Review Article

Identifying the shortfalls of crude protein-reduced, wheat-based broiler diets

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A B S T R A C T

The objective of this review is to identify the shortfalls of wheat-based, crude protein (CP)-reduced diets for broiler chickens as wheat is inferior to maize in this context but to inconsistent extents. Inherent factors in wheat may be compromising gut integrity; these include soluble non-starch polysaccharides (NSP), amylase trypsin inhibitors (ATI) and gluten. Soluble NSP in wheat induce increased gut viscosities, which can lead to compromised gut integrity, which is not entirely ameliorated by NSP-degrading feed enzymes. Wheat ATI probably compromise gut integrity and may also have the capacity to increase endogenous amino acid flows and decrease apparent starch and protein digestibilities. Gluten inclusions of 20 g/kg in a maize-soy diet depressed weight gain and feed intake and higher gluten inclusions have been shown to activate inflammatory cytokine-related genes in broiler chickens. Further research is required, perhaps particularly in relation to wheat ATI. The protein content of wheat is typically higher than maize; importantly, this results in higher inclusions of non-bound amino acids in CP-reduced broiler diets. These higher inclusions could trigger post-enteral amino acid imbalances, leading to the deamination of surplus amino acids and the generation of ammonia (NH_3) which, if not adequately detoxified, results in compromised growth performance from NH_3 overload. Thus, alternatives to non-bound amino acids to meet amino acid requirements in birds offered CP-reduced, wheat-based diets merit evaluation. The digestion of wheat starch is more rapid than that of maize starch which may be a disadvantage as the provision of some slowly digestible starch in broiler diets may enhance performance. Alternatively, slowly digestible starch may result in more de novo lipogenesis. Therefore, it may prove instructive to evaluate CP-reduced diets based on maize-wheat and/or sorghum–wheat blends rather than entirely wheat. This would reduce non-bound amino acid inclusions by lowering dietary CP derived from feed grains and may enhance starch digestive dynamics by retarding starch digestion rates. Also, the use of biomarkers to monitor gut integrity in broiler chickens is examined where calprotectin, ovo-transferrin and possibly citrulline appear to hold promise, but their validation requires further research.
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1. Introduction

There is an active quest to develop crude protein (CP)-reduced diets that will meet practical acceptance as this, in many countries of the world, would reduce the chicken-meat industry's dependence on imported soybean meal. CP-reduced diets have the potential to halve the industry's dependence on imported soybean meal; thereby, enhancing its sustainability. However, the idea of reducing dietary CP is certainly not new; perhaps unwittingly, it has

been practiced for decades following the introduction of synthetic D,L-methionine more than 60 years ago. The inclusion of 4.3 g/kg non-bound amino acids (lysine·HCl, methionine, threonine) was shown to reduce the CP content of a maize-soy diet from 356 to 200 g/kg with a radical reduction in the dietary soybean meal from 709 to 293 g/kg as demonstrated by Pesti (2009).

Moreover, CP-reduced broiler diets have the potential to provide additional advantages extending to the environment with reduced nitrogen and ammonia emissions, litter quality, bird welfare and flock health (Greenhalgh et al., 2020a) and may extend to food safety. The impacts of reducing dietary CP levels from 225 to 205 and 185 g/kg in diets on faecal microflora were investigated by Laudadio et al. (2012). Faecal concentrations of *Escherichia coli* were reduced by 23.7% (2.97 versus 3.89 log₁₀ colony forming units [CFU]/g) at 42 d post−hatch, which is advantageous from the standpoint of food safety.

Broiler diets are frequently based on maize or wheat; however, wheat is less suitable than maize as the basis of reduced CP (Chrystal et al., 2021), which complicates the tangible challenges to their further development (Chrystal et al., 2020a; Liu et al., 2021a). Thus, this review is an attempt to identify the factors underlying the shortfalls of wheat-based diets in relation to broiler growth performance following reductions in dietary CP concentrations, so that they may be addressed. Importantly, the effects of any shortfalls in wheat will be only amplified in CP-reduced diets because of increased dietary wheat and starch inclusions.

2. Background: maize versus wheat

Maize and wheat are the 2 commonly used feed grains in global chicken-meat production. However, wheat has a higher CP content (118 versus 91 g/kg) than maize and their amino acid profiles differ. Relative to lysine (100), wheat grown in Australia contains more glutamic acid (145) and glycine (117), but less aspartic acid (79), methionine (75), leucine (51) and alanine (47) than maize (Fickler et al., 2016). As a consequence of these differences, wheat-based, CP-reduced diets contain more non-bound amino acids with greater proportions relative to protein-bound amino acids, which is probably disadvantageous (Selle et al., 2022).

The impact of dietary CP concentrations in maize-based versus wheat-based diets have been directly compared in 2 studies (Chrystal et al., 2021; Greenhalgh et al., 2022a). In both studies, wheat supported better weight gain and FCR in standard CP diets, but this position was reversed following dietary CP-reductions, as shown in Table 1, although the reductions differed in magnitude. Maize was clearly superior to wheat in terms of growth performance, but not fat deposition, in Chrystal et al. (2021) following dietary CP reductions and the same pattern was evident, but to far less pronounced extents, in Greenhalgh et al. (2022). The dietary CP reduction in wheat-based diets from 222 to 165 g/kg compromised weight gain by 35.5% (1,549 versus 2,403 g/kg) and FCR by 26.6% (1.840 versus 1.453) in Chrystal et al. (2021). In contrast, the dietary CP reduction from 220 to 180 g/kg in wheat-based diets depressed weight gain by 7.72% (2,510 versus 2,720 g/bird) and FCR by 9.82% (1.443 versus 1.314) in Greenhalgh et al. (2022a). A non-starch polysaccharides (NSP)-degrading enzyme was included across all diets in the Greenhalgh et al. (2022a) study, which was not the case in the Chrystal et al. (2021) study, and this may have contributed to the differences in responses observed.

