Mycotoxins in poultry – External signs can give a hint

Part 3: Bone disorders and foot pad lesions

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Bone health is essential for animals and humans. Besides giving structural support, allowing movement, and protecting vital organs, the bones release hormones that are crucial for mineral homeostasis and acid balance and serve as reservoirs of energy and minerals (Guntur & Rosen, 2012; Rath, N.C. & Durairaj, 2022; Suchacki et al., 2017).

Bone disorders and foot pad lesions are considerable challenges in poultry production, especially for fast-growing birds with high final weights. Due to pain, the animals do not move, and dominant, healthy birds may restrict lame birds' access to feed and water. In consequence, these birds are often culled. Moreover, processing these birds is problematic, and often, they must be discarded or downgraded.

Foot pad lesions, another common issue in poultry production, can also have significant economic implications. On the one hand, pain restricts birds from eating and drinking and reduces weight gain. On the other hand, for many producers, chicken feet constitute a substantial part of the economic value of the bird; therefore, discarding them represents a significant financial loss. Additionally, to push poultry production in the right direction concerning animal health and welfare, a foot pad scoring system at the processing plant is in place in European countries.

Mycotoxins affect bones in different ways

Mycotoxins, depending on their target organs, can have diverse effects on the skeleton of birds. For example, mycotoxins that target the liver can disrupt calcium metabolism, which in turn affects the mineralization of the bones (rickets) and the impairment of chondrocytes can slow down bone growth (e.g., tibial dyschondroplasia). When the kidneys are impacted, urate clearance decreases, plasma uric acid consequently increases, and urate crystals form in the synovial fluid and tendon sheaths of various joints, particularly the hock joints. These examples highlight the complex and varied ways mycotoxins can impact poultry bone health.

Inadequate bone mineralization and strength – Rickets and layer cage fatigue

Sufficient bone mineralization is essential for the stability of the skeleton. Calcium (Ca), Vitamin D, and Phosphorous (P) deficiency leads to inadequate mineralization, weakens the bone, and can cause soft and bent bones or, in the case of layers, cage fatigue – a collapse of the spinal bone- and paralysis. Inadequate bone mineralization can be caused in different ways, among them:

1. Decrease in the availability of the nutrients necessary for mineralization. This can occur if the digestibility of these nutrients deteriorates
2. Impact on the Ca/P ratio—A ratio of 1 – 2:1 is vital for adequate bone development (Loughrill et al., 2016). Mycotoxins can alter absorption and transporters for one or both elements, altering their ratio.
3. Impact on the Vitamin D receptor, affecting its expression or the transporters for Ca and P.

Aflatoxins can impair bone mineralization by different modes of action. An important one is the impairment of the digestibility of Ca and P: Kermanshahi et al. (2007) fed broilers diets with high levels of aflatoxins (0.8 to 1.2 mg AFB1/kg feed) for three weeks, which resulted in a significant reduction of Ca and P digestibility. Other researchers, however, did not find an effect on Ca and P digestibility with lower aflatoxin levels: Bai et al. (2014) feeding diets contaminated with 96 (starter) and 157 µg Aflatoxins (grower) per kg of feed to broilers and Han et al. (2008) saw no impact on cherry valley ducks with levels of 20 and 40 µg AFB1/kg diet.

Indirectly, a decrease in the availability of Ca and P due to aflatoxin-contaminated feed can be shown by blood or tibia levels of these minerals, as demonstrated by Zhao et al. (2010): They conducted a trial with broilers, resulting in blood serum levels of Ca and P levels significantly ($P<0.05$) dropped with feed contaminated with 2 mg/kg of AFB1. Another trial conducted by Bai et al. (2014) showed decreased Ca in the tibia and reduced tibial break strength.

To get more information about the effect of mycotoxins on bone mineralization and the utilization of Ca, P, and Vit. D in animal organisms, Costanzo et al. (2015) challenged osteosarcoma cells with 5 and 50 ppb of aflatoxin B1. They asserted a significant down-modulation of the expression of the Vitamin D receptor. Furthermore, they assumed an interference of AFB1 with the actions of vitamin D on calcium-binding gene expression in the kidney and intestine. Paneru et al. (2024) could confirm this downregulation of the Vit D receptor and additionally of the Ca and P transporters in broilers with levels of ≥75 ppb AFB1. They also saw a significant reduction in tibial bone ash content at AFB1 levels >230 ppb, a decreased trabecular bone mineral content and density at AFB1 520 ppb, and a reduced bone volume and tissue volume of the cortical bone of the femur at the level of 230 ppb (see Figure 1). They concluded that AFB1 levels of already 230 ppb contribute to bone health issues in broilers.

