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Impact of non-fermented and fermented *Ginkgo biloba* leaves on cytokine and some histological markers in normal and oxidative stress broilers

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Abstract

Oxidative stress (OS) may occur when the body is exposed to internal or external factors that compromise the effectiveness of antioxidant mechanisms or amplify the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). The present study investigated the influence of *Ginkgo biloba* leaves on cytokines and histological parameters in male broiler chickens under hydrogen peroxide-induced oxidative stress and normal conditions.

A total of 216 Ross 307 strain male broiler chickens, aged one day, were employed in the study using a completely randomized design. The chickens were distributed into six groups, each containing an equal number of birds. Each group had three replicates, with 12 chicks in each replicate. The initial group, denoted as Group 1 or the control group, was provided with the standard diet. Group 2 was exposed to oxidative stress (OS) by introducing 0.5% ml of H_2O_2 (at a 50% concentration) per liter of water. In Group 3, OS was induced, and the chickens were administered 3.5 g/kg of *Ginkgo biloba* leaves (Gbl) that non-fermentation. Group 4 underwent OS and was treated with 3.5 g/kg of fermented Gbl. Group 5 comprised normal chickens treated with 3.5 g/kg of non-fermented Gbl, while Group 6 consisted of normal chickens treated with 3.5 g/kg of fermented Gbl. Throughout the 42-day experimental period, all groups received their respective treatments. The results indicated that the fermented group displayed a significant reduction ($P<0.01$) in interleukin (Il8) levels when compared to the control and other groups. However, the TNF-α level did not exhibit a significant decrease ($P>0.01$) when compared to the control group. In Group 6, there was a noteworthy increase ($P<0.01$) in jejunum villi height compared to all other groups, and no significant reduction ($P>0.01$) in crypt depth in comparison to the experimental groups. In conclusion, the fermentation approach for Gbl positively influenced the gut's ecological system and the immune response of the chickens against pathological diseases.

Keywords

Ginkgo biloba leaves, medicinal plant, immunity, histological, poultry.

1.Introduction

In 2006, the European Commission (EC) imposed restrictions on the utilization of antibiotics to encourage growth-promoting effects (Ferket et al., 2002). Animal nutrition experts have worked to identify extra feed additives that fulfill comparable functions to those of antimicrobial growth promoters (Pandey et al., 2019). The immune system of the recently hatched chick is not fully developed, immature, and ineffective, which greatly increases the bird's susceptibility to infectious present in its environments (Zhang et al., 2013). For ages, natural medicinal items sourced from fungi or herbs have been utilized as dietary enhancements in the field of ethnoveterinary medicine.

Belonging to the Ginkgoaceae family, *Ginkgo biloba* leaves are widely employed as a traditional remedy in China, their use extends to various countries globally, and they demonstrate strong physiological impacts in addressing diverse medical ailments (Gurib-Fakim, 2006). The main goals of the poultry sector include managing diseases, achieving high production rates, and delivering quality products while maintaining reasonable production expenses (Hafez and Attia, 2020). (Niu et al., 2017) documented that adding fermented *Ginkgo biloba* leaves to the diet of broiler chickens led to enhanced growth performance, meat quality, and antioxidant status. The most effective supplementation range of fermented *Ginkgo biloba* was found to be between 3.5 and 4.5 g/kg (El-Kasrawy et al., 2023). The enhanced growth observed due to *Ginkgo biloba* supplementation could be credited to the presence of flavonoids (mainly quercetin in the form of flavone glycosides), polysaccharides, and terpenoids (specifically ginkolides and bilobalides) in their composition (Saeed et al., 2022). An important advantage of the production of fermented is that the required technology is affordable and feasible even for small farms. *Ginkgo biloba* leaves are a good and cheap source and are known to be rich in flavonoids and polysaccharides. Information suggests that *Ginkgo biloba* leaves extract has potential applications in treating inflammatory conditions (Tao et al., 2019). When chickens encounter different triggers, such as pathogens or allergens, their bodies react by producing cytokines. These cytokines prompt cells to enact defense mechanisms against other cells and tissues, often leading to inflammation like sepsis. The main cytokines released in this process are IL-1β, IL-6, and TNF-α (Horvatić et al., 2019). In regular conditions, pro-inflammatory cytokines are not produced, but they are released when the immune system is triggered (Slawinska et al., 2021).

