

Article

Optical Characterization of *Alternaria* spp. Contaminated Wheat Grain and Its Influence in Early Broilers Nutrition on Oxidative Stress

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Abstract: The aim of this research was the visual characterization and investigating the effects of *Alternaria* spp. contaminated wheat grains in the starter stage of broilers nutrition on productive parameters and oxidative stress. The research was divided into two phases. Bunches of wheat in post-harvest period of year 2020 was collected from a various locality in Serbia and Albania. In the first phase, collected samples were visual characterized by *Alternaria* spp. presence by color measurement methods. Gained results are conferred in the range of the color properties of grain color properties of *Alternaria* toxins. Wheat grain samples were significantly different ($p < 0.05$) in terms of all measured color parameters (L^* , a^* , b^*). Classification of field fungi in analyzed wheat grain samples showed that the significant field fungi were *Rhizopus* spp., followed by *Alternaria* spp., and *Fusarium* spp. In the second phase, biological tests with chickens were carried out during the broiler chickens' dietary starter period in the first 14th days of age. At the beginning of the experiment, a total of 180-day-old Ross 308 strain broilers were equally distributed into three dietary treatments, with four replicates each. Dietary treatments in the experiments were as follows: basal diet without visual contamination of *Alternaria* spp. with 25% wheat (A1), a basal diet with visual contamination of *Alternaria* spp. with 25% wheat from Serbia (A2), basal diet with visual contamination of *Alternaria* spp. with 25% wheat from Albania (A3). The trial with chickens lasted for 14 days. After the first experimental week, wheat infected with *Alternaria* spp. in treatment A2 and A3 expressed adverse effects. The highest body weight of chickens of 140.40 g was recorded in broilers on control treatment A1 with statistically significant differences ($p < 0.05$) compared to treatments A2 (137.32 g) and A3 (135.35 g). At the end of the second week of test period, a statistically significant ($p < 0.05$) difference in body weight of broiler chickens could be noticed. The highest body weight of 352.68 g was recorded in control treatment A1, with statistically significant differences compared to other *Alternaria* spp. treatments. The lowest body weight of chickens was recorded in treatment A3 (335.93 g). Results of feed consumption and feed conversion ratio showed some numerical differences between treatments but without any statistically significant differences ($p > 0.05$). *Alternaria* spp. contaminated diet increased glutathione (GSH), glutathione reductase (GR), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) and decreased peroxidase (POD) and superoxide dismutase (SOD) serum levels, respectively. Built on the achieved results, it can be concluded that the wheat contaminated with *Alternaria* spp. in broilers nutrition negatively affected growth, decreased oxidative protection and interrupted chicken welfare in the first period of life.

Keywords: *Alternaria* spp.; mycotoxins; fungi; poultry production; welfare; oxidative stress

1. Introduction

Corn and wheat represent the primary energy source in the food animal's daily diet, while wheat has been considered the third most-produced feedstuff globally [1]. In the last ten years, studies and researchers have been struggling with the fungi of the genus *Alternaria*, which has grown to be the leading cause of wheat grains contamination [2]. The essential characteristics of *Alternaria* genera is the production of melanin and the host-specific plant–fungi/toxin interaction [3–5]. In addition, direct melanin emerges an indirect role in virulence, as well [6]. Melanin poses the ability to function as the shield in plant fungi protections versus ecological stress or unfavorable conditions, which gives fungus permanency and endurance. Furthermore, melanin promptly responds with free oxygen radicals, versus the pathogen's infiltration in the plant-host cells [7,8]. The blackening of the wheat grain lobes prior to cropping is typical indicator of contamination with *Alternaria* spp. [9]. At hand there is several forms of discoloration that can alter ordinary wheat (*Triticum aestivum* L.). In nearly all areas where wheat is cultivated, the black point is usually correlated by *Alternaria alternata* as a common discoloration of seed [10]. The staining usually appears in the external pericarp and internal grain tissue and could broaden beside its adaxial side. Such kinds of grain color changes differ drastically in frequency and seriousness depending on grain during the maturation. Biotic and abiotic stresses can cause wheat grain color changes, often caused by high humidity and high temperatures [11]. Those kinds of conditions are very favorable for fungi and mycotoxins occurrence in general [12,13]. Recently it has been confirmed that high humidity levels might stimulate the sporadic expansion of black point on wheat grain under controlled conditions [14]. *A. alternata* was the primary cause related to black point occurrence on wheat grain [15]. Likewise, pathogenicity and decrease of quality of wheat grains are influenced by a number of *Alternaria* spp. the producers of toxic secondary metabolites known as *Alternaria* mycotoxins [16,17]. *Alternaria* mycotoxins as alternariol (AOH) [18], tenuazonic acid (TzA) [19], alternariol monomethyl ether (AME) [20], altenuene (ALT) [2], altertoxin I (ATX-I) [21], altertoxin II (ATX-II) [18], and stemphylytoxin III (STTX-III) [22] could be toxic for animal health [23].

