymes (Bhatt and Maheshwari, 2020). Therefore, *B. megaterium* could have potential mycotoxins degradation activity.

The endophytic fungus *S. indica* (former *Piriformospora indica*) has the potential to offer various benefits (Verma *et al*., 1998). *Serendipita indica* has been experimentally proven to significantly improve water and nutrient/mineral absorption, early flowering, seed germination, plant photosynthetic capability, growth rates, especially in nutrient-deprived soils, alter the production of secondary metabolites and promote adaptation, tolerance and/or resistance to biotic and abiotic stressors (Abdelaziz *et al*., 2018; Sabra *et al*., 2018; Mensah *et al*., 2020). Moreover, it improves the plant's tolerance, against heavy metal toxicity (Sabra *et al*., 2018) and its use as abiotic remediation of bio-solid waste and sewage sludge compost minimized the health risk hazards affecting the human food chain, allowing for the different uses of sludge to be safer for the environment (Youssef *et al*., 2020).

Albizia lebbeck (L.) Benth (Mimosaceae), referred as a wooden tree, is developed in many parts of farmlands, along roadsides and rivers and as an ornamental plant in gardens because of its wonderful appearance. The plant has a surprising reputation because of its food, feed and medicinal values (Avoseh *et al*., 2021). It is considered a potent alexipharmic (Wati and Khabiruddin, 2017). *Albizia* sp. is rich in bioactive secondary metabolites, including flavonoids, tannins, saponins, terpenes and alkaloids. They are traditionally used to treat a variety of ailments like diarrhoea, cough, anxiety, depression, insomnia, rheumatism and wounds. They can also be beneficial in treating different inflammatory and oxidative stress-related disorders (Sobeh *et al*., 2017; 2019). *Leucaena leucocephala* (Fabaceae) is a small, fast-growing, multipurpose, nitrogen-fixing tree legume widely distributed throughout the tropics and subtropics (Hughes, 2010). Mohammed *et al.* (2015) studied the chemical constituents of *L. leucocephala* leaves and evaluated the antioxidant and antimicrobial activities of the extract and compounds. The structures of compounds were elucidated based on spectral analysis. It possesses the best antioxidant and antibacterial properties and could serve as free radical inhibitors or scavengers, possibly acting as primary antioxidants (Olckers, 2011). The phytochemical investigations of *A. lebbeck* and *L. leucocephala* revealed the presence of terpenes, flavonoids, coumarins and sterols (Mohamed *et al*., 2013; Hassan *et al*., 2014). The high abundance of phytochemicals in these plants qualifies them to be used as feed additives and in mycotoxins detoxification.

Considering the previous considerations, the present study aimed to investigate mycotoxin degradation using some microbial and plant extracts as potential feed additives. In addition, the safety of using these different new feed additives has been determined on brine shrimp (*Artemia*) to assess their LC_{50}, followed by a confirmatory experiment on Nile tilapia, *Oreochromis niloticus*, to evaluate the survivability of fish fed the treated diets with these additives.

MATERIALS AND METHODS

Feed additives preparation and characterization

Microbial feed additives

Bacterial strain *B. megaterium* (strain number EMCC1062) was obtained from the Bio-fertilization Unit, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The bacteria were reproduced on Luria Bertani (LB) medium comprising of (g/L) tryptone, 10; yeast

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extract, 5; NaCl, 5. The pH of the medium was adjusted to 7.2-7.4 using 1 N HCl or 1 N NaOH and sterilized by autoclaving at 121 °C for 15 min. The culture was maintained at 35 °C for 3 days. The bacterial count was measured by a hemocytometer slide to determine the bacterial count (CFU), which was 1.3×10^6 viable cells/mL in the broth media.

The fungal strain, *S. indica* (strain DSM11827, NCBI tax. ID: 1109443) was first isolated from the Thar Desert in India (Verma *et al*., 1998). The fungal strain was obtained from Erfurt Research Centre for Horticultural Crops, Germany and propagated in the Microbiology Lab, Faculty of Agriculture, Saba Basha, Alexandria University, Egypt. Hyphal plug was transferred to 250 mL flasks with complete liquid media (CM: 50 mL 20× salt solution (120 g $NaNO_3$, 10.4 g KCl, 10.4 g $MgSO_4 \cdot 7H_2O$ and 30 g KH_2PO_4 in 1 L), 20 g glucose, 2 g peptone, 1 g yeast extract, 1 g casamine acid and 1 mL microelements (6 g $MnCl_2$, 1.5 g H_3BO_3, 2.65 g $ZnSO_4 \cdot 7H_2O$, 750 mg KI, 2.4 mg $NaMO_4 \cdot 2H_2O$, 130 mg $CuSO_4 \cdot 5H_2O$ dissolved in 1 L), autoclaved at 121 °C for 20 min and incubated for around 20 days in the dark at 26 °C. The liquid suspension contains around 4×10^5 mL^{-1} of chlamydospores of *S. indica hyphae* and spores were added with the different concentrations to the fish feeds.

Plant extracts feed additives

Plant leaves of *L. leucocephala* and *A. lebbeck* were collected from Antoniadis Research Branch, Horticultural Research Institute, Ministry of Agriculture, Alexandria, Egypt. 20 g each were taken for mycotoxins estimation using multi-screening mycotoxins with some modification, according to Frisvad *et al.* (2007) and Probst *et al.* (2014). Only free mycotoxin leaves were used in this study.

