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The use of synthetic and natural vitamin D sources in pig diets to improve meat quality and vitamin D content

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Abstract

This study investigated the effects of synthetic and natural sources of vitamin D biofortification in pig diets on pork vitamin D activity and pork quality. One hundred and twenty pigs (60 male, 60 female) were assigned to one of four dietary treatments for a 55 d feeding period. The dietary treatments were (1)50 µg vitamin D_3/kg of feed; (2)50 µg of 25-hydroxvitamin D_3 /kg of feed (25-OH-D_3); (3)50 µg vitamin D_2/kg of feed; (4)50 µg vitamin D_2-enriched mushrooms/kg of feed (Mushroom D_2). The pigs offered the 25-OH-D_3 diet exhibited the highest ($P<0.001$) serum total 25-hydroxyvitamin D concentration and subsequently exhibited the highest ($P<0.05$) *Longissimus thoracis* (LT) total vitamin D activity. Mushroom D_2 and 25-OH-D_3 supplementation increased pork antioxidant status. The vitamin D_2-enriched mushrooms improved ($P<0.05$) pig performance, carcass weight, LT

colour. In conclusion, 25-OH-D_3 is the most successful source for increasing pork vitamin D activity, while Mushroom D_2 may be a new avenue to improve animal performance and pork quality.

Keywords: Antioxidant activity; Pork colour; Pork Quality; Vitamin D; 25-hydroxvitamin D_3; Vitamin D_2-enriched mushroom

1. Introduction

Vitamin D deficiency is now recognised as a public health priority within Europe (Cashman et al., 2016), with daily vitamin D intakes, less than the Estimated Average Requirement (EAR) for most individuals (Roman Viñas et al., 2011). Due to this wide-spread inadequacy of vitamin D intake, there is a need for more effective food-based strategic approaches to produce a wider range of sustainable natural vitamin D-enriched foods with potential to increase vitamin D dietary intakes (Calvo and Whiting, 2013; Cashman, 2015). Pork is one staple food that contains naturally occurring vitamin D (Schmid and Walther, 2013). The fortification of pig diets with vitamin D sources, in order to enhance the pork vitamin D content in indoor reared pigs (so called vitamin D biofortified pork), may have potential to be one natural food-based solution. In terms of enhancing the natural level of vitamin D in pork meat via biofortification, the choice of vitamer (i.e., vitamin D_2 or D_3) or the 25-hydroxvitamin D_3 metabolite for fortification purposes has been highlighted as an important consideration (Cashman, 2012). The use of vitamin D supplementation of swine diets is regulated by the European Food Safety Authority (EFSA, 2009), and the maximum inclusion of vitamin D sources, including cholecalciferol (vitamin D_3), 25-hydroxvitamin D_3 (25-OH-D_3) and ergocalciferol (vitamin D_2) is 50 µg/kg/feed or equivalent. This assumes equivalence between both vitamers as well as the 25-OH-D_3. Total vitamin D activity of pork meat from animals fed the metabolite or vitamin D_3-containing feed (50 µg per kg) showed a two-fold

higher activity in the former compared to the latter (1.42 versus 0.65 μg/100 g meat, respectively) (Burild et al., 2016). Likewise, serum 25(OH)D concentration was over twice as high in the animals fed the 25-OH-D_3- versus vitamin D_3-containing feed (168 v. 68 nmol/L, respectively) (Burild et al. 2016). Supplemental 25-OH-D_3 has been shown in healthy adults to possess five times higher activity in terms of raising serum 25-OH-D compared to an equivalent amount of supplemental vitamin D_3 (Cashman et al., 2012). There has also been a growing body of evidence from human nutrition to suggest that vitamin D_2 may be less effective in raising serum total 25-OH-D compared to an equivalent amount of vitamin D_3 (Armas et al., 2004; Houghton and Vieth, 2006; Tripkovic et al., 2012). This may also be the case for pigs, with implications for uptake into muscle, but has not received research attention to-date. If vitamin D_2 has equivalence with D_3 for the purposes of enhancement of pork, it is of note that many species of mushrooms contain a high ergosterol content which offers the potential to form vitamin D_2, if they are exposed to UVB radiation (Kalaras et al., 2012). Apart from being a novel natural and potentially more cost-effective sustainable source of vitamin D (Itkonen et al., 2016), research findings suggest that mushrooms are also a rich natural source of bioactive metabolites, such as polysaccharides and phenolic compounds (Reis et al., 2012), which may have the potential to improve animal performance, increase pork antioxidant status and in turn increase shelf-life of food. Naturally occurring antioxidants have developed ample interest in recent years in order to avoid or minimize the use of artificial food additives (Kumar et al., 2015).

