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RESEARCH ARTICLE

Effects of *Aspergillus niger* Organic Acid Filtrate on Growth, Immunity, Oxidative Balance, and Gut Health in Aflatoxin-Challenged Broiler Chickens

Nahla Alsayd Bouqellah[1*]

[1]Department of Biology, College of Science, Taibah University, Madinah, 42353, Saudi Arabia
*Corresponding author: Nbouqellah@taibahu.edu.sa

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ABSTRACT

Aflatoxin contamination of poultry feed is still a major concern worldwide and leads to huge economic losses due to poor growth, immunosuppression, hepatotoxicity, and oxidative stress, as well as food safety concerns. The search for effective, safe, and natural countermeasures is therefore urgent. This study evaluated an organic acids-enriched filtrate (OAF) of *Aspergillus niger* isolate as a dietary supplement in broilers challenged with aflatoxin (250ppb aflatoxin B_1 for 28 days). HPLC analysis of OAF revealed the presence of citric, oxalic, succinic, gluconic, malic and fumaric acids as the major organic acids. The filtrate showed dose-dependent (25-400mg/kg) antimicrobial activity against *Escherichia coli, Salmonella* Typhimurium*, Staphylococcus aureus,* and aflatoxigenic fungi*, Aspergillus flavus* and *Aspergillus parasiticus*, with MICs ranging from 62.5 to 250µg/mL, as well as it showed strong antioxidant activity against DPPḢ with IC_{50} of 85.3µg/mL. *In vivo*, OAF was tested at 50, 100 and 200mg/kg, in comparison to an antibiotic and combination group (OA 200+antibiotic). Aflatoxin challenge resulted in reduced body weight gain, increased feed conversion ratio and mortality (18%), impaired carcass traits (lower dressing and breast percentages, higher liver weight and abdominal fat), anemia and leukocytosis, increased liver enzymes and uric acid, decreased immune markers (IgG, IgM, lymphocyte proliferation, phagocytic index), induced oxidative stress (increased malondialdehyde, decreased superoxide dismutase/catalase/reduced glutathione), and disturbed cecal microbiota (higher *Escherichia coli* and total bacterial count, total yeast and molds, lower *Lactobacillus*). OAF (200mg/kg) significantly mitigated all these changes, restoring performance, immunity, antioxidant status, and microbial balance to levels close to controls, with efficacy comparable to antibiotic treatment. The combination OAF 200+ antibiotic showed additive benefits resulting in complete enhancement. In conclusion, organic acid filtrate of *A. niger* is a promising natural agent for the amelioration of aflatoxicosis in poultry.

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INTRODUCTION

Aflatoxins, mainly produced by *Aspergillus flavus*, are among the most potent naturally occurring mycotoxins in poultry feed and thus in birds' health and food safety. Aflatoxins and other mycotoxins impair growth, feed efficiency, immunity, vaccine response, product quality and overall production sustainability in broilers and layers (Attia *et al.,* 2025; Olariu *et al.,* 2025). The liver and kidneys are primary targets for these contaminants, which possess carcinogenic, mutagenic, teratogenic, hepatotoxic, and nephrotoxic properties (Kolawole *et al.,* 2022). Such toxins are widely distributed in animal feed, especially within the tropical climates of sub-Saharan Africa.

Contamination levels in these areas frequently exceed regulatory limits, resulting in the bioaccumulation of toxic substances in meat and eggs (Sharifi *et al.*, 2026). This not only compromises human health but also creates significant trade barriers. Affected livestock suffer from gastrointestinal damage and immunosuppression, leading to substantial economic losses for producers. These challenges are further compounded by concurrent

exposure to other toxicants or diseases, such as coccidiosis.

The primary mitigation strategies rely on physical or chemical detoxification of the feed or the inclusion of targeted feed additives (Koshiol *et al.*, 2026). Physical and chemical methods include washing, solvent extraction, heat treatment, microwave processing, irradiation, pulsed light, cold plasma, acids, enzymes, gases, and formaldehyde (Sipos *et al.,* 2021). These techniques significantly reduce aflatoxin concentrations in both animal feed and human food supplies. Additionally, incorporating organic and inorganic binders, such as bentonites, HSCAS, zeolites, and activated charcoal, alongside antioxidants into animal feed is a standard mitigation approach that reduces toxicity and supports hepatoprotection (Kolawole *et al.,* 2022).

Comparative studies indicate that inorganic binders are highly effective at reducing elevated liver enzymes in chickens exposed to aflatoxin (Kolawole *et al*., 2022). Alternative detoxification methods involve supplementing water or feed with biological substances, antioxidants, and immunomodulators. While these interventions can improve livestock performance, mitigate tissue damage, support immune function, and reduce the severity of mycotoxicosis, residual contaminant levels may still exceed regulatory thresholds (Nabarawy *et al*., 2020). Ultimately, the adoption of these methods is constrained by their variable efficacy, cost-effectiveness, and practical scalability in large-scale animal production.

Even after weeks of exposure, some chemical treatments leave toxic residues, and the animals often don't fully recover in performance (Orogu and Ehiwario, 2025). Consequently, research is increasingly pivoting toward bio-based solutions, which are widely recognized as safer and more sustainable alternatives to traditional chemical interventions. Lactic acid bacteria and *Bacillus* species are primary examples; they produce organic acids, volatile compounds, and polyphenols that exert potent inhibitory effects on *Aspergillus flavus*. These biogenic compounds not only suppress fungal proliferation but also inhibit up to 99.98% of aflatoxin B1 production. Notably, Abdel-Nasser *et al*. (2023) demonstrated that these metabolites disrupt the fungus' structural integrity, and subsequently *in vivo* trials confirmed their safety profile. Broadly, this aligns with a larger paradigm shift in agriculture: the use of fungal metabolites, such as terpenoids and polyketides, as eco-friendly biocontrol agents against agricultural pests and crop diseases. Ultimately, these biological interventions hold significant promise for ensuring the safety and sustainability of the animal feed supply chain (Jinna *et al*., 2025).

Organic acids, *i.e.,* propionic, butyric, sorbic, benzoic and acetic, are used in poultry as preservatives and growth promoters. Studies indicate that these acids can improve weight gain, nutrient absorption, gut health, and immune responses while decreasing microbial contamination (Salazar *et al.,* 2022). *In vitro* experiments have shown that several organic acids can completely eliminate A. flavus at low concentrations and attenuate aflatoxin production by silencing the genes responsible for toxin synthesis (Du *et al.,* 2023).

The combination of organic acids with essential oils makes the effect on *A. flavus* in corn even stronger at lower doses, making it a practical option for keeping poultry feed clean. One feeding trial found that a combination of heat treatment, organic acids, and yeast extracts reduced aflatoxin levels by about 65% in corn-based poultry feed (Orogu and Ehiwario, 2025). However, there is still no reliable, safe, and effective broad-spectrum control method for aflatoxins (Sipos *et al.,* 2021).

Several critical research gaps remain to be addressed. First, because most studies on fungal and bacterial secondary metabolites have been conducted in vitro, extensive in vivo validation is required to confirm their efficacy as aflatoxin biocontrol agents in commercial poultry feed systems (Abdel-Nasser *et al*., 2023; Jinna *et al*., 2025). The dual benefits of organic acids, specifically, the integration of their nutritional and gut-health advantages with their direct anti-aflatoxin properties, have not been fully evaluated under practical feeding regimens (Moon *et al*., 2018). Third, data regarding the optimal combinations and dosages of organic acids, microbial metabolites, and essential oils remain insufficient to guarantee detoxification without disrupting livestock performance or leaving undesirable chemical residues (Nabarawy *et al*., 2020). Furthermore, the precise timeline for complete physiological recovery in poultry following mycotoxin exposure remains undetermined, particularly given the persistent adverse effects observed for several weeks post-toxin cessation (Ochieng *et al*., 2021).

To address these limitations, the primary objective of this study was to develop and evaluate sustainable, bio-based methods for aflatoxin mitigation in poultry using secondary metabolites from selected micro-organisms and organic acids, both individually and in combination. Specifically, this investigation aimed to assess the antimicrobial, antioxidant, and therapeutic efficacy of an organic acid-enriched filtrate derived from *Aspergillus niger* in broiler chickens experimentally challenged with aflatoxin B_1.

MATERIALS AND METHODS

Production of organic acid filtrate from *Aspergillus niger*: *Aspergillus niger* was cultured in 250mL Erlenmeyer flasks containing 100mL of Czapek Dox broth (pH 6.2). The flasks were incubated at 28°C for 7 days under shaking conditions (120rpm). Following incubation, the culture broth was centrifuged at 10,000×g for 15 min at 4°C, and the supernatant was sequentially filtered through Whatman No. 1 filter paper and a 0.22 µm membrane filter (Millipore, USA). The resulting cell-free, sterile filtrate, designated as the organic acid filtrate (OAF), was stored at −20°C until further analysis.

