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MOLECULAR AND CELLULAR MARKERS IN CHICKENS EXPOSED TO STRESSORS AND THE ABILITY OF FEED SUPPLEMENTS TO OVERCOME NEGATIVE EFFECTS – A REVIEW

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Abstract

Performance of chickens is negatively impacted by stressors such as heat stress, infection with *Campylobacter jejuni*, the mycotoxins – aflatoxin B_1 and deoxynivalenol, the glucocorticoid dexethasone, insecticides and *Eimeria tenella*. Effects of stressors include reduced gastrointestinal function and induction of inflammation. Stressors influence the following molecular and cellular structures and functions: gastrointestinal integrity, villus height, crypt depth, mast cell number, expression of pro-inflammatory cytokines, concentrations of leukocytes, and antioxidant status. In multiple studies, adverse effects of multiple stressors were overcome partly by supplementation. The utility of these parameters is discussed and recommendations/guidelines for future research made.

Key words: stressors, supplements, chickens, immune parameters, gastrointestinal morphology

There are novel reports on positive effects of feed additives in human (Chuai et al., 2023; Liu et al., 2023; Liang et al., 2024; Zhen et al., 2024) and animals (Movahhedkhah et al., 2019; Chand et al., 2021; Osita et al., 2022; Das et al., 2023; Belali et al., 2024; Hosseintabar-Ghasemabad et al., 2024). The use of supplements in poultry production is generating much interest particularly with the moves away from antibiotic supplementation. Stressors such as heat stress, pathogens and toxicants reduce production efficiency, and these negative effects can be overcome by nutritional supplements. For instance, supplementation with betaine tended to both increase growth in coccidial inoculated chickens and reduce mortalities (Waldenstedt et al., 1999). Moreover, the depressed growth rate in heat stressed broiler chickens is reported to be overcome by either betaine (Attia and Hassan, 2017; Wen et al., 2021) or curcumin (Attia, 2018; Zhang et al., 2019) supplementation. Reducing dietary methionine was accompanied by depressed growth which was overcome by supplementation with either choline or the methyl donor, betaine (Attia et al., 2005; Waldroup et al., 2006). Similarly, weight gain in broiler chickens was improved with betaine supplementation when birds were on a low methionine feed (Rao et al., 2011).

The present communication focuses on molecular and cellular responses of chickens to stressors and the ability of feed supplementation to overcome these. It is argued that these are inflammatory and oxidative stress responses. The communication provides a critical assessment of the responses including the following: ability of supplements to reverse effects on gastrointestinal structures, mast cells, pro-inflammatory cytokines, immune and antioxidant status. Discussion will be limited to molecular and cellular responses to stressors in the presence or absence of supplementation under the following topics:

- Molecular and cellular responses in the gastrointestinal tract,
- Inflammatory responses,
- Antioxidant status,
- Leukocyte responses.

Production indices will not be covered except for in this introduction, neither will organ weights.

Molecular and cellular responses in the gastrointestinal tract

DNA concentrations in the small intestine

Supplements can overcome the negative effects of stressors on cell numbers as indicated by DNA concen-

trations in the gastrointestinal tract. There were decreases in the DNA concentration in both the duodenum and jejunum in heat-stressed adult male chickens (Attia et al., 2019). This effect was completely overcome with betaine supplementation (Attia et al., 2019). What is not known is effects of stressors and supplements to influence mucosal cell division and rates of cells sloughing off from the villus tips.

Villus height and crypt depth

Villus height and crypt depth are frequently employed characteristics of the gastrointestinal tract. Villus height is depressed while crypt depths are increased by stressors. For instance, villus heights were decreased while crypt depths were increased in both regions of the small intestine and ceca of young chickens challenged with the stressor, *Eimeria* (Table 1).

Supplements can overcome the negative effects of stressors on intestinal villus height. Table 2 summarizes the results of studies examining the effects of stressors on intestinal villus height and the ability of supplements to overcome these. In general, there were lower villus heights in regions of the gastrointestinal tract in chickens subjected to stressors such as the glucocorticoid, dexamethasone (Barekatain et al., 2019 a), *Eimeria tenella* infection (Zhou et al., 2020 a), challenges with *Campylobacter jejuni* infection (Awad et al., 2015; Munoz et al., 2023) and mycotoxins, aflatoxin B_1 (AFB_1) (Cheng et al., 2020) and deoxynivalenol (DON) (Awad et al., 2006) (Table 2).