Fermentation is employed as a valuable technique to create biologically active substances with health-promoting attributes from different fruits and vegetables (Meena et al., 2022). *Aspergillus niger*, a prominent probiotic fungal type employed in broiler farming, possesses the capability to generate various enzymes including hemicellulases, hydrolases, pectinases, protease, amylase, lipases, and tannases. Research discovered that incorporating *Aspergillus niger*-fermented *Ginkgo biloba* leaves (FG) products at a rate of 0.5% during the starter phase and 1.0% during the grower phase into diets yielded positive effects on intestinal structure, digestive processes, and nutrient absorption in broiler chickens, without detrimental consequences. The small intestine is a dynamic organ that could potentially give precedence to utilizing the scarce nutrients present in times of stress (Aboragah et al., 2023). Both villus height (VH) and crypt depth (CD) are significant indicators of the digestive well-being of birds, directly linked to the absorptive potential of the mucous membrane (Hosseini et al., 2016). The research investigated the impact of both non-fermented and fermented *Ginkgo biloba* leaves (Gbl) on cytokine enzymes and histological characteristics in male broiler chickens challenged to oxidative stress induced by H_2O_2 and normal conditions.

2. Materials and Methods

2.1 Experimental Design

This research involved 216 male broiler chickens of the Ross-308 strain at one day of age. The chicks were obtained from a nearby hatchery located in Erbil. They were housed in partially enclosed cages measuring 2m x 1.5m. The litter on the floor was around 5-6 cm thick. The housing facility underwent sterilization using formalin and potassium permanganate at a concentration of $0.5g/m^3$, and it was then sealed for a duration of three days. Detailed ratios were specified for heating, cooling, ventilation, and humidity control. The initial temperature was established at 32°C and was subsequently lowered by 2°C every week until it reached 22°C at the conclusion of the study. A mixture of 0.5% ml hydrogen peroxide (H_2O_2) with a concentration of 50%, per liter of drinking water, was introduced starting from the first week of the chicks' life. Both feed and water were made available without restriction. The chicken was put into six equal groups; every group consisted of three replicates. For each replicate, 12 chickens. Group 1 feeds the control standard diet and represents the control group. The second group was exposed to oxidative stress (OS) by 0.5 % ml (H_2O_2). The third group included induced OS chicken treated with 3.5 g/kg non-fermented *Ginkgo biloba* leaves supplemented to the standard diet, the fourth group comprised induced OS chicken treated with 3.5 g/kg of fermented Gbl supplemented to the standard diet, the fifth group contain normal chicken treated with 3.5 g/kg non-fermented Gbl supplemented to the standard diet. The last group included normal chicken given 3.5 g/kg of fermented Gbl supplemented with a standard diet.

2.2 Analysis of Interleukin 8 (IL-8) and TNF-α

Blood samples are procured and stored in containers treated with an anticoagulant agent such as EDTA or citrate. These samples undergo an initial stabilization process at ambient temperature for a duration ranging between 10 and 20 minutes, followed by a centrifugation phase at a speed ranging from 2000 to 3000 rpm for 20 minutes. Subsequently, the plasma samples are isolated with utmost precision from the supernatant fraction.

The analytical procedure employs a Sandwich-ELISA technique, utilizing a kit furnished with a Microelisa strip plate that has been pre-treated with an antibody specifically reactive to TNF-α and IL-8. The respective standards or samples are introduced into the designated wells of the strip plate, facilitating a reaction with the pertinent antibody. This is succeeded by the addition of a horseradish peroxidase (HRP)-linked antibody, which is specific to TNF-α and IL-8, to each well, initiating a period of incubation. Post incubation, non-bound elements are meticulously removed through a washing step.

Following this, a TMB substrate solution is applied to each well, instigating a colorimetric reaction. It is to be noted that only the wells housing the TNF-α and IL-8 alongside the conjugated HRP antibody undergo a color transformation, initially exhibiting a blue hue which later transitions to yellow upon the introduction of a stop solution. The culmination of the procedure involves a spectrophotometric analysis, where the optical density (OD) is quantified at a wavelength of 450 nm.