Again, remarkably different outcomes were recorded in 2 similar studies when broiler chickens were offered CP-reduced diets based on wheat, as shown in Table 2. However, it is relevant that both exogenous phytase and xylanase were included across all dietary treatments in the 2 studies. In Yin et al. (2020), the dietary CP reduction from 215 to 165 g/kg fractionally increased weight gain by

0.48% (2,106 versus 2,096 g/bird), compromised FCR by 4.74% (1.546 versus 1.476) and increased relative fat-pad weights by 21.2% (8.87 versus 7.32 g/kg). In contrast, the dietary CP reduction from 197.5 to 162.5 g/kg depressed weight gain by 48.4% (1,010 versus 1,958 g/bird), negatively impacted FCR by 44.1% (2.426 versus 1.684) and decreased relative fat-pad weights by 18.9% (6.79 versus 8.37 g/kg) in Greenhalgh et al. (submitted for publication). It may be deduced from 3 of the above studies that a CP reduction from 210 to 160 g/kg would trigger a 33.8% increase in dietary wheat inclusions from 568 to 760 g/kg on the basis of least-cost formulations, so the high and low CP diets have substantially different compositions.

The immensely different outcomes in the 2 sets of feeding studies are intriguing and an explanation should prove instructive. It was proposed that 'ammonia (NH_3) overload' may have contributed to the extraordinarily poor performance in birds offered 180.0 and 162.5 g/kg CP, wheat-based diets in the Greenhalgh et al. (2020b) study. The superiority of maize versus wheat in the Chrystal et al. (2021) study may have also been due partially to 'NH₃ overload', triggered by the higher inclusions of non-bound amino acids in reduced-CP, wheat-based diets. As discussed later, this proposal was validated to some extent by Selle et al. (2021a), with retrospective analyses of uric acid concentrations in excreta from the Chrystal et al. (2021) study. The more modest advantages held by maize over wheat in Greenhalgh et al. (2022) and the quite reasonable performance of birds offered 165 g/kg CP, wheat-based diets in Yin et al. (2020) suggest that NH₃ overload may not have been involved and that other factors may have been engaged in relatively moderately depressed growth performance.

The inclusion of antibiotic growth promoters (AGP) in poultry diets is declining, which was initiated by the banning of the practice in Europe in 2006. The declining global usage of AGP represents a challenge to the sustainability of chicken-meat production (Cervantes, 2015). This situation has prompted a search for viable alternatives to AGP and has increased the focus on 'gut integrity' in broiler chickens. Indeed, Adedokun and Olojede (2019) contended that maintaining gut integrity in broiler chickens would continue to be a challenge for the foreseeable future. It is possible that wheat-based, CP-reduced diets compromise gut integrity to greater extents than maize. Therefore, in this review, initial consideration is given to the inherent factors in wheat which may be compromising gut integrity, which include soluble NSP, amylase trypsin inhibitors (ATI) and gluten. Therefore, the feasibility of using biomarkers to monitor gut integrity in broiler chickens is examined. This is followed by a consideration of the relatively high protein content of wheat and its implications, including NH₃ overload, and starch digestive dynamics.

3. Influential factors and biomarkers for gut integrity

Since the AGP ban in Europe, the chicken-meat industry has experienced a rise in gut integrity problems under the rather nebulous collective description of 'dysbacteriosis'. Dysbacteriosis has been defined as the presence of abnormal microbiota in the proximal small intestine, capable of reducing nutrient digestibility, impairing intestinal barrier function and increasing the risk of inflammatory responses (Teirlynck et al., 2011). Interestingly, NSP were given priority as a feed component that triggers gut inflammation in poultry by Dal Pont et al. (2020), the genesis of which appears to stem mainly from increases in gut viscosity. In this section the focus is on soluble NSP, ATI and gluten contents in wheat.

3.1. Soluble non-starch polysaccharides

Soluble NSP are hydrocolloids that strongly interact with water and effectively act as a thickening agent (Karaman et al., 2014). In

Table 1
Impact of dietary crude protein (CP) concentrations in maize-based diets relative to wheat-based diets on weight gain, FCR and relative abdominal fat-pad weight from d 7 to 35 post−hatch.

CP, g/kg	Weight gain, g/bird			FCR			Relative fat-pad weight, g/kg		
	Maize	Wheat	Response	Maize	Wheat	Response	Maize	Wheat	Response
222[1]	2,214	2,403	−7.87%	1.453	1.453	0.00%	6.40	6.40	0.00%
193	2,396	2,386	+0.42%	1.415	1.471	−3.81%	11.10	8.50	+30.60%
165	2,370	1,549	+53.00%	1.473	1.840	−19.90%	12.80	7.50	+70.70%
220[2]	2,690	2,720	−1.10%	1.338	1.314	+1.83%	8.02	6.19	+29.60%
180	2,598	2,510	+3.51%	1.385	1.443	−4.02%	10.16	7.78	+30.60%

[1] From Chrystal et al. (2021).
[2] From Greenhalgh et al. (2022a).

Table 2
Impact of dietary crude protein concentrations in wheat-based diets on weight gain, FCR and relative abdominal fat-pad weight from d 14 to 35 post−hatch.