Table 1. Effects of a stressor, *Eimeria* (challenged for 6 d) on villus height and crypt depth in different regions of the small intestine

Items	Control	Stressed	Bird type	Reference
Villus height (μm)				
duodenum	1433 a	898 b	16 d old pullets	Sharma et al., 2022
jejunum	829 a	525 b	16 d old pullets	Sharma et al., 2022
ileum	585 a	386 b	16 d old pullets	Sharma et al., 2022
ceca	135 a	64.9 b	Males of layer line	Zhou et al., 2020 a
Crypt depth (μm)				
duodenum	183 a	308 b	16 d old pullets	Sharma et al., 2022
jejunum	143 a	235 b	16 d old pullets	Sharma et al., 2022
ileum	121	162	16 d old pullets	Sharma et al., 2022
ceca	75 a	142 b	Males of layer line	Zhou et al., 2020 a

a, b – different letters indicate difference $P<0.05$.

Table 2. Examples of effects of stressors in the presence or absence of supplements on villus heights and crypt depths in small intestine of chickens

Items	Control	Stressed	Stressed + Supplement	Bird type	Reference
1	2	3	4	5	6
Villus height (μm)					
Duodenum					
Campylobacter jejuni challenge (d 21) +/– yeast cell wall (d 28)	1715	1814	1856	Broilers	Munoz et al., 2023
Campylobacter jejuni challenge (d 21) +/– yeast cell wall (d 42)	1789	1714	1871	Broilers	Munoz et al., 2023
Aflatoxin B_1 challenge +/– curcumin (d 28)	1271 a	1120 b	1409 c	Broilers	Cheng et al., 2020
Mixed *Eimeria* challenged for 6 d +/– *Artemisia annua*	1827 a	1111 b	1230 b	♀ adult	Sharma et al., 2024
Jejunum					
Heat stressed 21–41 d +/– glutamine supplement	1546 a	1314 b	1526 a	Broilers	Wu et al., 2018
Heat stress +/– resveratrol	1298	1058	1186	Broilers	Zhang et al., 2017
Heat stress +/– resveratrol	1543 a	1334 b	1524 a	Broilers	Wang et al., 2021
Deoxynivalenol +/– probiotic	1,064 a	976 b	1,050 a	Broilers	Awad et al., 2006
Aflatoxin B_1 +/– curcumin	1271 a	1020 b	1409 a	Broilers	Cheng et al., 2020
Mixed *Eimeria* challenged for 6 d +/– *Artemisia annua*	1100 a	932 b	912 b	♀ adult	Sharma et al., 2024
Salmonella jejuni challenge	889 a	662 b	NA	Broilers	Awad et al., 2015
Ileum					
Heat for 6 d, supplement Probiotic, *Bacillus licheniformis*	630 a	565b	611 a	♀ adult	Deng et al., 2012
Heat for 12 d, supplement Probiotic, *Bacillus licheniformis*	615	587	605	♀ adult	Deng et al., 2012
Heat stressed 21–41 d +/– glutamine supplement	900 a	762 b	899 a	Broilers	Wu et al., 2018

Table 2 – contd.

1	2	3	4	5	6
Intermittent heat stress for 35 d Probiotic	NA	1168 a	1411 b	35 d old broilers	Elbaz et al., 2023
Intermittent heat stress for 35 d Clove essential oils	NA	1168 a	1366 b	35 d old broilers	Elbaz et al., 2023
Intermittent heat stress for 35 d Probiotic, clove essential oils	NA	1168 a	1756 b	35 d old broilers	Elbaz et al., 2023
Campylobacter jejuni challenge +/– yeast cell wall (d 28)	451	441	452	Broilers	Munoz et al., 2023
Campylobacter jejuni challenge +/– yeast cell wall (d 42)	500	393	447	Broilers	Munoz et al., 2023
Mixed *Eimeria* challenged for 6 d +/– *Artemisia annua*	686	739	694	♀ adult	Sharma et al., 2024
Crypt depth (μm)					
Duodenum					
Mixed *Eimeria* challenged for 6 d +/– *Artemisia annua*	121 a	449 b	406 b	♀ adult	Sharma et al., 2024
Jejunum					
Heat stress +/– Resveratrol	160 a	221 b	184 a	Broilers	Wang et al., 2021
Heat stressed 21–41 d +/– glutamine supplement	182 a	200 b	180 a	Broilers	Wu et al., 2018
Mixed *Eimeria* challenged for 6 d +/– *Artemisia annua*	97 a	416 b	351 b	♀ adult	Sharma et al., 2024
Ileum					
Heat for 6 d	144 a	156 b	149 a	♀ adult	Deng et al., 2012
Heat for 12 d	139 a	152 b	151 b	♀ adult	Deng et al., 2012
Intermittent heat stress for 35 d Probiotic	NA	204 a	216 b	35 d old broilers	Elbaz et al., 2023
Intermittent heat stress for 35 d Clove essential oils	NA	204	207	35 d old broilers	Elbaz et al., 2023
Intermittent heat stress for 35 d Probiotic, clove essential oils	NA	204 a	219 b	35 d old broilers	Elbaz et al., 2023
Heat stressed 21–41 d +/– glutamine supplement	154 a	179 b	154 a	Broilers	Wu et al., 2018
Mixed *Eimeria* challenged for 6 d +/– *Artemisia annua*	84 a	335 c	246 b	♀ adult	Sharma et al., 2024

NA – not available.
a, b, c – different letters indicate difference $P<0.05$.