HPLC analysis of organic acids: The organic acid profile of the OAF was determined by a modified HPLC method. The filtrate was diluted 1:5 with ultrapure water and passed through a 0.45µM syringe filter. The sample was then injected into a reversed-phase C18 HPLC column (250×4.6mm, 5µm particle size; Phenomenex, Torrance, CA, USA). The system was operated using isocratic elution with 0.01M H_2SO_4 as the mobile phase, at a flow rate of 0.6mL/min, and a UV detector was used to monitor the effluent at 210 nm. Citric, oxalic, succinic,

gluconic, malic, and fumaric acids were identified and quantified by comparing their retention times and peak areas with those of commercial standards obtained from Sigma-Aldrich (St. Louis, MO, USA) (Tormo and Izco, 2004).

***In vitro* antimicrobial activity:** The OAF was evaluated against pathogenic bacteria (*E. coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, and aflatoxin-producing fungi (*Aspergillus flavus*, *Aspergillus parasiticus*). Preliminary screening was performed by the agar well diffusion method, and MIC was determined by the broth microdilution method, according to the protocols validated by Klančnik *et al*. (2010). Agar well diffusion: Mueller-Hinton agar plates were prepared for bacteria and Sabouraud dextrose agar plates for the fungi.

Wells (6mM diameter) were bored in the agar and filled with 100µL of OA filtrate at concentrations of 25, 50, 100, 200, and 400µg/mL. Plates were incubated at 37°C for 24h (bacteria) or at 28°C for 48h (fungi). The diameters of the inhibition zones were measured in millimetres using a digital caliper. All tests were performed in triplicate. MICs were determined by standard broth microdilution in 96-well plates using Mueller-Hinton broth (for bacteria) or Sabouraud dextrose broth (for fungi). Two-fold serial dilutions of the OA filtrate from 7.8 to 1000µg/mL were prepared. After incubation of the plates, 20µL of 0.5% 2,3,5-triphenyltetrazolium chloride (TTC) per well was used as a viability indicator; a pink color indicated aerobic bacterial growth. The minimum inhibitory concentration (MIC) was recorded as the lowest concentration that completely inhibited visible growth of the microbe according to Klančnik *et al.* (2010).

***In vitro* antioxidant activity:** The antioxidant activity of the OAF (25, 50, 100, 200, and 400ug/mL) was assessed using a DPPH spectrophotometric method. Each dilution (0.5 mL) was mixed with 2.5mL of ethanolic DPPH. The samples were incubated at room temperature in the dark for 30 min, and absorbance was measured at 517nM. The percentage of scavenging activity was determined as [(A0 - A1) / A0]* 100 (Jang *et al.*, 2010).

Birds and housing conditions: 240 mixed-sex Ross 308 broiler chicks were obtained from a local commercial hatchery and randomly divided into eight groups of 10, with triplicate measurements (n=10). Sample size was determined a priori using a power analysis for a one-way ANOVA with 8 groups, assuming a large effect size (Cohen's f = 0.40), a statistical power of 0.80, and an alpha level of 0.05, which indicated a minimum of 8 birds per replicate; this was increased to 10 birds per replicate to account for potential attrition, resulting in 30 birds per treatment group across the three replicate pens. The experimental unit for body weight, hematological, biochemical, immunological, and oxidative stress parameters was the individual bird, whereas the pen served as the experimental unit for feed intake, feed conversion ratio, and mortality, as these measurements were recorded per pen. The chicks were housed in stainless-steel brooders in a temperature-controlled environment. Environmental conditions were maintained as follows: room temperature was set at 33°C for the first week, then gradually decreased by 3°C per week until reaching 24°C at week 4. Relative humidity was kept at 55–65%. A 23-h light and 1-h dark lighting schedule was provided throughout the entire study period. Birds had *ad libitum* access to feed and fresh water. All experimental procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC). The experiment spanned 28 days (from day 1 to day 28 of age).

Experimental design: The experiment included eight treatment groups. The negative control group was not challenged with aflatoxin. The aflatoxin challenge group was exposed to aflatoxin but remained untreated. Three groups received OAF at different doses following aflatoxin challenge: OAF 50 (50mg/kg body weight/day), OAF 100 (100mg/kg/day), and OAF 200 (200mg/kg/day). The selected doses were justified based on the *in vitro* MIC values obtained in this study (which ranged between 50–200µg/mL against the tested pathogens) and on a subsequent pilot *in vivo* trial that demonstrated a graded protective response; 50mg/kg was chosen as the minimum effective dose to evaluate baseline efficacy, 200mg/kg as the maximum safe dose that did not impair performance in healthy birds, and 100mg/kg as a mid-range dose to assess dose-dependency. The positive group was challenged and treated with antibiotics. A combined group received both the aflatoxin challenge and the OAF at 200mg/kg/day, together with an antibiotic (OAF 200 + antibiotic). Finally, the antibiotic group was not challenged with aflatoxin but received the antibiotic as a safety control.

Aflatoxin challenge: Aflatoxin B_1 (AFB_1) was purified from *Aspergillus flavus* culture and verified by HPLC to contain ≥98% AFB_1 with no detectable contamination by other aflatoxins (AFB_2, AFG_1, AFG_2). The purified AFB_1 was dissolved in 100% ethanol (1mg/mL stock solution) and incorporated into the basal broiler starter diet (crude protein 22%, metabolizable energy 3,050kcal/kg) to achieve a final concentration of 250ppb (µg/kg). Feed contaminated with 250ppb AFB_1 was freshly prepared each week, pelleted, and stored at 4°C in airtight, opaque containers to shield the toxin from degradation. This diet was fed continuously to the broilers from day 1 to day 28. This inclusion level was chosen based on Liu *et al*. (2018) as it induces measurable sublethal effects on broiler growth and physiology without inducing acute mortality. Feed samples were periodically analyzed throughout the trial to confirm that AFB_1 concentrations were consistent.

Treatment administration: OAF was concentrated, lyophilized, and reconstituted in distilled water, then administered orally by gavage once daily at the indicated doses (50, 100, or 200mg/kg body weight). The antibiotic used was enrofloxacin (Baytril® 10%, Bayer HealthCare, Germany), given orally at the therapeutic dose of 10 mg/kg body weight once daily for 7 consecutive days (days 1–7 of the challenge period). The combination group (OAF-200 + Antibiotic) received the OAF-200 filtrate (200mg/kg/day) and enrofloxacin (10 mg/kg/day) simultaneously. All treatments were continued for the full 28-day experimental period (Fig. 1).

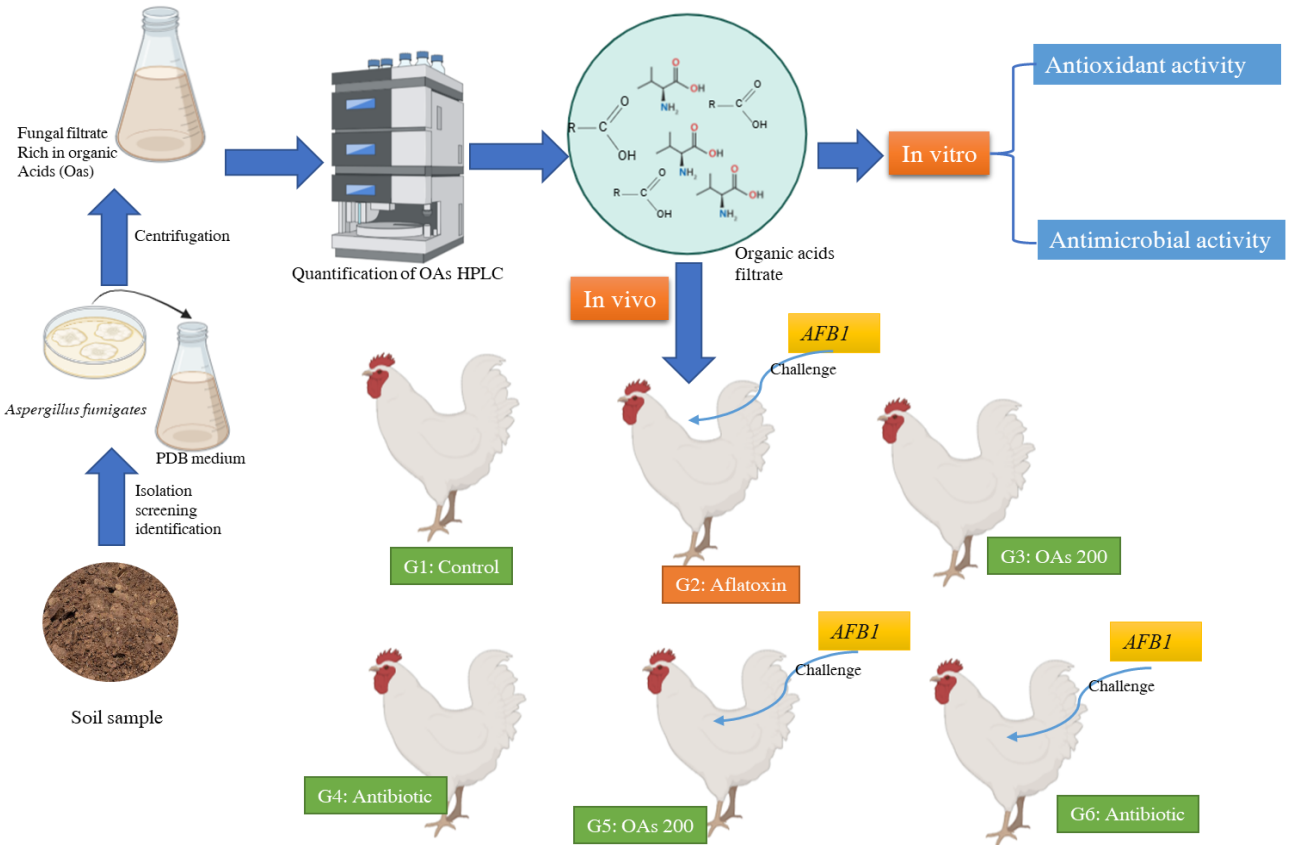

Fig. 1: Experimental design of the effect of *Aspergillus niger*- organic acid filtrate on aflatoxin challenge in broilers.