Some of the previous mentioned toxins could cause a serious health damages in animals when ingested, between them, for instance, fetotoxicity and somatic or functional deficiencies in the fetus when the mother is exposed to toxins [23]. *A. alternata*, as a separate mycotoxin, is mutagenic and clastogenic in various in vitro systems [24]. Moreover, it has been recommended that *Alternaria* toxins in grains be accountable for gullet pipe cancer [25]. Consequently, because of toxic effects, *Alternaria* toxins are of concern for public and animal health [26]. The European Commission (EC), and European Food Safety Authority (EFSA) were therefore engaged to give a technical view on the hazards for community and animal wellbeing associated with the occurrence of *Alternaria* mycotoxins in the commodities for human and animal daily nutrition. Subsequently, *A. alternata* have been chemically characterized, and incidence in feed was recorded [27]. Nevertheless, more than a few other *Alternaria* toxins have been classified as well, respectively [28].

Assessment of *Alternaria* toxins consumption by food animals through daily feeding have been restricted to broilers since poultry have been single one animal race where certain information about mycotoxin toxicity is appropriate for hazard evaluation [29–31]. Given that the incidence of feed data was lacking for the majority of the *Alternaria* toxins, the exposure assessments have been restricted to AOH toxin. Estimated lower bound and upper bound introductions to alternariol (AOH) were around 0.003 mg/day and 0.006 mg/day, for chickens and layers, respectively.

Broilers in production conditions are subjected to a variety of stressors [32]. The additional reactive oxygen species (ROS) and reactive nitrogen species (RNS) production

and oxidative stress are the essential harmful outcomes [33]. In the evolutionary process, antioxidant defense mechanism were built in birds to be able to stay alive in an oxygenated atmosphere [34]. They consist of a dense system of inside integrated antioxidant enzymes, for instance, glutathione (GSH), coenzyme Q (CoQ), and outwardly provided by vitamins, carotenoids, and antioxidants [34]. Furthermore, all antioxidants in the body work together to sustain the best oxidoreduction equilibrium [35]. This equilibrium is a crucial component in supplying the required preconditions for cells indicating, stress adjustment, and homeostasis upkeep [36]. While ROS and RNS are critical signaling molecules, their presence have been rigorously controlled by the antioxidant defense system linked with various transcript components and vitagenes [37]. Physiology shows that change from optimum inner and outer circumstances causes stress [38].

Additionally, a complicated flow of controlling systems is implicated in the stress reaction, causing the metabolic alterations triggering weakened live performance in broilers [39]. When the ROS and RNS construction outstrips the antioxidant defense mechanism ability to neutralize them, oxidative stress arises [38]. That includes polyunsaturated fatty acids (PAFAs), proteins, and DNA [40], take the lead to damaging outcomes in wellbeing, progress, development, and overall animal welfare [41].

Contemplating lacking research results and significant information's on *Alternaria* mycotoxins and that the biochemical composition of more than a few is identified, this research's precise aim was to visually characterize and investigate the effects of *Alternaria* spp. contaminated wheat grains in broiler chicken nutrition in the starter stage on productive parameters, oxidative stress, and overall welfare of this species of food animals. Obtained results from this research can serve in the future as the reference material for creating the new up-to-date guidelines on *Alternaria* toxins in foodstuffs and feedstuffs.

2. Materials and Methods

2.1. Wheat Samples

Wheat grain samples (*Triticum aestivum*) were collected in post-harvest time in the season of 2020 from the region of Serbia (Vojvodina) and Albania (Durrës). Obtained samples were collected with the appropriate equipment, such as a probe for stationary grain and a diverter-type mechanical sampler, using a sampling pattern and procedures designed to collect samples from all areas of the lot. The appropriate size of wheat grain between 1.5 and 2.5 kg sample was taken from a truck with adequate identified and labeled bags. Collected samples were handled in such a way as to maintain representativeness. Samples were stored in a cool and dry place in triple lined paper breathable bags to avoid mold growth and increase of sample moisture level over 14%.