There were increased villus heights with treatments anticipated to promote well-being of poultry such as the following (also see Table 2): dried olive pulp (Tufarelli et al., 2022), arginine in protein restriction (Barekatain et al., 2019 a), natural betaine (Awad et al., 2022), fructo-ligo-saccharide (FOS) (Xu et al., 2003) and raffinose *in ovo* (Berrocoso et al., 2017). In addition, villus heights in the small intestine were increased in chickens receiving the following supplementations: *Bacillus subtilis* (small intestine: Xie et al., 2020), fermented feed (jejunum: Xie et al., 2020), and zinc oxide together with montmorillonite (duodenum, jejunum and ileum: 26). Effects of stressors on villus height were reversed by supplements such as the following (also see Table 2): the mycotoxin DON overcome by probiotics (Awad et al., 2006); and another mycotoxin, AFB_1, overcome by curcumin (Cheng et al., 2020).

It should be noted that there were, however, exceptions. For instance, supplementation with oregano was accompanied by decreased jejunal crypt depth while tending to decrease villus height in broiler chickens challenged with *Eimeria* (Silva et al., 2009). Moreover, there were no effects of either supplementation with yeast cell walls or antibiotics or inoculations with *Campylobacter jejuni* on villus height in the duodenum and jejunum (Munoz et al., 2023). In another study, there was no effect of lipase on small intestine villus height (Al-Marzooqi and Leeson, 2000). Similarly, there was no effect of salinmysin or direct-fed microbials on either jejunal or ileal villus heights (Chichlowski et al., 2007). There were no effects of supplementation with either probiotics or synbiotics on duodenal villus heights (Awad et al., 2009). However, duodenal villus height was higher in broiler chickens on an antibiotic-free diet compared to one containing antibiotics (Koltes et al., 2017).

The coefficients of variance for villus height and crypt depths in the duodenum, jejunum and ileum between studies were relatively high. However, differences with treatment across studies are small (Tables 2 and 3). It is unclear the basis for these differences in a simple measure of length. It is possible these reflect differences in genetics or other management practices. However, it is argued that these are artifactual reflecting differences in fixation and other aspects of histological analysis (Ta-

bles 2 and 3). Determination of villus height, particularly in the jejunum, remains a very useful parameter indicative of small intestine health. It is recommended that this parameter be employed in studies of the effects of supplements and/or stressors and that investigators report on intra- and inter-study coefficients of variance.

Table 3. Characteristics of villus heights and crypt depths across multiple studies

Region of GI tract	Villus height in μm ± (n =) SEM [CoV]
Villus height	
Duodenum	1407 ± (14) 86.0 [22.9 %]
Jejunum	918 ± (13) 75.2 [29.6 %]
Ileum	616 ± (14) 40.1 [26.7 %]
	% Decrease in villus height in response to challenge % ± (n =) SEM [P <]
Duodenum	12.5 ± (6) 7.0 [P < 0.1]
Jejunum	16.6 ± (7) 2.05 [P < 0.001]
Ileum	7.7 ± (6) 4.2
	% Increase in villus height by supplementation compared to response to challenge % ± (n =) SEM [P <]
Duodenum	12.5 ± (6) 4.3 [P < 0.05]
Jejunum	14.3 ± (6) 5.5 [P < 0.05]
Ileum	14.2 + (9) 5.4 [P < 0.05]
	Crypt depths in μm ± (n =) SEM [CoV]
Jejunum	146 ± (3) 2.5
Ileum	130 ± (4) 15.7
	% Increase in crypt depths in response to challenge % ± (n =) SEM [P <]
Jejunum	126 ± (3) 102
Ileum	83.1 ± (4) 71.9
	% Increase in crypt depths by supplementation compared to response to challenge % ± (n =) SEM [P <]
Jejunum	14.1 ± (3) 2.1 [P < 0.01]
Ileum	4.4 ± (7) 4.6

Based on data in Supplementary Tables 1 and 2.
GI: Gastrointestinal.

A conclusion based on the above is that villus height in chickens appears to be a sensitive indicator of gastrointestinal integrity and, hence, digestibility and absorption (Scanes et al., 2022). Villus height in the duodenum has been demonstrated to be markedly greater in birds than in mammals (Scanes et al., 2022). Villus height reflects the balance between multiplication of mucosal cells in the crypt and loss of mucosal cells sloughed off from the tips of villi due, at least in part, to abrasion from ingesta (Cera et al., 1988; Silva et al., 2009). However, this is an over-simplification.