Growth performance and mortality: Body weight gain (BWG) was measured individually at week 1, 2, 3, and at the end of the experiment (day 28) using a digital scale (±1g). Feed intake (FI) was recorded daily for each replicate, and feed conversion ratio (FCR) was calculated as FI divided by body weight gain (FI/gain). Mortality was recorded daily, and the cumulative mortality percentage was calculated for each group (Kraieski *et al.*, 2017).

Carcass properties: At day 28, following an overnight fast (12 h), 10 birds per group (randomly selected, two birds per replicate) were euthanized by cervical dislocation. Birds were bled, scalded, defeathered, and eviscerated. Dressing percentage was calculated as [eviscerated carcass weight (excluding head, feet, and abdominal fat) / live body weight] × 100. The breast muscle (pectoralis major and minor) was excised and weighed, and the breast percentage (%) was calculated as (breast weight / live weight) × 100. Relative liver weight (%) was calculated as (liver weight / live weight) × 100. Abdominal fat (the fat pad surrounding the gizzard, cloaca, and abdominal wall) was excised and weighed, and abdominal fat percentage (%) was calculated as (abdominal fat weight / live weight) × 100 (Zou *et al.*, 2023).

Hematology: Blood samples (2mL per bird) were collected from the brachial wing vein into Vacutainer tubes containing lithium heparin as an anticoagulant. Blood collection was performed on days 14 and 28 of the experiment, between 8:00 and 10:00 AM to avoid diurnal variation. Hematological parameters, including red blood cell (RBC $\times 10^6/\mu L$) , white blood cell (WBC$\times 10^3/\mu L$) count, hemoglobin (Hb, g/dL) concentration, and hematocrit (HCT %) count, were analyzed within 4 hours of collection using an automated hematology analyzer (Mindray BC-5000 Vet, Shenzhen, China), calibrated specifically for avian blood according to the manufacturer's instructions. Hemoglobin was measured using the cyanmethemoglobin method. Hematocrit was determined by the microhematocrit centrifugation method (12,000×g for 5 minutes) (Kilany *et al.*, 2020).

Blood biochemistry: Serum samples were obtained by collecting blood (3mL per bird) in plain Vacutainer tubes, allowing the blood to clot for 30 minutes at room temperature, and centrifuging at 3,000×g for 10 minutes at 4°C. Serum was separated and stored at −20°C until analysis. Biochemical parameters were determined using commercial colorimetric kits (Randox Laboratories Ltd., Crumlin, UK; or BioVision Inc., Milpitas, CA, USA) with a semi-automated spectrophotometer. Aspartate aminotransferase (AST, U/L) and alanine aminotransferase (ALT, U/L) activities were measured using the International Federation of Clinical Chemistry (IFCC) kinetic method, with results expressed in U/L. Total protein (TP) was determined using the Biuret method (absorbance at 546nM) and expressed in g/dL. Uric acid (UA) was measured using the uricase-peroxidase method (absorbance at 520nm) and expressed in mg/dL (Kilany *et al.*, 2020).

Immunological markers: To measure serum immunoglobulins (IgG and IgM, mg/ml), commercial chicken-specific ELISA kits (MyBioSource, USA; Bethyl

Laboratories, USA) were used according to the provided instructions. Serum was diluted (1:200 for IgG, 1:100 for IgM) and incubated in anti-chicken IgG- or IgM-coated 96-well plates for 1h at 37°C. After washing, HRP-conjugated detection antibodies were added, and the signal was developed with TMB substrate. After stopping the reaction with H_2SO_4 (2N), we read the optical density at 450nm using a BioTek ELx800 microplate reader. Immunoglobulin concentrations were then calculated against standard curves from purified chicken IgG and IgM (Kundu *et al*., 2016).

Lymphocyte proliferation assay (MTT method): Density gradient centrifugation (Histopaque 1077, Sigma-Aldrich; 1,500 × g for 20 min) was used to harvest PBMCs from fresh heparinized blood. Once isolated, the cells were washed twice with PBS and resuspended in RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine, and antibiotics (100U/mL penicillin, 100µg/mL streptomycin). The plates were seeded (2 × 10^5 cells/well in a 96-well flat-bottom format) and stimulated in triplicate with 5 µg/mL concanavalin A (Con A, Sigma-Aldrich). Control wells received no mitogen. Plates were incubated for 72 hours at 39°C in a 5% CO_2 humidified atmosphere. MTT solution (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5mg/mL, 20µL/well) was added 4 hours before the end of incubation. Formazan crystals were dissolved in 150µL dimethyl sulfoxide (DMSO), and absorbance was measured at 570nm using a microplate reader (Hou *et al.*, 2022). The stimulation index (SI) was calculated as: SI = (absorbance of stimulated wells) / (absorbance of unstimulated control wells).

Carbon clearance assay for phagocytic index: The phagocytic activity of the reticuloendothelial system (RES) was assessed using the carbon clearance method. On day 28, each bird (n = 8 per group) was injected intravenously (via the wing vein) with colloidal carbon suspension (Pelikan Fount India ink, Günther Wagner, Germany) at a dose of 0.1mL per 100g body weight. Blood samples (20 µL) were collected from the opposite wing vein at 2-minute intervals for 10 minutes (T = 2, 4, 6, 8, 10 min) and immediately mixed with 2 mL of 0.1% Na_2CO_3 solution. The absorbance of the resulting solution was measured at 675nm (Bhatti *et al.*, 2021). The carbon clearance rate (K) was calculated from the slope of the natural logarithm of carbon concentration versus time. The phagocytic index (α) was calculated as $\alpha = K^{1/3} \times$ (body weight/liver + spleen weight).

Oxidative stress markers: Serum levels of malondialdehyde (MDA, nmol/mL), superoxide dismutase (SOD, U/mL), catalase (CAT, U/mL), and reduced glutathione (GSH, umol/L) were measured on day 28.

Malondialdehyde (MDA): Lipid peroxidation was assessed by measuring thiobarbituric acid-reactive substances (TBARS) as described by Mujahid *et al.* (2007). Serum (0.5mL) was mixed with 1mL of 20% trichloroacetic acid (TCA) containing 0.01% butylated hydroxytoluene (BHT) and centrifuged at 3,000 × g for 10 min. The supernatant was mixed with an equal volume of 0.67% thiobarbituric acid (TBA) and incubated at 100 °C for 15 min. After cooling, the absorbance was measured at 532 nm. MDA concentration was calculated using a standard curve of 1,1,3,3-tetramethoxypropane and expressed as nmol/mL.

Superoxide dismutase (SOD): SOD activity was measured using the nitroblue tetrazolium (NBT) reduction method. The assay mixture contained 0.1mL of serum, 1.2mL of 0.05 M phosphate buffer (pH 7.8), 0.1mL of 0.1 mM EDTA, 0.1mL of 1.5mM NBT, and 0.1mL of 0.12 mM riboflavin. The reaction was initiated by illuminating the mixture with a fluorescent lamp for 15 minutes. The absorbance was measured at 560nM. One unit of SOD was defined as the amount of enzyme required to inhibit NBT reduction by 50%. Results were expressed as U/mL.

Catalase (CAT): The activity of the enzyme was determined spectrophotometrically by the rate of H_2O_2 decomposition at 240nM. The assay mixture contained 50µL of serum, 1.95mL of 50mM phosphate buffer (pH 7.0), and 1mL of 30mM H_2O_2. The decrease in absorbance was monitored for 3 min. One unit of CAT was defined as the amount of enzyme that catalyzes the decomposition of 1µmol of H_2O_2/min at 25°C. The results were expressed as U/mL.

Reduced glutathione (GSH): Its concentration was determined using the DTNB method. Serum (0.5mL) was deproteinized by 0.5mL of 10% TCA and then centrifuged at 3000 × g for 10 min. Then, 0.2 mL of supernatant was mixed with 2.6mL of 0.1 M phosphate buffer (pH 8.0) and 0.2mL of 0.01 M DTNB. The solution turned yellow, and the absorbance was measured at 412nm. The final results were calculated from a reduced glutathione standard curve and expressed as µmol/L. The procedures for SOD, CAT, and GSH were adapted from standard protocols previously applied in avian species (Xu and Diplock, 1983).