As well as increasing the vitamin D content of pork, the supplementation of pigs with vitamin D may also have the potential to enhance additional pork quality properties, as vitamin D supplementation of beef cattle enhanced post-mortem proteolysis, reduced shear force values and improved beef tenderness (Karges et al., 2001; Montgomery et al., 2000). Therefore, the objective of the current study was to investigate the effect of inclusion of

synthetic and natural vitamin D sources in pig diets on total vitamin D activity of the pork meat as well as on content of individual vitamers and their 25-hydroxymetabolites, and on meat quality and antioxidant status. It is hypothesised that pigs supplemented with 25-OH-D_3 enriched diets will have increased pork vitamin D content and improved pork quality, while the supplementation of 25-OH-D_3 and vitamin D_2-enriched mushrooms may produce pork with higher antioxidant activity than that of pigs offered the vitamin D_3 and vitamin D_2 dietary treatments.

2. Materials and methods

All experimental procedures described in this work were approved under University College Dublin Animal Research Ethics Committee (AREC-13-79-O'Doherty) and conducted under experimental license from the Department of Health in accordance with the cruelty to animal act 1876 and the European communities (amendments of cruelty to animal act, 1876) regulations (1994).

2.1 Experimental design and animal management

The experiment was designed as a complete randomised block. One hundred and twenty (60 males, 60 females) pigs (Meat line boars × Large White × Landrace sows) (Hermitage, Co. Kilkenny, Ireland) with initial mean live weight of 58.0 kg (SD 4.6) were blocked according to live weight and sex, within each block assigned to one of four dietary treatments (n = 30). Dietary treatments consisted of ; (1) 50 µg vitamin D_3/kg of feed (Vit D_3); (2) 50 µg of 25-OH-D_3/kg of feed (25-OH-D_3); (3) 50 µg vitamin D_2/kg of feed (Vit D_2); (4) 50 µg vitamin D_2-enriched mushrooms/kg of feed (Mushroom D_2). The vitamin D levels in the experimental diets were chosen to comply with EU regulations. The inclusion of 50 µg of Vit D_3, 25-OH-D_3 and Vit D_2 sources is the maximum inclusion rate permitted in the EU for swine diets (EFSA, 2009). The Vitamin D_3 and 25-OH-D_3 (Hy-D®) was sourced from DSM,

Nutritional Products Limited, UK. The Vitamin D_2 was sourced from A & Z Food Additives, Zhejiang, China. The vitamin D_2-enriched mushrooms were sourced from Monaghan Mushrooms, Ireland. The mushroom vitamin D content was naturally enhanced, using synthetic UVB exposure, as previously described by (Stepien et al., 2013). The dried mushroom powder was included at 0.89 g/kg of feed. This inclusion level was added to obtain 50 µg of Vit D_2/kg of feed. The vitamin D_2-enriched mushroom powder was analysed for vitamin D concentration by high performance liquid chromatography, as described by Mattila et al. (1994), prior to diet manufacture. The diets were provided *ad libitum* in a meal form and water was available *ad libitum* from nipple drinkers. The diets were formulated to have similar digestible energy (14 MJ/kg) and standardised ileal digestible lysine (8.5 g/kg). All amino acid requirements were met relative to lysine (NRC, 2012). Detailed ingredient composition and chemical analysis of the diets are presented in supplementary text Table S1 and S2 respectively.

The pigs were grouped in mixed sex (50:50) groups of ten with a space allowance of 0.75 m^2 per pig (three pens per treatment). The pens were equipped with single space computerised feeders (Mastleistungsprufung MLP-RAP; Schauer Agrotronic AG, Sursee, Switzerland), as described by Varley et al. (2011), which allowed individual *ad libitum* feeding and daily recording of individual dietary intake. The house was mechanically ventilated and temperature was maintained at 18 °C and there was no sunlight exposure inside the pig barn. Pigs were weighed at the beginning of the experiment day 0, day 28 and day 55 (slaughter). Feed samples were collected at diet manufacture and throughout the experimental period and stored at - 20 °C for chemical analysis.

2.2 Slaughter procedure

The pigs were slaughtered by bleeding after carbon dioxide stunning at a nearby abattoir when the average live weight exceeded 108 kg. A blood sample for serum analysis was taken immediately after slaughter via the jugular vein using lithium/heparin vacutainers (BD Plymouth, UK). The blood was stored overnight at 4 °C and centrifuged at 4720 *g* for 20 min at 4 °C (40R centrifuge, Thermo-Fisher Scientific, Ireland) after which the serum layer was removed from the blood cell layer and stored at –20 °C until required for further 25-OH-D analysis. The back-fat thickness was measured as described by Egan et al. (2015) using the Hennessy grading probe (Hennessy and Chong, Auckland, New Zealand). The lean meat content was estimated as described by Walsh et al. (2013), using the following equation:

Estimate lean meat content (g/kg) = 543.1 – 7.86x + 2.66y (Eq.1)

Where x is fat depth (mm) and y is muscle depth (mm).