CP, g/kg	Weight gain		FCR		Fat-pad weight	
	g/bird	Response	FCR	Response	g/kg	Response
215.0[1]	2,096		1.476		7.32	
165.0	2,106	+0.48%	1.546	+4.74%	8.87	+21.20%
197.5[2]	1,958		1.684		8.37	
180.0	1,451	−25.90%	1.878	+11.50%	8.14	−2.75%
162.5	1,010	−48.40%	2.426	+44.10%	6.79	−18.90%

[1] From Yin et al. (2020).
[2] From Greenhalgh et al. (2020b).

broiler chickens this water holding capacity increases digesta viscosity along the gastrointestinal tract. Decades ago, β-glucan, a soluble NSP in barley, was shown to increase digesta viscosity and compromise broiler performance but this could be counteracted by exogenous enzymes as demonstrated by Burnett (1966). Wheat, rye, barley and oats have high soluble NSP levels which increase digesta viscosity, decrease digesta passage depress digestive enzymatic activities and nutrient digestibility and compromise growth performance (Yegani and Korver, 2008). Increased digesta retention time facilitates bacterial colonisation and activity in the small intestine (Vahjen et al., 1998).

Importantly, wheat contains higher levels of soluble NSP than maize by a 2.8-fold factor of (25 versus 9 g/kg) on a dry matter basis (Bach Knudsen, 1997) and this is a critical difference between the 2 feed grains. Extraordinary efforts have been devoted to the anti-nutritive effects of soluble NSP in wheat in broiler chickens and, ostensibly, their anti-nutritive effects can be counteracted by inclusions of NSP-degrading feed enzymes, which is routinely practiced. The capacity of soluble NSP to increase ileal viscosity linearly ($P = 0.011$) was recently reported by Nguyen et al. (2021). However, it is noteworthy that Munyaka et al. (2016) found that wheat-based diets generated higher average gut viscosities by 61.3% (3.08 versus 1.91 mPa·s) than maize-based diets in broiler chickens, irrespective of NSP-degrading enzyme inclusions. Therefore, while the routine inclusions of NSP-degrading enzymes in reduced-CP, wheat-based diets will attenuate the anti-nutritive properties of soluble NSP and reduce gut viscosity, the likelihood is that corresponding maize-based diets will retain an advantage in this respect.

Both the morphology of the gastrointestinal tract and the gut microbiota in boiler chickens are impacted by dietary NSP (Nguyen et al., 2022). The inclusion of an NSP-degrading enzyme in wheat-based diets offered to broiler chickens significantly increased intestinal villus height by 22.6% (1,668 versus 1,360 μm) and decreased crypt depth by 6.09% (108 versus 115 μm) as reported by Yaghobfar and Kalantar (2017). Enzyme inclusion significantly reduced digesta viscosity by 26.3% (1.60 versus 2.17 cP; 1 cP = 0.001 Pa·s), significantly increased feed intake by 2.14% (4,253 versus 4,164 g/bird) and numerically improved weight gain and FCR from

1 to 42 d post−hatch in this study. Thus, soluble NSP have the capacity to alter gut morphometry, presumably by increased gut viscosity. Elevating dietary soluble NSP levels increased ileal viscosity in broilers by 24.3% (3.493 versus 4.343 cP) in Nguyen et al. (2021), which was associated with an increase (7.541 versus 7.247 $\log_{10}$ genomic data commons DNA copies/g; 1 cP = 0.001 Pa·s) in *Lactobacillus* in ileal digesta. Interestingly, *Lactobacilli* constituted 84.2% of total anaerobic bacteria in the ileum in this study. A similar *Lactobacilli* majority in ileal digesta was in birds offered wheat-based diets was reported by Munyaka et al. (2016).

The likelihood is that increased gut viscosities are associated with increases in gut microbiota and in the small intestine where *Lactobacilli* are dominant. Broilers offered rye-based diets, a more 'viscous' cereal grain than maize, had greater ileal anaerobe counts than maize-based diets by 2 or 3 logarithmic cycles in Wagner and Thomas (1977). Accordingly, Hübener et al. (2002) found that a wheat/rye diet supported more CFU of mucosa associated bacteria in comparison to a maize diet with notable increases in enterobacteria and enterococci CFU. These researchers concluded that dietary cereals producing high intestinal viscosities lead to increased overall bacterial activity in the small intestine. Rye inclusions of 50 and 100 g/kg in maize-based broiler diets were investigated by Van Krimpen et al. (2017). The study reported that ileal gut morphology, microbiota composition of jejunal digesta, and jejunal gene expression profiles were impacted by rye inclusions. Also, Choct et al. (1996) found that there was extensive small intestinal fermentation in birds offered NSP-enriched diets from microscopic examinations and considered that this is partly responsible for the anti-nutritive effects soluble NSP.