Cecal microbial count: On day 28, after euthanasia, the cecum was aseptically excised from 10 birds per group (two per replicate). The cecal contents (approximately 1g) were collected into sterile tubes, weighed, serially diluted (10^{-1} to 10^{-8}) in sterile 0.1% peptone water, and vigorously vortexed for 30 seconds to ensure homogenization (Zhang *et al.*, 2022). Aliquots of 0.1mL from appropriate dilutions were spread onto the following selective media:

Microbiological analysis of the cecal contents was performed using selective and non-selective media under appropriate incubation conditions. Total aerobic bacterial counts were determined on nutrient agar plates, which were incubated aerobically at 37°C for 24h. *Escherichia coli* was enumerated on MacConkey agar after aerobic incubation at 37°C for 24–48h. Total yeast and mold count was assessed using Dichloran Rose Bengal Chloramphenicol (DRBC) agar incubated at 28°C for 7 days. *Lactobacillus* spp. were counted on De Man, Rogosa, and Sharpe (MRS) agar, incubated at 37°C for 48h under anaerobic or microaerophilic conditions (5% CO_2). References for the applied methods are provided in

the relevant sections. After incubation, colonies were counted using a digital colony counter, and results were expressed as $\log_{10}$ colony-forming units per gram of cecal content ($\log_{10}$ CFU/g).

Statistical analysis: All data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS software (version 26.0, IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) was used to compare means among the eight experimental groups. When ANOVA indicated significant differences ($P<0.05$), Tukey's honestly significant difference (HSD) post hoc test was used for pairwise comparisons. The assumptions of normality (Shapiro-Wilk test, $P>0.05$) and homogeneity of variances (Levene's test, $P>0.05$) were verified prior to ANOVA. Mortality data were analyzed using the Chi-square (χ^2) test. Significance was declared at $P<0.05$.

RESULTS

Organic acid composition of *Aspergillus niger* filtrate: HPLC analysis of the filtrate revealed the 6 main organic acids, *i.e.,* citric acid at 342.5mg/L, followed by oxalic (187.2mg/L), succinic (112.3mg/L), gluconic (98.4mg/L), malic (45.7mg/L), and fumaric (28.9mg/L). These acids constituted the bioactive components in the fungal filtrate and may exhibit anti-aflatoxin activity (Table 1).

Table 1: HPLC analysis of *Aspergillus niger*- organic acid filtrate

Organic Acid	Concentration (mg/L)	Retention Time (min)
Citric acid	342.5±12.3a	3.45
Oxalic acid	187.2±8.1b	4.12
Gluconic acid	98.4±5.6	5.87
Malic acid	45.7±3.2	6.23
Fumaric acid	28.9±1.9	7.58
Succinic acid	112.3±6.7	8.04

Data are presented mean ± SD n=3. Lowercase letters in the same column indicate significant differences at P<0.05

***In vitro* antimicrobial activity:** The consortia of organic acids (25–400ug/mL) showed concentration-dependent antibacterial activity against all tested pathogens. *S. aureus* was the most sensitive with IZDs of 15.6mM and 40.2 mm at 25 and 400mg/kg, respectively, and a minimum inhibitory concentration (MIC) of 62.5µg/mL. *E. coli* showed moderate susceptibility (MIC = 125µg/mL) while *S.* Typhimurium was less affected (MIC = 250µg/mL). The filtrate also inhibited aflatoxin-producing fungi with MICs of 200 and 250µg/mL against *Aspergillus flavus* and *A. parasiticus*, respectively, and the inhibition zones increased proportionally with concentration (Table 2).

Table 2: Antimicrobial activity of *Aspergillus niger*- organic acid filtrate against pathogenic microorganisms

Micro-organisms	Concentrations (ug/mL) / Inhibition zones diameters (mm)					MIC µg/mL
	25	50	100	200	400	
Pathogenic bacteria						
E. coli	8.2±0.5e	12.4±0.6d	18.7±0.7c	24.3±0.8b	30.1±0.9a	125c
S. Typhimurium	6.5±0.4e	10.1±0.5d	14.8±0.6c	20.2±0.7b	26.5±0.8a	250a
S. aureus	15.6±0.6e	22.3±0.7d	28.9±0.8c	35.4±0.9b	40.2±1.0a	62.5d
Aflatoxin-producing fungi						
A. flavus	9.3±0.5e	14.2±0.6d	19.8±0.7c	26.4±0.8b	33.1±0.9a	200b
A. parasiticus	8.8±0.5e	13.5±0.6d	18.9±0.7c	24.7±0.8b	31.0±0.9a	250a

Data are presented mean ± SD n=3. Lowercase letters in the same row indicate significant differences at P<0.05.

***In vitro* antioxidant activity:** OAF showed a considerable radical scavenging activity in a dose dependent manner (25-400µg/mL). DPPH scavenging increased from 22.5 to 88.3% with an IC 50 of 85.3µg/mL. ABTS scavenging was between 28.3 and 89.5% (IC 50 = 78.6µg/mL). Ferric reducing antioxidant power (FRAP) increased from 0.42 to 2.42mM Fe^{2+} /g indicating a strong electron donating capacity (Fig. 2 A, B).

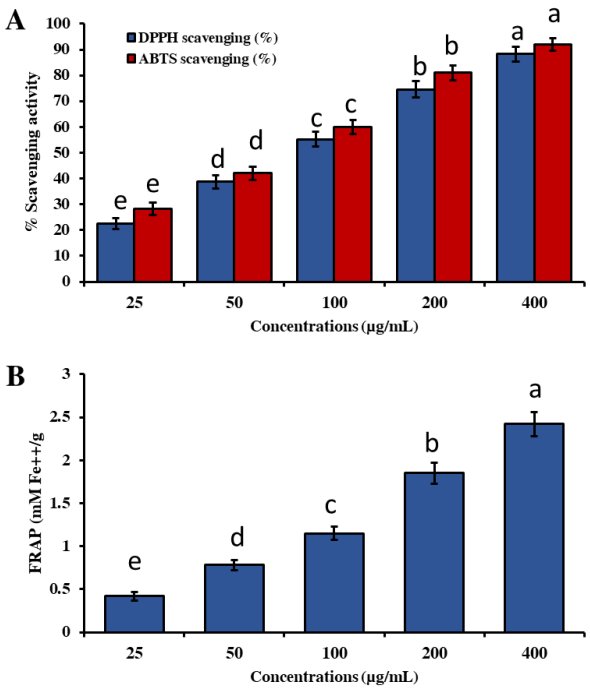

Fig. 2: Antioxidant activity of *Aspergillus niger*- organic acid filtrate enriched in organic acids against (A) DPPH, ABTS, and (B) FRAP. Lowercase letters above columns indicate significant difference at P<0.05, Data are presented as mean ± SD, n=3.

Growth performance and mortality: Aflatoxin challenge (250 ppb in feed) for 28 days significantly reduced body weight gain (1120g vs. 1850g in control) and increased the feed conversion ratio (FCR: 2.35 vs. 1.62), while increasing mortality to 18 % ($P<0.05$). OAF supplementation at 50, 100 and 200mg/kg dose dependently improved these parameters. The OAF (200 mg/kg) recorded body weight gain (1720g) and FCR (1.70) near control levels and reduced mortality to 2%. The combination OAF 200 + antibiotic showed enhanced performance parameters (body weight gain: 1830g; FCR: 1.60; 0% death), comparable to the antibiotic only and negative control groups (Table 3).

Table 3: The effect of *Aspergillus niger*- organic acid filtrate on Growth performance and mortality rate of aflatoxin-challenged broilers

Groups	Body weight gain (g)	Feed conversion ratio (FCR)	Mortality (%)
Control	1850±45^{a}	1.62±0.03^{a}	0^{a}
Aflatoxin challenge	1120±38^{e}	2.35±0.07^{e}	18^{b}
OAF-50	1280±42^{d}	2.15±0.06^{d}	14^{b}
OAF-100	1480±40^{c}	1.92±0.05^{c}	8^{c}
OAF-200	1720±39^{b}	1.70±0.04^{b}	2^{a}
Antibiotic	1700±41^{b}	1.72±0.04^{b}	3^{a}
OAF-200 + Antibiotic	1830±43^{a}	1.60±0.03^{a}	0^{a}
Antibiotic only	1860±44^{a}	1.61±0.03^{a}	0^{a}

Data are presented as the mean ± SD, n=10. Different superscript letters (a–e) indicate significant differences (P<0.05). Organic acid filtrate (OAF)

Carcass properties: Aflatoxin challenge decreased dressing percentage (61.2 vs. 72.5% in control) and breast percentage (18.5 vs. 24.3%), while increasing relative liver weight (4.8 vs. 2.1%) and abdominal fat (3.4 vs. 1.9%). OAF treatment at 200mg/kg significantly improved dressing (70.8%), breast (23.5%), liver (2.5%), and abdominal fat (2.1%). The combination OA 200 + antibiotic enhanced all carcass traits (dressing: 73.1%; breast: 24.8%; liver: 2.0%; abdominal fat: 1.8%) to values statistically indistinguishable from controls (Table 4).