Further carcass data were determined using the following equations:

Carcass weight (kg) = hot carcass weight × 0.98 (Eq.2)

Kill out proportion (%) = carcass weight / live weight (Eq.3)

Following overnight chilling of the carcasses at 4 °C, the *Longissimus thoracis* (LT) muscle was excised from twelve randomly selected pigs / treatment (2 males and 2 females from 3 pens), and subdivided into eight boneless pork steaks / pig (approximately 1.5cm in thickness). Five boneless pork steaks (approximately 1.5cm in thickness) were placed in vacuum pack pouches (200 × 300 mm, Mc Donnells Ltd, Dublin, Ireland) and flushed with 80% O_2: 20% CO_2 (modified atmosphere packs, MAP technology), as described by Rajauria et al. (2016) using a single chamber vacuum-sealing unit (Webomatic vacuum packaging

system, C10 H with a Km100-3M gas mixer, Germany) for colour and antioxidant analysis. The LT steaks in MAP were stored for up to 14 days at 4 °C for antioxidant and colour analysis. The gas atmosphere (oxygen and carbon dioxide) in the MAP was checked using a handheld gas analyser (Checkpoint handheld gas analyser, PBI Dansensor, Denmark). The remaining LT samples were vacuum packed using a single chamber vacuum-sealing unit and were stored at - 20 ºC until required for vitamin D analysis, water holding capacity and Warner Bratzler shear force (WBSF) analysis.

2.3 Chemical analysis

Feed samples were analysed for dry matter (DM), ash, nitrogen (N), gross energy (GE) and ether extract (EE) according to the methods described in our previous publication (Duffy et al., 2017). The concentration of P in feed samples was determined spectrophotometrically using the method of Cavell (1955). A series of concentrations of P ranging from 10 to 50 $\mu g.g^{-1}$ were used for a calibration curve and digested samples were diluted to fit this curve. The Ca concentration of feed samples was determined using an atomic absorption spectrophotometer (Varian '50,' Varian, Santa Clara, CA, USA) using the method of Ramakrishna et al. (1968). All samples were measured in duplicate.

2.4 Vitamin D analysis of LT, serum, experimental diets and dried vitamin D_2-enriched mushroom powder

Vitamers (vitamin D_3 and vitamin D_2) and their respective 25-hydroxy metabolites (25-OH-D_3 and 25-OH-D_2) in the experimental diets and in LT steaks (n=5 to 7/group) separated into lean meat without any subcutaneous fat, were analysed. Three of the four groups were analysed at the Technical University of Denmark (TUD) using modifications of a sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS), as described by Burild et al. (2014) and Roseland et al. (2016). For technical reasons, vitamin D and metabolites in LT

steaks from the 25-OH-D group were measured by a LC-MS/MS method at University College Cork (UCC) by a comparable extraction procedure (see Online Supplemental information, including Supplemental Tables S3 and S4). The comparability of the two LC-MS/MS measurement methods were tested using six pork steak samples which were analysed by both laboratories, and while in general there was good agreement, the UCC estimates were positively biased by 21% and 10% for vitamin D_3 and 25-OH-D_3 relative to those from the TUD estimates (see Supplemental Table S5). Accordingly, the UCC estimates were adjusted for this bias. Total vitamin D activity of LT was defined as [vitamin D_3 + (25-OH-D_3 × 5) + vitamin D_2 + (25-OH-D_2 × 5)], except where the values of vitamin D_2 or 25-OH-D_2 were below the limit of quantification, or not measured for this reason, and were not included in the calculation. The conversion factor of 5 is applied to the 25-OH-D_3 content on the basis of efficacy data from a randomized controlled trial with oral vitamin D_3 and 25-OH-D_3 in healthy adults (Cashman et al., 2012), and is a factor commonly used in several food-composition tables (Finglas et al., 2015; Saxholt et al., 2008). It should be noted however that equivalent data does not exist for vitamin D_2 and 25-OH-D_2, so we assumed a conversion factor of 5 in our calculation of total vitamin D activity of LT. Furthermore, the content of fat was assessed to 2.2 ± 0.6% by the analyses of randomly selected six lean meat samples. Serum 25-OH-D_3 and 25-OH-D_2 was analysed by LC-MS/MS, as described by Cashman et al. (2013). Serum total 25-OH-D concentration was calculated as [25-OH-D_3 + 25-OH-D_2].