Villus crypts are the major site of both cell division and apoptosis (reviewed: Pritchard and Watson, 1996). There is also possibly some apoptosis at the villus tips (reviewed: Pritchard and Watson, 1996) and perhaps along the crypt–villus axis. There is evidence that there are increases in both villus cell apoptosis and shedding of intestinal epithelial cells in heat stressed rats (Gao et al., 2013). DON injury or heat stress negatively influences intestinal stem cell expansion along the crypt–villus axis *in vitro* (Zhou et al., 2020 b; 2022). Moreover, supplementation with methionine reduced the effects on DON induced cell injury *in vitro* (Zhou et al., 2019).

In conclusion, what are needed are *in vivo* studies determining the rates of the following: cell division, progression along the crypt–villus axis, apoptosis and shedding of mucosal cells into the lumen of the duodenum, jejunum, ileum, and ceca of chickens receiving supplements and/or challenged with stressors. In addition, the effect of feed supplements on gut health, villi height and crypt depth are different among various studies and affected by feed additives type, stressors, age and type of chickens that warrant further research.

Gastrointestinal integrity and leakiness (permeability)

Loss of gastrointestinal integrity allows the entry of pathogens and other microorganisms into the bloodstream with concomitant ill effects. Intestinal permeability can be estimated by transfer of fluorescein isothiocyanate dextran 4 kD from the intestinal lumen into the blood *in vivo* (Barekatain et al., 2019 a, b; Sharma et al., 2024) or *in vitro* (Song et al., 2014). Mucosal-to-serosal flux of fluorescein isothiocyanate dextran 4 kDa *in vitro* was depressed in heat stressed broiler chickens (Song et al., 2014). Stress increases intestinal permeability as is evidenced by the increased transfer of fluorescein isothiocyanate dextran 4 kD across the intestinal mucosa in broiler chickens receiving dexamethasone, the synthetic analogue of stress related glucocorticoid hormones (Barekatain et al., 2019 b). This was referred to as the "dexamethasone induced model of leaky gut" (Barekatain et al., 2019 a). Moreover, the marked loss of gastrointestinal integrity was indicated by a 48-fold increase in permeability in laying hens challenged with *Eimeria* (Table 4) (Sharma et al., 2024). There are also effects of pathogens on conductance, arguably reflecting permeability with increases in conductance in the jejunal and ceca following *Campylobacter jejuni* challenge (Awad et al., 2015).

It is recommended that consideration be given to determining gastrointestinal integrity and permeability as a parameter in studies of stressors and dietary supplement.

Table 4. Effects of stressors on gut permeability and pro-inflammatory cytokines and the ability of supplements to modulate these effects

Items	Control	Stressed	Stressed + Supplement	Bird type	Reference
Gut permeability					
Mixed *Eimeria* challenged for 6 d +/– *Artemisia annua*	0.01 a	0.48 b	0.36 b	♀ adult	Sharma et al., 2024
Gastrointestinal expression of inflammatory cytokines					
IL-10					
Jejunum					
Heat stressed 21–41 d +/– glutamine supplement	2.8 a	2.2 b	3.1 a	Broilers	Wu et al., 2018
Mixed *Eimeria* challenged for 6 d +/– *Artemisia annua*	1.0 a	4.1 b	4.0 b	♀ adult	Sharma et al., 2024
Ileum					
Heat stressed 21–41 d +/– glutamine supplement	2.4	2.1	3.2	Broilers	Wu et al., 2018
IFN-γ					
Jejunum					
Mixed *Eimeria* challenged for 6 d +/– *Artemisia annua*	1.0 a	10.4 b	10.0 b	♀ adult	Sharma et al., 2024
TNF-α					
Jejunum					
Heat stressed 21–41 d +/– glutamine supplement	1.0	2.0 b	1.1 a	Broilers	Wu et al., 2018
Mixed *Eimeria* challenged for 6 d +/– *Artemisia annua*	1.0 a	0.67 b	0.59 b	♀ adult	Sharma et al., 2024
Ileum					
Heat stressed 21–41 d +/– glutamine supplement	0.90	1.63	0.90	Broilers	Wu et al., 2018
Plasma concentrations ng mL^{-1}					
TNF-α					
LPS challenged +/– glutamine supplement	0.58 a	1.35 b	0.62 a	Broilers	Zhang et al., 2022
IL-1β					
LPS challenged +/– glutamine supplement	0.16 a	0.34 b	0.16 a	Broilers	Zhang et al., 2022

a, b – different letters in a row indicate difference ($P<0.05$).
LPS: lipopolysaccharide.
IL-1β: interleukin-1β.
IFN-γ: IL-10, interferon-γ.
TNF-α: tumor necrosis factor-α.

Tight junctions in the gastrointestinal tract

Tight junctions between cells form a physical barrier such as across the mucosa of the small intestine and ceca (reviewed: Awad et al., 2022). Tight junctions are composed of a series of specific (ZO) proteins including claudins, occludin, tricellulin, tight junction-associated ZO proteins and junction-adhesion molecules. The tight junctions between the mucosal cells on the surface of the villi prevent pathogens or toxicants passing from the lumen of the gastrointestinal tract into the hepatic portal blood vessels.