2.3 Microorganism's culture

The microorganism used in this study was *Aspergillus niger*, acquired from the research center within Koya University's Faculty of Science and Health. *Aspergillus niger* was cultivated by Oxoid Ltd., located in Basingstoke, UK, using Sabouraud dextrose agar. The agar culture was then placed in an incubator at a temperature of 24 °C for a duration of 7 days. To harvest spores of *Aspergillus niger*, the culture dish was flipped upside down and tapped repeatedly. A total of 4.0×10^6 spores or 0.25 g of spores were collected using the Fuchs-Rosenthal technique.

2.4 Medicinal plant fermentation

The *Ginkgo biloba leaves* were split into two parts for the experiment. One portion was left untreated, while the other was subjected to fermentation through the use of *Aspergillus niger*. The fermentation process involved employing a solid medium made up of a combination of Radix astragali-ginkgo leaf, wheat bran, and corncob at a ratio of 8:1.5:0.5, with a total weight of 10g. Moreover, this medium was supplemented with 16mL of a nutrient solution containing $MgSO4 \cdot 7H2O$, KH_2PO4, peptone, $(NH4)_2SO4$, urea, and glucose in proportions of 1:4:1:6:2:4. The medium was inoculated with 0.1% of *Aspergillus niger* seed and subsequently placed in a temperature range of 28–30°C for two days. The resulting mixture was lightly compacted, covered with adhesive film, and sealed within a plastic container. Afterward, the mixture was spread on a polythene sheet and air-dried at room temperature. Around six days later, approximately 900 g/kg of dried material was obtained, crushed, and sifted through a 0.5 mm screen.

3. Statistical Analysis

The experimental data were subjected to analysis using the Statistical (SPSS) software. A one-way ANOVA test was conducted on the data (SPSS version 26, 2019). Descriptive statistics were employed to aid in the data analysis process. The means and standard errors were calculated, and in line with Duncan's method from 1955, the Duncan test was utilized to assess whether the differences among various parameters were statistically significant at the 0.05 significance level.

4.Results

4.1 Interleukin IL-8 Analysis

The analysis revealed a notable elevation in the IL-8 serum levels in the group of chicks subjected to oxidative stress induced by a 0.5% ml H2O2 component in their drinking water, with a significant increment ($P < 0.01$), registering at 88 Pg/ml, in comparison to the baseline levels observed in the non-treated control group, which was 64 Pg/ml. Furthermore, when the standard diet of the oxidative stress-induced chicks' group was supplemented with 3.5 g/kg of unfermented Gbl, a significant rise ($P < 0.01$) was observed in IL-8 levels, reaching 80 Pg/ml, contrasting with the 64 Pg/ml noted in the non-treated control group. Conversely, the group administered with 3.5 g/kg of unfermented Gbl under normal conditions exhibited a non-significant alteration ($P > 0.01$) in IL-8 levels, recording at 71 Pg/ml, in comparison to the non-treated control group at 64 Pg/ml. Notably, the group treated with 3.5 g/kg of fermented Gbl

demonstrated a significant decrement ($P < 0.01$) in IL-8 levels, falling to 56 Pg/ml, when compared to the non-treated control group at 64 Pg/ml.

4.2 Analysis of Tumor Necrosis Factor (TNF-α)

The data on the TNF-α, a prominent tumor necrosis factor, indicated a non-significant fluctuation ($P > 0.01$) in the group experiencing oxidative stress induced by a 0.5% ml H2O2 component, with levels registering at 49.594 Pg/ml, as opposed to the 54.429 Pg/ml recorded in the control group. Moreover, the study illustrated a non-significant deviation ($P > 0.01$) in the TNF-α serum levels in chicks supplemented with 3.5 g/kg of both fermented and unfermented Gbl amidst H2O2 induced oxidative stress, with concentrations ranging between 42.896 and 43.261 Pg/ml respectively, in relation to the 49.594 Pg/ml observed in the non-treated control group.

change ($P < 0.01$) in the TNF-α levels, registering at a markedly different concentration of 50.832 Pg/ml, a noticeable contrast when pitted against the 49.594 Pg/ml documented in the non-treated control group.

This indicates that the supplementation with unfermented Gbl at a The observations indicate a statistically significant elevation ($P < 0.01$) in the TNF-α concentration, reaching 60.639 Pg/ml, when the subjects were administered with a regimen incorporating 3.5 g/kg of unfermented Gbl, as opposed to the baseline value of 49.594 Pg/ml seen in the control group not subjected to any treatment. This highlights the substantial influence the unfermented Gbl concentration has on escalating the TNF-α levels in chickens, thereby hinting at its pivotal role in steering certain biological reactions within the subjects.