2.5 Colour measurement

The surface colour (L*, a* and b*) of the LT steaks was measured using a colorimeter (CR-400 handheld Chroma meter, Konica Minolta, Co, Oska, Japan), as described by Rajauria et al. (2016). Colour measurements were recorded on steaks following storage in MAP at 4 °C for 0, 4, 7, 11 and 14 days. Values were recorded as the mean of three measurements made on

non-overlapping regions of the LT steak in triplicate (averaged from three locations) from each side of the cut of LT.

2.6 Warner Bratzler shear force and cook loss percentage

Warner Bratzler shear force analysis was carried out according to Wheeler et al. (1996) with some modifications. Briefly, LT samples were de-frosted overnight at 4 °C, trimmed of external fat, weighed and cooked in open vacuum bags in a circulating water bath (Grant Instruments Ltd., Barrington, Cambridge CB2 IBR, UK) set at 72 °C, until an internal temperature of 70 °C was achieved. Internal temperature was monitored by placing a thermocouple in the geometric centre of each steak, four steaks were cooked per water bath to ensure water circulation was consistent around all samples. All juices was poured out of bag immediately after removing from water bath. The LT steaks were cooled to room temperature, dabbed gently with tissue paper to absorb excess moisture and reweighed. The % cook loss was then determined using the equation:

$$\text{Cook loss } \% = \frac{(X-Y)}{X} \times 100 \qquad \text{(Eq. 4)}$$

Where X = raw weight of LT and Y = cooked weight of LT.

The LT steaks were allowed to cool to room temperature, placed in storage bags (to prevent dehydration) and stored at 4 °C overnight. Coring was carried out on chilled samples after 24 as described by Duffy et al. (2017).

2.7 Water holding capacity

Water holding capacity was carried out according to Honikel (1998). Briefly samples were weighed and suspended using a hook and placed in a polythene bag, which was tied to prevent dehydration. Samples were hung freely inside the bag to ensure exudate did not remain in contact with the meat. The bag was hung freely for 48 h at 4 °C. LT steaks were

removed from the bag, gently blotted dry using tissue paper and reweighed. Water holding capacity was expressed as a percentage of the final weight. Water holding capacity was calculated using the equation:

$$\text{Water holding capacity} = \frac{(W1-W2)}{(W1)} \times 100 \qquad \text{(Eq. 5)}$$

Where W_1 = initial weight and W_2 = weight after 48 h.

2.8 Antioxidant activity analysis

For antioxidant analysis, meat samples were homogenised with phosphate buffer (0.05 M, pH 7) and collected meat supernatant was tested for lipid peroxidation (LPO), total phenolic content (TPC) and total antioxidant status (TAS) by analysing DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capacity and ferric reducing antioxidant power (FRAP) according to the methods described in previous publications (Rajauria et al., 2016; Qwele et al., 2013).

2.9 Statistical analysis

The data was checked for normality and homogeneity of variance by histograms, qq plots and formal statistical tests as part of the UNIVARIATE procedure of SAS (SAS, 2006). The growth performance, carcass characteristics, total vitamin D activity of the meat and serum total 25-OH-D concentration, shear force, cook loss and water holding capacity were analysed using PROC MIXED procedure of SAS (Littell et al, 1996). The model included the fixed effects of treatment, sex and the associated two-way interaction while the random effect was pen and animal within pen. Initial body weight was used as a covariate for growth performance data. The colour variations and antioxidant activity was analysed by repeated measures analysis using the PROC MIXED procedure of SAS (Littell et al, 1996). The statistical model included the fixed effects of treatment, sex, time of sampling and the

associated two and three way interactions while the random effect was pig. Pearson correlation coefficients amongst serum total 25-OH-D concentration and pork total vitamin D activity were determined using the correlation procedure of SAS. The mean values were considered to be significantly different when $P < 0.05$ and considered a numerical tendency when $P < 0.10$ but > 0.05. Least square means are reported with pooled standard errors.

3. Results

3.1 Growth performance, dietary intake and carcass characteristics

The effect of dietary treatment on pig performance and carcass characteristics are presented in Table 1 and 2. Pigs offered the Mushroom D_2 diet had a higher final body weight ($P < 0.01$), increased average daily gain (ADG) ($P < 0.01$), improved feed conversion ratio (FCR) ($P < 0.01$) and a higher carcass weight ($P < 0.05$) compared to pigs offered the other dietary treatments during the experiment (day 0-55). There was no significant difference ($P > 0.05$) in final body weight, ADG and FCR between pigs offered the Vit D_3, 25-OH-D_3 and Vit D_2 treatment groups. Male pigs had a higher ($P < 0.05$) final body weight, ADG, ADFI and a lower FCR than female pigs. Pigs fed the Vit D_2 and Mushroom D_2 had higher ($P < 0.05$) back fat depth compared to the Vit D_3 treatment.