2.2. Proximate Analyses of Compound Feed for Broilers Chickens

Compound feed for broiler chickens in each experimental treatment were analyzed for moisture, crude ash, crude protein, crude fat, and crude fiber. All analyses were performed in triplicate. The moisture content was determined according to AOAC (Association of Official Analytical Chemists) [42] Method 934.01. Crude protein content was determined by Kjeldahl method according to the AOAC Method 978.04, crude ash, according to AOAC Method 942.05, crude fat, according to AOAC Method 920.39 and crude fiber according to AOAC Method 978.10 (AOAC, 1998). Concentration of total phosphorus (P) and calcium (Ca) as well as metabolizable energy (ME), was calculated within licenced feed formulation software.

2.3. Visual Scale Establishing and Color Measurement

Instrumental methods were used to measure the color of wheat grains. The wheat grain samples color was measured with Minolta Chroma Meter CR-400, and the attachment CR-A50, respectively. The color space defined by the International Commission on Illumination (CIELAB) L^* (lightness), a^* (red-green), and b^* (yellow-blue), and dominant wavelength (DWL) was determined using a D65 light source and the observer angle at

2°. The tristimulus values of L^* , a^* and b^* readings were calibrated against a standard white plate ($Y = 84.8$; $x = 0.3199$; $y = 0.3377$). Each wheat ear sample was divided into four subgroups, and the color of one hundred ears from each subgroup (400 ears from one sample) was measured on 5 locations. Samples of wheat grains was divided into 4 subgroups, and 5 repetitions measure were applied in each subgroup (20 repetitions per sample in total) [43].

2.4. Wheat Grain Infection Confirmation

Precisely one hundred wheat grains were counted randomly and used in all the treatments. Therefore, wheat grain samples must be cleaned with 0.4% NaOCl, and washed with clean water for 2 min. After cleaning process samples of wheat was placed on Petri dishes in 4 replicates (25 grains/Petri) containing potato dextrose culture medium. Grains were incubated at 25 °C, in total of 7 days, after which concentration of contamination was evaluated. For validation of fungi species from each Petri dish 5 randomly wheat grain was taken out by microscopic inspection after finishing the incubation period [44].

2.5. In Vivo Experimental Part with Broiler Chickens

The test with the broilers was conducted in the wake of the EU legislation and tenet of the 3Rs contained by Directive 2010/63/EU, as well with the approval of Ethic Commission for the Protection and Welfare of Experimental Animals EK-I-2020-01. At the beginning of the investigation, a total of 180-day-old Ross 308 strain broilers were equally distributed into three dietary treatments, with four replicates each. Dietary treatments in the experiments were as follows: basal diet without visual contamination of *Alternaria* spp. with 25% wheat (A1), a basal diet with visual contamination of *Alternaria* spp. with 25% wheat from Serbia (A2), and basal diet with visual contamination of *Alternaria* spp. with 25% wheat from Albania (A3). Wheat contamination of *Alternaria* spp. from control treatment A1 was prepared as 1/2 mixture of wheat samples from both Serbia and Albania. During the test period broilers was given feed and water by will e.g., ad libitum, with regularly monitored and maintained environmental conditions provided by broilers producer. Broilers were kept on the ground bedding system with the pelleted wheat straw. To control the productive results of broilers, body weight, feed consumption, and feed utilization were monitored.

2.6. Blood Samples Collection and Hemolysate Preparation

The broiler chickens' blood was collected by the trained veterinarian from the broilers' heart by puncture into heparinized sterile tubes. Blood samples were immediately delivered to the laboratory and centrifugated for 10 min at 1507 g and 4 °C. Plasma was removed, following the erythrocytes rinsing in saline three times. The obtained red blood cell pellet was held in same amount of two filtered water and vortexed afterward. Following incubation for 60 min at 25 °C, the hemolysate was centrifugated during fifteen minutes at 1507 g, after which obtained buoyant was gathered for additional examination [45].

2.7. Determination of Glutathione and Enzymatic Determination

To determine glutathione (GSH) concentration, proteins from hemolysates were divided by increasing half amount of 10% sulfosalicylic acid and centrifugated at 3075 g, for five minutes, at 4 °C. The buoyant was deposited at 4 °C, and GSH was determined the next day. The GSH concentration in the blood hemolysate was determined from the quantity of sulfhydryl residues [45].