There are stress induced shifts in either the concentrations or expression of proteins that make up tight junctions between cells. The synthetic glucocorticoid, dexamethasone, increased the expression of Claudin3 and ZO-2 and reduced that of Claudin1 and junctional adhesion molecule 2 (Barekatain et al., 2019 a; b). Moreover, heat stress depressed jejunal expression of claudin-1, occludin and ZO-1 with the effects on claudin-1 and ZO-1 reversed by resveratrol supplementation (Zhang et al., 2017). Similarly, there is decreased expression of cell tight junction protein genes in the small intestine following administration of lipopolysaccharide (LPS), a major membrane component of Gram-negative bacteria and the effect is overcome at least partially by glutamine supplementation (Figure 1) (Zhang et al., 2022). In addition, there were depressed concentrations of occludin and ZO-1 in jejunal mucosa in heat stressed broiler chickens (Song et al., 2014).

It is suggested that consideration be given to employing expression of tight junction proteins as a parameter in studies of stressors and dietary supplement.

Goblet cells and mucus production

Another aspect of intestinal integrity is the protective effect of mucus produced by the goblet cells. Both goblet cell number and mucin-2 expression in the jejunum are depressed by heat stress and the effect is overcome at least partially by resveratrol supplementation (Zhang et al., 2017). Supplementation with betaine depressed mucus secretion, as indicated by sialic acid released and goblet cell number (dos Santosa et al., 2019). Moreover, the relative staining density of goblet cells in the ceca was markedly depressed (from 1.0 to 0.23) in broiler chickens infected with *Eimeria* compared to uninfected controls *(*Zhou et al., 2020).

Inflammatory responses

Overview of inflammatory responses

It would seem axiomatic that inflammatory in the gastrointestinal tract would be disadvantageous to the animal with problems such as reductions in digestibility and passage of pathogens and toxicants into the hepatic portal vein. There was increased growth rate in broiler chickens receiving feed supplemented with acetylsalicylic acid (aspirin) (Tavakoli et al., 2022), this being an anti-inflammatory compound (reviewed e.g. Jack, 1997; Montinari et al., 2019; Werz et al., 2024).

Cytokines

There are effects of stressors or stressors plus supplements on pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-10, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). Table 4 summarizes studies reporting on effects of stressors in the presence or absence of supplements on the gastrointestinal expression and circulating concentrations of pro-inflammatory cytokines. Plasma concentrations of TNF-α and IL-1β were elevated in LPS challenged broiler chickens with the effect overcome by supplementation with glutamine (Zhang et al., 2022) (Table 4). Heat stress in broiler chickens was followed by decreased jejunal and ileal expression of IL-10 Moreover, challenge with mixed *Eimeria* was followed by shifts in jejunal expression of IL-10 (increased), IFN-γ and TNF-α (decreased) in laying hens (Sharma et al., 2024) (Table 4). In contrast, supplementation with the botanic, *Artemisia annua*, did not influence the effect on the expression of pro-inflammatory cytokines in birds subjected to stressors (Sharma et al., 2024) (Table 4).

Both splenic TNF-α immuno-reactivity and expression of IL-1β were increased following imidacloprid challenge while expression of IL-10 was decreased (Eleiwa et al., 2023). These effects were overcome by addition of curcumin to the feed (Eleiwa et al., 2023).

It is recommended that pro-cytokine expression and protein concentrations be determined in studies of stressors and dietary supplement.

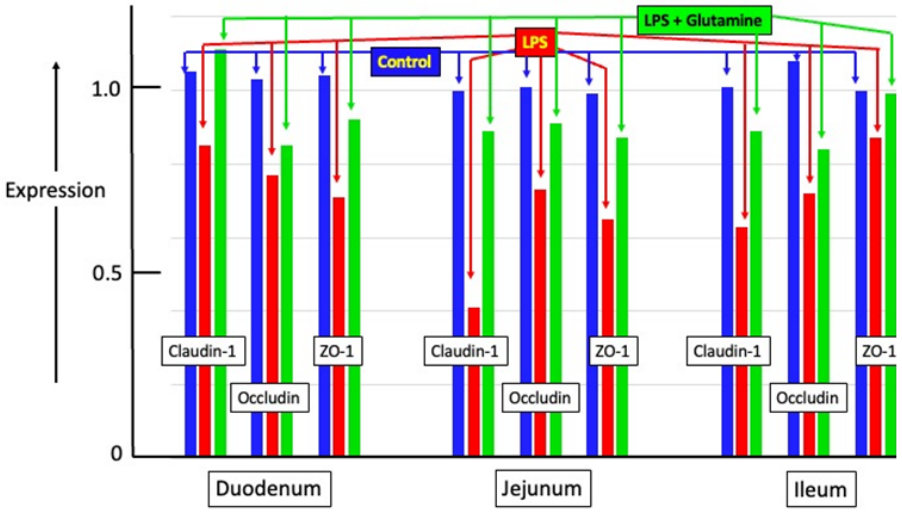

Figure 1. Effects of the stressor, lipopolysaccharide (LPS), alone or with glutamine supplementation on expression of tight junction proteins, claudin-1, occludin and ZO-1 (Zonula occludens 1) in broiler chickens (data from Zhang et al., 2022)