That soluble NSP in wheat-based diets increase gut viscosities and, in turn, increase gut microbiota, which has important implications for the apparent digestibility coefficients of amino acids. Importantly, CP-reduced diets generate perturbations in apparent amino acid digestibility coefficients (Liu et al., 2021a) and any increases in microbial amino acids in distal ileal digesta, relative to dietary and endogenous amino acids, would compromise apparent digestibility coefficients. In Greenhalgh et al. (2022), where 180 g/kg CP maize-based diets outperformed wheat, maize supported higher apparent amino acid digestibilities than wheat. This appeared to be related to estimated increases microbial amino acid proportions in distal ileal digesta in birds offered wheat-based diets as deduced by the Duvaux et al. (1990) model. Significant, negative Pearson correlations between microbial amino acid proportions in distal ileal digesta and apparent ileal digestibility coefficients of all 16 amino acids assessed were detected. Pearson correlations ranged from proline ($r = -0.421$; $P = 0.001$) to alanine ($r = -0.833$; $P < 0.001$) in the Greenhalgh et al. (2022) study in which both phytate- and NSP-degrading enzymes were included across all dietary treatments. Thus, it appears that soluble NSP in wheat-based, CP-reduced diets, despite NSP-degrading enzyme inclusions, remain a threat to gut integrity and may have a negative impact on

amino acid digestibilities. Soluble NSP can compromise amino acid digestibilities directly because increased digesta viscosity in the small intestine impedes the digestion of protein and absorption of amino acids and indirectly from increases in microbial amino acids in distal ileal digesta (Ravindran et al., 1999; Selle et al., 2009). Nevertheless, the use of NSP-degrading enzyme should remain routine and the most appropriate inclusion rates should be given consideration.

3.2. Amylase trypsin inhibitors

The capacity of wheat ATI to inhibit mammalian digestive enzymes, specifically α-amylase and trypsin, under in vitro conditions has been reported by Cuccioloni et al. (2016). Amylase trypsin inhibitors were detected and quantified in a wide range of wheat cultivars via discovery proteomics and data mining by Bose et al. (2020) and the synthesis and accumulation of amylase-trypsin inhibitors during grain development of bread wheat has been documented by Call et al. (2021). Amylase trypsin inhibitors are low-molecular-weight proteins (12,000 to 15,000 Da) and make up about 4% of the wheat proteome. Wheat ATI were reported to drive intestinal inflammation via activation of toll-like receptor 4 (TLR4) in human and murine cells by Junker et al. (2012). Similarly, Zevallos et al. (2017) found that wheat ATI activate TLR4 and increase intestinal inflammation by activating gut and mesenteric lymph node myeloid cells in mice. These researchers also reported that gluten-containing cereals have by far the highest concentrations of ATI and orally ingested ATI are largely resistant to proteases.

In theory, wheat ATI could cause compensatory increases in pancreatic secretions of amylase and trypsin which would increase endogenous amino acid flows and may even depress starch and protein digestibility. Soy trypsin inhibitor activity has been shown to depress digestibilities of all essential and non-essential amino acids assessed in a linear manner in broiler chickens (Kuenz et al., 2022). The inherent phytase activity of wheat is denatured by pepsin in the acidic conditions of the proventriculus (Phillippy, 1999), but it appear that this does not apply to wheat ATI and soy trypsin inhibitors. It appears that wheat ATI are resistant to both thermal processing and proteolysis (Call et al., 2019). It is likely that wheat ATI trigger gut inflammation in poultry and it is possible that ATI depress starch and protein digestibility given sufficient concentrations in wheat. Arguably, both aspects merit further investigations.

3.3. Gluten

Gluten is an insoluble storage protein, or prolamin, in wheat and is subdivided into gliadins and glutenins. Gluten comprises 80% to 85% of the wheat proteome (Van Der Borght et al., 2005), so wheat would typically contain in the order of 100 g/kg glutelin. The amino acid composition of gluten, glutenin and gliadin have been determined by Rumbouts et al. (2009), as shown in Table 3, where it is evident that gluten contains a paucity of lysine. Gluten contains relatively high levels of glutamate, glutamine, serine, proline and phenylalanine in respect of amino acid requirements for broiler chickens.

Ingestion of wheat gluten elicits an immune response and small intestinal inflammation in genetically predisposed human subjects (Schuppan et al., 2009), which has been the subject of considerable research. However, more specifically, gliadin has the capacity to compromise the integrity of small intestinal tight junctions by inducing inflammation and increasing intestinal permeability (De Punder and Pruimboom, 2013). Gliadin, a component of gluten, has been demonstrated to increase permeability in human Caco-2

Table 3
Amino acid concentrations of wheat gluten, glutelin and gliadin, and amino acid profile relative to lysine (100).[1]

Amino acid, μmol/g	Gluten	Glutelin	Gliadin	Gluten profile
Arginine	245	240	215	223
Histidine	130	135	125	118
Isoleucine	315	305	345	286
Leucine	550	550	580	500
Lysine	110	155	45	100
Methionine	100	105	90	91
Phenylalanine	335	310	380	305
Threonine	215	255	170	196
Valine	415	425	395	377
Alanine	270	315	225	245
Aspartate/asparagine	215	250	170	196
Cysteine	170	150	180	155
Glutamate/glutamine	2450	2180	2510	2227
Glycine	420	560	215	382
Proline	1080	965	1275	982
Serine	440	620	320	400
Tyrosine	220	235	195	200

[1] From Rombouts et al. (2009).

intestinal epithelial cells to small molecules (4 kDa) by reorganizing actin filaments and altering expression of junctional complex proteins in humans with Coeliac disease (Sander et al., 2005).