Extracts preparation

The leaves were surface sterilized; 20 mL of ethanol was added for each 6 g plant leaves according to Farahmandfar *et al.* (2017; 2019) and kept in the dark for 16 h, then blended in a sterilized electric blender. Afterward, the mixture was filtered through a sterilized sheath cloth. Samples were kept in sterilized bottles at -4 °C until their use for further analysis.

Phytochemical characterization of plant leaves extracts

Determination of total phenolics

The total phenolic contents of the plant leaf extracts were determined using the Foline-Ciocalteu method (Turkmen *et al.*, 2006; Dehghan *et al.*, 2016; Farahmandfar *et al.*, 2019) using 0.5 mL of extract (250 mg/mL, DW) mixed with 1 mL water and 0.5 mL of 1 mol/L Folin-Ciocalteau reagent. After three min, 2 mL of sodium carbonate Na_2CO_3 solution (7.5%) adjusted to 50 mL water was added and incubated at room temperature for 10 min. The sample was read at 765 nm using a spectrophotometer (Kerper boulevard Du Buque, Iowa, USA). The phenolic content was expressed as mg gallic acid equivalents per gram of the extract.

Detection of flavonoids of the tested plant extracts

The global concentrations of flavonoids were quantified using a colorimetric assay method with some modifications (Zhao *et al.*, 2018); 1 g of leaves added to 10 mL ethanol (60%) was used to extract flavonoids using supersonic ultra-sonicator equipment for 30 min and re-centrifuged at 3000 rpm. The supernatant was transferred to a volumetric flask (25 mL) and further fixation was carried out using 25 mL of ethanol 60%. 1.5 mL extract plus 4.5 mL of distilled water mixed with 1 mL of $NaNO_2$ solution (5%). After 6 min of incubation, 1 mL of the $Al(NO_3)_3$ solutions (10%) was added to the mixture. Then kept for 6 min before further addition of 10 mL of NaOH solutions (4%) and fixed with 25 mL ethanol 60%. The absorbance was measured by spectrophotometer (Kerper boulevard Du Buque, Iowa, USA) at 765 nm against a blank containing 5 mL of extraction solvent. The mean of three tests was used, and the total flavonoid content was expressed as mg per g dry weight (DW).

Determination of antioxidant activities

The antioxidant activity (DPPH assay) was measured by the radical scavenging ability of 1,1 diphenyl,2 picrylhydrazyl (DPPH) radical. An aliquot of the extract was combined with 1 mL of a reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate); the method was described by Farahmandfar *et al.* (2017) with some modifications. Samples were added in tubes containing 5.9 mL of 0.1 mM methanolic DPPH solution. The reaction mixtures were shaken, then kept in the dark and measured by absorbance at 517 nm. The results were calculated using the following equation:

Radical scavenging capacity (%) = (Blank absorption - Sample absorption) × 100

Plant extracts toxicity assay

This experiment was carried out to test the toxicity of the tested treatments using brine shrimp larvae *Artemia salina* Leach.

Brine shrimp lethality assay

For each tested concentration of extract, the bacterial and fungal broth was dissolved and diluted with artificial seawater NaCl (3.8%). Newly hatched *A. salina* eggs were allocated into Petri dishes (10 individuals; three replicates per each treatment) containing seawater plus the tested extracts and broths and controls as in the hatching assay (Otang *et al.*, 2013). Dry yeast suspension was added as food for the brine shrimps (Gadir, 2012). Negative control was prepared using ethanol and distilled

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water. Non-treated control was used too. The Petri dishes were aerated under constant lighting. The dead larvae were manually calculated by observing the larvae inside the Petri dish under a lamp for 24 h with a magnifying glass or loop and measuring the number of dead larvae of *A. salina* Leach. The mortality percentage (%) was calculated as the following:

Mortality (%) = (Total HAS - Living HAS)/(Total HAS) × 100

Fish feed collection and mycotoxins screening

Ten fish feed samples (5 kg each) were used for mycotoxins screening (20 g each) using reverse phase high-performance liquid chromatography (RP-HPLC) apparatus with a photodiode array (PDA), fluorescence detector (FLD) and photochemical reactor for mycotoxin enhancing detection (PHRED) and post-column derivatization according to Soleimany *et al.* (2011) with some modifications. Highly mycotoxin content was selected for carrying out the experiment of detoxification.

Proximate analysis of fish feeds

Leaves samples and fish feed samples as a non-treated control and treated fish feed with all the tested treatments were proximately analyzed to investigate the proximate chemical composition and any change in their nutrient value after treatment. Moisture, crude protein, fat, ash and crude fiber were determined according to the Association of Official Analytical Chemists methods (Latimer, 2016). Total carbohydrate content was estimated by difference. The moisture content of the plant leaves samples was estimated by calculating the difference before and after drying for 2 h using a hot air oven (105 °C). Crude protein content was determined in Kjeldhal digestor and automatic distillation system by following the Macro Kjeldahl method (Stoscheck, 1990). Fat content was measured using the soxhlet extraction method. The crude fiber was determined by acid and base extraction methods. The following formula determined the total carbohydrate content:

Carbohydrate% = 100 - (Crude protein% + Crude fiber% + Fat% + Ash%)