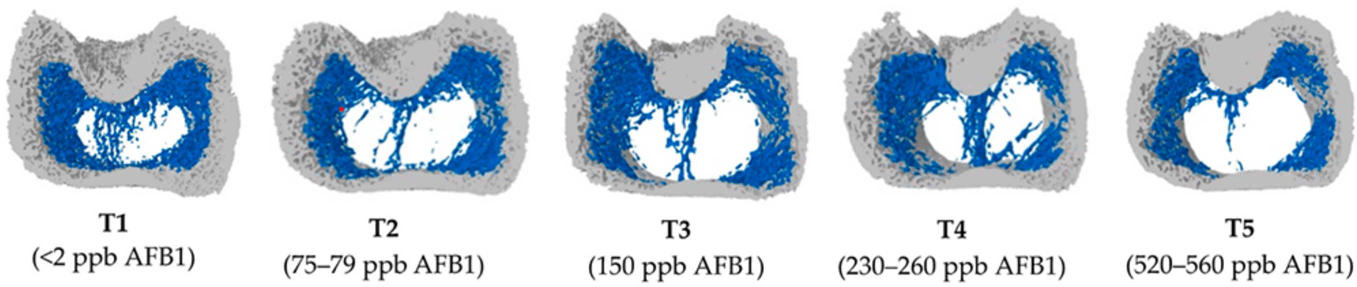

Figure 1: Increasing doses of AFB1 (<2 ppb – 560 ppb) deteriorate bone quality (Paneru, 2024): Cross-sectional images of femoral metaphysis with increasing AFB1 levels (left to right). The outer cortical bone is shown in light grey, and the inner trabecular bone in blue. Higher levels of AFB1 (T4 and T5) show a disruption of the trabecular bone pattern (less dense blue pattern with thinner and more fragmented bone strands and with wide spaces between the trabecular bone) (shown in white).

All experiments strongly suggest that aflatoxins harm bone homeostasis. Additional liver damage, oxidative stress, and impaired cellular processes can exacerbate bone health issues**.**

Trichothecenes also negatively impact bone mineralization. Depending on the mycotoxin, they may affect the gut, decreasing the absorption of Ca and P and probably provoking an imbalance in the Ca/P ratio.

For instance, when **T-2 toxin** was fed to Yangzhou goslings at 0.4, 0.6, and 0.8 mg/kg of diet, it decreased the Ca levels (halved at 0.8 mg/kg) and increased the P levels in the blood serum, so the Ca/P ratio decreased from the adequate ratio of 1 – 2 to 0.85, 0.66, and 0.59 ($P<0.05$) (Gu et al., 2023). The alterations of the Ca and P levels, the resulting decreasing Ca/P ratio, and an additional increase in alkaline phosphatase (ALP) suggest that T-2 toxin negatively impacts Ca absorption, increases ALP, and, therefore, disturbs calcification and bone development.

Other studies show that serum P levels decreased in broilers fed DON-contaminated feed with levels of only 2.5 mg/kg (Keçi et al., 2019). One reason for the lower P level is probably the lower dry matter intake, affecting Ca and P intake. Ca serum level is not typically reduced, which can be explained by the fact that Ca plays many critical physiological roles (e.g., nerve communication, blood coagulation, hormonal regulation), so the body keeps the blood levels by reducing bone mineralization. Another explanation is delivered by Li et al. (2020): After their trial with broilers, they stated that dietary P deficiency is more critical for bone development than Ca deficiency or Ca & P deficiency. The results of the trial conducted by Keçi et al. with DON (see above) were reduced bone mineralization, affected bone density, ash content, and ash density in the femur and tibiotarsus with a stronger impact on the tibiotarsus than on the femur.

In line with trichothecenes effects in Ca and P absorption, Ledoux et al. (1992) suppose that diarrhea caused by intake of fumonisins leads to malabsorption or maldigestion of vitamin D, calcium and phosphorus, having birds with rickets as a secondary effect.

Ochratoxin A (OTA) impairs kidney function, negatively affects vitamin D metabolism, reduces Ca absorption, and contributes to deteriorated bone strength (Devegowda and Ravikiran, 2009). Indications from Huff et al. (1980) show decreased tibia strength after feeding chickens OTA levels of 2, 4, and 8 µ/g, and Duff et al. (1987) report similar results also in turkey poults.

A further mycotoxin possibly contributing to leg weakness is cyclopiazonic acid produced by Aspergillus and Penicillium. This mycotoxin is known for leading to eggs with thin or visibly racked shells, indicating an impairment of calcium metabolism (Devegowda and Ravikiran, 2009). Tran et al. (2023) also showed this fact with multiple mycotoxins.

The co-occurrence of different mycotoxins in the feed – the standard in praxis – increases the risk of leg issues. A trial with broiler chickens conducted by Raju and Devegowda (2000) showed a bone ash-decreasing effect of AFB1 (300 µg/kg), OTA (2 mg/kg), and T-2 toxin (3 mg/kg), fed individually but an incomparable higher effect when fed in combination.

Impairment of bone growth – tibial

dyschondroplasia (TD)

In TD, the development of long bones is impaired, and abnormal cartilage development occurs. It is frequent in broilers, with a higher incidence in males than females. It happens when the bone grows, as the soft cartilage tissue is not adequately replaced by hard bone tissue. Some mycotoxins have been related to this condition: According to Sokolović et al. (2008), actively dividing cells such as bone marrow are susceptible to T-2 toxin, including the tibial growth plates, which regulate chondrocyte formation, maturation, and turnover.

T-2 toxin: In a study with primary cultures of chicken tibial growth plate chondrocytes (GPCs) and three different concentrations of T-2 toxin (5, 50, and 500 nM), He et al. (2011) found that T-2 toxin decreased cell viability, alkaline phosphatase activity, and glutathione content ($P < 0.05$). Additionally, it increased the level of reactive oxygen species and malondialdehyde in a dose-dependent way, which could be partly recompensated by adding an antioxidant (N-acetyl-cysteine). They concluded that T-2 toxin inhibits the proliferation and differentiation of GPCs and contributes, therefore, to the development of TD, altering cellular homeostasis. Antioxidants may help to reduce these effects.