In broiler chickens, dietary additions of 50 and 75 g/kg gluten have been shown to depress growth performance (Afshar and Moslehi, 2006). In this study, 75 g/kg gluten depressed weight gain by 38.2% (1,373 versus 2,220 g/bird) to 49 d post–hatch. The addition of 20 g/kg gluten to a maize-soy broiler diet was investigated by Fang et al. (2017). Gluten significantly depressed feed intake by 6.21% (3,656 versus 3,898 g/bird) and numerically depressed weight gain by 8.37% (1,882 versus 2,054 g/bird) and FCR by 2.65% (1.94 versus 1.89) from 1 to 42 d post–hatch. In the Chrystal et al. (2021) study, the stepwise reduction of dietary CP from 222 to 193 and 165 g/kg CP increased wheat inclusions from 525 to 637 and 751 g/kg. This would correspond to approximate increases of 21% and 43% glutelin in the 2 reduced-CP diets; thus, the Fang et al. (2017) assessment of 20 g/kg gluten is relevant. Substantially higher gluten inclusions in broiler diets of 0, 250 and 500 g/kg were investigated by Kang et al. (2019) from d 1 to 7 and from d 21 to 28 post–hatch. Growth performance was seriously compromised and was accompanied by increased mRNA expression of genes related to inflammation including interleukins and tumour necrosis factor-α (TNF-α). This infers that gluten may have the capacity to trigger gut inflammation and compromise growth performance in poultry.

3.4. Biomarkers for gut integrity

The identification of biomarkers to monitor gut integrity in broiler chickens would be a distinct advantage (Ducatelle et al., 2018). This probably particularly applies when gut integrity is challenged by the inherent contents of soluble NSP, ATI and gluten in wheat. However, Banerjee (2014) argued that a void still exists for a robust gastrointestinal damage marker in poultry, despite the very real need for such agents, and Niewold (2015) suggested that a greater research effort needs to be invested in this field. Biomarkers of gastrointestinal functionality in poultry and livestock were considered by Celi et al. (2019). Their contention was that the use of a single biomarker might not be feasible because of the complexity of the interactions between the key components of gastrointestinal functionality. Therefore, the development of a suite of biomarkers of may be required to monitor the functionality and health of the gastrointestinal tract, but also of animal performance, health and welfare.

The capacity of enteric pathogens to disrupt the intestinal barrier via the alteration of tight junctions in chickens has been reviewed by Awad et al. (2017). De Meyer et al. (2019) sought to identify intestinal biomarkers in a gut leakage model in broilers. Twelve candidate protein biomarkers were identified in ileal contents and ten candidates in colonic contents. Serum endotoxin and α1-acid glycoprotein may be potential biomarkers for gut barrier health (Chen et al., 2015). Fibronectin, intestinal alkaline phosphatase and lipocalin-2 were reported to show promise by Barekatain et al. (2020) as biomarkers in this context and are non-invasive and quantifiable in excreta by ELISA kits. Inflammatory metabolites in excreta that could be potential biomarkers nominated by Shini and Bryden (2021) include ovotransferrin, cloacal immunoglobulin A, fibronectin, intestinal alkaline phosphatase and lipocalin-2.

Ovotransferrin is an acute phase, hepatic protein in chickens (Xie et al., 2002a) and serum ovotransferrin levels in chickens have been shown to respond to experimentally induced inflammation (Xie et al., 2002b). A subsequent study by Rath et al. (2009) showed that when chickens were infected with *E. coli*, or *Eimeria maxima* and *Eimeria tenella*, there were significant increases in ovotransferrin serum concentrations. The researchers concluded that ovotransferrin could be used as a diagnostic marker of infection and inflammation in chickens. More recently, Goossens et al. (2018) proposed that elevated ovotransferrin levels in excreta are indicative of intestinal barrier failure in broiler chickens. In this study, faecal ovotransferrin concentrations were correlated to the severity of intestinal barrier failure induced by either Coccidiosis or Necrotic Enteritis.

The potential role of citrulline as a gastrointestinal biomarker in human medicine has attracted substantial interest (Crenn et al., 2000, 2003; Kaore and Kaore, 2014; Fragkos and Forbes, 2018). Initial research into the source and fate of circulating citrulline was completed by Windmueller and Spaeth (1981) and citrulline has been reviewed in considerable detail by Maric et al. (2021). Citrulline is a non-proteinogenic amino acid which is released into the circulation from duodenal and jejunal enterocytes where it is synthesised de novo from glutamine derived from the gut lumen and/or the arterial blood supply to the gut mucosa (Curis et al., 2007). The kidneys take up the circulating citrulline and convert it into arginine and it is recognised that citrulline has an arginine sparing effect in poultry (Dao and Swick, 2021). Maric et al. (2021) concluded that citrulline is a suitable functional biomarker for severe intestinal disease and intestinal function in human patients. Physiological increases in citrulline were associated with improved intestinal absorption capacity and any condition associated with reduced absorptive intestinal capacities was associated with reduced citrulline levels.

In broiler chickens, citrulline was 1 of 3 candidates considered indicative of intestinal inflammation by Baxter et al. (2019) in an investigation into plasma biomarkers for intestinal integrity in a malabsorption model. Birds were offered diets based on maize, rye or maize—rye combinations where maize supported superior 20 d weight gains to rye by 81.7% (716 versus 394 g/bird); however, plasma citrulline levels in maize-based (0.09 ng/mL) and rye-based birds (6.67 ng/mL) were remarkably different. These researchers attributed the higher citrulline plasma concentrations observed to a higher epithelial turnover and an increase in villus height to compensate for poor digestibility of the rye-based diet. However, Niewold (2015) expressed reservations about the use of citrulline as a biomarker in poultry, which stemmed from work completed by Wu et al. (1995). These researchers found that glutamine was metabolised to glutamate, alanine and aspartate in chick enterocytes, but not to citrulline, arginine, proline and ornithine. The lack of citrulline synthesis from glutamine in avian enterocytes was attributed to the absence pyrroline-5-carboxylase synthase in these cells. This raises the obvious question as to the source of the elevated plasma citrulline levels in the Baxter et al. (2019) study.