Superoxide dismutase (SOD) activity was determined by the spectrophotometric method based on the inhibition of adrenaline reduction to adrenochrome at pH 10.2 [45]. The activity of glutathione reductase (GR) was determined from the rate of nicotinamide adenine dinucleotide phosphate (NADPH) oxidation measured at the absorbance at 340 nm [46]. The concentration of lipid peroxides (LPx) was determined by the thio-barbituric acid (TBA) assessment [47]. The oxidation of cellular membrane lipids was measured through the reaction of lipid peroxides with TBA [47]. The determination of

peroxidase (POD) activity was based on the catalytic oxidation of guayacol by H₂O₂ as an electron acceptor [45]. The reaction of xanthine oxidation of uric acid was used for the determination of xanthine oxidase (XOD) activity. Spectrophotometric measurement was performed in 0.1 mmol/dm³ phosphate buffer at pH 7.5, at the absorbance at 295 nm [45].

2.8. Serum Biochemical Analyses

The serum activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined in serum samples. Analysis of the serum samples was measured by an automatic biochemistry analyzer (Beckman Synchron CX4 PRO, Fullerton, CA, USA) [48].

2.9. Statistical Analyses of Data

The data acquired in the conducted examination were evaluated by one-way analysis of variance (ANOVA) using the software package Statistica 13. Once the analysis of variance exhibited statistical significance, Duncan's MRT was employed. A significant difference was registered at $p < 0.05$.

3. Results and Discussion

Results of proximate analysis of compound feed used in daily nutrition of broiler chickens during the experiment are presented in Table 1.

Table 1. Proximate composition and diet ingredients of compound feed, %.

Nutrients	Treatments		
	A1—Control	A2—Serbia	A3—Albania
Dry matter	89.6	89.5	89.6
Moisture	10.4	10.5	10.4
Crude protein	22.0	22.1	22.1
Crude fat	5.1	5.0	5.2
Crude fiber	3.5	3.4	3.5
Crude ash	6.3	6.4	6.2
Ca	1.0	1.1	0.9
P	0.8	0.8	0.8
Metabolizable Energy, MJ/kg *	12.5	12.5	12.5
Diet ingredients			
Corn	35.4	35.6	35.5
Wheat	25.0	25.0	25.0
Soybean meal	19.5	19.5	19.5
Sunflower meal	2.0	2.0	2.0
Soy protein isolate	8.8	8.6	8.5
Corn gluten	2.0	2.0	2.0
Yeast	1.5	1.5	1.5
Limestone	1.8	1.8	1.8
Premix	4.0	4.0	4.0

* Values were calculated.

Gained results are conferred in the range of the color properties of grain color properties of *Alternaria*. Gained results are conferred in the range of the color properties of *Alternaria* spp. contaminated wheat grains [49]. Wheat grain samples were significantly different ($p < 0.05$) in terms of all measured color parameters (L^* , a^* , b^*). Control wheat grain (A1) samples were significantly different in terms of lightness and dominant wavelength, compared to wheat grain samples (A2) and (A3), which have shown significant difference ($p < 0.05$) compared to A1, but without any statistically significant difference ($p > 0.05$) between themselves, nevertheless numerical differences (Figure 1), respectively.