Gu et al. (2023) investigated the closely bodyweight-related shank length and the tibia development in Yangzhou goslings fed feed with six different levels (0 to 2.0 mg/kg) of T-2 toxin for 21 days. They determined a clear dose-dependent slowed tibial length and weight growth ($p<0.05$), as well as abnormal morphological structures in the tibial growth plate. As tibial growth and shank length are closely related to weight gain (Gu et al., 2023; Gao et al., 2010; Ukwu et al., 2014; Yu et al., 2022), their slowdown indicates lower growth performance.

Fumonisin B1 is also a potential cause of this kind of leg issue. Feeding 100 and 200 mg/kg to day-old turkey poults for 21 days led to the development of TD (Weibking et al., 1993). Possible explanations are the reduced viability of chondrocytes, as found by Chu et al. (1995) after 48 h of exposure, or the toxicity of FB1 to splenocytes and chondrocytes, which was shown in different primary cell cultures from chicken (Wu et al., 1995).

Bacterial chondronecrosis with osteomyelitis lameness (BCO) can be triggered by DON and FUM

BCO presents a highly critical health and welfare issue in broiler production worldwide, and it is estimated that 1-2 % of condemnations in birds at the marketing age result from this disease. What is the reason? Today's fast-growing broilers are susceptible to stress. This enables pathogenic bacteria to compromise epithelial barriers, translocate from the gastrointestinal tract or the pulmonary system into the bloodstream, and colonize osteochondrotic microfractures in the growth plate of the long bone. This can lead to bone necrosis and subsequent lameness.

In their experiment with DON and FUM in broilers, Alharbi et al. (2024) showed that these mycotoxins reduce the gut's barrier strength and trigger immunosuppressive effects. They used contaminations of 0.76, 1.04, 0.94, and 0.93 mg DON/kg of feed and 2.40, 3.40, 3.20, and 3.50 mg FUM/kg diet in the starter, grower, finisher, and withdrawal phases, respectively. The team observed lameness on day 35; the mycotoxin groups always showed a significantly ($P<0.05$) higher incidence of cumulative lameness.

The increase in uric acid leads to gout

In general, mycotoxins, which damage the kidneys and, therefore, impact the renal excretion of uric acid, are potentially a factor for gout appearance.

One of these mycotoxins is T-2 toxin. With the trial mentioned before (Yangzhou goslings, 21 days of exposure), Gu et al. (2023) showed that the highest dosage of the toxin (2.0 mg/kg) significantly increased uric acid in the blood ($P<0.05$), possibly leading to the deposit of uric acid crystals in the joints and to gout.

Huff et al. (1975) applied Ochratoxin to chicks at 0, 0.5, 1.0, 2.0, 4.0, and 8.0 µg/g of feed during the first three weeks of life. They found ochratoxin A as a severe nephrotoxin in young broilers as it caused damage to the kidneys with doses of 1.0 µg/g and higher. At 4.0 and 8.0 µg/g doses, uric acid increased by 38 and 48%, respectively (see Figure 2). Page et al. (1980) also reported increased uric acid after feeding 0.5 or 1.0 mg/kg of Ochratoxin A to adult white Leghorn chickens.

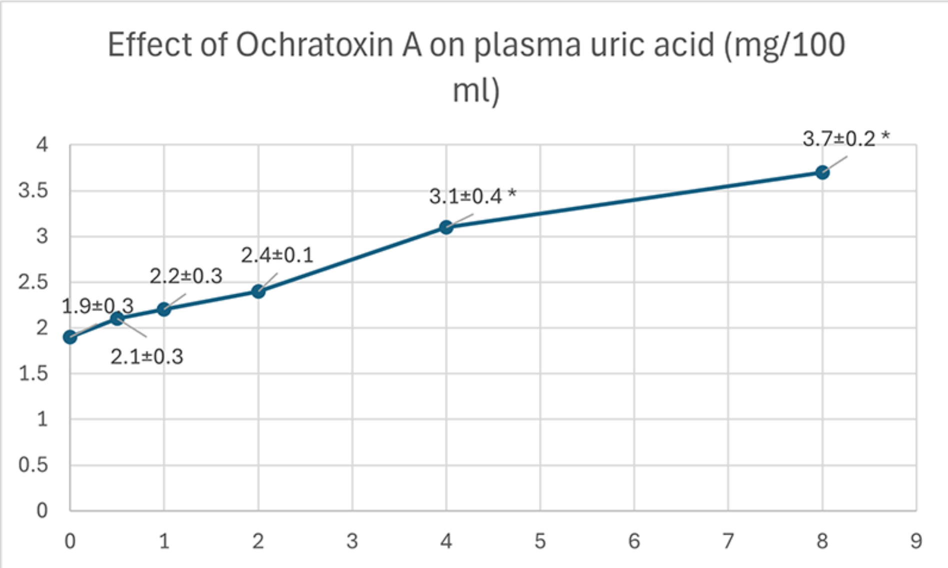

Figure 2: Effect of Ochratoxin A on plasma uric acid (mg/100 ml) (according to Huff et al., 1975)