Calprotectin was identified by Dal Pont et al. (2021) as a potential biomarker for chronic intestinal inflammation in poultry and calprotectin has been used for the same purpose in human subjects (Canani et al., 2008; Bjarnason, 2017). Calprotectin is a soluble protein that originates from neutrophil activation in an acute inflammatory response and calprotectin has a regulatory role in inflammatory processes. Following inflammatory damage to the intestinal mucosa, calprotectin levels increase and it is released into the intestinal lumen. Therefore, calprotectin may have potential as a non-invasive biomarker of intestinal inflammation as it is stable in faeces (Chang et al., 2014). Stříž and Trebichavský (2004) suggested that calprotectin plasma concentrations may be a very sensitive non-specific inflammatory marker. In the Dal Pont et al. (2021) study, broiler chickens were subject to intestinal challenges including dextran sodium sulphate (DSS) to induce intestinal inflammation and NSP derived from rice bran. At 14 d post—hatch, DSS significantly increased plasma calprotectin concentrations by 135% (39.68 versus 16.86 ng/mL) and NSP resulted in a numerical increase of 60.9% (27.13 versus 16.86 ng/mL) in calprotectin concentrations.

Thus, the quest to find an appropriate biomarker for gut integrity in poultry is complicated and may prove elusive. However, calprotectin and ovotransferrin do appear to merit further investigation as confirmation of their validity is required. Also, despite the reservations expressed, citrulline remains of interest as citrulline could be synthesised from glutamine within enterocytes. Glutamine is an important energy substrate in avian enterocytes (Watford et al., 1979; Porteous, 1980) and it may be that increased glutamine plasma concentrations are indicative of the extent of amino acid catabolism within enterocytes to generate energy for gut function. Therefore, citrulline could possibly serve as a marker for this key parameter because amino acid that are catabolised by the gut mucosa are denied entry to the portal and systemic circulations.

4. Protein content of wheat and its consequences

As mentioned, the protein content of wheat typically exceeds that of maize. As a result, there are higher concentrations of non-bound amino acids and lower concentrations of 'intact' soy protein in wheat-based diets than in corresponding maize-based dies pursuant to dietary CP reductions. For example, in Chrystal et al. (2021) wheat and maize contained 107 and 81 g/kg CP, respectively. The transition from 222 to 165 g/kg CP in maize-based diets resulted in an increase in non-bound amino acid inclusions from 7.2 to 38.5 g/kg and a decrease in soybean meal inclusions from 334 to 113 g/kg. In wheat-based diets the same transition resulted in corresponding increases from 7.5 to 49.4 g/kg and decreases from 300 to 48 g/kg. Thus, the CP-reduced, wheat-based diet contained 57.5% less soybean meal, and 28.3% more non-bound amino acids, than the nutritionally-equivalent maize-based diet. The different ratios of non-bound to protein-bound amino acids would not be an issue if both forms were bioequivalent, but this is probably unlikely (Selle et al., 2022). It may be argued that just the more rapid intestinal uptakes of non-bound amino acids effectively preclude bioequivalence. In broilers fed on a once daily basis, Zamani et al. (2021) found that utilisation of protein-bound methionine was better than either non-bound methionine or a methionine dipeptide, which indicates that the 3 forms of methionine were not bioequivalent in restricted fed birds. Thus, rather ironically, the relatively high protein content of wheat could be seen as a shortfall in the context of CP-reduced diets as the higher inclusion levels of

non-bound amino acids may ultimately lead to NH_3 overload, as discussed in the next section.

Wheat and sorghum were compared as the feed grain basis of 187.5 g/kg CP diets with standard and elevated branch-chained amino acid (BCAA) levels by Greenhalgh et al. (submitted for publication). Relative to lysine (100), isoleucine was increased from 65 to 75, leucine from 105 to 150 and valine from 75 to 85. Elevated BCAA levels in sorghum-based diets significantly increased weight gain by 9.26% (1,451 versus 1,328 g/bird) and numerically improved FCR by 0.86% (1.378 versus 1.390) from 7 to 28 d post−hatch. In contrast, elevated BCAA additions to wheat-based diets significantly compromised both weight gain by 9.49% (1,288 versus 1,423 g/bird) and FCR by 8.33% (1.665 versus 1.537). There was noticeably higher non-bound BCAA inclusions in the elevated wheat-based diet than the sorghum-based diet (17.84 versus 11.09 g/kg), which may have contributed to these marked differences in growth performance in response to elevated BCAA inclusions because of a lack of bioequivalence. It is noteworthy in the Greenhalgh et al. (submitted for publication) study that wheat-based diets generated lighter relative fat-pad weights by 57.9% (5.68 versus 13.48 g/kg) than in birds offered sorghum-based diets. It is then relevant that sorghum is more similar to maize than wheat in respect of protein concentration, amino acid profile, starch digestion rate and soluble NSP content.

The ratio of non-bound to protein-bound amino acids will be higher in wheat-based, CP-reduced diets than those based on maize or sorghum and this probably impacts on starch and protein digestive dynamics. The likelihood is that the higher ratios in wheat-based diets may place wheat at a disadvantage. The higher inclusions of non-bound amino acids in wheat-based diets may become a problem as their inclusions may become excessive (Liu et al., 2021b). An equilateral triangular response surface design was used by Macelline et al. (2022) to evaluate 3 wheat-based diets offered to broiler from 14 to 35 d post−hatch. Different protein sources and non-bound amino acid inclusions, which ranged from 6.75 to 19.43 and 66.84 g/kg, were incorporated into the formulations of the 3 apical diets which all had true protein concentrations of 203 g/kg. Maximum weight gain and minimal FCR were supported by the diet containing 13.41 g/kg non-bound amino acids. While not conclusive, this relatively moderate level suggests that there may be a 'ceiling' on non-bound amino acid inclusions in CP-reduced, wheat-based diets above which growth performance is compromised. One implication is that non-bound and protein-bound amino acids are not fully bioequivalent and that high inclusions of non-bound amino acids generate post-enteral imbalances to the detriment of broiler performance.