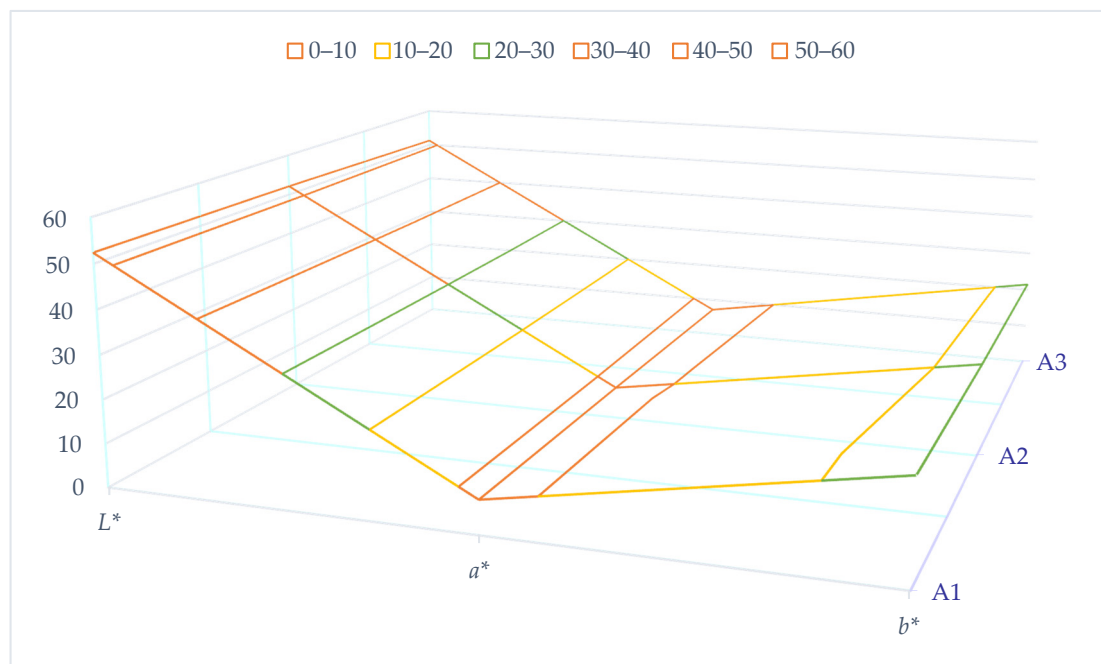


Figure 1. Color parameters of different samples of wheat grains. A¹—wheat grain without visual contamination of *Alternaria* spp.; A²—wheat grain with visual contamination of *Alternaria* spp. (Serbia); A³—wheat grain with visual contamination of *Alternaria* spp. (Albania); L*—lightness; a*—red/green value; b*—blue/yellow value.

The results presented in Figure 1 show that all wheat grain samples belong to the different groups by dominant wavelength values. Contemplating all stated, it can be concluded that infection entered the grain in a higher amount in some wheat samples (A2 and A3). Simultaneously, there were samples without visible infection and color changes on the grain (A1). Wheat grain samples without visible dark spots were commonly described by higher lightness and more prominent yellow tones [50].

All wheat grain samples collected from the field and previously instrumentally analyzed were disinfected with 0.4% NaOCl and placed for incubation (Section 2.4) for seven days. Results of fungi genera confirmation were carried out by microscopic examination, and the results have been shown in Table 2.

Table 2. Incidence of some genera of fungi in wheat grain samples, %.

Treatment/Sample	Fungi spp.			Other Fungi spp.
	<i>Alternaria</i>	<i>Rhizopus</i>	<i>Fusarium</i>	Not Identified
A1—Control	25.2 ^b	72.2 ^a	1.0 ^b	1.6
A2—Serbia	37.9 ^a	48.3 ^b	3.5 ^a	10.3
A3—Albania	39.2 ^a	46.6 ^b	3.9 ^a	10.3
<i>p-value</i>	0.023	0.016	0.003	

Values in the same row marked with the different letters are significantly different at the significance level of $p < 0.05$.

Classification of field fungi in analyzed wheat grain samples showed that the significant field fungi were *Rhizopus* spp., followed by *Alternaria* spp., and *Fusarium* spp. The ratio of contamination of wheat grain samples by *Alternaria* spp. was the highest in A3 samples without significant difference ($p > 0.05$) compared to A2, as previously stated. Differences in percentages between A2 and A3 could be explained by the fact that *Alternaria* spp. produce melanin pigments of dark color, which can cause the differentiation in determination with instrumental measurement, respectively. Due to fungi growth in the field even at low temperatures, they are also responsible for spoilage of commodities during refrigerated transport and storage. Several *Alternaria* species are known producers of toxic