In contrast, the group that received a standard diet supplemented with 3.5 g/kg of fermented Gbl exhibited non-significant deviations ($P > 0.01$) in the TNF-α levels when compared to the untreated control group holding steady at 49.594 Pg/ml. This suggests a potentially differential impact on the TNF-α levels by the fermented variant of Gbl, warranting further investigations to delineate the possible implications and the mechanistic insights behind these observed disparities in the TNF-α concentrations.

Table 1: Effect of *Ginkgo biloba* leave on cytokines in normal and (H_2O_2) induced oxidative stress in broiler chick.

Parameters	Control group	H_2O_2 oxidative stress group	Unfermented Gbl H_2O_2 stress group	Fermented GBL H_2O_2 stress group	Unfermented Gbl group	Fermented Gbl group	P. value
IL-8 Pg/ml	64.774± 1.306^{c}	88.553± 2.362^{a}	80.519± 2.607^{b}	71.985± 1.093^{c}	71.711± 3.569^{c}	56.366± 2.811^{d}	0.01
TNF-α Pg/ml	49.594± 3.322^{bc}	54.429± 1.562^{ab}	43.261± 1.502^{c}	42.896± 2.655^{c}	60.639± 2.829^{a}	45.144± 2.974^{c}	0.01

4.3 Histological effects of GBL in oxidative stress and normal broiler chicken

Intestinal Histology

The histological section of jejunum showed that induced oxidative stress by %0.5 mill H_2O_2 (con %50) / lit. with drinking water was significantly reduced ($P < 0.01$), the jejunum villi heigh (681

µm), as compared to non-treated control group (755 µm) and all other experimental groups. There was a significant decrease ($P < 0.01$) in the heigh villi in groups supplemented with 3.5 g / kg of unfermented Gbl, (706 µm) in induced oxidative stress by %0.5 mill H_2O_2, as compared to its villi heigh in non-treated control group (755 µm). Also, the histological study revealed that normal chicks of standard diet supplemented with 3.5 g / kg of fermented Gbl showed significant increase ($P < 0.01$) in the mean value of the jejunum villi height (790 µm), as compared to non-treated control group (755 µm). Meanwhile the group add 3.5 g/kg unfermented Gbl showed non-significant change ($P > 0.01$) in villi heigh (759 µm) as compared to control group (755 µm).

The histological section of jejunum showed that induced oxidative stress by %0.5 mill H_2O_2, was tended to decreased but non-significantly ($P > 0.01$), in crypt depth (91 µm), as compared to non-treated control group (93 µm). There was non-significant change ($P < 0.01$) in the crypt depth (96 -94 µm) respectively in groups supplemented 3.5 g / kg of fermented and unfermented Gbl, in induced oxidative stress by %0.5 mill H_2O_2, as compared to its crypt depth in non-treated control group (93µm). Also, the histological study revealed that normal chicks supplemented 3.5 g / kg of fermented Gbl tended to decrease in villi heigh but non dramatically ($P > 0.01$) (90 µm) as compared to control group (93 µm). Group supplemented unfermented Gbl showed non-significantly change ($P > 0.01$) in the mean value of the jejunum (crypt depth) (93.01 µm), as compared to non-treated control group (93.52 µm).

Table 2: Effect of *Ginkgo biloba* leave in normal and (H_2O_2) induced oxidative stress on morphology small intestine jejunum, villus heigh and crypt depth in male broiler chicks (µm).

Parameters	**Control group**	**H_2O_2 oxidative stress group**	**Unfermented GBL H_2O_2 stress g**	**Fermented GBL H_2O_2 stress g**	**Unfermented GBL group**	**Fermented GBL g**	**P. value**
Villus heigh	755.329± 5.563^{b}	681.340± 8.327^{d}	706.725± 6.386^{c}	765.939± 10.604^{b}	759.778± 5.875^{b}	790.725± 4.591^{a}	<0.001
Crypt depth	93.524± 1.536^{abc}	91.165± 0.863^{bc}	94.932± 1.515^{ab}	96.774± 1.364^{a}	93.015± 1.504^{abc}	90.355± 1.774^{c}	0.030

5. Discussion

The gastrointestinal tract is a vital area for absorbing nutrients. Insights into the health of the gut can be ascertained by scrutinizing the morphological characteristics of the intestinal mucosa (Celi et al., 2017). Due to the intimate contact between the mucosal surface and intestinal contents, alterations in the intestinal mucosa can rapidly occur in response to stressors within the digesta (Han et al., 2012).