4.1. Consequences: 'ammonia overload'

The higher non-bound amino acid inclusions in wheat-based, reduced-CP diets, because of wheat's higher protein content, are probably pivotal in triggering NH_3 overload, which is more likely to be declared in birds offered wheat-based diets. Importantly, the intravenous 50% lethal dose of ammonium acetate in broiler chickens is half that of mice (2.72 versus 5.64 mmol/kg), as recorded by Wilson et al. (1968), which suggests that poultry may be more susceptible to NH_3 overload than mammalian species.

From first principles, intestinal uptakes of non-bound amino acids are more rapid than their protein-bound counterparts (Wu, 2009) and this was demonstrated in broilers offered sorghum-based diets by Liu et al. (2013). Non-bound amino acids are more likely to be subject to postprandial oxidation because of their rapid intestinal uptakes and post-enteral amino acid imbalances (Schreurs et al., 1997; Bujko et al., 2007; Nolles et al., 2009), which involves either deamination or decarboxylation. Deamination

generates NH_3, which is inherently toxic (Stern and Mozdziak, 2019); however, NH_3 is detoxified and ultimately $NH_3−N$ is excreted in urine as uric acid-N. Initially, NH_3 and glutamic acid are condensed by glutamine synthetase to generate glutamine (Minet et al., 1997) and then glutamine enters the Krebs uric acid cycle, in which glycine is a prerequisite, and uric acid is excreted (Salway, 2018). Instructively, the need to fortify CP-reduced diets with glycine or glycine equivalents is established (Siegert and Rodehutscord, 2019).

Plasma NH_3 concentrations have been determined in 3 broiler studies with maize-based diets (Namroud et al., 2008; Ospina-Rojas et al., 2013, 2014) where increasing NH_3 concentrations were associated with compromised growth performance. The transition from 220 to 190 g/kg CP diets in Ospina-Rojas et al. (2014) increased plasma NH_3 concentrations from 4.56 to 7.27 mg/dL, which was associated with a depressed weight gain (781 versus 909 g/bird) and compromised FCR (1.57 versus 1.43) from 1 to 21 d post−hatch. Namroud et al. (2008) concluded that high inclusions of non-bound essential amino acids in reduced-CP diets increased plasma NH_3 concentrations, which, due to the negative effects of NH_3 on tissue metabolism, contributed to retarded growth and depressed feed intake in diets containing less than 190 g/kg CP. It was suggested in both the Greenhalgh et al. (2020b) and Chrystal et al. (2021) studies that NH_3 overload contributed to the highly unsatisfactory growth performance observed. As discussed, this proposal was supported by Selle et al. (2021a) in which retrospective determinations of uric acid concentrations in excreta from the Chrystal et al. (2021) study were completed. In Selle et al. (2021a) linear relationships between proportions of uric acid-N to total N in excreta in birds offered the three 165 g/kg CP diets were detected for weight gain ($r = 0.587$; $P = 0.010$), feed intake ($r = 0.526$; $P = 0.025$) and feed conversion ratios ($r = 0.635$; $P = 0.005$). It was suggested that increasing uric acid-N proportions in excreta were indicative of excessive systemic NH_3 levels compromising growth performance. The three 165 g/kg CP diets in question contained 38.5 g/kg non-bound amino acids when based on maize but 49.4 g/kg non-bound amino acids when based on either all ground wheat or diets with 150 g/kg whole wheat. It is likely that the higher non-bound amino acid inclusions in reduced-CP wheat-based triggered more deamination and higher NH_3 levels. The proportion of uric acid N of total excreta N in standard, 165 g/kg CP, ground-wheat diets was significantly higher by a factor of 1.39 (38.0% versus 27.4%) than in the corresponding maize-based diet.

Indirect support for the NH_3 overload proposal is provided in the review by Visek (1978) of the mode of action of APG. This researcher argued that AGP suppress the generation of NH_3 in the gut lumen by microbiota and that this is fundamental to their mode of action. Thus, the identification of a biomarker that is indicative of NH_3 intoxication would be advantageous. This is because taking blood samples for accurate determinations of plasma NH_3 concentrations require special care. Blood samples should be chilled immediately they are taken, centrifuged as quickly as possible and plasma samples held at −80 °C prior to analysis, otherwise NH_3 concentrations will increase spontaneously at room temperature (Da Fonseca-Wollheim, 1990) and confound the results.

5. Starch digestive dynamics

The starch digestion rates for several feed grains are presented in Table 4 where rates in unprocessed grains were determined in vitro (Giuberti et al., 2012) or in vivo after incorporation into standard diets offered to broiler chickens (Selle et al., 2021b). As can be gleaned from Table 4, wheat starch was more rapidly digested than maize starch by 106% in vitro and by 36.0% in broilers offered

Table 4
Starch digestion rates (per minute) recorded for several feed grains under in vitro and in vivo conditions in poultry.

