Assessment of caecal parameters in layer hens fed diets containing wheat distillers dried grains with solubles.

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Abstract

1. There is much interest in quantifying the nutritional value of UK Wheat Distillers Dried Grains with Solubles (W-DDGS) for livestock species. A study was designed to evaluate caecal parameters (pH, Short Chain Fatty Acids; SCFAs and bacterial diversity) in layer hens fed balanced diets containing graded levels of W-DDGS.
2. Thirty two layer hens (Bovan Brown strain at 27 weeks of age) were randomly allocated to one of four dietary treatments containing W-DDGS at 0, 60, 120 or 180 g/kg. Each treatment was fed to eight replicate individually housed layer hens over a 5-day acclimatisation period, followed by a 4 week trial. Individual feed intakes were monitored and all eggs were collected daily for weeks 2, 3 and 4 of the trial, weighed and an assessment of eggshell 'dirtiness' made. All hens were culled on day 29 and caecal pH and short chain fatty acids (SCFAs) measured. Polymerase Chain Reaction Denaturing Gradient Gel Electrophoresis (PCR-DGGE) of the bacterial 16S rDNA gene was used to assess total bacterial diversity of luminal caecal content from hens fed the 0 and 180g W-DDGS/kg diets. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrograms were generated from DGGE banding patterns.
3. Increasing W-DDGS dietary levels resulted in a more acidic caecal environment ($P<0.001$). Caecal SCFAs were unaffected by diet aside from a quadratic effect (<0.05) for molar proportions of iso-butyric acid. Diversity profiles of the bacterial 16S rRNA gene from luminal caecal contents were unaffected by W-DDGS inclusion.
4. The results of the current study suggest that W-DDGS can be successfully formulated into nutritionally balanced layer diets (supplemented with xylanase and phytase) at up to 180g/kg with no detrimental effects to the caecal environment.

INTRODUCTION

With increasing global demand for the production of cleaner, renewable sources of energy, there is considerable interest in the production of ethanol from fermentation of cereal grains. This interest has led to significant expansion in the bioethanol industry over recent years, particularly in the US, although there has also been support for biofuel production from the European Union (Directive, 2003/30/EC). As a result of the greater production of bioethanol, there has also been a concurrent increase in the amount of co-products produced from the process that are entering the market. These co-products are generating much debate in terms of their potential nutritional value as a feed raw material for livestock. Wheat Distillers Dried Grains with Solubles (W-DDGS) is the main co-product produced from the UK bioethanol industry. Despite being potentially a rich source of nutrients, there are limitations regarding the general use of DDGS in animal diets; of particular note is the high probability of heat damage during the production process, with concomitant effects on lysine content and digestibility (Ergul *et al.*, 2003, Fastinger *et al.*, 2006).

Another limitation is the high fibre content of W-DDGS. This has traditionally limited W-DDGS dietary inclusion mainly to ruminants. However, there is now significant interest in evaluating the nutritional potential of W-DDGS for use in non-ruminant diets. Evidence in the scientific literature suggests DDGS (of either wheat or maize origin) is typically formulated at 50-80 g/kg in starter diets for broilers and turkeys, and 100-150 g/kg in grower/finisher diets for broilers, turkeys and laying hens (Swiątkiewicz and Koreleski, 2008). Much of the published data evaluating DDGS in poultry studies are from maize DDGS, due to its predominance from bioethanol production in the US. By contrast, there are few comparable poultry studies with W-DDGS.

Another consideration when formulating W-DDGS in poultry diets is the negative effect of Non-starch polysaccharides (NSP) in the gastrointestinal tract. In wheat, water soluble arabinoxylans (pentosans) can result in increased viscosity within the intestinal lumen, resulting in reduced protein, fat and starch digestibility and low feed efficiency (Annison and Choct, 1991, Khattak *et al.*, 2006). With no endogenous enzymes to hydrolyse NSPs in the poultry digestive tract, these carbohydrates are typically fermented by the endogenous microbiota. The primary objective of the current study was to address concerns from the commercial poultry sector that the inclusion of W-DDGS at levels above 50 g/kg in layer diets would result in an increased level of fermentation (primarily of pentosans) within the avian ceca. It was postulated that any increased fermentation would be associated with concomitant changes to the avian caecal environment and bacterial diversity. This increased level of fermentation could result in the recognised problem of 'dirty' (stained) eggs.