secondary metabolites known as *Alternaria* mycotoxins [23]. *A. alternata* produces several mycotoxins. TeA is harmful to several animal species, e.g., mice, chickens, and dogs [23]. Many *Alternaria* metabolites have been reported to occur naturally in cereals [5,12]. Alternariol, alternariol monomethyl ether, and tenuazonic acid were frequently detected in sorghum, wheat, and edible oils [23]. Xu et al. [51] have reported the importance and danger of exposure to *Alternaria* toxins from grain and grain-based products because of its relation to human esophageal cancer in China. In their study, a total of 370 freshly harvested wheat grain samples were analyzed for the four *Alternaria* toxins TeA, TEN, AOH, and AME. Field contaminated samples (95%) of the wheat grains were positive for more than one type of *Alternaria* toxins [51]. Li and Yoshizawa [52] reported the first report of the natural occurrence of *Alternaria* mycotoxins in Chinese wheat. Their wheat grains were significantly infested by *Alternaria* species, mainly *A. alternata*, with a median infection rate of 87.3%. The grains with low quality which is acceptable in some cases was researched in post-harvest period to investigate if the *Alternaria* or *Fusarium* influenced in adverse quality of the grains [53]. The distribution of *Alternaria* and *Fusarium* spp. they were varied significantly in samples of reduced rate compared with acceptable samples. The results of Kosiak et al. [53] revealed a negative interaction between *F. graminearum* and *Alternaria* spp. as well as between *F. graminearum* and another *Fusarium* spp. *Fusarium* and *Alternaria* fungi naturally occurring on the ears and the formation of their mycotoxins in the ripe grains. Müller et al. [9] investigated the fluorescent pseudomonads colonizing wheat ears, which have a high antagonistic potential against phytopathogenic fungi. Unfortunately, the results of their findings indicate that extensive biological management of mycotoxin development by naturally arising pseudomonads with incompatible activity is very doubtful [9].

Based on the gained results in the second phase of the experiment with the live broiler chickens, after the first experimental week, it could be noticed that the addition of wheat infected with *Alternaria* spp. in the amount of 25% in treatment A2 and A3 expressed adverse effects. The highest body weight of chickens of 140.40 g was recorded in broilers on control treatment A1 with statistically significant differences ($p < 0.05$) compared to treatments A2 (137.32 g) and A3 (135.35 g).

At the end of the second week of test period, a statistically significant ($p < 0.05$) difference in body weight of broiler chickens could be noticed. The highest body weight of 352.68 g was recorded in control treatment A1, with statistically significant differences compared to other *Alternaria* spp. treatments. The lowest body weight of chickens was recorded in treatment A3 (335.93 g), while significant differences ($p > 0.05$) between chickens in *Alternaria* spp. treatments were not recorded (Table 3). The low broiler chicken body weight observed in *Alternaria* spp. contaminated diet than control could be due to *Alternaria* spp. toxin tenuazonic acid which was firstly described in 1987 [54].

Table 3. Broiler chickens body weight in the experiment, g.

Age	Treatments in Test			Pooled	
	A1	A2	A3	SE	<i>p</i>
0 day	35.52 ^a ± 2.82	35.38 ^a ± 2.66	34.97 ^a ± 2.77	0.16	0.098
7 day	140.40 ^a ± 9.61	137.32 ^b ± 8.13	135.35 ^b ± 8.19	0.47	0.000
14 day	352.68 ^a ± 18.44	341.85 ^b ± 23.30	335.93 ^b ± 22.42	1.29	0.000

Values in the same row marked with the different letters are significantly different at the significance level of $p < 0.05$.

Numerous researches have registered a broad array of serious wellbeing impacts and medical indications after food animals was subjected to the elevated amount of toxins. Nevertheless, not a lot is seen concerning the wellbeing impacts of toxins at small amounts [55]. Kolawole et al. [55] conducted a long-term feeding trial in order to investigate the impact of small amounts of toxin combinations on the production of poultry fed with naturally contaminated complete feed. Total of eighteen tests with poultry production

was performed, with closely of 2200 one-day-old Ross-308 birds per each test. As food animals are frequently subjected to low doses of mycotoxin, a cumulative risk evaluation in quantifying and alleviating counter to the economic, welfare, and health influences is necessary for mycotoxins. Hessel-Pras et al. [56] stated that once *Alternaria* mycotoxins passes the intestinal barrier, they can reach the liver to exert yet uncharacterized molecular effects. Hence, the same group of authors used hepatic in vitro systems to examine selected *Alternaria* mycotoxins for their induction of metabolism-dependent cytotoxicity, phosphorylation of the histone H₂AX surrogate marker for DNA double-strand breaks, and relevant marker genes for hepatotoxicity. They have found evidence that 50 µmol/L of AOH, AME, TeA, and TEN might be associated with hepatotoxic effects, necrosis, and the development of diseases like cholestasis and phospholipidosis [56]. Kemboi et al. [57] discovered that other developing toxins and metabolites, counting *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium* toxins, were discovered at differing concentrations during their research. Such co-occurrences of mycotoxins could trigger synergistic and additive health effects, impeding the food animal production sectors worldwide.