***In vitro*: Detoxification of mycotoxins by the tested feed additives**

The treatments with different concentrations (0.5%, 0.75% and 1%) (v/w) of plant extracts, *S. indica* and *B. megatieum* broth were added to estimate the biodegradation of mycotoxins naturally contaminated fish feed (FF). The experiment was carried out using the sample that revealed the highest mycotoxins content. Fourteen finely ground FF subsamples, 100 g each, were used. The tested substances (extracts and broths) were added in three concentrations (0.5%, 0.75% and 1%). Control non-treated samples and feed samples treated with 1% ethanol were used as a blank. The moisture of samples was measured before and after treatment, in addition to using Motomco apparatus (serial n° = K26242, U.S.A). Then the moisture was adjusted to 13% in all samples by adding a calculated sterilized deionized water volume according to the tested sample using the approved AACC method by the American Association of Cereals (AACC, 1962). The desired added water volume was calculated according to the AACC equation:

S° = (Required moisture content - Initial moisture content)/(100 - Required moisture) × 100

Where: S° = x mL water for 100 g

All treatments in triplicates were homogenized using a homogenizer blender, Seward Stomacher, 400 samples were transferred to sterilized glass bottles and incubated for fifteen days at room temperature (25 °C). Finally, samples were taken for mycotoxins determination.

***In vitro*: Determination of the feed additives detoxification**

A total of 840 Nile tilapia (*Oreochromis niloticus*) fingerlings (4.0 ± 1.10 g mean body weights) were used. The fish were randomly allotted into 42 aquaria (3 aquariums/treatment) with 20 fish per aquarium and all were subjected to the same environmental conditions. Glass aquaria were used (100 × 40 × 30 cm each) with a capacity of 120 L and adjusted to contain 100 L. Continuous aeration was maintained in each aquarium using an electric air pump. In all treatments, the dissolved oxygen concentration was 7 ± 0.51 mg/L, pH was 7 ± 0.30, unionized ammonia concentration was 0.002 ± 0.009 mg/L, salinity was 0.4 ± 0.1 g/L, the temperature was 28 ± 0.20 °C and the light regime was set at 12 h light/12 h dark. The experiment continued for one week. Fish were hand-fed at 5% of wet body weight with the composited feed twice daily. The diets after mycotoxins biodegradation were used in this experiment. Accordingly, there are 14-diets, including a non-treated diet (control) and treated diet with alcohol (blank), and composited diets with plant extracts, bacterial and fungal broth at different concentrations (0.5, 0.75 and 1 mg/mL) were used to determine the mortality of fish fry and to calculate the LC_{50}.

Determining the half-minimal lethal dose (LC_{50}) of the tested treatments using probity regression

The toxicological value of the plant leaves extracts, fungal and bacterial suspension broths was valorized and calculated using mortality percentages and their conversion to probit values according to Fenny's table (1952; 1971) was done (Table 1). The toxicity of each leaf plant extract and fungal and bacterial broth concentrations expressed as LC_{50} values are evaluated

Table 1: Finney's table for the transformation of the percentage of mortality to probit values (Finney, 1952; 1971).

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%	0	1	2	3	4	5	6	7	8	9
0	__	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.25	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
__	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

Table 2: The toxicity classification models (Benfenati *et al*., 2020).

LC_{50}	Class of toxicity	Degree of toxicity for aquatic organisms
<1 mg/L	Class 1	Very toxic
1-10 mg/L	Class 2	Toxic
10-100 mg/L	Class 3	Harmful to aquatic organisms
>100 mg/L	Class 4	May cause long-term adverse effects on aquatic organisms

twice by comparison to Meyer's toxicity index. According to Meyer's toxicity index, extracts with LC_{50}<1000 µg/mL are considered toxic, while treatments with LC_{50}>1000 µg/mL are considered non-toxic (Meyer *et al*., 1982; Hamidi *et al*., 2014; Maposa *et al*., 2020).

After the calculation of the mortality percentages, the relationship between mortality and the concentration of each tested treatment was drawn in Excel, and the y and R^2 values were determined.

The mortality percentage values were transformed to a probit value and the log of concentration was calculated according to the scientific method- probit (Pum, 2019) with certain modifications and using the Y values in the linear regression coefficient calculated by Excel, the intercept and the slope. The same step was repeated in the case of fish after the confirmatory test was done. The LC_{50} was calculated by two equations as follows:

Log of each treatment concentration = (Probit value - intercept)/Slope = X mg/L

$LC_{50} = 10^x$ mg/L

Toxicity determination criteria

The degree of toxicity was registered according to the Fish Toxicity Classification Model (SARpy/IRFMN) (Benfenati *et al*., 2020), which divided the acute toxicity (LC_{50}) 96 h towards fish into four classes as shown in Table 2.

Statistical design

The complete randomized mycotoxin experiment was statically analyzed using a one-way analysis of variance (ANOVA, p<0.05). The fish and shrimp experiment were carried out in three replicates using a Costat computer program (CoHort Software, Berkeley, CA, USA). The pairwise comparison of means was made using the Least Significant Difference ($LSD_{0.05}$). The experimental mycotoxin data was statically analyzed using one-way completely randomized complete blocks ANOVA (p<0.005, Tukey test). The fish and shrimp experiment were carried out in three replicates using a Costat computer program (CoHort Software, Berkeley, CA, USA). The statistical significance of each experiment was calculated using the Tukey test ANOVA (p<0.001) and the unpaired t-test (p<0.05).