Foot pad lesions – a further hint of mycotoxicosis

Foot pad lesions often result from wet litter, originating from diarrhea due to harmed gut integrity. Frequently, mycotoxins impact the intestinal tract and create ideal conditions for the proliferation of diarrhea-causing microorganisms and, therefore, secondary infections. Some also negatively impact the immune defense system, allowing pathogens to settle down or aggravate existing bacterial or viral parasitic diseases. In general, mycotoxins affect the physical (intestinal cell proliferation, cell viability, cell apoptosis), chemical (mucins, AMPs), immunological, and microbial barriers of the gut, as reported by Gao et al. (2020). Here are some examples of the adverse effects of mycotoxins leading to intestinal disorders and diarrhea:

- Mycotoxins can modulate intestinal epithelial integrity and the renewal and repair of epithelial cells, negatively impacting the intestinal barrier's intrinsic components; for instance, DON can significantly reduce the transepithelial electrical resistance (TEER)(Grenier and Applegate, 2013). A higher permeability of the epithelium and a decreased absorption of dietary proteins can lead to higher protein in the digesta in the small intestine, which serves as a nutrient for pathogens including *perfringens* (Antonissen et al., 2014; Antonissen et al., 2015).
- The application of Ochratoxin A (3 mg/kg) increased the number of S. typhimurium in the duodenum and ceca of White Leghorn chickens (Fukata et al., 1996). Another trial with broiler chicks at a concentration of 2 mg/kg aggravated the symptoms due to an infection by S. gallinarum (Gupta et al., 2005).
- In a trial by Grenier et al., 2016, feed contaminated with DON (1.5 mg/kg), Fumonisin B (20 mg/kg), or both mycotoxins aggravated lesions caused by coccidia.
- DON impacts the mucus layer composition by downregulating the expression of the gene coding for MUC2, as shown in a trial with human goblet cells (Pinton et al., 2015). The mucus layer prevents pathogenic bacteria in the intestinal lumen from contacting the intestinal epithelium (McGuckin et al., 2011).
- Furthermore, DON and other mycotoxins decrease the populations of lactic acid-producing bacteria, indicating a shift in the microbial balance (Antonissen et al., 2016).
- FB1 causes intestinal disturbances such as diarrhea, although it is poorly absorbed in the intestine. According to Bouhet and Oswald (2007), the main toxicological effect ascertained in

vivo and in vitro is the accumulation of sphingoid bases associated with the depletion of complex sphingolipids. This negative impact on the sphingolipid biosynthesis pathway could explain other adverse effects, such as reduced intestinal epithelial cell viability and proliferation, modification of cytokine production, and impairment of intestinal physical barrier function.

- T-2 toxin can disrupt the immune response, enhance the proliferation of *coli* in the gut, and increase its efflux (Zhang et al., 2022).

All these mycotoxins can cause foot pad lesions by impacting gut integrity or damaging the gut mucosa. They promote pathogenic organisms and, thus, provoke diarrhea and wet litter.

Mitigating the negative impact of mycotoxins on bones and feet is crucial for performance

Healthy bones and feet are essential for animal welfare and performance. Mycotoxins can be obstructive. Consequently, the first step to protecting your animals is to monitor their feed. If the analyses show the occurrence of mycotoxins at risky levels, proactive measures must be taken to mitigate the issues and ensure the health and productivity of your poultry.

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Decoding the connection between stress, endotoxins, and poultry health

By Technical Team, EW Nutrition

Stress can be defined as any factor causing disruptions to homeostasis, which triggers a biological response to regain equilibrium. We can distinguish four major types of stressors in the poultry industry:

- Technological: related with management events and conditions
- Nutritional: involving nutritional disbalances, feed quality and feed management
- Pathogenic: comprising health challenges.
- Environmental: changes in environment conditions

In practical poultry production, multiple stress factors occur simultaneously. Their effects are also additive, leading to chronic stress. The animals are not regaining homeostasis and continuously deviate the use of resources through inflammation and the gut barrier-function, thus leading to microbiome alteration. As a consequence, welfare, health, and productivity are compromised.

What are endotoxins?

Bacterial lipopolysaccharides (LPS), also known as endotoxins, are the main components of the outer membrane of all Gram-negative bacteria and are essential for their survival. LPS have direct contact with the bacteria's surroundings and function as a protection mechanism against the host's immunological response and chemical attacks from bile salts, lysozymes, or other antimicrobial agents.

Gram-negative bacteria are part of animals' microbiota; thus, there are always LPS in the intestine. Under optimal conditions, this does not affect the animals, because intestinal epithelial cells are not responsive to LPS when stimulated from the apical side. In stress situations, the intestinal barrier function is impaired, allowing the passage of endotoxins into the blood stream. When LPS are detected by the immune system either in the blood or in the basolateral side of the intestine, inflammation and changes in the gut epithelial structure and functionality occur.

The gut is critically affected by stress

Even when there is no direct injury to the gut, signals from the brain can modify different functions of the intestinal tract, including immunity. Stress can lead to functional disorders, as well as to inflammation and infections of the intestinal tract. Downstream signals act via the brain–gut axis, trigger the formation of reactive oxygen and nitrogen species as well as local inflammatory factors, and circulating cytokines, affecting intestinal homeostasis, microbiome, and barrier integrity.

Stress then results in cell injury, apoptosis, and compromised tight junctions. For this reason, luminal substances, including toxins and pathogens, leak into the bloodstream. Additionally, under stress, the gut microbiome shows and increment on Gram-negative bacteria (GNB). For instance, a study by Minghui Wang and collaborators (2020) found an increase of 24% in GNB and lower richness, in the cecum of pullets subjected to mild heat stress (increase in ambient temperature from 24 to 30°C).