3.2 Serum total 25-OH-D, phosphorous and calcium analysis

The effect of dietary treatment on serum 25-OH-D_3, 25-OH-D_2, and total serum 25-OH-D (ie. 25-OH-D_3 + 25-OH-D_2), as well as serum P and Ca concentrations are presented in Table 3. Pigs offered the 25-OH-D_3 exhibited the highest ($P < 0.001$) serum total 25-OH-D concentration compared to the other dietary treatments. Serum total 25-OH-D did not differ ($P > 0.05$) between Vit D_2 and the Mushroom D_2 treatments, however it was lower in both these groups ($P < 0.05$) compared to the Vit D_3 treatment. The pigs offered the 25-OH-D_3

diet exhibited the highest ($P < 0.001$) serum 25-OH-D_3, while pigs offered the Vit D_2 had the highest ($P < 0.001$) serum 25-OH-D_2 concentration compared to other dietary treatments. Both serum 25-OH-D_2 and 25-OH-D significantly differed across all four dietary treatment groups. There was no dietary treatment effect ($P > 0.05$) observed for serum Ca concentration, however, pigs offered the 25-OH-D_3 had a lower serum P content compared to all other dietary treatments. There was no effect of sex ($P > 0.05$) on serum 25-OH-D_3, 25-OH-D_2 metabolites, total serum 25-OH-D, P or Ca concentrations.

3.3 LT steak total vitamin D activity

The effect of dietary treatment on LT total vitamin D activity (i.e. vitamin D_3 + [25-OH-D_3 × 5] + vitamin D_2 + [25-OH-D_2 × 5]) as well as on vitamin D_3, 25-OH-D_3, vitamin D_2 and 25-OH-D_2, individually are presented in Table 4. The pigs offered the 25-OH-D_3 diet had the highest ($P < 0.001$) LT total vitamin D activity compared to all other treatments. The LT total vitamin D activity was positively related to both total serum 25-OH-D ($R = 0.75$, $P < 0.001$) and LT 25-OH-D_3 content ($R = 0.98$, $P < 0.01$). There was no difference ($P > 0.05$) in total LT vitamin D activity between the Vit D_2 and Mushroom D_2 treatments, however, both were significant lower ($P < 0.05$) than that from the Vit D_3 treatment. Pigs offered the Vit D_3 had the highest ($P < 0.001$) LT vitamin D_3, while pigs offered the 25-OH-D_3 treatment exhibited the highest ($P < 0.001$) LT 25-OH-D_3 content. The LT 25-OH-D_3 was positively related to serum 25-OH-D_3 content ($R = 0.85$, $P < 0.001$). The Vit D_2 treatment exhibited the highest ($P < 0.001$) LT vitamin D_2 and 25-OH-D_2 content compared to other treatments. There was no effect of sex ($P > 0.05$) on LT total vitamin D activity or LT vitamin $D_{2/3}$ or their metabolite contents.

3.4 Pork Cook loss %, water holding capacity and WBSF

The effect of dietary treatment on the pork quality parameters including cook loss %, water holding capacity and WBSF is presented in Table 5. There was no effect of dietary treatment or sex on cook loss %, water holding capacity and WBSF values.

3.5 Colour stability of fresh LT muscle

The value of colour parameters L*, a* and b* of the pork LT steaks are presented in Fig. 1. (a - c). There was no sex or treatment × time interaction ($P > 0.05$) observed for L*, a* or b* values indicating lightness, redness and yellowness respectively. However, there was a significant treatment ($P < 0.05$) and time effect ($P < 0.05$) over the 14 day storage period for 'L*, a* and b*' values. The L* values increased ($P < 0.001$) in fresh pork samples in all treatments over the 14 day storage period. The Mushroom D_2 had an overall lower ($P < 0.01$) L* value compared to the other dietary treatments (Fig. 1.a). Furthermore, all treatments exhibited a significant increment ($P < 0.05$) in a* value up to storage day 4, however they decreased thereafter. Among the treatments, the Mushroom D_2 diet exhibited the highest red colour with a higher ($P < 0.05$) a* value compared to all dietary treatments (Fig. 1.b). Pigs supplemented with 25-OH-D_3 exhibited higher a* value compared to the Vit D_2 and Vit D_3 treatment groups. The b* value of the LT steaks increased ($P < 0.01$) in the fresh muscle over the storage period. The 25-OH-D_3 dietary treatment exhibited higher ($P < 0.01$) b* value compared to other dietary treatments (Fig. 1.c). The Mushroom D_2 exhibited the lowest ($P < 0.01$) yellowness (b*) value, compared to the other treatments.