Results of feed consumption and feed conversion ratio are shown in Tables 4 and 5. *Alternaria* spp. contaminated wheat grain showed some numerical differences between treatments but without any statistically significant differences in broiler chickens' life stage of life.

Table 4. Feed consumption of broiler chickens, g.

Age	Treatments in Test			Pooled	
	A1	A2	A3	SE	<i>p</i>
7 day	163.57 ^a ± 30.51	152.63 ^a ± 20.01	162.71 ^a ± 29.50	9.62	0.564
14 day	292.33 ^a ± 10.16	293.77 ^a ± 11.66	284.11 ^a ± 17.03	4.72	0.689

Values in the same row marked with the different letters are significantly different at the significance level of $p < 0.05$.

Table 5. The feed conversion ratio of broiler chickens, kg/kg.

Age	Treatments in Test			Pooled	
	A1	A2	A3	SE	<i>p</i>
7 day	1.16 ^a ± 0.21	1.11 ^a ± 0.15	1.21 ^a ± 0.22	0.07	0.454
14 day	1.29 ^a ± 0.09	1.29 ^a ± 0.07	1.33 ^a ± 0.08	0.04	0.555

Values in the same row marked with the different letters are significantly different at the significance level of $p < 0.05$.

In addition to wheat, corn is the main feed ingredient used in poultry nutrition. As a wheat grain, the corn can also be naturally infected with mycotoxins, especially with *Alternaria* spp. Topi et al. [58] have investigated the presence of *Alternaria* mycotoxins in grains from Albania: alternariol, alternariol monomethyl ether, tenuazonic acid, and tentoxin. They have concluded that the contribution of AOH and AME originating from wheat was 0–31.7 ng/kg body weight per day. In contrast, the contribution of *Alternaria* toxins through maize consumption was significantly lower.

Changes from optimal internal and external conditions lead to stress from a physiological point of view. Between the main stressors in broiler production, nutritional stressors have a significant role, and within them, the leading role is mycotoxins feed contamination [34].

The highly probable clarification for the remarked results presented in Table 6 is that the pathological modifications strengthen free radical processes by promoting catalytic activities of enzymes engaged in the antioxidative protection, POD, and GR. Still, through the disease phase, lipolysis from the lipid depots could be increased due to reduced feed consumption, which is not the case in our research. Moreover, tiredness of the organism could lead to escalation of free radical processes and higher amounts of lipid peroxides in

blood. To defend himself, the body initiates its antioxidative safety mechanisms. Decrease of SOD activity was anticipated and is in accordance with other research [59,60].

Table 6. GSH and LPx content and the activity of POD, SOD, GR, and XOD in blood hemolysates, $\mu\text{mol/g Hb min}$.

Treatment	GSH	LPx	POD	SOD	GR	XOD
A1	5.1 ^b \pm 0.9	0.3 ^a \pm 0.2	65.2 ^a \pm 4.4	82.6 ^a \pm 6.1	11.3 ^b \pm 5.9	26.3 ^a \pm 3.6
A2	5.8 ^a \pm 0.3	0.3 ^a \pm 0.1	55.1 ^b \pm 7.2	25.5 ^b \pm 3.5	21.1 ^a \pm 7.2	27.5 ^a \pm 2.9
A3	6.2 ^a \pm 1.1	0.4 ^a \pm 0.1	59.3 ^b \pm 3.4	29.1 ^b \pm 8.9	19.8 ^a \pm 9.6	26.7 ^a \pm 4.1
<i>p-value</i>	0.032	0.089	0.038	0.001	0.004	0.341

Values in the same row marked with the different letters are significantly different at the significance level of $p < 0.05$.