Both these factors, barrier damage and alterations in the microbiome, facilitate the passage of endotoxins into the blood stream, which promotes systemic chronic inflammation.

What categories of stress factors trigger luminal endotoxins' passage into the bloodstream?

Technological stress

Various management practices and events can be taken as stressors by the animals' organism. One of the most common examples is **stocking density**, defined as the number of birds or the total live weight of birds in a fixed space. High levels are associated with stress and loss of performance.

A study from the Chung-Ang University in 2019 found that broilers with a stocking density of 30 birds/m^2 presented two times more blood LPS than birds kept at half of this stocking density. Moreover, the body weight of the birds in the high-density group was 200g lower than the birds of the low-density group. The study concluded that high stocking density is a factor that can disrupt the intestinal barrier.

Nutritional stress

The feed supplied to production animals is designed to contribute to express their genetic potential, though some feed components are also continuous inflammatory triggers. **Anti-nutritional factors, oxidized lipids, and mycotoxins** induce a low-grade inflammatory response.

For instance, when mycotoxins are ingested and absorbed, they trigger stress and impair immunity in animals. Their effects start in gastrointestinal tract and extend from disrupting immunity to impairing the intestinal barrier function, prompting secondary infections. Mycotoxins can increase the risk of endotoxins in several ways:

- By inducing changes in the intestinal microbiota that increase gram-negative bacteria
- By disrupting the intestinal barrier function, allowing endotoxins (as well as other toxins and pathogens) to cross the gut barrier and pass into the bloodstream
- By alterations in the immune response, low doses of mycotoxins, such as trichothecenes, induce the upregulation of pro-inflammatory cytokines. A possible synergy can be inferred as when they are together, the effects may be prolonged and require a lower dosage to be triggered.

A study conducted by EW Nutrition (Figure 1) shows an increase in intestinal lesions and blood endotoxins after a mycotoxin challenge of 200pbb of Aflatoxin B1 + 360ppb Ochratoxin in broilers at 21 days of age. The challenged birds show two times more lesions and blood endotoxins than the ones in the unchallenged control. The use of the right mitigation strategy, a product based on bentonite, yeast cell walls, and phytogenics (EW Nutrition GmbH) successfully prevented these effects as it not only mitigates mycotoxins, but also targets endotoxins in the gut.

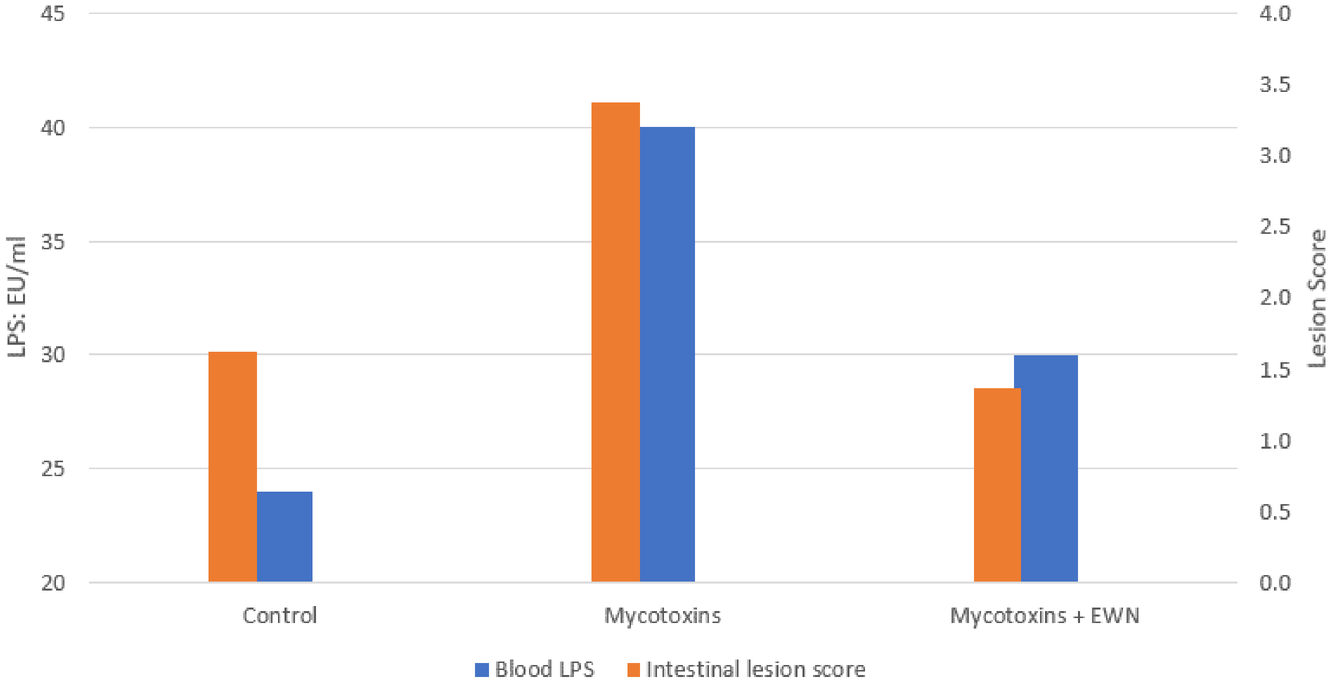


Figure 1 Blood LPS and intestinal lesion score of broilers challenged with 200ppb AFB1 + 350 ppb OTA from 1 to 21 days of age without and with an anti-toxin product from EW Nutrition GmbH (adapted from Caballero et al., 2021)

Pathogenic stress

Intestinal disease induces changes in the microbiome, reducing diversity and allowing pathogens to thrive. In clinical and subclinical necrotic enteritis (NE), the intestinal populations of GNB, including Salmonella and E.coli also increases. The lesions associated with the pathogen compromise the epithelial permeability and the intestinal barrier function, resulting in translocation of bacteria and LPS (Figure 5) into the bloodstream and internal organs.