A plausible explanation for the observed elevation in villi height and reduction in crypt depth in chickens nourished with a 3.5g/kg diet of fermented Gbl might be attributed to the enhanced concentrations of total polysaccharides, crude protein, and amino acids found in fermented Ginkgo biloba L, when compared to its unfermented counterpart and the standard control group. Other research has suggested that plant-derived water-soluble polysaccharides can function similarly to prebiotics, fostering intestinal functionality (He et al., 2016). The leaves of

Ginkgo biloba contain ginkgolic acid, a component that can potentially be harmful to the broiler's digestive system due to its toxic properties (Ren et al., 2018). This risk seems to be mitigated following fermentation, a process that breaks down ginkgolic acid and antigenic nutrients, thereby promoting better gut health (Boateng, 2022). These positive modifications can be attributed to the potent compounds found in Ginkgo biloba, which harbor a rich assortment of essential oils that facilitate a balanced intestinal microbial ecosystem (Al-Tememy, 2022).

TNF-α is well-acknowledged for its crucial regulatory functions, primarily its ability to facilitate inflammatory responses that assist the host in combating microbial infections (Croft and Siegel, 2017). Flavonoids are hydroxylated polyphenols that consist of at least two interconnected aromatic rings linked by a heterocyclic pyran ring, and feature one or more aromatic hydroxyl groups (Catarino et al., 2016). Moreover, fermentation processes have been shown to breakdown large antigenic proteins into smaller peptides, enhancing their digestibility (Hong et al., 2004). The fungus Aspergillus niger is capable of producing a variety of enzymes that aid in this process (Mathivanan et al., 2006; Dei et al., 2008).

Groups subjected to oxidative stress demonstrated notable changes in intestinal morphology, including a decrease in villi height (681 μm) compared to the control group (755 μm), and an increase in crypt depth. Such morphological changes, characterized by shorter villi and deeper crypts, can be indicative of toxin presence or elevated tissue turnover rates, potentially impairing growth performance (Yason et al., 1987; Miles et al., 2006). Crypts, which house stem cells, act as production sites for villi; deeper crypts suggest accelerated tissue turnover and greater nutrient requirements for tissue regeneration.

In the current study, the group administered with 3.5 g/kg of fermented Gbl alongside oxidative stress exhibited improvements in intestinal ecology, with minor variations in villi height and crypt depth in the jejunum when compared to the control group. The fermentation process potentially facilitates the breakdown of large peptides into smaller units, enhancing nutrient digestibility and resulting in more efficient dietary utilization, thereby optimizing the feed-to-growth ratio in broilers (Mansoori et al., 2007; Zhang et al., 2012).

TNF, a multifunctional cytokine, plays a pivotal role in modulating immune responses and inflammatory processes. Originating from various immune cells, it engages in a wide array of biological functions, encompassing apoptosis, immune cell activation, inflammation regulation, and cell growth control. Our investigation revealed a significant decrease in IL-8 levels (56 Pg/ml) in the group treated with 3.5 g/kg fermented Gbl, compared to the control group (64 Pg/ml). Concurrently, a trend towards a non-significant reduction in TNF-α serum levels (45 Pg/ml) was observed compared to the control group (49 Pg/ml).

This study aligns with earlier research highlighting the anti-inflammatory properties of flavonoids in both rat models and macrophages (Hämäläinen et al., 2007). It is posited that oxidative stress and TNF-α can trigger and enhance NF-KB activation. In vitro analyses have suggested that Ginkgo biloba extracts can effectively suppress the production of several cytokines, including TNF-α, potentially through the attenuation of NK-AP-1 signaling pathway activity.

6. Conclusion

Fermented *Ginkgo biloba* leaves have been shown to have a positive impact on broiler immunity. This is attributed to the presence of flavonoid compounds in *Ginkgo biloba* leaves that become more bioavailable after fermentation, making them easily absorbed in the chicken's gut. Improved absorption is facilitated by the increased intestinal morphological changes, including the elevation of the villus height to crypt depth ratio. These changes contribute to a heightened immunological response and an overall improvement in the health of broilers.

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