RESULTS

Determination of total phenol contents, flavonoids and antioxidant activity of the two tested plant leaves ethanol extract

The protein content (%) of the tested plant leaves is shown in Table 3. The results recorded that the leaves of *A. Lebbeck* contained 26.045%, followed by *L. leucocephala* with 23.94%. Moreover, the content of carbohydrates was higher in *A. lebbeck* than *L. leucocephala* leaves (44.56% and 40.76%, respectively). The same trend was recorded for ash, where *A. lebbeck* had the highest ash compared to *L. leucocephala* (6.38% and 4.43%, respectively). In addition, Table 3 showed that the flavonoids (µg/100 g) content was much higher in *L. leucocephala* compared to *A. lebbeck*. Flavonoids analysis revealed that *L. leucocephala* contained 123.35 µg/100 g compared to 40.11 µg/100 g for *A. lebbeck.* Also, the total phenols content was 188.11 µg/100 g and 56.31 µg/100 g for *L. leucocephala* and *A. lebbeck*, respectively. Moreover, the antioxidant capacity in *L. leucocephala* recorded 83.6% and 57.46% for *A. lebbeck*.

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Table 3: Proximate chemical analysis and phytochemical profile of *A. lebbeck* and *L. leucocephala* leaf extracts.

Proximate chemical analysis of plant leaves					
Tested plant	Protein (%)	Carbohydrates (%)	Ash (%)	Fibers (%)	Fats (%)
A. lebbeck	26.05	44.56	6.98	8.38	7.93
L. leucocephala	23.94	40.76	4.43	2.09	7.76
Phytochemical profile					
Plant material	Flavonoids (µg/100g)	Total phenols (µg/100g)	Antioxidants (%)		
A. lebbeck	40.11	56.31	57.46		
L. leucocephala	123.35	188.11	83.60		

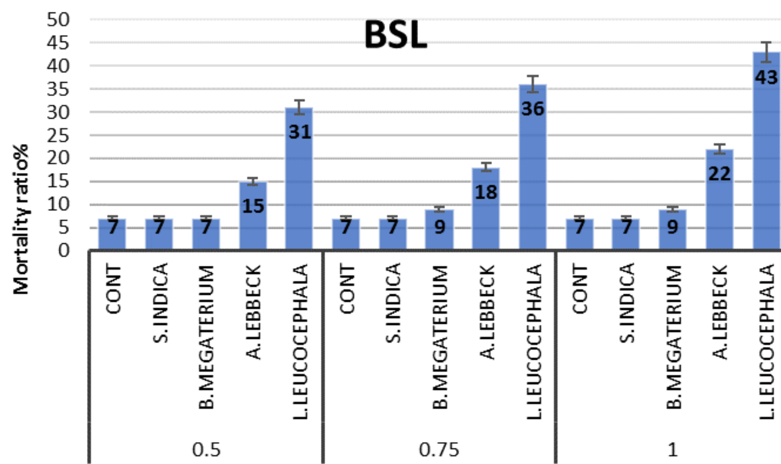

Figure 1: Mortality ratios percentage in brine shrimp larvae (BSL) under the different treatments.

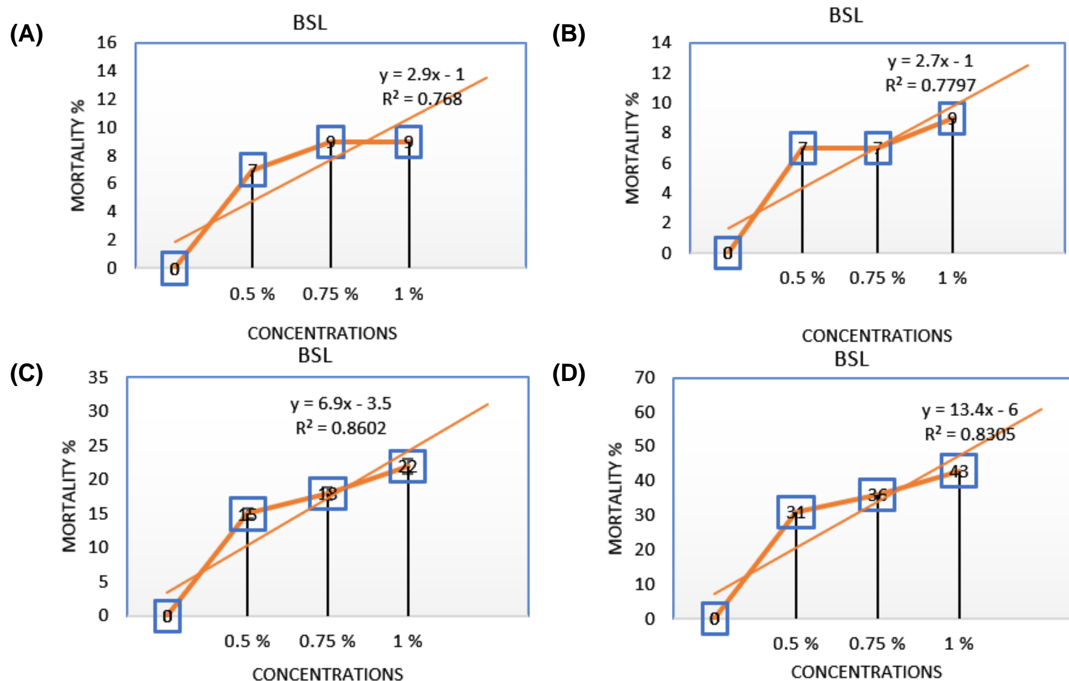

Figure 2: Linear regression relation between mortality % and treatments with different concentrations of (A) *Serendipita indica*, (B) *Bacillus megaterium*, (C) *Albizzia lebbeck* and (D) *Leuceana leucocephala* in brine shrimp larvae (BSL) experiment.