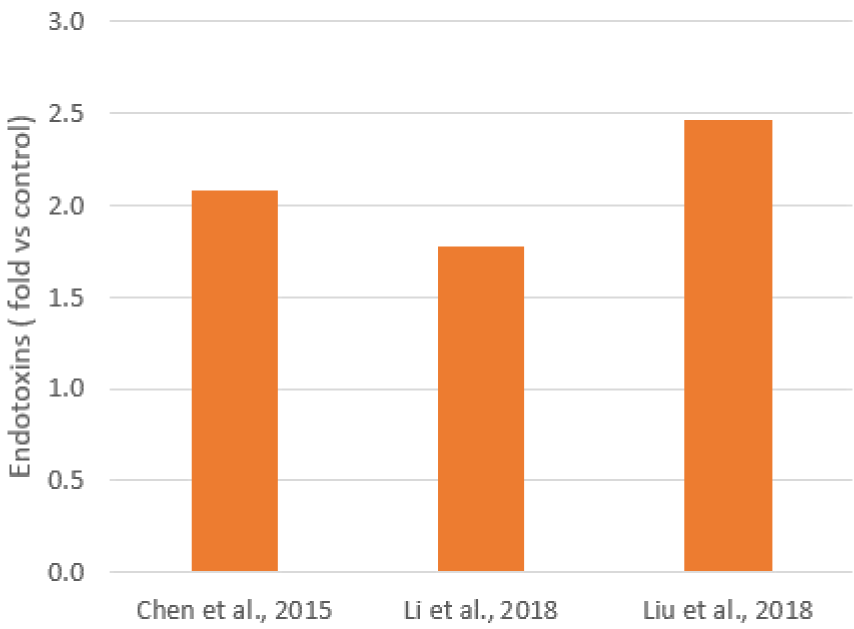


Figure 2 Increase in systemic LPS (vs a healthy control) after a NE challenge (adapted from Chen et al., 2015, Li et al., 2018 & Liu at al., 2018)

Environmental stress

Acute and chronic heat and cold stress increases gut permeability, by increasing intestinal oxidative stress and disrupting the expression of tight junction proteins. This results in the damage and destruction of intestinal cells, inflammation, and imbalance of the microbiota. An increased release and passage of endotoxins has been demonstrated in heat stress (Figure 3), as well as a higher expression of TLR-4 and inflammation.