Feed grain	In vitro[1]		In vivo[2]	
	Digestion rate	Number of samples	Digestion rate	Number of samples
Maize	0.017	14	0.086	2
Barley	0.024	14	0.104	3
Wheat	0.035	12	0.117	4
Triticale	0.036	11	0.093	2
Sorghum	0.018	11	0.075	7

[1] Form Giuberti et al. (2012).
[2] From Selle et al. (2021b).

standard diets. That the digestion rate of wheat starch is more rapid than maize starch is an important distinction as there is evidence that the dietary provision of some slowly digestible starch is advantageous (Weurding et al., 2003a; Herwig et al., 2019). Moreover, starch digestive dynamics inevitably impact starch-protein digestive dynamics that are critical to broiler growth performance (Liu and Selle, 2015, 2017).

There are indications that slowly digestible starch spares amino acids from catabolism in the gut mucosa in broiler chickens (Weurding et al., 2003b; Enting et al., 2005). In rats, glucose and glutamine provide similar proportions of energy to the gut mucosa (Fleming et al., 1997); thus, the inference is that the sites of glucose absorption along the small intestine impact on the catabolism of glucose relative to amino acids for energy provision. Slowly digestible maize starch may spare amino acids from catabolism in the posterior small intestine which would increase their post-enteral availability and the likelihood is that glucose is a more efficiently utilised energy source (Fleming et al., 1997). Consequently, the relatively rapid wheat starch digestion rate is probably disadvantageous in this respect.

There is the distinct possibility that glucose and amino acids compete for intestinal uptakes via their respective Na^+-dependent transport systems. In maize-based, CP-reduced diets with high starch levels, Moss et al. (2018) detected numerous negative relationships between apparent digestibility coefficients of glucose and amino acids along the small intestine. For example, starch digestibility coefficients were negatively correlated with digestibility coefficients of twelve amino acids to significant extents in the proximal ileum. In this study it was also suggested that non-bound amino acids may be less prone to undergo catabolism in the gut mucosa. The extent to which the findings of the Moss et al. (2018) study apply to CP-reduced broiler diets with more typical starch concentrations is problematic.

Curiously, wheat enjoys an advantage over maize in the context of CP-reduced diets in that it generates less fat deposition, as is evident in Table 1. For example, the transition from 210 to 165 g/kg CP in maize-based diets prompted an increase of 69.2% (14.62 versus 8.64 g/kg) in relative abdominal fat-pad weights in Chrystal et al. (2020b), which were quadratically ($r = 0.606$; $P = 0.003$) associated with compromised FCR. The likelihood is that slowly digestible maize starch increases fat deposition via de novo lipogenesis to greater extents than rapidly digestible wheat starch. Essentially, starch is digested in the gut lumen and absorbed as glucose. The metabolic disposal of glucose involves direct oxidation, glycogen synthesis and hepatic de novo lipogenesis (Jéquier, 1994). Glucose can be stored as glycogen but carbohydrate overfeeding in humans has been shown to trigger de novo lipogenesis once glycogen stores in liver and skeletal muscle have been saturated (Acheson et al., 1988). In hepatic de novo lipogenesis glucose

is catabolised to acetyl-CoA which is converted into fatty acids and cholesterol. Cholesterol and triacylglycerol are incorporated into very low-density lipoproteins and transported to adipose via the circulation (Wang et al., 2017). Thus, excess carbohydrate is converted into fatty acids that are then esterified to storage triacylglycerols (Ameer et al., 2014). It seems possible that the more sustained glucose and insulin blood levels generated by maize-based diets is promoting more de novo lipogenesis than in birds offered wheat-based diets containing more rapidly digestible starch. Glucose derived from rapidly digestible starch may be directly catabolised for energy provision; whereas, glucose from slowly digestible starch is being converted to glycogen and then fat via de novo lipogenesis to greater extents. However, any consideration of starch digestive dynamics is frustrated by an imperfect comprehension of the starch–glucose–insulin axis in poultry relative to mammalian species (Tesseraud et al., 2007).

6. Conclusions

The successful development of CP-reduced diets is a tangible challenge, which is only complicated by the selection of the feed grain on which these diets are based. There is certainly a real need to identify the shortfalls of wheat in the context of CP-reduced diets for countries in which wheat is the dominant feed grain. However, a substantial research effort is clearly required if this objective is to be realised. It appears that the presence of soluble NSP, even with NSP-degrading enzyme inclusions, wheat ATI and gluten may all constitute shortfalls by compromising gut integrity in wheat-based, CP-reduced diets. There is also the possibility wheat ATI may increase endogenous flows of amino acids and compromise starch and protein digestibility. The recognition of viable biomarkers to monitor gut integrity would be ideal and calprotectin, ovo-transferrin and citrulline appear to be among the candidates that merit further investigations. Relatively high non-bound amino acid inclusions in CP-reduced, wheat-based diets appear to be another shortfall as, in worse case scenarios, they may trigger NH_3 overload, thereby compromising broiler growth performance. Therefore, alternatives to non-bound amino acids to meet amino acid requirements in birds offered CP-reduced, wheat-based diets merit evaluation and such alternatives include protein isolates or concentrates and oligopeptides. Carnosine, a histidine–alanine dipeptide, is one example of an oligopeptide that deserves evaluation. One advantage reduced-CP, wheat-based diets hold is that they generate less fat deposition than corresponding diets based on maize and sorghum. Consequently, it may prove fruitful to evaluate CP-reduced diets based on maize-wheat and/or sorghum–wheat blends.

Author contributions

All co-authors contributed to the compilation of this review and were involved in generating the research on which this review is based.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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