Analysis of the intestinal microbiota based on laboratory culture is difficult as it has been reported that only 0.20 of human-associated gut bacterial species have been cultivated (Eckburg *et al*., 2005). These difficulties may in part be overcome through application of Polymerase Chain Reaction - Denaturing Gradient Gel Electrophoresis (PCR-DGGE), a molecular approach through which regions of the universal bacterial 16S rDNA gene at which the DNA sequence varies between species are specifically amplified from DNA isolated from intestinal content (or other sample of interest) using PCR and detected by DGGE, thus removing the requirement for culture (Muyzer *et al*., 1993; Hume *et al*., 2003; Ercolini, 2004). Different DNA sequences have dissimilar migratory properties when subjected to electrophoresis on DGGE gels and correspondingly discreet PCR amplicons visualized on the DGGE gel are representative of different bacterial species. Bacterial communities of different compositions therefore generate different banding patterns

analogous to a community fingerprint. As such, DGGE was employed in the current study to compare bacterial diversity within the avian caecal environment.

The aim of the current study accordingly was to evaluate the potential of feeding graded levels of W-DDGS in layer hen diets. The study was designed to confirm whether W-DDGS could be included in balanced layer hen diets with no detrimental effects on the caecal environment.

MATERIALS AND METHODS

All animal protocols and procedures were conducted under both National and Institutional guidelines as approved in advance of the programme by the Ethical Review Committee of the School of Biosciences, University of Nottingham, UK.

Diets

Two diets were originally formulated containing either 0 or 180 g W-DDGS/kg; subsequently termed D0 and D180 (W-DDGS supplied by Ensus Ltd, Teesside, UK). Table 1 shows analysed composition of the W-DDGS used and Table 2 shows experimental formulations of diets D0 and D180. These two diets were then blended to generate two additional experimental diets, ultimately resulting in four trial diets containing 0, 60, 120 or 180 g W-DDGS/kg respectively (termed D0, D60, D120 and D180; analysed composition of all diets given in Table 3). All dietary treatments (formulated by AB Vista, Marlborough, UK, manufactured by Target Feeds, Whitchurch, Shropshire, UK and analysed by Sciantec Analytical Services, North Yorkshire, UK) were formulated to be iso-energetic (apparent metabolisable energy 11.72 MJ/kg) and balanced for crude protein and standard ileal digestible amino acids (data reported in Masey O'Neill *et al*., 2014). All diets contained exogenous enzymes (ABVista Feed Ingredients, Marlborough, UK) to

126 replicate commercial practice. Finase EC (5000 phytase units per gram, fed at 0.06 g/kg
127 of feed) provided 300 phytase units per kg of feed. One phytase unit is defined as the
128 amount of enzyme required to release 1 μmol of inorganic P per minute from sodium
129 phytate at 37 °C and pH 5.5. Econase XT 25P (160,000 XU per gram, fed at 0.075 g/kg of
130 feed) provided 12,000 XU of endo-1,4- β -xylanase activity (EC 3.2.1.8) per kg of feed.
131 One unit of xylanase (XU) is defined as the amount of enzyme that liberates 1 nmol
132 reducing sugars from birchwood xylan, measured as xylose equivalents, at pH 5.3 and 50
133 °C. Exogenous enzyme inclusion was verified by analysis of all diets prior to
134 commencement of the study (Enzyme Services and Consultancy, Ystrad Mynach, Wales) -
135 see Table 3. Titanium dioxide was added to all diets (5 g/kg) as an indigestible marker.
136
137 **Trial design**
138 A total of 32 layer hens (Bovan Brown strain in early lay at 27 weeks of age) were obtained
139 from a commercial supplier (Noble Foods Ltd, Tring, Hertfordshire, UK), housed
140 individually and allocated to one of four dietary treatments in a completely randomised
141 design. Environmental parameters were a lighting regime of 15 hr light: 9 hr dark, with a
142 light intensity of 15 LUX and environmental temperature maintained at 21ºC throughout
143 the study period. Hens were allocated to experimental diets for an initial period of 5 days
144 (to allow acclimatisation to the new environmental surroundings) before the 4 week trial
145 period commenced. At all times, feed and water were provided on an *ad-libitum* basis.
146 During the trial period, feed intakes were monitored and all eggs were collected daily,
147 weighed and assessed for incidence of 'dirty' eggs by a senior colleague (Noble Foods
148 Ltd, Hertfordshire, UK) who was blinded to the dietary treatments.
149
150 During days 15-17 of the trial, excreta were collected for subsequent assessment of
151 coefficient of apparent N metabolisability (CAM_N). At day 29, all hens were euthanised by