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Table 4: Proximate chemical composition of fish feed (FF) before and after treatment with different feed additives.

Substrates	Concentration (%)	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	Fiber (%)	Carbohydrates (%)
Fish feed (FF)		10.2	28.10	6.70	8.70	3.40	42.90
FF + *L. leucocephala*	0.5	10.20	28.62	6.74	8.72	3.80	41.92
	0.75	10.30	28.68	6.75	8.73	3.82	41.72
	1	10.36	28.74	6.78	8.74	3.83	41.13
FF + *A. lebbeck*	0.5	10.50	28.73	6.99	8.74	3.90	41.14
	0.75	10.30	28.79	7.21	8.75	3.90	41.05
	1	10.36	29.01	7.48	8.79	3.90	40.46
FF + *B. megaterium*	0.5	10.35	28.10	6.76	8.71	3.41	42.67
	0.75	10.42	28.65	6.78	8.72	3.42	42.01
	1	10.80	28.85	6.80	8.72	3.44	41.39
FF + *S. indica*	0.5	10.35	28.60	6.77	8.72	3.44	42.12
	0.75	10.42	28.62	6.82	8.81	3.46	41.87
	1	10.80	29.40	6.90	9.28	3.50	40.12

Plant extracts toxicity assays

Determination of the half-minimal lethal dose (LC_{50}) of the tested treatments using probit regression in BSL according to their mortality ratios

Transformation of the mortality percentages to a probit value (Probit regression determination) was done. The probit regression of each treatment was determined using five concentrations; three of them were used during the detoxification experiment and two proposed concentrations (0% and 99% mortality). The LC_{50} determination was carried out using the Y equation.

Mortality % in both shrimp and fish experiments

The mortality ratios of each experiment were calculated as illustrated in Figure 1. The mortality ratios in fish were higher than in brine shrimp larvae (BSL) which indicates the sensitivity of fish than shrimp to these additives.

Determination of Y equation and R^2 values using linear regression

The Y and R^2 equation of each treatment in both experiments were evaluated using linear regression equations by Excel program, as shown in Figure 2.

Effect of feed additives on diet composition

Table 4 showed that the FF + *S. indica* at 1% concentration contained the highest protein content (29.4%) as compared to FF + *S. indica* with concentrations of 0.5% and 0.75 %. Moreover, the protein content (%) of FF + *B. megaterium* with concentrations of 0.5%, 0.75% and 1% was 28.10%, 2.65% and 28.85%, respectively. In addition, the fish feed with both *A. lebbeck* or *L. leucocephala* had 29.01 and 28.10% protein content, respectively. From the data illustrated in Table 4, it could be observed that the combination with *S. indica* fungi had the most proximate analysis values (ash, fiber, carbohydrate and fat %) compared with FF (control) under different concentrations. However, the treatment FF + *L. leucocephala* recorded ash content 8.72%, 8.73% and 8.74% with concentrations of 0.5%, 0.75% and 1%, respectively. The lowest ash content was found at FF (control), which was 8.7%.

Fiber (%) of the fish feed resulted in the highest content (3.9%) with the treatment FF + *A. lebbeck*, compared with the treatment FF + *L. leucocephala*. It was found that fiber contents were 3.44%, 3.46% and 3.5% for treatment FF +*S. indica* with concentrations 0.5%, 0.75% and 1%, respectively. The lowest fiber content was with FF (control). It was detected that the treatment FF contained the highest carbohydrate content, 42.95%, compared with treatment FF + *L. leucocephala* with concentrations of 0.5%, 0.75% and 1% they were 41.92%, 41.72% and 41.13%, respectively. However, carbohydrate content was 42.67%, 42.01% and 41.39% for FF + *B. megaterium* with concentrations of 0.5%, 0.75% and 1%, respectively. While the fat content (%) for FF + *B. megaterium* with concentration 0.5%, 0.75% and 1%, they were 6.76%, 6.78% and 6.8%, respectively.

Table 5: The mycotoxin contents of the feed sample used in the detoxification experiment.

Mycotoxin type	Abbreviation	Concentration (ppb)
Aflatoxin B_1	aflaB1	17.89
Aflatoxin G_1	aflaG1	17.97
Cyclopiazonic acid	CPA	24.16

Detection of mycotoxins in the obtained samples

The mycotoxins content in all obtained samples was determined. The predominant mycotoxin occurring in the ten tested feed samples was the aflatoxins B (B or B_1) and G_1 followed by cyclopiazonic acid CPA alone or with other aflatoxins (Table 5). The highest mycotoxin's feed sample content was taken for carrying out the experiment of detoxification.

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Table 6: Effect of the tested feed additives on minimizing mycotoxins content of fish feed.