Table 4: The effect of *Aspergillus niger*- organic acid filtrate on carcass properties of aflatoxin-challenged broilers

Group	Dressing (%)	Breast (%)	Liver (%)[1]	Abdominal fat (%)
Control	72.5±1.2^{a}	24.3±0.8^{a}	2.1±0.1^{e}	1.9±0.2^{cd}
Aflatoxin challenge	61.2±1.5^{d}	18.5±0.9^{d}	4.8±0.3^{a}	3.4±0.3^{a}
OAF-50	63.8±1.4^{cd}	19.8±0.8^{cd}	4.1±0.2^{b}	3.1±0.2^{ab}
OAF-100	67.5±1.3^{bc}	21.5±0.7^{bc}	3.2±0.2^{c}	2.6±0.2^{bc}
OAF-200	70.8±1.3^{ab}	23.5±0.7^{ab}	2.5±0.2^{de}	2.1±0.2^{cd}
Antibiotic	71.5±1.2^{a}	23.8±0.7^{ab}	2.3±0.2^{e}	2.0±0.2^{cd}
OAF-200 + Antibiotic	73.1±1.1^{a}	24.8±0.6^{a}	2.0±0.1^{e}	1.8±0.2^{d}
Antibiotic only	73.0±1.2^{a}	24.5±0.7^{a}	2.1±0.1^{e}	1.8±0.2^{d}

Data are presented as the mean ±SD, n=10. [1]Relative liver weight (%). Different superscript letters (a–e) indicate significant differences (P<0.05).

Hematology: Aflatoxin induced microcytic hypochromic anemia, evidenced by reduced RBC (2.15×10^{6}/µL vs. 3.15×10^{6}/µL), hemoglobin (8.2 vs. 11.8g/dL), and hematocrit (26.5% vs. 38.2%), together with leukocytosis (WBC: 35.8 vs. 22.5×10^{3}/µL). OAF (200 mg/kg) improved hematology with RBC (3.02×10^{6}/µL), Hb (11.4g/dL), HCT (36.9%) and WBC (24.1×10^{3}/µL) values close to control values. The combination OAF (200) + antibiotic showed enhanced hematology (RBC: 3.18×10^{6}/µL; Hb: 12.0 g/dL; HCT: 38.5%; WBC: 21.8×10^{3}/µL), close to the antibiotic-only group (Table 5).

Table 5: The effect of *Aspergillus niger*- organic acid filtrate on Hematology properties of aflatoxin-challenged broilers

Group	RBC ($\times10^{6}$/µL)	Hb (g/dL)	HCT (%)	WBC ($\times10^{3}$/µL)
Control	3.15±0.12^{a}	11.8±0.4^{a}	38.2±1.2^{a}	22.5±1.2^{d}
Aflatoxin challenge	2.15±0.10^{d}	8.2±0.3^{d}	26.5±1.4^{d}	35.8±1.8^{a}
OAF-50	2.35±0.11^{cd}	8.8±0.4^{cd}	28.4±1.3^{cd}	33.2±1.6^{a}
OAF-100	2.68±0.12^{bc}	9.9±0.4^{bc}	31.5±1.3^{bc}	29.4±1.5^{b}
OAF-200	3.02±0.11^{a}	11.4±0.4^{ab}	36.9±1.3^{ab}	24.1±1.3^{cd}
Antibiotic	3.00±0.10^{a}	11.3±0.4^{ab}	36.5±1.2^{ab}	24.5±1.4^{cd}
OAF-200 + Antibiotic	3.18±0.10^{a}	12.0±0.4^{a}	38.5±1.1^{a}	21.8±1.2^{d}
Antibiotic only	3.20±0.11^{a}	12.1±0.4^{a}	38.8±1.2^{a}	21.5±1.1^{d}

Data are presented as the mean ± SD, n=10. Different superscript letters (a–e) indicate significant differences (P<0.05).

Blood biochemistry: Aflatoxin caused severe hepatocellular damage and kidney dysfunction, with elevated AST (385 vs. 185U/L), ALT (98 vs. 42U/L) and uric acid (12.5 vs. 5.2mg/dL) and reduced total protein (2.6 vs. 4.2g/dL). OA 200 significantly reduced AST (210 U/L), ALT (52U/L), and uric acid (6.1mg/dL), while increasing total protein (3.9g/dL). The combination OA 200 + antibiotic enhanced all biomarkers close to control levels (AST: 190 U/L; ALT: 44U/L; uric acid: 5.0mg/dL; total protein: 4.3g/dL) (Fig. 3 A, B, C).

Immunological markers: Aflatoxin severely suppressed humoral and cell mediated immunity, reducing IgG (3.5 vs. 8.2 mg/mL), IgM (0.6 vs. 1.4mg/mL), lymphocyte proliferation index (0.92 vs. 1.85), phagocytic index (34% vs. 68%). OA 200 significantly enhanced immune functions (IgG: 7.6mg/mL, IgM: 1.3mg/mL, proliferation: 1.72, phagocytic index: 63%) similar to antibiotic treatment (IgG: 7.4mg/mL, IgM: 1.2mg/mL, proliferation: 1.68, phagocytic index: 61%). The combination of OA 200 + antibiotic enhanced immunity markers comparable to the control levels (IgG: 8.4mg/mL, phagocytic index: 70%), demonstrating synergistic immunomodulation (Table 6).

Table 6: The effect of *Aspergillus niger*- organic acid filtrate on Immune markers of aflatoxin-challenged broilers

Group	IgG (mg/mL)	IgM (mg/mL)	Lymphocyte proliferation (SI)	Phagocytic index (%)
Control	8.2±0.4^{a}	1.4±0.1^{a}	1.85±0.12^{a}	68±3^{a}
Aflatoxin challenge	3.5±0.3^{d}	0.6±0.1^{d}	0.92±0.08^{d}	34±4^{d}
OAF-50	4.2±0.3^{cd}	0.7±0.1^{cd}	1.08±0.09^{cd}	41±3^{cd}
OAF-100	5.6±0.3^{bc}	0.9±0.1^{bc}	1.32±0.10^{bc}	51±3^{bc}
OAF-200	7.6±0.4^{a}	1.3±0.1^{ab}	1.72±0.10^{ab}	63±3^{ab}
Antibiotic	7.4±0.4^{a}	1.2±0.1^{ab}	1.68±0.10^{ab}	61±3^{ab}
OAF-200 + Antibiotic	8.4±0.4^{a}	1.5±0.1^{a}	1.88±0.11^{a}	70±3^{a}
Antibiotic only	8.5±0.4^{a}	1.5±0.1^{a}	1.90±0.10^{a}	71±3^{a}

Data are presented as the mean ± SD, n=10. Different superscript letters (a–e) indicate significant differences (P<0.05).

Oxidative stress markers: Aflatoxin challenge increased lipid peroxidation (MDA: 6.8 vs. 2.1nmol/mL) and decreased antioxidant defence (SOD: 42 vs. 85U/mL; CAT: 38 vs. 72U/mL; GSH: 7.2 vs. 18.5µmol/L). OA 200 reduced MDA (2.8nmol/mL) and increased SOD (78U/mL), CAT (66U/mL) and GSH (16.8µmol/L). The combination OA 200 + antibiotic completely enhanced oxidative stress (MDA: 2.0nmol/mL; SOD: 87U/mL; CAT: 74U/mL; GSH: 19.2µmol/L), similar to that of the negative control and antibiotic only groups (Table 7).

Table 7: The effect of *Aspergillus niger*- organic acid filtrate on Oxidative markers of aflatoxin-challenged broilers

Group	MDA (nmol/mL)	SOD (U/mL)	CAT (U/mL)	GSH (µmol/L)
Control	2.1±0.2^{d}	85±4^{a}	72±3^{a}	18.5±1.2^{a}
Aflatoxin challenge	6.8±0.5^{a}	42±5^{d}	38±4^{d}	7.2±0.8^{d}
OAF-50	5.9±0.4^{b}	50±4^{cd}	44±4^{cd}	9.5±0.9^{cd}
OAF-100	4.5±0.4^{c}	62±4^{bc}	53±4^{bc}	12.8±1.0^{bc}
OAF-200	2.8±0.3^{d}	78±4^{ab}	66±3^{ab}	16.8±1.1^{ab}
Antibiotic	2.9±0.3^{d}	76±4^{ab}	64±3^{ab}	16.2±1.0^{ab}
OAF-200 + Antibiotic	2.0±0.2^{d}	87±3^{a}	74±3^{a}	19.2±1.2^{a}
Antibiotic only	1.9±0.2^{d}	88±3^{a}	75±3^{a}	19.5±1.1^{a}

Data are presented as the mean ± SD, n=10. Different superscript letters (a–e) indicate significant differences (P<0.05)

Cecal microbial counts: Aflatoxin interrupted the gut microbiota, increasing total aerobes (8.9 vs. 7.2 log CFU/g), *E. coli* (8.2 vs. 6.1), and total yeast and molds count (7.1 vs. 4.5), while decreasing beneficial *Lactobacillus* (5.2 vs. 8.4). OA-200 partially restored microbial balance (total aerobes: 7.5; *E. coli*: 6.5; *Lactobacillus*: 7.9). The combination OA-200 + antibiotic achieved the best modulation, with *E. coli* (5.5) and TYMC (4.0) even lower than the negative control, and *Lactobacillus* (8.6) significantly higher, indicating prebiotic-like effects (Fig. 4).