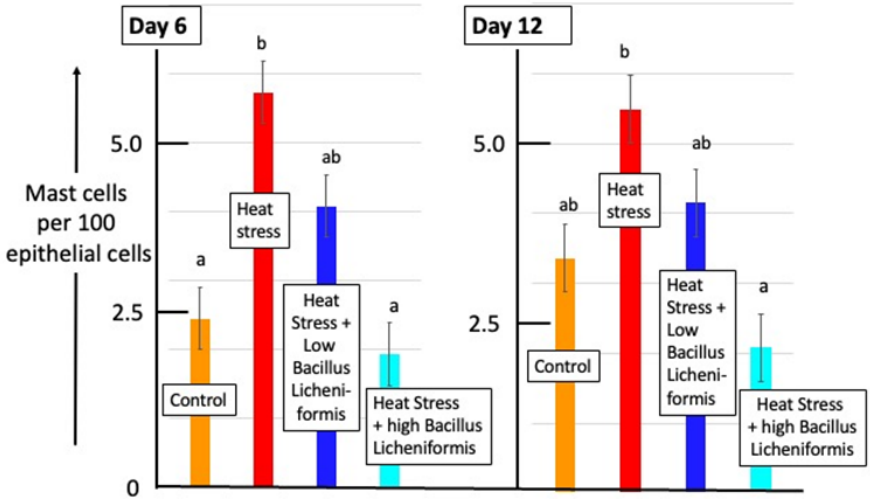

Figure 2. Effects of heat stress and high and low concentrations of probiotic on the numerical density of mast cells in the ileal epithelium (data from Deng et al., 2012)

Mast cells

Mast cell numerical densities were increased in heat stressed chickens with probiotics ameliorating these (Figure 2) (Deng et al., 2012). Mast cells have an important role in evoking inflammation releasing histamine and pro-inflammatory cytokines including TNF-α, IL-10 and IFN-α, IFN-β, IFN-γ (reviewed: Theoharides et al., 2012; Galli et al., 2020). It is suggested that future studies include intestinal concentrations of histamine as this may be a useful measure of inflammatory effects of stressors and the ability of supplements to overcome these. It should be emphasized that there are marked age related increases and major regional differences in histamine concentrations in the small intestine and ceca in chickens (Lyte et al., 2022).

Toll-like receptor 4 (TLR-4)

Another aspect of inflammation is Toll-like receptor 4 (TLR-4). There was an increase in TLR-4 immune reactivity in the spleen in chickens chronically challenged with the insecticide imidacloprid, and the effect was partially overcome by a feed supplemented with curcumin (Eleiwa et al., 2023). It is noted that the inflammatory responses of young turkeys to a *Salmonella typhimurium* challenge was markedly reduced when birds received antibodies against TLR-4 (Scanes et al., 2009).

It is suggested that consideration be given to employing expression of TLR-4 as a parameter in studies of stressors and dietary supplement.

Antioxidant status

Overview of antioxidant status

There are marked effects of both stressors and supplements on antioxidant status. Figure 3 summarizes the antioxidant/oxidant system including reduced and oxidized glutathione. For instance, there were increases in the glutathione concentration in the uterus in hens supplanted with betaine (Guo et al., 2023). Moreover, liver glutathione peroxidase activities were elevated in chickens supplemented with betaine or antioxidants (vitamin E and selenium) with considerably greater effects in cyclically heat stressed chickens (Shakeri et al., 2018).

Oxidative damage of lipids results in the formation of malondialdehyde (MDA) and, hence, concentrations of MDA are a marker of oxidative stress. Glutathione together with glutathione peroxidase (GPX) act as antioxidant scavengers. In young chickens receiving aflatoxin B_1 (AFB_1) in their feed, there are increases in duodenal concentrations of a product of lipid peroxidation, MDA (Figure 3) and 8-hydroxy-2′-deoxyguanosine from 2′-deoxyguanosine (Cheng et al., 2020). Moreover, there were decreases in the duodenal activities of both superoxide dismutase (SOD) and catalase (CAT) (Cheng et al., 2020), these being part of the pathway for breakdown of hydrogen peroxide (Figure 3). These changes in these markers of oxidative damage were completely overcome when the chickens also received supplementation with curcumin (Cheng et al., 2020). Curcumin is a polyphenol derived from turmeric, a rhizomatous plant (*Curcuma longa*) (reviewed: Hewlings and Kalman, 2017). Curcumin is established to decrease oxidative and inflammatory damage (reviewed: Hewlings and Kalman, 2017).