asphyxiation with carbon dioxide and cervical dislocation to confirm death. Within 1 min of death, the caeca were dissected out, and pH of caecal digesta was measured using a digital pH meter (Hanna Instruments, Bedfordshire, UK). Samples of caecal digesta were also collected and stored at -80˚C prior to SCFA analysis. Additionally, caecal digesta samples from the hens on the two dietary extremes (D0 and D180 diets) were subjected to assessment of microbial diversity by PCR-DGGE.

Chemical analyses and calculations

All analyses were conducted in duplicate with repetition if variation was >5%. Diet and excreta samples were dried to a constant weight in a forced air convection oven at 100 ºC. Ground dried samples of diet and excreta (40-50 mg) were analysed in duplicate for N content, using the Dumas method. Subsequently, the concentration of titanium dioxide, employed as an inert marker, was determined in diet and excreta samples using the method of Short *et al.* (1996). These chemical analyses allowed CAM_N to be calculated, using the following equation:

$$CAM_N = 1 - [(N^E \times M^D) / (M^E \times N^D)]$$

Where:

N^E = N concentration in excreta (g/kg DM)

M^D = marker concentration in the diet (g/kg DM)

M^E = marker concentration in excreta (g/kg DM)

N^D = N concentration in the diet (g/kg DM)

Determination of caecal SCFAs

A standard solution of 1 ml/l formic, acetic acid ^{12}C, acetic acid ^{13}C, propionic, butyric, isobutyric and valeric acid was prepared and used as 5.5 ml aliquots in 20 ml headspace

vials. All reagents were sourced from Sigma-Aldrich Co. Ltd., Dorset, UK. Samples were prepared by mixing ~0.2 g caecal content with 3.75 ml H_2O and adding internal standard (acetic acid ^{13}C) to a final concentration of 1 ml/l. The pH of the sample preparation was lowered to pH 2-3 through addition of dilute phosphoric acid. Sealed vials were incubated at 30 °C for 5 min before headspace volatiles were sampled using a 50/30 µm DVB/Carboxen/PDMS StableFlex SPME fibre (Sigma-Aldrich) for a further 5 min at 30 °C. Volatiles adhered to the SPME fibre were then transferred onto a ZB-FFAP column (30 mm x 0.25 mm ID, x 1 µm film thickness; (Phenomenex, Cheshire, UK) and chromatogram with Helium as the carrier gas at 18 psi. Gas chromatography starting temperature was 60°C, held for 1 min and increased to 180 °C at 8 °C/min. All compounds were detected with a DSQ Mass spectrometer (Thermo fisher Scientific, Cheshire, UK) in scan mode, 20-150 m/z.

Determination of caecal microbial diversity using PCR-DGGE

DNA was extracted from ~0.2 g aliquots of caecal content using a QIAamp DNA Stool Mini Kit (Qiagen Ltd., Manchester, UK) with the incorporation of a bead beating stage (0.2 g 0.1 mm glass beads for 1 min at 6,000 rpm using a MagNalyser cell disruptor (Roche Diagnostics Ltd., West Sussex, UK). Extracted DNA was quantified and assessed for purity using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Leics., UK) and stored at -20 ºC prior to PCR-DGGE analysis.

Extracted DNA was diluted to 15 ng/µl with nuclease-free water and used as a template for PCR amplification of the eubacterial 16S rRNA gene using universal primer pairs targeting either the V3 region; 341f (5′-CCTACGGGAGGCAGCAG-3′) and 518r (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer *et al*., 1993) or the V6-V8 region; 968f (5'- AA CGC GAA GAA CCT TAC-3') and 1401r (5'-CGG TGT GTA CAA GAC CC-3'). A 40-bp