DISCUSSION

Following the European Union's implementation of Regulation (EC) No 1831/2003, which culminated in a

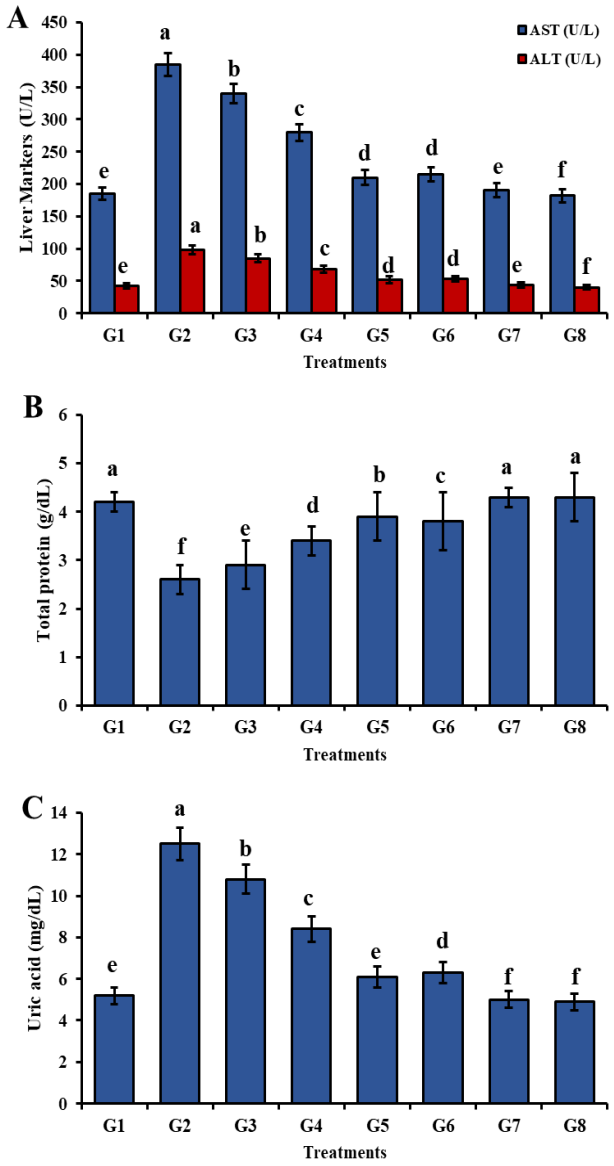

Fig. 3: The effect of the *Aspergillus niger*- organic acid filtrate on (A) liver markers (ALT and AST), (B) protein content, and (C) uric acid. Lowercase letters above columns indicate a significant difference at P<0.05. Data are presented as mean ±SD, n=3.

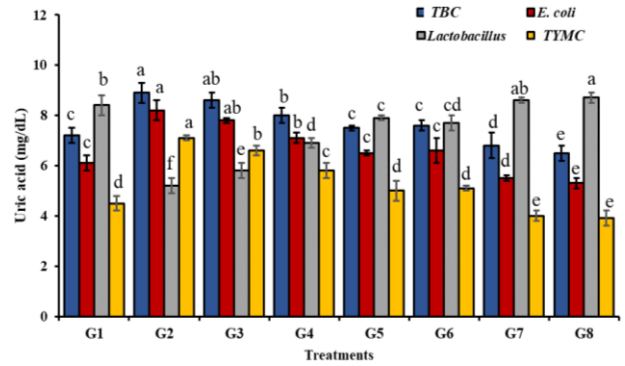

Fig. 4: The effect of the *Aspergillus niger*- organic acid filtrate on different microbial count. Lowercase letters above columns indicate a significant difference at P<0.05.

strict ban on antibiotic growth promoters in 2006, the livestock industry underwent a profound operational transition. While the ban represented a significant advancement for public health, it initially left poultry populations vulnerable to enteric diseases and performance deficits that were previously managed through subtherapeutic antibiotic use. To mitigate these challenges, subsequent research focused on identifying viable alternatives to antibiotics. These efforts led to the integration of prebiotics, phytogenic extracts, and immunomodulators into standard formulation strategies. Today, these additives are essential components for maintaining flock health, welfare, and productivity in antibiotic-free production systems (Lalev *et al.*, 2022; Akan *et al.*, 2025).

Organic acids (OAs) have attracted significant interest as alternative compounds, mainly because they have traditionally been used as preservatives for perishable foods, prolonging their shelf life (Coban, 2020). Organic acids are weak acids that contain a carboxylic group (R-COOH). These compounds are naturally produced as intermediates during the breakdown of major macromolecules into active compounds with lower molecular weights, such as fatty acids and carboxylic acids (Cherrington *et al.,* 1991). Their strong antimicrobial effects are well known, and they help to control pathogenic bacteria. They also reduce gut pH and improve both nutrient absorption and feed efficiency (Okey, 2023).

The reported results support the conclusion that the *Aspergillus niger* filtrate is a biologically active organic-acid mixture with antimicrobial, antioxidant, and anti-aflatoxicosis effects in broilers, and they align well with both recent poultry studies and older work on fungal metabolites, aflatoxin toxicity, and organic-acid feed additives. Aflatoxin B1 is consistently described as the most toxic and most relevant aflatoxin in poultry, with well-established effects on growth depression, liver injury, oxidative stress, and immunosuppression, so the broad pattern of damage in the challenged birds is expected rather than anomalous (Rajput *et al.*, 2017; Nabi *et al.*, 2022; Sang *et al.*, 2023).

The HPLC profile showing citric acid as the dominant component, followed by oxalic, succinic, gluconic, malic, and fumaric acids, is plausible for a fungal-derived filtrate and is consistent with the wider literature showing that fungi and mushrooms commonly accumulate organic acids as prominent bioactive metabolites (Kostić *et al.*, 2023; Shah *et al.*, 2024). The most common low-molecular-weight organic acids (LMWOAs) in macromycetes and filamentous fungal systems are citric, oxalic, and malic acids. This universality indicates that these compounds are not merely incidental metabolic byproducts but functional components of fungal metabolism with specific biological activities (Kostić et al., 2020; Khan *et al.*, 2022).

These acids may play a role in reducing aflatoxin, as organic acids are acknowledged in poultry nutrition for their antimicrobial, immune-enhancing, and growth-promoting properties, with their effectiveness being significantly influenced by the type of acid, pKa, concentration, and target microorganism (Khan *et al.*, 2022). Prior research has demonstrated that organic acids function by luminal acidification, membrane permeabilization, intracellular pH disruption, and microbiota regulation, collectively suppressing pathogenic bacteria and indirectly curtailing fungal proliferation or

toxin-induced dysbiosis (Kumar *et al.*, 2021; Dai *et al.*, 2022; Mekonnen *et al.*, 2024).

The concentration-dependent antibacterial activity against *S. aureus, E. coli,* and *S. typhimurium* is strongly consistent with the known mode of action of organic acids in poultry and food microbiology. Organic acids can enter microbial cells in the undissociated form and dissociate in the more alkaline cytoplasm, lowering intracellular pH and disrupting metabolism, which explains why susceptibility often differs across bacterial species (Kumar *et al.,* 2021; Khan *et al.,* 2022). The heightened sensitivity of Gram-positive *S. aureus* compared to Gram-negative enteric bacteria in the current findings is justifiable, as antimicrobial spectra differ significantly among acids and target species (Khan *et al.,* 2022).

The antifungal activity against *A. flavus* and *A. parasiticus* is especially relevant because these are the principal aflatoxin-producing fungi in feeds and feed ingredients (Rajput *et al.,* 2017; Rashidi *et al.*, 2020). The concept that biologically derived metabolites can suppress both bacterial and fungal targets has been previously supported by various studies. Fungal extracts from mushrooms and endophytes frequently exhibit antibacterial, antibiofilm, and antioxidant activities, and probiotic microorganisms with antifungal properties have also demonstrated strong inhibition of A. flavus growth and AFB1 detoxification potential (Chen *et al.,* 2021; Kostić *et al.,* 2023; Shah *et al.,* 2024). This does not prove that the six measured acids alone account for all inhibition, but it supports the conclusion that the filtrate contains a functional antimicrobial consortium rather than a single active principle.