Treatments	Concentration dose (%)	Mycotoxin concentration (ppb)			Mycotoxin inhibition ratio (%)		
		$AflaB_1$	$AflaG_1$	CPA	$AflaB_1$	$AflaG_1$	CPA
Control (Fish feed)		18.02^{a}	18.82^{a}	24.46^{a}			
Control + Ethyl	1	17.47^{b}	17.82^{b}	20.16^{b}	3.05	5.31	17.72
Serendipita indica	0.5	6.53^{f}	3.19^{j}	6.48^{f}	63.76	83.05	73.51
	0.75	2.59^{k}	1.73^{l}	ND^{m}	85.63	90.81	100
	1	6.05^{h}	4.54^{f}	0.87^{l}	66.43	75.88	96.44
Bacillus megaterium	0.5	9.96^{d}	6.05^{d}	14.76^{d}	44.73	67.85	39.66
	0.75	2.16^{m}	2.46^{k}	17.28^{c}	88.01	86.93	29.35
	1	2.52^{l}	4.14^{h}	7.28^{e}	86.015	78.00	70.24
Leucaena leucocephala	0.5	7.78^{e}	1.22^{m}	ND^{m}	56.82	93.52	100
	0.75	15.99^{c}	3.86^{i}	3.5^{j}	11.26	79.49	85.53
	1	3.24^{j}	8.28^{c}	5.99^{g}	82.02	56.00	75.51
Albizia lebbeck	0.5	2.16^{m}	1.15^{n}	1.74^{k}	88.01	93.89	92.89
	0.75	3.53^{i}	5.18^{e}	5.40^{h}	80.41	72.48	77.92
	1	6.12^{g}	4.22^{g}	4.70^{i}	66.04	77.58	80.78
$LSD_{0.05}$		0.0216	0.0511	0.0514			

*Data with same letters are not significant, N.B: ND≤0.012 ng/g according to Soleimany *et al.* (2011), ^{a,b} Letters means ± SD, indicate statistically significant differences, while the identical letters denote no statistical differences calculated using one-way ANOVA ($p<0.005$, Tukey test). Three independent samples were conducted for each treatment.

Table 7: Class of treatments toxicity for both tested organisms.

Treatments (Feed additives)	Concentrations (%)	LC_{50} Brine shrimp larvae (mg/L) (A)	LC_{50} Fish (mg/L) (B)	Toxicity to aquatic organisms
Serendipita indica	0.5	17.175	21.78	Harmful for A and B
	0.75	17.175	21.78	Harmful for A and B
	1	53.198	23.82	Harmful for A and B
Bacillus megaterium	0.5	36.19	17.49	Harmful for A and B
	0.75	40.45	18.99	Harmful for A and B
	1	40.45	18.99	Harmful for A and B
Albizia Lebbeck	0.5	12.05	7.92	Harmful for A and toxic for B
	0.75	12.55	8.16	Harmful for A and toxic for B
	1	13.19	8.47	Harmful for A and toxic for B
Leuceana leucocephala	0.5	6.075	4.598	Toxic for A and B
	0.75	6.22	4.686	Toxic for A and B
	1	6.41	4.842	Toxic for A and B

Determination of the effects of the treatments on minimizing mycotoxins contaminated fish feed

The effect of the tested feed additives in reducing mycotoxins content was determined as exhibited in Table 6, where it is observed that general mycotoxin concentrations were reduced by the treatments. Both *A. lebbeck* and *S. indica* realized the best results in mycotoxin degradations at concentrations of 0.5% and 0.75% successively. On the other hand, mycotoxin concentrations and mycotoxin inhibition ratio in the control treatment of FF without any additives had the highest mycotoxin concentration, especially for CPA (24.46 ppb). Moreover, the fish feed treated with both microorganisms (*B. megaterium* and *S. indica*) at 75% concentration recorded the lowest mycotoxin concentrations (2.16, 2.46, and 17.28 ppb) and (2.59, 1.73 ppb and not detected) for *B. megaterium* and *S. indica*, respectively.

Mortality % in both shrimps and fish experiments

The mortality ratios of each experiment were calculated as illustrated in Figure 3. The mortality ratios in fish were higher than in brine shrimp larvae (BSL) which indicates the sensitivity of fish than shrimp to these additives.

Determination of the half-minimal lethal dose (LC_{50}) of the tested treatments using probit regression in fish according to their mortality ratios

Data in Figures 3 and 4 illustrated that the fish are less tolerant or more sensitive to the tested treatments than BSL. Our data also illustrated a high correlation between treatment concentration and mortality ratio. This correlation depended on the type of treatment because it was higher in plant leaf extract than in probiotics extracts.

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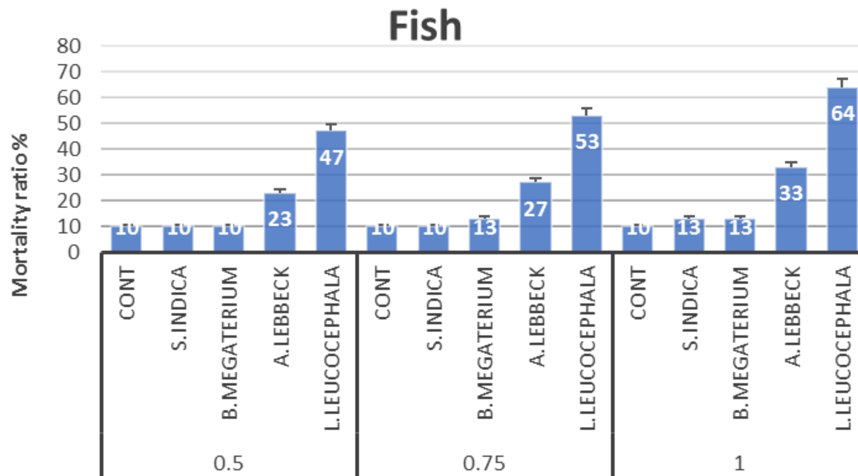

Figure 3: Mortality ratios percentage in fish under the different treatments.