GC-rich sequence (GC-clamp) was added to the forward primer at its 5' end as described by Muyzer *et al*. (1993). After visual confirmation of the PCR products by agarose gel (10 g/l) electrophoresis, both V3 and V6-V8 16S rDNA amplicons were analysed by DGGE using a Dcode vertical electrophoresis unit (Bio-Rad Labs., Hertfordshire, UK). Separation of the V3 amplicons was achieved using a 1x TAE buffer/ polyacrylamide gel (80 ml/l; 37.5:1 acrylamide:bisacrylamide; Severn Biotech Ltd., Worcestershire., UK) containing a linearly increasing 30%:55% urea-formamide denaturing gradient, in which the 100% denaturant stock solution contained 7 M urea (Severn Biotech Ltd) and 400 ml/l formamide (Severn Biotech Ltd). V6-V8 amplicons were separated using polyacrylamide gel (60 ml/l); all other conditions were unchanged. Electrophoresis was performed at 60 ºC in 1x TAE electrophoresis buffer for 10 min at 40 V followed by 6 h at 170 V. DNA amplicons were stained in 1:10,000 GelStar Nucleic Acid gel stain solution (Lonza, Maryland, USA) in 1 x TAE for 30 mins and visualised under UV transillumination.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using a fully randomised design Genstat v14 (VSN, International Ltd, Hemel Hempstead, UK) with diet as the main factor, with linear and non-linear contrasts to account for the incremental increase in W-DDGS. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrograms of DGGE banding patterns were generated by FPQuest Software Version 4.5 (BIO-RAD Laboratories) using the Dice coefficient. Analysis of molecular variance (AMOVA) was performed to compare the DGGE patterns of bacteria communities at a selected similarity level as according to Excoffier *et al.* (1992) using GenAIEX v6.5 software as described by Peakall and Smouse (2012). The Shannon-Wiener diversity index (*H*) was used to describe bacterial diversity as detected by DGGE (Scanlan *et al.*, 2006, Shannon, 1948). This index was calculated by the following equation:

$$Shannon\ Wiener\ index = \sum_{i=1}^{s}(P_i)(lnP_i)$$

where:

s = number of species/DGGE bands in the sample

P_i = proportion of species/DGGE bands for the *i*th species/DGGE band in the sample

Student's t-test was performed on the species richness and Shannon-Wiener index of each group using –GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, USA, www.graphpad.com).

RESULTS

Hens took longer than expected to acclimatise to the trial environment which was reflected in a low laying percentage over the first week of the study. Therefore egg parameters presented are from weeks 2, 3 and 4.

Caecal pH and SCFA

Mean caecal data (pH and SCFA values) are shown in Table 4. Caecal pH was significantly affected by diet ($P<0.001$) with values ranging from 7.3 for hens on the D0 diet to 6.1 for those on the D180 treatment. This linear effect was highly significant ($P<0.001$) with increasing W-DDGS dietary levels associated with a more acidic caecal environment. SCFA analysis revealed a significant quadratic effect for molar proportions of iso-butyric ($P<0.05$) but no other significant dietary effects were observed for molar proportions of acetic, propionic, butryric, valeric acid or total SCFAs within the caecum.

Caecal microbial diversity analysis by PCR-DGGE

Total bacterial diversity of luminal caecal content from hens on diets with and without W-DDGS (180 g/kg) was compared by PCR-DGGE. Surveys were made of both the V3 and V6-V8 regions of the universal bacterial 16S rRNA gene with visualization of the amplicons allowing the determination of different bacterial community structures. To determine whether specific changes in diversity were promoted through addition of W-DDGS, UPGMA analysis was performed to determine similarities in bacterial community fingerprints. UPGMA analysis of profiles of the V3 16S rRNA gene region revealed that community fingerprints were distributed in two significant clusters ($P < 0.05$, AMOVA; Figure 1A), however clustering did not relate to dietary inclusion of W-DDGS. DGGE banding patterns for the V6-V8 16S rRNA gene region were distributed into three significant clusters ($P < 0.05$, AMOVA; Figure 1B) that were again independent of W-DDGS inclusion.

Species richness was resolved by enumeration of bands in each DGGE profile. In profiles generated from surveys of either region of the 16s rRNA gene (V3 or V6-V8) species richness was not influenced by W-DDGS inclusion ($P < 0.05$, Student's *t* test, Table 5). The Shannon-Wiener index is a measure of species diversity in a community that considers both the number and evenness of species. Shannon-Wiener index values calculated for hens with and without W-DDGS from profiles of the V3 and V6-V8 16S rRNA gene regions were not significantly affected, ($P < 0.05$, Student's *t* test, Table 5).