The antioxidant profiles exhibit a high degree of validity and consistency. The filtrate's potent electron-donating and radical-quenching capacity is confirmed by its robust DPPH and ABTS radical-scavenging activities and elevated FRAP values. These results are consistent with the established literature on fungal bioactivity, which indicates that extracts that are abundant in phenolic metabolites and organic acids consistently demonstrate significant antioxidant efficacy (Kaptan, 2024; Puia *et al.*, 2025). Endophytic fungal metabolites have shown measurable DPPH scavenging, including compounds such as kojic acid with IC_{50} values (Shah et al., 2024) agreeing with the current study. Because aflatoxin toxicity is tightly linked to oxidative stress, the filtrate's *in vitro* antioxidant capacity provides a mechanistic bridge to its in vivo protective effects (Rajput et al., 2017).

The *in vivo* results under 250ppb aflatoxin challenge align closely with the established pathology of aflatoxicosis in broilers. The previous studies have shown that AFB1 exposure reduces growth performance, worsens feed conversion, impairs liver function, lowers immunity, and increases oxidative stress, with the liver consistently identified as a primary target organ (Zou *et al.*, 2023). Even marginal dietary concentrations of aflatoxin B_1 (AFB_1) are sufficient to compromise systemic antioxidant capacity and elevate serum aspartate aminotransferase (AST) levels, whereas elevated concentrations further exacerbate feed conversion ratios and organ pathology (Zou *et al.,* 2023).

The marked reduction in body weight gain, deterioration in FCR, and increased mortality in the challenged untreated group (Rashidi *et al.,* 2020; Sang *et al.,* 2023). The dose-dependent physiological recovery observed with the administration of the organic-acid filtrate, particularly at the 200mg/kg dose, is consistent with previous literature indicating that organic acids improve the growth performance, intestinal integrity, and immune function of broilers during periods of metabolic stress or infectious challenge (Dai *et al.,* 2022; Khan *et al.,* 2022). The significant enhancement with the OAF+ antibiotic coadministration suggests additive or synergistic effects, which is reasonable because prior studies have found interactions between organic acids and other microbial-control interventions on immune and microbiological outcomes (Ebeid *et al.*, 2021; Mekonnen *et al.,* 2024).

The improvement in carcass yield and the reduction in relative liver weight also align with prior knowledge. Aflatoxin exposure impairs carcass traits and produces liver enlargement and damage, whereas successful protective interventions restore productive tissue deposition and reduce liver burden (Zou et al., 2023; Zhang *et al.*, 2024). The present improvement of liver weight is especially important because the liver is the main organ of AFB1 accumulation and injury in chickens (Nabi *et al.,* 2022).

The hematological and biochemical corrections are equally persuasive. Aflatoxicosis is known to reduce total protein and albumin, elevate uric acid, and increase liver enzymes such as AST and ALT, reflecting hepatocellular injury and altered protein metabolism (Rashidi *et al.,* 2020; Sang *et al.,* 2023). OAF-treated groups, particularly OAF 200 and OAF 200+ antibiotic, reversed these changes in parallel with better performance, suggesting true physiological protection rather than a superficial growth-promoting effect. Similar reversals have been reported with other anti-aflatoxicosis interventions, such as *Lactobacillus salivarius,* curcumin, taraxasterol, grape seed proanthocyanidins, *Spirulina*, and *Penthorum chinense* extract (Chen *et al.,* 2021; Zhang *et al.,* 2024).

The recovery of IgG, IgM, lymphocyte proliferation, and phagocytic index is highly consistent with the established immunosuppressive nature of AFB1. AFB1 reduces immunoglobulin production, damages immune organs, suppresses cell-mediated immunity, and increases susceptibility to infections in poultry (Rashidi *et al.,* 2020; Chen *et al.,* 2021; Nabi *et al.,* 2022). Prior intervention studies have shown that protective feed additives can reverse these deficits, including *Lactobacillus salivarius*, probiotics plus organic acids, and antioxidant plant extracts (Rajput *et al.,* 2017; Ebeid *et al.,* 2021). Consequently, the observed immune restoration supports the paradigm that the filtrate functions dualistically as both an antimicrobial acidifier and an immunomodulatory anti-stress additive.

Furthermore, the oxidative stress profiles provide a robust mechanistic foundation for the complete dataset. Aflatoxin B_1 (AFB_1) induces reactive oxygen species (ROS) accumulation and lipid peroxidation, as evidenced by elevated malondialdehyde (MDA) levels and the concurrent depletion of superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), and related antioxidant enzymes in broiler chickens (Rajput *et al.,* 2017; Sang *et al.,* 2023). Several recent studies show that

successful mitigation of aflatoxicosis is commonly accompanied by decreased MDA and restoration of antioxidant defenses, often through Nrf2-related signaling and anti-apoptotic pathways (Nabi *et al.,* 2022; Zhang *et al.,* 2024). The reduction of MDA from 6.8 to 2.8 nmol/mL with OAF 200, and its improvement in the combination group, is therefore biologically coherent and likely central to the observed improvements in liver function, immunity, and growth.

The cecal microbiota findings also align with the modern understanding of how organic acids function in broilers. Organic acids consistently reduce gut pH, suppress enteropathogens, improve barrier integrity, and favor more beneficial microbial communities, particularly lactic acid bacteria and related taxa (Dai *et al.,* 2022; Mekonnen *et al.,* 2024). In challenged broilers, OA supplementation can restore disordered microbiota, increase short-chain fatty acids, improve tight-junction expression, and reduce inflammatory signaling (Dai *et al.,* 2022). Other OA-containing interventions similarly lower cecal *E. coli* load, improve serum IgG, and alleviate gut injury during *E. coli* challenge (Pham *et al.*, 2022). The current finding strongly supports a microbiota-mediated component of protection.

Overall, these results indicate that the *A. niger* organic-acid filtrate acts through several converging mechanisms: direct antimicrobial activity against bacteria and aflatoxigenic fungi, antioxidant radical scavenging, reduction of aflatoxin-induced oxidative and hepatic injury, recovery of immune competence, and partial restoration of gut microbial homeostasis (Dai *et al.,* 2022; Khan *et al.,* 2022). The main limitation is that the current evidence does not isolate the contribution of each individual acid, so the active principle is best interpreted as a consortium effect rather than proof for citric or oxalic acid alone. Even so, when discussed against recent and earlier studies across fungal bioactives, aflatoxicosis models, and organic-acid poultry nutrition, the results are coherent, mechanistically plausible, and well supported by the field (Rashidi *et al.,* 2020; Kumar *et al.,* 2021).

Conclusions: Dietary organic acids (OAs) are increasingly recognized as viable alternatives to traditional antibiotic growth promoters for enhancing poultry performance and health. By acidifying the feed, OAs lower the pH of the gastrointestinal tract, thereby optimizing nutrient utilization and suppressing pathogenic microorganisms. However, their efficacy is contingent upon the specific acid profile and dietary inclusion levels. While the majority of dietary OA supplementations demonstrate measurable improvements in livestock performance and health status, some inconsistencies persist across current literature. Consequently, further systematic research is required to elucidate their precise mechanisms of action, determine optimal dosages, and evaluate their comprehensive effects on productivity, systemic health, and end-product quality. Furthermore, integrating advanced molecular, biotechnological, and nanotechnological approaches could further enhance the efficacy of OAs, thereby expanding their functional applications in sustainable poultry production.

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Authors contribution: NAB: Conceptualization, visualization, methodology, writing the original draft, writing-review, and editing.

REFERENCES

Abdel-Nasser A, Hathout A, Badr A, *et al.,* 2023. Extraction and characterization of bioactive secondary metabolites from lactic acid bacteria and evaluating their antifungal and antiaflatoxigenic activity. Biotechnology Reports 38:799- 808.

Akan M, Diker KS, Özlü S, *et al.,* 2025. Effects of a diet supplemented with a blend of protected organic acids and essential oilson growth performance and cecal microbiota composition in broilers. Turkish Journal of Veterinary Animal Sciences 49:11-18.

Attia Y, Ebeid T, Shafi M, *et al.,* 2025. Strategies for decontamination and alleviation of mycotoxins for sustainable poultry farming – A review. Annals of Animal Science 25:139-157.

Bhatti SA, Khan MZ, Saleemi MK, *et al.,* 2021. Combating immunotoxicity of aflatoxin B1 by dietary carbon supplementation in broiler chickens. Environmental Science and Pollution Research 28:49089-49101.

Chen X, Ishfaq M, Wang J, 2021. Effects of *Lactobacillus salivarius* supplementation on the growth performance, liver function, meat quality, immune responses and *Salmonella Pullorum* infection resistance of broilers challenged with Aflatoxin B1. Poultry Science 101(3):101651.

Coban HB, 2020. Organic acids as antimicrobial food agents: applications and microbial productions. Bioprocess and Biosystems Engineering 43:569-591.

Dai D, Qi G, Wang J, *et al.,* 2022. Dietary organic acids ameliorate high stocking density stress-induced intestinal inflammation through the restoration of intestinal microbiota in broilers. Journal of Animal Science and Biotechnology 13(1):124.

Du H, Sarwar I, Ahmad S, *et al.,* 2023. Organic acids in poultry industry: a review of nutritional advancements and health benefits. World's Poultry Science Journal 80:133-153.