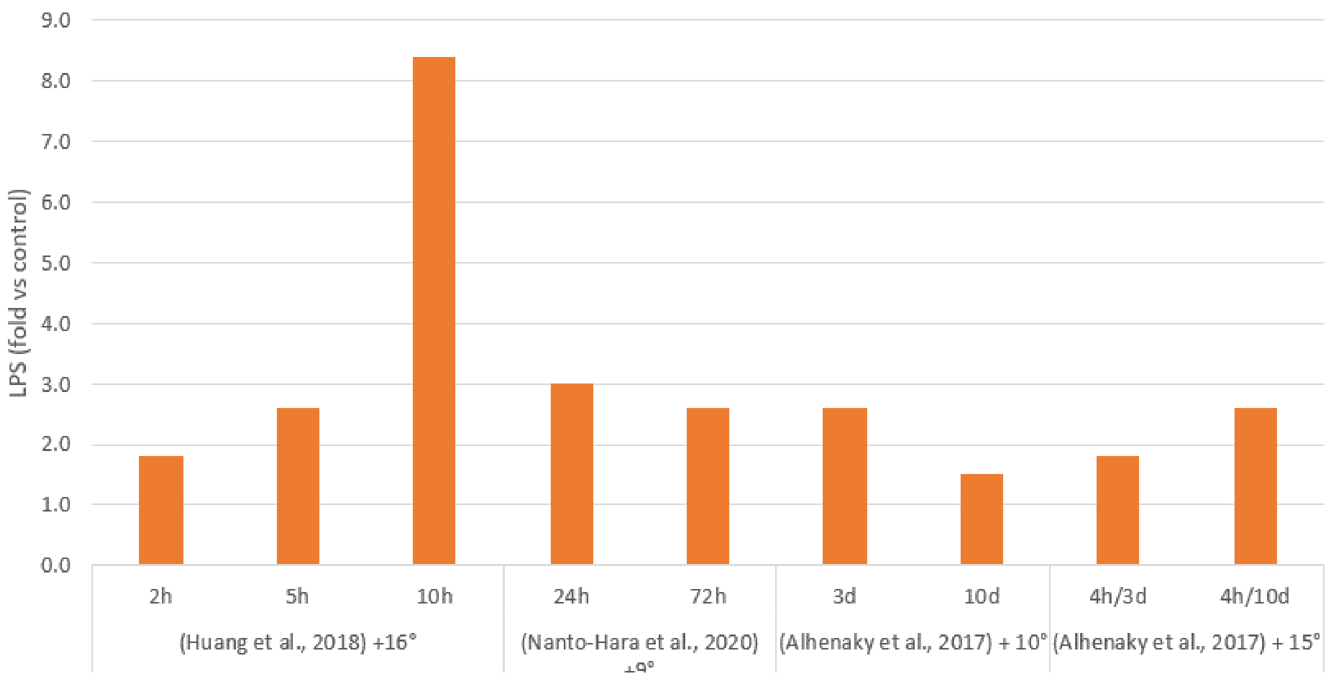
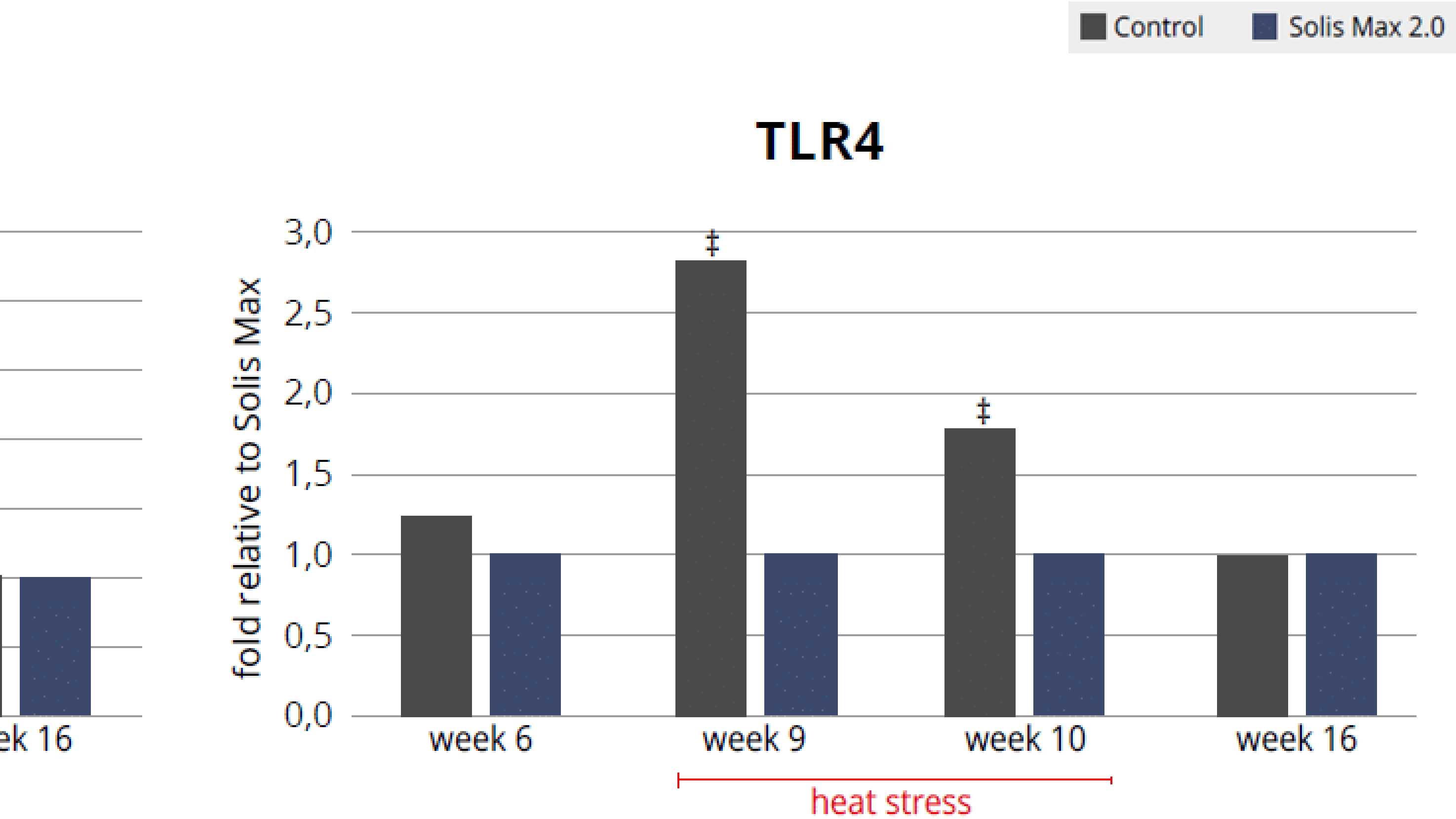


Figure 3 Systemic LPS increase (in comparison with a non-stressed control) after different heat stress challenges in broilers: 16°C increased for 2, 5 and 10 hours (Huang et al., 2018); 9°C increased for 24 and 72 hours (Nanto-Hara et al., 2020); 10°C continuously for 3 and 10 days, and 15°C 4 hours daily for 3 and 10 days (Alhenaky et al., 2017)

Zhou and collaborators (2021) showed that 72 hours of low temperature treatment in young broilers increased intestinal inflammation and expression of tight junction proteins, while higher blood endotoxins indicate a disruption of the intestinal barrier. As a consequence, the stress decreased body gain and increased the feed conversion rate.