Performance Parameters

Although the primary objective of the study was an assessment of the influence of increasing levels of dietary W-DDGS on caecal parameters, the protocol adopted allowed a preliminary evaluation of influences on general egg production (g egg/hen/day) and eggshell cleanliness. Over the 4 week trial period, there was no evidence of any 'dirty'

eggs. In total, n = 13 / 615 eggs were soft-shelled and 5 / 615 eggs were broken in cage over weeks 2-4 inclusive of the trial. Dietary effects on mean feed intake and CAM_N were also recorded and are shown in Table 4. Feed intakes and CAM_N were unaffected by dietary treatment.

DISCUSSION

Data evaluating the caecal environment revealed a highly significant dietary effect ($P<0.001$) for caecal pH, with more acidic luminal contents associated with increasing W-DDGS dietary inclusion. This observed difference in caecal acidity could be explained by the reasoning that an increasing rate of inclusion of W-DDGS across the four dietary formulations would be associated with an accompanying increase in dietary fibre level (evident in Table 3). This increased fibre would result in differing levels of NSP fermentation by the avian caecal microbiota, given that the predominant fermentation chambers within the avian gastrointestinal tract are the caeca (Józefiak *et al.*, 2004). An increased level of caecal fermentation would probably result in increased molar proportions of SCFAs and a more acidic caecal environment. Although not statistically significant, molar proportions of total SCFAs did increase as inclusion rate of W-DDGS increased which could explain the increasing caecal acidity. Similarly, the indicative increase in molar proportions of butyric acid would probably contribute to the increased caecal acidity (as well as suggesting some degree of change to the caecal microbiota). The authors also postulate that the variation in caecal pH between dietary treatments could be at least partly due to an intrinsic property of the experimental diets themselves, such as pH levels. This would seem a reasonable assumption given that DDGS is an acidic material with a pH value typically between 3.6 and 5.0 (Shurson and Alghandi, 2008) although it might be expected that other variables (gizzard activity, intestinal buffers etc.) would have more of an influence on caecal acidity in poultry.

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308 It was postulated that changes in caecal fermentation levels across treatments would
309 be associated with differences in the diversity of bacterial species. However, assessment
310 of bacterial diversity within the caeca, detected using PCR-DGGE, revealed no significant
311 changes in bacterial population structure between the hens on the D0 and D180 diets
312 (Figure 1 and Table 5). Although clustering was apparent for both the V3 and V6-V8
313 regions of the 16S rRNA gene, it was not linked to diet (as evidenced by the even
314 distribution of D0 and D180 diets within clusters). PCR-based 16S rDNA techniques have
315 been applied successfully to detect changes in poultry microbial populations (Hume *et al*.,
316 2003; Amit-Romach *et al*., 2004; Waters *et al*., 2005) with 16S rDNA gene V3 and V6-V8
317 hypervariable regions shown to be appropriate for fingerprinting the diversity of intestinal
318 bacteria (Yu and Morrison, 2004). The chicken caecum is colonised by a highly numerous
319 and species rich bacterial community (Barnes, 1979, Bjerrum *et al.*, 2006). A diverse
320 microbial population is associated with several host benefits. Aside from contributing to
321 feed conversion by generation of substrates (SCFAs) through fermentation of host
322 indigestible carbohydrates (van der Wielen *et al.*, 2000), competition for resources
323 between the bacterial community of the gastrointestinal tract can exclude pathogens such
324 as *Salmonella* (Impey *et al.*, 1987, Nava *et al.*, 2005). Additionally, the presence of SCFAs
325 in the avian caeca have been reported to have bacteriostatic effects (van der Wielen *et al*.,
326 2000).

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328 It is somewhat difficult to draw firm conclusions from the caecal environment data as
329 the significant changes observed in caecal pH (along with indicative changes in molar
330 proportions of butyric and total SCFAs) would suggest differing levels of caecal
331 fermentation across diets which would have been expected to be reflected in changes to
332 the caecal microbiota. However, the lack of a shift in caecal bacterial diversity measured

in the current study, detected using PCR-DGGE, suggests that W-DDGS at 180 g/kg does not cause a gross change in bacterial population structure within the avian caeca. Given this, it may be beneficial to also employ the use of other techniques (Next-generation DNA sequencing etc.) to aid in the interpretation of any future work of a similar nature.