Ebeid T, Al-Homidan I, Fathi M, *et al.,* 2021. Impact of probiotics and/or organic acids supplementation on growth performance, microbiota, antioxidative status, and immune response of broilers. Italian Journal of Animal Science 20:2263-2273.

Hou L, Qiu H, Li A, *et al.,* 2022. Effects of aflatoxin B1 on growth performance, antioxidant status, immune response, and pro-inflammatory cytokine mRNA expression in ISA chicks. Frontiers in Veterinary Science 9:993039.

Jang HD, Chang KS, Chang TC, *et al.,* 2010. Antioxidant potentials of buntan pumelo (*Citrus grandis* Osbeck) and its ethanolic and acetified fermentation products. Food Chemistry 118:554-558.

Jinna Z, Khan T, Kanwal N, *et al.,* 2025. Diversity and biological functions of fungal secondary metabolites: Biocontrol agents for sustainable agriculture. A review. Cellular and Molecular Biology 71(5):43-48.

Kaptan B, 2024. Antioxidative, Antimicrobial activities and fatty acid compositions of four agarics. Turkish Journal of Agriculture - Food Science and Technology 12(s2): 2257-2265.

Khan R, Naz S, Raziq F, *et al.,* 2022. Prospects of organic acids as safe alternative to antibiotics in broiler chickens diet. Environmental Science and Pollution Research International 29:32594-32604.

Koshiol, J., Yadav, A., Groopman, J., & Dutta, U. (2026). Aflatoxins and human health: global exposure, disease burden, and one health strategies. Toxins 18(2): 90.

Kilany OE, Helmi RA, Fares I, *et al.,* 2020. Effects of chemical and biological anti-mycotoxins on performance, haematological, biochemical and immunological parameters of broiler chickens during aflatoxicosis. Egyptian Academic Journal of Biological Sciences, F. Toxicology and Pest Control 12:141-162.

Klančnik A, Piskernik S, Jeršek B, *et al.,* 2010. Evaluation of diffusion and dilution methods to determine the antibacterial activity of plant extracts. Journal of Microbiological Methods 81:121-126.

Kolawole O, Siri-Anusornsak W, Petchkongkaw A, *et al.,* 2022. The efficacy of additives for the mitigation of aflatoxins in animal feed: a

systematic review and network meta-analysis. Toxins 14(10):707-718.

Kostić M, Ivanov M, Fernandes Â, *et al.,* 2020. Antioxidant extracts of three russula genus species express diverse biological activity. Molecules 25(18): 4336.

Kostić M, Ivanov M, Fernandes Â, *et al.,* 2023. A comparative study of lactarius mushrooms: chemical characterization, antibacterial, antibiofilm, antioxidant and cytotoxic activity. Journal of Fungi 9(1): 70.

Kraieski A, Hayashi R, Sanches A, *et al.,* 2017. Effect of aflatoxin experimental ingestion and Eimeira vaccine challenges on intestinal histopathology and immune cellular dynamic of broilers: applying an Intestinal Health Index. Poultry Science 96:1078-1087.

Kumar A, Toghyani M, Kheravii S, *et al.,* 2021. Organic acid blends improve intestinal integrity, modulate short-chain fatty acids profiles and alter microbiota of broilers under necrotic enteritis challenge. Animal Nutrition 8:82-90.

Kundu A, Singh D, Mohapatra S, 2016. Immunocompetence based scoring index for evaluating disease resistance status in indigenous and exotic breeds of chicken. Indian Journal of Animal Science 86:861-864.

Lalev M, Mincheva N, Hristakieva P, *et al.,* 2022. Performance of laying hens fed diets supplemented with probiotics and prebiotics. Journal of Hygienic Engineering Design 39: 143-148.

Liu N, Wang J, Jia S, *et al.,* 2018. Effect of yeast cell wall on the growth performance and gut health of broilers challenged with aflatoxin B1 and necrotic enteritis. Poultry Science 97:477-484.

Mekonnen YT, Savini F, Indio V, *et al.,* 2024. Systematic review on microbiome-related nutritional interventions interfering with the colonization of foodborne pathogens in broiler gut to prevent contamination of poultry meat. Poultry Science 103(5): 103607.

Moon Y, Kim H-M, Chun H, *et al.,* 2018. Organic acids suppress aflatoxin production via lowering expression of aflatoxin biosynthesis-related genes in *Aspergillus flavus*. Food Control 88: 207-216.

Mujahid A, Pumford NR, Bottje W, *et al.,* 2007. Mitochondrial oxidative damage in chicken skeletal muscle induced by acute heat stress. The Journal of Poultry Science 44:439-445.

Nabarawy AE, Madian K, Shaheed I, *et al.,* 2020. Comparative evaluation of different antimycotoxins for controlling mycotoxicosis in broiler chickens. Journal of World's Poultry Research 10(2s):223-234.

Nabi F, Tao W, Ye Ry, *et al.,* 2022. *Penthorum chinense* pursh extract alleviates aflatoxin B1-induced liver injury and oxidative stress through mitochondrial pathways in broilers. Frontiers in Veterinary Science 9:822259.

Ochieng P, Scippo M, Kemboi D, *et al.,* 2021. Mycotoxins in poultry feed and feed ingredients from sub-saharan Africa and their impact on the production of broiler and layer chickens: A Review. Toxins 13(9): 633.

Olariu RM, Fiț N, Bouari C, *et al.,* 2025. Mycotoxins in broiler production: impacts on growth, immunity, vaccine efficacy and food safety. Toxins 17(6): 261-282.

Orogu J, Ehiwario N, 2025. Microbial and physicochemical evaluation of aflatoxin reduction strategies in maize-based poultry feeds. IPS Journal of Applied Microbiology and Biotechnology 4(1):144-146.

Pham VH, Abbas W, Huang J, *et al.,* 2022. Dietary coated essential oil and organic acid mixture supplementation improves health of broilers infected with avian pathogenic *Escherichia coli*. Animal Nutrition 12:245-262.

Puia A, Pandrea S, Cruceru J, *et al.,* 2025. Phenolic compounds with antimicrobial properties in mushrooms frequently encountered in temperate deciduous forests. Life 15(11): 1653.

Rajput SA, Sun L, Zhang N, *et al.,* 2017. Ameliorative effects of grape seed proanthocyanidin extract on growth performance, immune function, antioxidant capacity, biochemical constituents, liver histopathology and aflatoxin residues in broilers exposed to aflatoxin B1. Toxins 9(11): 371.

Rashidi N, Khatibjoo A, Taherpour K, *et al.,* 2020. Effects of licorice extract, probiotic, toxin binder and poultry litter biochar on performance, immune function, blood indices and liver histopathology of broilers exposed to aflatoxin-B1. Poultry Science 99:5896-5906.

Salazar B, Ortiz A, Keswani C, *et al.,* 2022. *Bacillus* spp. as bio-factories for antifungal secondary metabolites: innovation beyond whole organism formulations. Microbial Ecology 86:1-24.

Sang R, Ge B, Li H, *et al.,* 2023. Taraxasterol alleviates aflatoxin B1-induced liver damage in broiler chickens via regulation of oxidative stress, apoptosis and autophagy. Ecotoxicology and Environmental Safety 251:114546.

Shah W, Khan W, Nisa S, *et al.,* 2024. HPLC, NMR based characterization, antioxidant and anticancer activities of chemical constituents from therapeutically active fungal endophytes. Journal of Microbiology and Biotechnology 34:1452-1463.

Sharifi, S. D., Ghaemmaghami, S., Rouhanipour, H., Najafi, A., Rasi, H., & Baeelashaki, R. (2026). Aflatoxin health risk in the feed source: Integrated strategies for feed safety and sustainable One Health. One Health 22: 101436.

Sipos P, Peles F, Brassó DL, *et al.,* 2021. Physical and chemical methods for reduction in aflatoxin content of feed and food. Toxins 13(3): 204.

Tormo M and Izco J, 2004. Alternative reversed-phase high-performance liquid chromatography method to analyse organic acids in dairy products. Journal of Chromatography A 1033:305-310.

Xu GL and Diplock AT, 1983. Glutathione peroxidase (EC 1.11.1.9), glutathione-S-transferase (EC 2.5.1.13), superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6) activities in tissues of ducklings deprived of vitamin E and selenium. The British Journal of Nutrition 50:437-444.

Zhang J, Sun X, Chai X, *et al.,* 2024. Curcumin mitigates oxidative damage in broiler liver and ileum caused by aflatoxin B1-contaminated feed through Nrf2 signaling pathway. Animals 14: 409.

Zhang ZF, Xi Y, Wang ST, *et al.,* 2022. Effects of Chinese gallnut tannic acid on growth performance, blood parameters, antioxidative status, intestinal histomorphology, and cecal microbial shedding in broilers challenged with aflatoxin B1. Journal of Animal Science 100:skac099.

Zou Y, Liu S-B, Zhang Q, *et al.,* 2023. Effects of Aflatoxin B1 on growth performance, carcass traits, organ index, blood biochemistry and oxidative status in Chinese yellow chickens. Journal of Veterinary Medical Science 85:1015-1022.