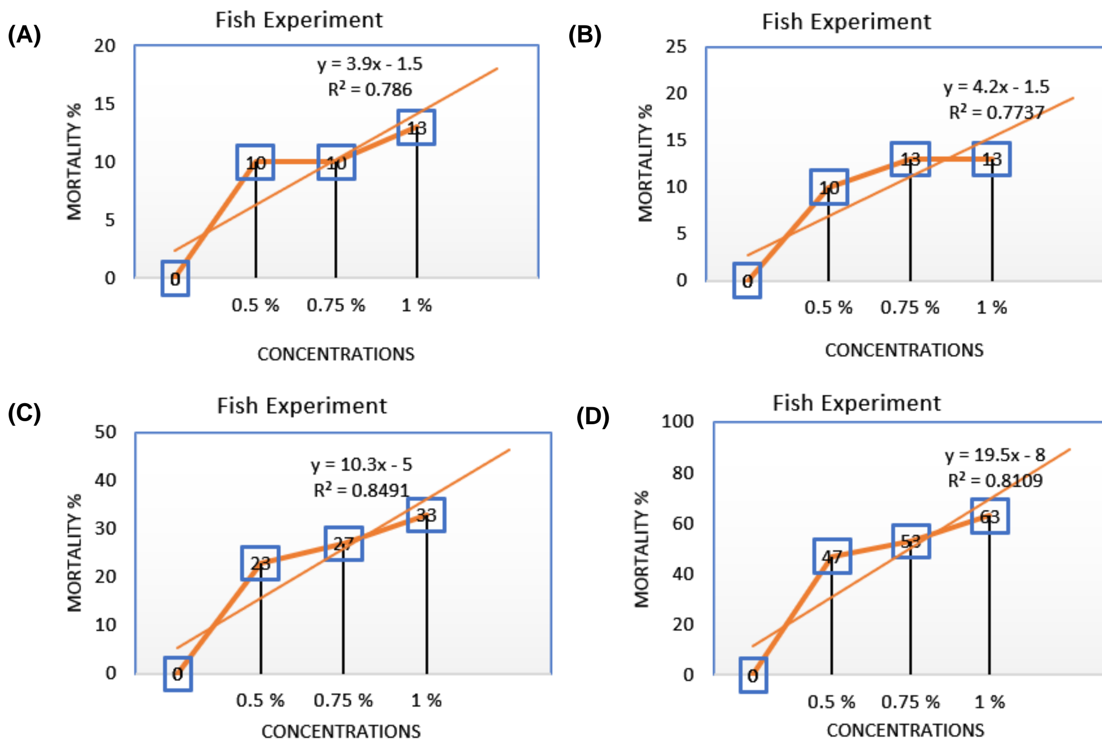

Figure 4: Linear regression relation between mortality % and treatments with different concentrations of (A) *Serendipita indica*, (B) *Bacillus megaterium*, (C) *Albizzia lebbeck* and (D) *Leuceana leucocephala* in fish experiment.

Classification of the treatment's toxicity according to their LC_{50}

The toxicity class of each treatment was valorized using the Fish Toxicity Classification Model (SARpy/IRFMN) v-1.0.2 (2020). Data exhibited in Table 7 indicates that all the tested treatments are not toxic for BSL except *L. leucocephala* at the three tested concentrations. Meanwhile, both *L. leucocephala and A. lebbeck* concentrations are toxic for fish and BSL. That may be partially due to the mimosine found in *L. leucocephala*, which contains around 16% and 2.57% (Adeneye, 1991).

DISCUSSION

The two tested plant leaves' total phenol contents, flavonoids and antioxidant activity were determined. The results of our study partially coincided with those of

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(Hassan *et al*., 2014; Sharma and Chaurasia, 2015; Chatchanayuenyong *et al*., 2018). Mohammed *et al.* (2015) also reported that *L. Leucocephala* leaves are an excellent source of phenolics and flavonoid contents, which were suggested to be responsible for high antioxidant potential. Wati and Khabiruddin (2017) showed that total phenolics, flavonoids and tocopherol contents were higher in methanol *A. lebbeck* extract. In addition, the seed composition of *Albizia lebeck* had the highest values of ash, protein and fiber content in *A. anthelmintica* (Sobeh *et al*., 2019).

The effect of feed additives on the fish diet composition was evaluated. Our proximate analysis of *A. lebbeck* and *L. leucocephala* leaves extract concerning *L. leucocephala* leaves were in harmony with those of (Alabi *et al*., 2018) and are relatively in harmony with those of (Atawodi *et al*., 2008), who reported that the concentration of the added treatment affects the composition of feed and augments the values of proteins, carbohydrates, fats, fibers and ash. While our phytochemical profile and the proximate analysis are not in agreement with those of Mohammed *et al.* (2015) who reported that the different proximate analysis of *A. lebbeck* may be due to the plant origin or the climate change and time of collection.

The mycotoxins content of the feed sample used in the detoxification experiment was investigated. The current study results are in accordance with those of Alasmari and Sakran (2020) and Mwihia *et al.* (2018), who detected that 48% of feeds sampled collected from 70 farms and 8 feed manufacturing were positive for aflatoxins. Feeds containing maize bran and fishmeal had significantly higher aflatoxin levels than those without these ingredients.