The glutathione has a vital position in reducing the acute toxicity of xenobiotics and products of lipid peroxidation. A statistically significant decrease of POD activity compared to the A1 control treatment was expected since POD catalyzes various proton donors' oxidation with hydrogen peroxide. Having in mind that mycotoxins are classified as hepatotoxins, nephrotoxins, neurotoxins, immunotoxins, and that there are to date, 400 mycotoxins identified and the most critical species producing mycotoxins belong to *Aspergillus*, *Penicillium*, *Alternaria*, and *Fusarium* genera, Ülger et al. [61] have described their genotoxic effects on the organism. Uric acid increased accumulation, and reduced excretion is closely related to the pathogenesis of gout and hyperuricemia. Higher plants produce different metabolites, which might impede XOD, so disallow the oxidation of hypoxanthine to xanthine then to uric acid in the purine metabolism. Nevertheless, microorganisms generate a group of degrading enzymes, which catalyze uric acid degradation to ammonia. Xanthine oxidoreductase (XOR) has two forms; xanthine oxidase (XOD) and xanthine dehydrogenase (XDH), both of them catalyze the oxidation of hypoxanthine to xanthines, then to uric acid in the purine metabolism [62]. Hafez et al. [63] presented an analysis with the incidence of uric acid in plants and other organisms, especially microorganisms, in addition to the mechanisms by which plant extracts, metabolites, and enzymes could reduce uric acid in the blood. Overactivity of both enzymes (XOD and XDH) cause the accumulation of uric acid in the animal body and form a pathogenesis condition called gout [64]. Additionally, XOD serves as a valuable biological source of oxygen free radicals that participate in various damages of animal tissues leading to many pathological states [65], which could be caused by multiple stress triggers, e.g., mycotoxins [66–68].

Serum biochemical parameters were significantly affected by *Alternaria* spp. wheat in both treatments compared to control treatment during the starter dietary phase (Table 7). Even though the *Alternaria* spp. contaminated wheat had no significant effect on growth performance in broiler chicks, it induced the typical clinical signs of hepatic injury, including increased activities of AST and ALT, during the starter dietary period what is in accordance with results of other researchers [48,69,70].

Table 7. Aminotransferase (AST) and alanine aminotransferase (ALT) activity in serum of broiler chickens, U/L.

Treatment	AST	ALT
A1	182.1 ^b \pm 32.1	1.1 ^b \pm 0.1
A2	268.8 ^a \pm 41.3	1.7 ^a \pm 0.3
A3	271.5 ^a \pm 33.7	1.6 ^a \pm 0.2
<i>p-value</i>	0.075	0.039

Values in the same row marked with the different letters are significantly different at the significance level of $p < 0.05$.

Oxidative stress plays an important role in the development of many animal diseases and it has been shown that have significant implications for the well-being and overall welfare of nonruminants [71]. Various studies have shown that oxidative stress has a funda-

mental role in the etiopathogenesis of several acute and chronic diseases which are causally related to animal welfare [72]. Over the years oxidative stress has been deeply investigated in human, while in poultry production the data are yet less uneven [73]. Poultry welfare is fundamental in maintaining correct health and a good level of mental and physical well-being of the animal [74]. In our study increased content of total glutathione levels in chicken dietary treatments (5.8 and 6.2 $\mu\text{mol/g Hb min}$) with addition of blackpoint wheat, indicates that chickens had increased antioxidant defense. These results are directly related with the impaired welfare of chickens. Likewise, certain indicators of impaired welfare of chickens in our expert are increased activity of GR (21.1 and 19.8 $\mu\text{mol/g Hb min}$), and decreased activity of SOD (25.5 and 29.1 $\mu\text{mol/g Hb min}$), respectively. The similar results were obtained by Brambilla et al. [75] in their research related to influence of oxidative stress markers reactive oxygen metabolites (ROM) and anti-oxidant power (OXY) in swine welfare. Stresses in commercial poultry result from many various factors which negatively impact poultry health, production, and welfare [76]. Oxidative stress is downstream of all these stresses. Oxidative stress in the cells results from an imbalance between free radical production and endogenous antioxidant defense [77]. It is well documented that poultry feed is often contaminated with a wide range of environmental toxicants, bacterial and fungal toxins, and known to affect the health and welfare of poultry [78]. Mycotoxins usually generates reactive oxygen species which induces lipid peroxidation, alters the cellular redox signaling, antioxidant status, and membrane integrity of the cells [79]. Mycotoxins increase cellular apoptosis and affect poultry health, production, and welfare.

4. Conclusions

Based on the gained results, it can be concluded that identifying field fungi in all analyzed wheat grain samples showed that the dominant mycotoxigenic fungus was *Rhizopus* spp., followed by *Alternaria* spp., and *Fusarium* spp. Usage of 25% wheat in complete feed for broiler chickens in the first 14 days of life has shown adverse effects reflected on body weight gain without significant influence on feed consumption and utilization. Concerning oxidative stress, it can be concluded that *Alternaria* spp. causes high oxidative stress in chickens at a young age negatively influences production and overall broiler chicken's welfare.

Further research on the influence of *Alternaria* spp. on animal production and genotoxicity is still essential.

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