An experiment conducted by EW Nutrition GmbH with the objective of evaluating the ability of a toxin mitigation product to ameliorate heat-stress induced LPS. For the experiment, 1760 Cobb 500 pullets were divided into two groups, and each was placed in 11 pens of 80 hens, in a single house. One of the groups received feed containing 2kg/ton of the product from the first day. From week 8 to week 12, the temperature of the house was raised 10°C for 8 hours every day.

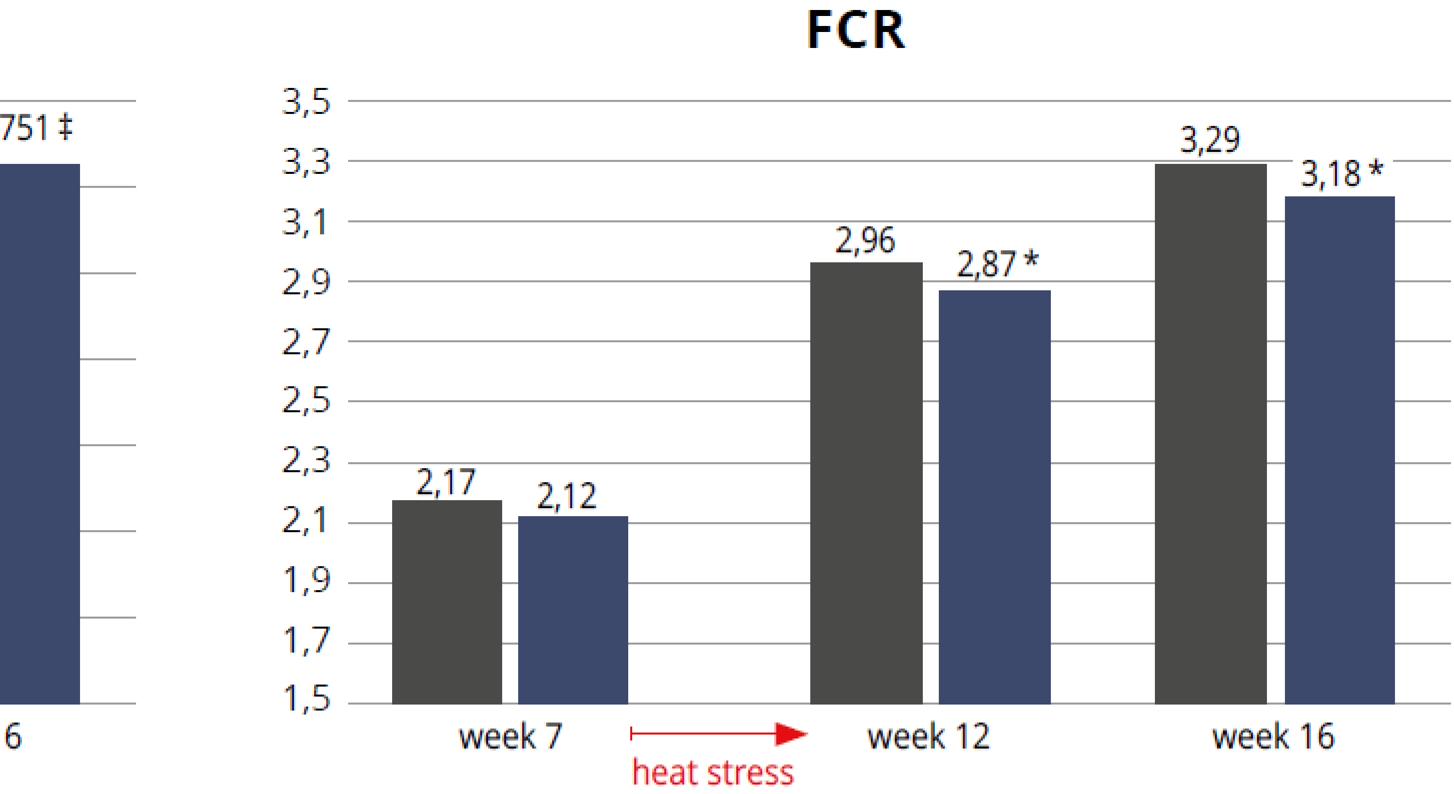
Throughout the heat stress period, blood LPS (Fig 4) was lower in the pullets receiving the product, which allowed lower inflammation, as evidenced by the lower expression of TLR4 (Fig. 5). Oxidative stress was also mitigated with the help of the combination of phytomolecules in the product, obtaining 8.5% improvement on serum total antioxidant capacity (TAC), supported by an increase in in superoxide dismutase (SOD glutathione peroxidase (GSH) and a decrease in malondialdehyde (MDH).



es of pullets before (wk 6) and during heat stress (wk 9 and 10). (*) indicates significant differences (P<0,05), and

In practice: there is no silver bullet

In commercial poultry production, a myriad stressors may occur at the same time and some factors trigger a chain of events that work to the detriment of animal health and productivity. Reducing the solution to the mitigation of LPS is a deceitfully simplistic approach. However, this should be part of a strategy to achieve better animal health and performance. In fact, EW Nutrition's toxin mitigation product alone helped the pullets to achieve 3% improvement in body weight and 9 points lower cumulative feed conversion (Figure 6).



Keeping the animals as free of stress as possible is a true priority for poultry producers, as it promotes animal health as well as the integrity and function of the intestinal barrier. Biosecurity, good environment, nutrition and good management practices are crucial; the use of feed additives to reduce the consequences of unavoidable stress also critically supports the profitability of poultry operations.