3.6 Total antioxidant and lipid peroxidation status of LT muscle

The effect of source of vitamin D on TPC, total antioxidant status (DPPH and FRAP) and lipid peroxidation in LT muscle samples stored in MAP at 4 °C over 14 storage days is presented in Fig. 2a-d. There was no significant ($P > 0.05$) sex or treatment × time interaction

seen for TPC (Fig. 4a). There was significant treatment ($P < 0.05$) and time effects ($P < 0.01$) on TPC. The pigs offered the Mushroom D_2 had higher ($P < 0.05$) TPC activity compared to the Vit D_2 and the Vit D_3 treatments but did not differ ($P > 0.05$) from the 25-OH-D_3 treatment. There was an interaction between dietary treatment and time of sampling ($P < 0.05$) on DPPH scavenging capacity, FRAP content and lipid peroxidation of meat samples (Fig. 1.b-d). The Vit D_3 samples had a higher DPPH scavenging capacity on storage days 0, 7, 11 and 14 compared to other treatments and the Mushroom D_2 exhibited the lowest ($P < 0.05$) DPPH capacity on storage days 4, 7, 11 and 14 compared to all other treatments. The supplementation of Mushroom D_2 and 25-OH-D_3 exhibited higher ($P < 0.01$) FRAP content compared to the Vit D_3 and Vit D_2 treatments on storage days 4 and 7. The supplementation of Vit D_2 resulted in a rapid increase ($P < 0.01$) in lipid peroxidation over the 14 day storage compared to all other treatments. The Mushroom D_2 and the 25-OH-D_3 delayed ($P < 0.05$) the lipid peroxidation of meat samples on day 4 and 7 compared to the Vit D_3 and Vit D_2 treatments, however on day 14 the Vit D_3 exhibited lower ($P < 0.05$) lipid peroxidation in the meat samples.

4. Discussion

The biofortification approach for improving the total vitamin D activity in natural food sources through vitamin D fortification of livestock feeds is now attracting consumer attention (Schmid and Walther, 2013; Cashman and Hayes, 2017). Previous research investigating vitamin D supplementation in pigs has looked at the inclusion of synthetic vitamin D_3 and 25-OH-D_3 (Burild et al., 2016) and sunlight exposure (Alexander et al., 2017; Larson-Meyer et al., 2017) to increase vitamin D levels with mixed findings. However, there is a paucity of research examining the supplementation of natural and synthetic forms of vitamin D_2 on production efficiency, pork vitamin D activity and meat quality.

We choose to measure the total vitamin D activity of pork in the LT muscle (without subcutaneous fat), since it is representative of a major muscle consumed by humans. The LT total vitamin D activity [vitamin D_3 + (25-OH-D_3 × 5) + vitamin D_2 + (25-OH-D_2 × 5)] was highest in LT from pigs offered the 25-OH-D_3 treatment, and then decreased in the order Vit D_3>Vit D_2=Mushroom D_2. While not reported, calculation of total vitamin D activity of pork lean meat from animals fed 25-OH-D_3 or vitamin D_3-containing feed (50 µg per kg) showed a two-fold higher activity in the former compared to the latter (1.42 versus 0.70 µg/100 g meat, respectively) in the study by Burild et al. (2016). Another older pig study from the same Danish group, and while not reported, the calculated total vitamin D activity of pork meat from animals fed 25-OH-D_3 or vitamin D_3 (50 µg per kg diet) showed only a slight difference (0.41 versus 0.56 µg/100g meat, respectively) (Jakobsen et al. 2007). Burild et al. (2016) have stressed an important consideration in comparing absolute levels of vitamin D compounds in pork meat from these different studies, due to differences in study design and experimental animals, but also importantly that the analytical methods used were identical for the two studies.

Of importance, the study also showed that supplementation of livestock feeds with 25-OH-D_3 led to significantly greater (68 - 177 %) total vitamin D activity of the resulting pork meat than that from Vit D_3,Vit D_2 or Mushroom D_2 treatment sources. The supplementation of 25-OH-D_3 may be a cost effective and feasible option for indoor swine production and associated vitamin D- enriched pork. It is important to stress that while there were no differences in carcass characteristics, albeit slightly, but significantly, better animal performance in the Mushroom D_2–supplemented animals, and in this sense supports the European Food Safety Authority's *Panel on Additives and Products or Substances used in Animal Feed* assumption of equivalence of vitamin D_2, D_3 and 25-OH-D_3, from a human nutrition perspective there are differences. The meat from the vitamin D_2 supplemented