There is growing interest in evaluating the nutritional value of feeding W-DDGS to layers, as reflected by the generation of prediction equations of energy values of W-DDGS for poultry (Cozannet *et al.*, 2010). The analysed composition of the W-DDGS used in the current study (Table 1) appears typical of that reported elsewhere; nutrient profiles from a range of W-DDGS samples from European ethanol plants by Cozannet et al, (2010) included DM ranging between 890-940 g/kg, CP (326-389 g/kg), CF (62-109 g/kg) and ash (43-67 g/kg).

As a general indication of the production level of the birds, data from the current study suggest that W-DDGS can be included at levels of up to 180 g/kg in layer diets containing exogenous enzymes, with no detrimental effects to egg production. These results are in good agreement with a similar, larger study (Niemiec *et al.*, 2012) where inclusion levels of W-DDGS of up to 200 g/kg were successfully fed in balanced diets with no dietary effect on laying performance .

An assessment of eggshell cleanliness was undertaken in the current trial but no dirty eggs were observed. The supplementation of layer diets with exogenous enzymes can overcome the negative effects of NSPs, by decreasing intestinal viscosity and reducing the incidence of stained/dirty eggs (Lazaro *et al*., 2003; Khattak *et al*., 2006). Diet formulations in the current study included phytase (at 600-700 U/kg) and xylanase (15400 to 17400 U/kg). These exogenous enzymes were formulated in the diets to reflect commercial

practice. The lack of difference in CAM_N values is expected, given that the four diets were formulated to be both iso-energetic and balanced for crude protein and standard ileal digestible amino acids.

The results of the current study provide valuable evidence that W-DDGS can be formulated into nutritionally balanced layer diets containing NSP enzymes and phytase at inclusion levels of up to 180 g/kg with no detrimental effects to the microbial diversity of the caecal microbiota. These results, taken together with the preliminary egg performance data should instil a greater degree of confidence in the use of W-DDGS in layer diets.

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469

470

471 **Table 1.** *Analysed composition of Wheat Distillers Dried Grains with Solubles (g/kg as-fed*

472 *unless otherwise stated)*

Dry Matter	884
Crude Protein	326
AME (MJ/kg)[1]	10.04
Crude Fibre	80
NDF [2]	389
ADF[3]	223
Total Oil	72.5
Ash	46
Indispensable AA	
Lysine	5.9
Methionine	4.7
Cysteine	11.8
Methionine and Cysteine	16.5
Threonine	10.8
Isoleucine	11.1
Valine	14.5
Leucine	23.2
Histidine	6.5
Phenylalanine	16.0
Arginine	13.0

473

474 [1] Apparent Metabolisable Energy

475 [2] Neutral Detergent Fibre

476 [3] Acid Detergent Fibre

477

478 **Table 2.** *Experimental diet formulations (g/kg as-fed)*

	Diet	
	D0[1]	D180[2]
Wheat DDGS	0	180
Wheat	588	528
Hipro Soya bean meal	143	54
Corn Glutenmeal	40	40
Sunflower meal	75	40
Soy oil	41	45
Limestone	91	93
Salt	2.0	1.0
Sodium Bicarbonate	2.0	0.3
DL Methionine	0.9	1.1
Lysine HCl	1.6	4.0
Dicalcium Phosphate	11.5	9.0
Monosodium Phosphate	0.06	-
Vitamin/Mineral Premix[3]	4.9	4.9
Finase EC	0.06	0.06
Econase XT 25P	0.075	0.075

479

480 DDGS = Distillers Dried Grains with Solubles

481

482 [1] Formulated to provide the following quantities (g) per kilogram complete diet: D-Lysine, 7.7; D-Methionine, 3.6; D-
483 Cysteine, 3.0; D-Methionine + Cysteine, 6.6; D-Threonine, 5.7; D-Tryptophan, 1.9; D-Isoleucine, 6.4; D-Leucine, 13.7; D-
484 Valine, 7.4; D-Histidine, 4.0; D-Arginine, 9.7.

485

486 [2] Formulated to provide the following quantities (g) per kilogram complete diet: D-Lysine, 7.7; D-Methionine, 3.6; D-
487 Cysteine, 3.1; D-Methionine + Cysteine, 6.7; D-Threonine, 5.4; D-Tryptophan, 1.8; D-Isoleucine, 6.3; D-Leucine, 13.3; D-
488 Valine, 7.4; D-Histidine, 3.9; D-Arginine, 7.6.