It was vital to evaluate the efficiency of using the tested feed additives in the mycotoxins content reduction. Our data are highly in agreement with those of Youssef *et al.* (2020), who reported that *S. indica* degraded mycotoxins during carbon starvation due to the competition of *S. indica* with the other flora to accomplish their needs of carbon sources. We propose that understanding how mycotoxin levels are organized by microbial combinations can offer novel insights for mycotoxin control in food and feed (Venkatesh and Keller, 2019; Makhuvele *et al*., 2020). Avoseh *et al.* (2021) reported that *A. zygia* then *A. lebbeck* leave extracts completely inhibited aflatoxin B_1 production *in vitro* at a concentration of 1 mg/mL. As mentioned above, we observed that *S. indica* was more effective in reducing all the occurred mycotoxins. Meanwhile, *B. megaterium* at 0.75% and 1% illustrated the highest and the same aflatoxin B_1 degradation of *A. lebbeck* at a concentration of 0.5%. Our result highly coincided with those of Kong *et al.* (2014), who found that *B. megaterium* inhibited the production of CPA and B_1. The treated fish feed with *L. leucocephala* at a concentration of 0.5%, achieved the best degradation percentage for both aflatoxin G_1 and CPA only. Our results can relatively match those of Loi *et al.* (2020), who declared that plant bioactive compounds are shown to be effective in reducing aflatoxin. Furthermore, each tested extract did not have the same effect against each mycotoxin. Our data were closely matching those of Youssef (2019), who reported that mycotoxins differed in their sensitivity against the same plant extract. Adding some microorganisms as a binding agent to AFB_1 contaminated diets could help in controlling AFB_1 (Oguz, 2012; Oguz *et al*., 2018). Rahman *et al.* (2017) investigated Nile tilapia (*O. niloticus*), fed on supplemented diets with fennel essential oil (FEO) and *Saccharomyces cerevisiae* and treated with different concentrations of aflatoxins for 30 days. Their results proved that FEO or *S. cerevisiae* protected fish target organs from the destructive effect of AFB_1. While Mwihia *et al.* (2018) reported that aflatoxin contamination of fish feeds is prevalent in Nyeri and may be the cause of adverse health effects in fish in this region. Fish feeds containing maize bran and fish meal had significantly higher aflatoxin levels than those without these ingredients. Many studies have focused on aflatoxin removal from food or feed, especially via microbe-mediated mechanisms either adsorption or degradation. The bacterium cell wall such as lactic acid bacteria, *Lactobacillus rhamnosus,* efficiently binds aflatoxin B_1 and a pep-tidoglycan. This ability of *L. rhamnosus* should be applied to the removal of aflatoxin B_1 (Kim *et al*., 2017).

Studying toxicity is essential as the initial step in drug safety assurance. It consists of acute, subchronic and chronic toxicity tests. The brine shrimp lethality test (BSLT) is one of the acute toxicity methods used in determining the toxic effects of a plant. This method generally identifies toxicity for natural substances (Sadino *et al*., 2017). The toxicity class of each treatment was detected. *L. leucocephala and A. lebbeck* concentrations are toxic for fish and BSL. That may be partially due to their mimosine, as found in *L. leucocephala* contains around 16% and 2.57% (Adeneye, 1991). So there appears to be no disturbing toxicity due to mimosine, although leaves are free of toxins and tannins (Azani *et al*., 2017). Our fish farmer workers noticed that fish did not prefer the diet supplied with *A. lebbeck* or *L. Leucocephala.* Our team observations are closely in harmony with those of (Azani *et al*., 2017), who mentioned that some animals refuse to eat diets containing *A. lebbeck* such as cattle. *L. leucocephala* has a high presence of tannins and glycosides and a moderate presence of saponin (Sharma and Chaurasia, 2015).

Data in Table 7 are in agreement with Patel *et al.* (2018; 2020), who reported that *A. lebbeck* leaves addition to fish meal was a good and economical source of protein and augmented the weight of fish and increased the milk yield and the animal body weight and increase the digestibility. Our findings also coincided with those of Atawodi *et al.* (2008), who reported that *L. leucocephala* leaves might only be useful as a feed supplement in egg-laying hens at low levels (5-10%) of supplementation. Furthermore, Both *A. lebbeck and L. leucocephala* at 0.5-0.6% are used in human drugs for pathological disturbances (Chatchanayuenyong *et al*., 2018).

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Although the high nutrient value of *A. lebbeck* and *L. leucocephala* leaves extracts and their good effectiveness in detoxifying mycotoxins contaminated feed, their usage as feed additives is not recommended because of their toxic effects. Our results recorded that the addition of *S. indica* as a feed additive with a concentration of 0.75% had very significant results with mycotoxins contaminated fish diet, which recorded low mycotoxin to the fish after treatment. Recommended to achieve the use of good feed approximately free of mycotoxins with efficacy ratios of 85.65%, 90.81% and 100% for $AflaB_1$, $AflaG_1$ and CPA, respectively. The addition of *B. megaterium* with a concentration of 0.75% was safe for fish and recommended for detoxifying feed contaminated with $aflaG_1$ and/or CPA only, with efficacy ratios 88.01% and 86.93% for $AflaB_1$ and $AflaG_1$, respectively because its effectiveness in reducing CPA was moderately low 29.35%.

CONCLUSION

Our results suggest that mycotoxins degradation can happen *in vitro* and *in vivo* by the application of new fish feed additives in the fish diet.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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