animals was less, such that a typical UK average serving size of pork loin chop (177 g) arising from the LT of 25-OH-D_3, Vit D_3, Vit D_2 and Mushroom D_2–supplemented groups would have 2.4, 1.4, 0.9 and 0.8 μg of total vitamin D activity, respectively. Consumption of a typical serving size of 25-OH-D_3-biofortified pork would contribute 24% to the current vitamin D, EAR of 10 μg/d and may contribute between 12-16% of an individual's Recommended Dietary Allowance (RDA) of 15-20 μg/d, dependent on age (Institute of Medicine, 2011). Despite less overall compared to that arising from 25-OH-D_3 or vitamin D_3 biofortified pork, there was no difference in total vitamin D activity in LT of animals supplemented with Vit D_2 and Mushroom D_2, and consumption of typical serving size of pork loin chop from these would contribute ~9 and ~8% to the EAR, respectively. This pig study, to the author's knowledge, is the first to use and test dried UVB-exposed mushroom as a natural novel feed ingredient.

The higher effectiveness of 25-OH-D_3 supplementation relative to vitamin D_3 in terms of resulting total vitamin D activity in the pork is likely to be associated with the more than doubling of serum total 25-OH-D in 25-OH-D_3 treatment groups compared to that of the vitamin D_3-supplemented group. Burild et al. (2016) in their pig study also showed that serum total 25-OH-D concentration was over twice as high in the animals fed the 25-OH-D_3 treatment compared to the vitamin D_3 treatment (168 v. 68 nmol/L, respectively). It also agrees with other studies of pigs which were supplemented with 25-OH-D_3 (Witschi et al. 2011; Coffey et al. 2012). This would have consequences for uptake into the muscle tissue. LT total vitamin D activity was positively related to a higher total serum 25-OH-D content ($R = 0.75$). On the other hand, the lower effectiveness of vitamin D_2 supplementation, be it as synthetic or via the more natural UVB-exposed mushroom source, in terms of resulting total vitamin D activity in the pork is likely to be associated with the significantly lower (by 28-

37%) serum total 25-OH-D in these two treatment groups compared to that of the vitamin D_3-supplemented group, again with consequences for uptake into the muscle tissue.

While the inclusion of vitamin D_3, 25-OH-D_3 and vitamin D_2 did not affect pig performance during the experimental period. However, pigs offered the vitamin D_2-enriched mushroom diet had increased animal performance and carcass weight compared to all other treatments. The improved performance is probably related to the polysaccharides present in mushrooms rather than vitamin D_2. Mushrooms contain β-glucans (Aida et al., 2009) which have been shown to improve gastrointestinal health and the composition of intestinal microbiota (Sweeney and O'Doherty, 2016). This improvement in gastrointestinal health will aid digestion and directly support the immune activities of the gut, thereby improving growth and feed efficiency in animals. Similar results have been shown in broilers with *Pleurotus eryngii* mushroom stalk residues (Lee et al., 2012). The numerically increased back fat depth in the Mushroom D_2 diet may be attributed to a higher final body weight in this treatment group at slaughter.

One of the most important quality traits of fresh pork determined from a consumer perception is colour (Risvik, 1994), with fresh pork expected by the consumer to have a homogeneous reddish-pink colour (Van Oeckel et al., 1999). The speed of fresh pork discoloration has been related to both pigment oxidation, by the activity of the oxidative processes and their effect on the oxygen content present at the meat surface (Faustman et al., 1989) with oxidation beginning immediately post slaughter. The present study demonstrated that lipid peroxidation in LT steaks of all dietary treatments began at storage day 0 and increased over the 14 day refrigerated period. The Vit D_2 treatment exhibited a rapid deterioration from day 0 of storage which may have contributed to the lower redness (a*) value observed in LT steaks. On storage day 7, Mushroom D_2 and 25-OH-D_3 supplementation delayed the lipid

peroxidation of muscle samples which may also have had a positive effect on colour stability of LT samples with an increased redness value. The decrease in lipid peroxidation due to inclusion of 25-OH-D_3 and the Mushroom D_2 treatment may be due to a higher antioxidant activity (FRAP and TPC), which demonstrates higher ability to quench free radicals and thus protect the phospholipid content against lipid peroxidation which resulted in improved colour stability (Kumar et al., 2015). Research investigating the supplementation of vitamin D_3 to finishing pigs and the effect on colour stability are contrasting, Wilborn et al. (2004) demonstrated that vitamin D supplementation of 2,000 µg of vitamin D_3 did not affect a* or b* values, but resulted in lower L* values in pork than the control pigs (0 µg of vitamin D_3). While Wiegand et al. (2002) demonstrated that the supplementation of 125,000 µg at 7 and 14 days post-mortem, lowered L* values, increased a* values and did not affect b* values compared to control animals.