In a similar manner, heat stressed broiler chickens had increased hepatic concentrations of MDA but reduced concentrations of glutathione together with lower activities of both SOD and GPX (Wen et al., 2021). Betaine supplementation reversed these changes in antioxidant markers (Wen et al., 2021). Similarly, antioxidative status (SOD, CAT, GPX activities together with MDA) was depressed in broiler chickens treated with imidacloprid with these effects being overcome, at least partially, by supplementation with curcumin (Herrero-Encinas et al., 2020).

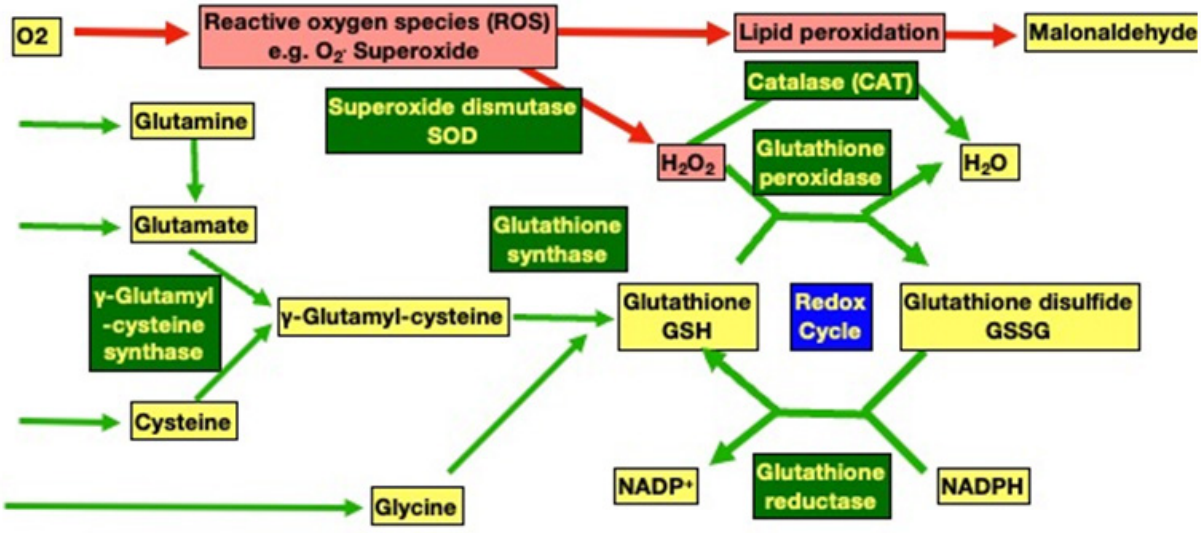

Figure 3. Schematic diagram of glutathione and antioxidant status

Table 5. Ability of betaine supplementation to overcome the effects of stressors on the immune system in chickens

Parameter	Treatment		
	control	stressed	
Items		control	betaine
		***Eimeria acervuline* challenged**	
Leukocytes/villus^{-1} in duodenum[P]	10.4 a	24.0 b	32.0 c
Leukocytes in lamina propria under 10 villi^{-1} in duodenum[P]	41.6 a	77.7 b	81.3 b
		Heat stressed	
Spleen weight %[R]	0.140 a	0.107 b	0.124 a
Erythrocyte concentration 10^6 mm^{-3}[S]	1.52 a	1.25 b	1.47 a
Leucocyte concentration 10^3 mm^{-3}[S]	26.3 a	22.5 c	24.3 b
Heterophil/Lymphocyte ratio[S]	0.74 a	0.91 b	0.81 a

a, b, c – different letters in a row indicate difference (P<0.05).
[P]Data from Klasing et al. (2002).
[R]Data from adult laying hens from Attia et al. (2016).
[S]Data from adult male chickens from Attia et al. (2016).