489

490 [3] Provided the following per kg of diet: retinol, 1.8 mg; cholecalciferol, 75 μg; α-tocopherol, 5 mg; riboflavin, 0.8 mg;
491 cyanocobalamin, 25 μg; niacin, 10 mg; pantothenic acid, 4 mg; folic acid, 0.3 mg; Fe, 10 mg; Mn, 79.6 mg; Cu, 5 mg; Zn,
492 59.9 mg; I, 0.99 mg; Se, 0.15 mg; Ca, 0.25 mg.

493

494 Diets were blended to produce two additional experimental dietary treatments giving four in total: D0, D60, D120 and
495 D180 representing diets containing 0, 60, 120 and 180 g W-DDGS/kg respectively.

Table 3. *Determined analysis of experimental layer diets (g/kg as-fed unless otherwise stated)*

	Diet			
	D0	D60	D120	D180
Dry Matter	897	896	897	900
Crude Protein	166	169	171	171
Crude Fibre	41	37	40	39
ADF[1]	47	54	63	69
NDF[2]	96	110	129	143
Total Oil	62	65	67	73
Ash	144	127	126	133
Calcium	48	39	42	39
Phosphorus	5.0	4.8	5.0	4.3
Starch	364	357	349	337
Finase EC (U/kg)	600	635	674	696
Econase XT 25P (U/kg)	16800	15400	16300	17400

[1] Acid Detergent Fibre

[2] Neutral Detergent Fibre

D0, D60, D120 and D180 represent diets containing 0, 60, 120 and 180g W-DDGS/kg respectively

514 **Table 4.** *Effect of increasing level of Wheat Distillers Dried Grains with Solubles on caecal*

515 *and performance parameters of layer hens (from 27-31 weeks of age)*

	Diet					*P*		
	D0	D60	D120	D180	Sed	Diet	Linear	Quadratic
Caecal parameters[1]								
Caecal pH	7.3	7.0	6.3	6.1	0.28	<0.001	<0.001	0.202
SCFA (mmol/L)								
Acetic	78	88	88	89	9.9	0.647	0.313	0.451
Propionic	30	34	35	37	5.6	0.638	0.301	0.452
Butyric	14	19	20	23	3.9	0.130	0.074	0.138
Iso-Butyric	3.8	2.7	3.2	2.0	0.73	0.115	0.319	0.036
Valeric	9.6	9.1	11.6	10.2	2.01	0.626	0.268	0.506
Total SCFA	135.4	152.8	157.8	161.2	19.38	0.534	0.210	0.451
Performance parameters								
Egg production [2]								
g egg/hen/day	49	49	49	48	2.9	0.967	0.945	0.766
Feed Intake (g/day)	112	112	110	116	3.5	0.353	0.927	0.215
CAM_N[3]	0.399	0.349	0.391	0.361	0.0438	0.631	0.926	0.209

516

517 [1] Data collected from birds at slaughter (at 31 weeks of age)

518 [2] Data from weeks 2, 3 and 4 of the trial

519 [3] Coefficient of Apparent Metabolisability of Nitrogen

520 D0, D60, D120 and D180 represent diets containing 0, 60, 120 and 180g W-DDGS/kg respectively

521 Sed = Standard error of the difference

522

Table 5. Species richness and Shannon-Wiener index determined from 16S rRNA genes amplified from luminal caecal content of layer hens on diets with or without Wheat DDGS (W-DDGS).

	16S rRNA gene target							
	V3				V6-V8			
	D0	D180	Sed	*P*	D0	D180	Sed	P
Richness indexes (*S*)	13.5	13	1.5	>0.05	9.9	10.5	0.8	>0.05
Shannon-Wiener index (*H*)	2.3	2.3	0.1	>0.05	2.2	2.3	0.1	>0.05

Figure 1. *UPMGA dendrogram of DGGE profiles of 16S rRNA genes amplified from luminal caecal content of layer hens on diets with or without Wheat DDGS (W-DDGS).*

UPMGA clustering dendrograms (3% positional tolerance) describing the relatedness of the DGGE-profiles of bacterial diversity determined by analysis of V3 (A) and V6-V8 regions (B) of the 16S rRNA gene. Open figure indicates diet (D0 and D180 contain 0 and 180 g W-DDGS/kg respectively), closed figure indicates hen identifier. For V3 region of 16S rRNA gene (A) the bacteria communities belonging to Clusters A and B are significantly different ($P < 0.05$; AMOVA). For V6-V8 region bacterial communities belonging to Clusters A. B and C are significantly different ($P < 0.05$; AMOVA).