Table 6. Examples of the ability of supplementation to overcome the effects of oxidative stressors in chickens

Items	Control	Stressor	Stressor + Supplement	Bird type	Reference
Liver		**Heat**	**Heat + Betaine**		
GSH mg g protein^{-1}	9.6 a	7.8 b	9.1 a	Broilers	Wen et al., 2021
GSSG mg g protein^{-1}	1.8	2.0	1.9	Broilers	Wen et al., 2021
MDA nmole mg protein^{-1}	3.0 b	4.7 a	3.3 b	Broilers	Wen et al., 2021
SOD units mg protein^{-1}	48.3 a	40.1 b	47.0 a	Broilers	Wen et al., 2021
GPX units mg protein^{-1}	49.9 ab	43.1 b	56.9 a	Broilers	Wen et al., 2021
GR units mg protein^{-1}	8.1	7.7	7.7	Broilers	Wen et al., 2021
Spleen		**Imidacloprid**	**Imidacloprid + Curcumin**		
TAC mM	0.51 a	0.22 c	0.28 b	Broilers	Eleiwa et al., 2023
MDA nmole g^{-1}	39.3 a	70.7 c	48.3 a	Broilers	Eleiwa et al., 2023
CAT units g^{-1}	5.7 a	2.0 b	4.0 a	Broilers	Eleiwa et al., 2023
SOD units g^{-1}	277 a	229 c	251 b	Broilers	Eleiwa et al., 2023
GPX units g^{-1}	28.1 a	13.9 c	20.7 b	Broilers	Eleiwa et al., 2023

a, b, c – different letters in a row indicate difference (P<0.05).
GSH: plasma glutathione peroxidase, GSSG: glutathione disulfide, MDA: malondialdehyde, SOD: superoxide dismutase, GPX: glutathione peroxidase, GR: glucocorticoid receptors, CAT: catalase activity, TAC: T cell antigen coupler.

Serum concentrations of MDA and oxidized glutathione were depressed in chickens supplemented with curcumin (Zhang et al., 2019). However, reduced glutathione concentrations in the jejunum were increased in chickens supplemented with curcumin (Zhang et al., 2019). Similarly, there were increases in reduced glutathione concentrations in both the liver and jejunum in birds receiving bisdemethoxycurcumin supplementation (Zhang et al., 2019).

In other studies, there were no effects of supplementation with lysine and/or phytase on anti-oxidant status in laying hens as indicated by serum activities of GPX and total SOD activity together with concentrations of MDA (Baghban-Kanani et al., 2018). Moreover, despite increased consumption of feed, there were no effects of supplementation with a spectrum of digestive enzymes on plasma glutathione peroxidase (GSH) activities or MDA concentrations in hens (Baghban-Kanani et al., 2020).

It is suggested that consideration be given to employing anti-oxidative parameters in studies of stressors and dietary supplements.

Cytochrome (CYP) P450 enzymes

Cytochrome (CYP) P450 enzymes play an important role in oxidation and detoxification of xenobiotics (reviewed: Shankar and Mehendale, 2014). Aflatoxin B_1 administration to young chickens was accompanied by increases in the duodenal concentrations of CYP3A4, CYP2A6, CYP1A1, and CYP1A2 (Cheng et al., 2020). All these increased were blocked by supplementation with curcumin (Cheng et al., 2020).

Leukocytes

Some stressors affect the numbers of leukocytes in the circulation. For instance, heat stress depressed both the numbers of leukocytes and the percentage of lymphocytes in adult male chickens while also increased the percentage of heterophils (Table 5) (Attia et al., 2019). These effects of heat stress were reversed by supplementation with betaine supplementation (Attia et al., 2019). The ratio of heterophils to lymphocytes (H:L) is a measure of stress (Gross and Siegel, 1983). As might be expected, the H:L ratio is increased with heat stress with this effect being overcome by betaine supplementation (Table 5) (Attia et al., 2019). Similarly, challenge with the insecticide imidacloprid was followed by shifts in the H:L ratios based on the relative concentrations of heterophils (increased) and lymphocytes (decreased) with the effects overcome by feeding curcumin as a supplement (Eleiwa et al., 2023).

Another example of a protective effect with betaine is with *Eimeria acervuline*. Betaine supplementation *per se* had no effect on the number of leukocytes in the duodenal villi and lamina propria (Table 5) (Klasing et al., 2002). There were marked increases in the number of leukocytes in the duodenal villi and lamina propria in young chickens challenged with *Eimeria acervuline* (Table 5) (Klasing et al., 2002). In chicks receiving betaine supplementation, there were greater numbers of leukocytes in the duodenal villi (days 2 and 7) and lamina propria (days 2 and 4) (Table 5) (Klasing et al., 2002).

It is suggested that consideration be given to employing number of leukocytes as a parameter in studies of stressors and dietary supplement.

Conclusions

In conclusion, stressors exert cellular and molecular effects on the gastrointestinal tract including villus height, crypt depths, expression of tight junction protein genes, pro-inflammatory cytokines, number and functioning of mast cells, goblet cells and leukocytes and the antioxidant system. It is recommended that research on supplements focus on stressed poultry. It is also suggested that where poultry are raised under "less than ideal" conditions, supplementation is a useful approach for the poultry industry.

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Author contributions

A.S. and C.G.S coordinated the project. A.S. and C.G.S wrote the main part of the principal draft, to which B.H-G.; F.Z.; O.V.K.; E.V.S. and G.K.D had contributed. All authors approved the final draft. All authors have read and agreed to the published version of the manuscript.

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