Pigs offered the Vit D_3 treatment yielded the highest DPPH antioxidant activity, over all storage days while the Mushroom D_2 showed the lowest, indicating that the 25-OH-D_3, Vit D_2 and Mushroom D_2 portrayed less scavenging capacity against DPPH radicals. Furthermore, the Mushroom D_2 treatment group exhibited a higher TPC content while the Mushroom D_2 and 25-OH-D_3 treatments portrayed higher FRAP content on storage days 4 and 7, which concurs with previous findings that both the active metabolite 1,25-hydroxvitamin D_3 and mushrooms exhibit high antioxidant properties (Wiseman, 1993, Faccin et al., 2007). This finding also agrees with Duffy et al. (2017) who demonstrated that hens supplemented with 25-OH-D_3 resulted in eggs with improved antioxidant capacity. The lower lightness, yellowness and a higher redness values portrayed by the Mushroom D_2 diet in this study is an encouraging finding and may be a novel and natural way of improving fresh meat quality for human consumption while also adding natural antioxidant properties.

Irrespective of source of vitamin D, there was no effect on pork quality parameters including the water holding capacity of LT muscles, cook loss % or WBSF values. In pork production the water holding capacity of pork is a vital quality characteristic (Van Moeseke and De Smet, 1999), with many physical properties including colour and texture dependent on it (Shin et al., 2008). Previous work reported that pigs supplemented with high levels (1000 – 2000 µg) of vitamin D_3 tended to have a lower drip loss after 8 days of storage (Wilborn et al. (2004). However in the present study drip loss was only measured over 48 h, due to the fact that the greatest amount of drip loss is generally lost in the first 24 - 48 h (Van Moeseke and De Smet, 1999). Previous research in beef cattle has shown that the supplementation of vitamin D_3 has the potential to increase serum and muscle Ca concentrations, resulting in enhanced levels of post-mortem proteolysis and improving beef tenderness (Montgomery et al., 2000). For the present study instrumental shear force values were similar for all treatments regardless of source and concur with earlier work by Wiegand et al. (2002), who reported that feeding 125, 000 µg of vitamin D_3 to pigs prior to slaughter did not improve the tenderness of loin chops. Studies evaluating the supplementation of allowable levels of vitamin D sources and pork quality are limited and need further investigation, however from a vitamin D biofortification perspective, no negative alteration in pork quality was observed which is an encouraging finding.

5. Conclusion

The present study concludes that among the four enriched dietary treatments, the enrichment of the upper allowable limit of 50 µg/kg feed of 25-OH-D_3 exhibited the highest LT muscle total vitamin D activity, and serum total 25-OH-D concentration. The biofortification of pigs with levels of 50 µg/kg feed of 25-OH-D_3 has the potential to contribute up to 24% (per 177g pork intake) of an individual's EAR for vitamin D. Additionally, the addition of 50 µg of

vitamin D_2-enriched mushrooms improved ADG, improved FCR and increased the final weight and carcass weight of pigs. The supplementation of 25-OH-D_3 and Mushroom D_2 increased the antioxidant status of the pork. The addition of vitamin D_2-enriched mushrooms could be a useful natural antioxidant to help improve the fresh pork quality for human consumption. The supplementation of Mushroom D_2 also improved the overall colour stability of fresh pork. These findings could provide a new avenue for developing new animal based diets to help improve pig performance and pork quality. Moreover, improving the total vitamin D activity of pork through biofortification may be one strategic approach for tackling vitamin D deficiencies and of importance not only to the population as a whole, but in particular in Europe and northern latitudes where a high prevalence of vitamin D deficiency has been detected (Cashman et al., 2016).

Conflict of interest

The authors wish to confirm that there are no known conflicts of interest associated with this publication.

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Fig. 1. Effect of dietary treatment on surface colorimeter values (a) 'L*' lightness, (b) 'a*' redness and (c) 'b*' yellowness of fresh *Longissimus thoracis* (LT) stored in modified atmosphere packs (80% O_2: 20% CO_2) for up to 14 days of storage at 4°C.

Fig. 2. The effect of dietary treatment on (a) total phenol content (TPC) and total antioxidant activity including (b) DPPH scavenging capacity (c) Ferric reducing antioxidant power

(FRAP) and (d) Lipid peroxidation, in fresh *Longissimus thoracis* (LT) stored in modified atmospheric packs (80% O_2; 20% CO_2) at 4 °C for up to 14 days of storage.

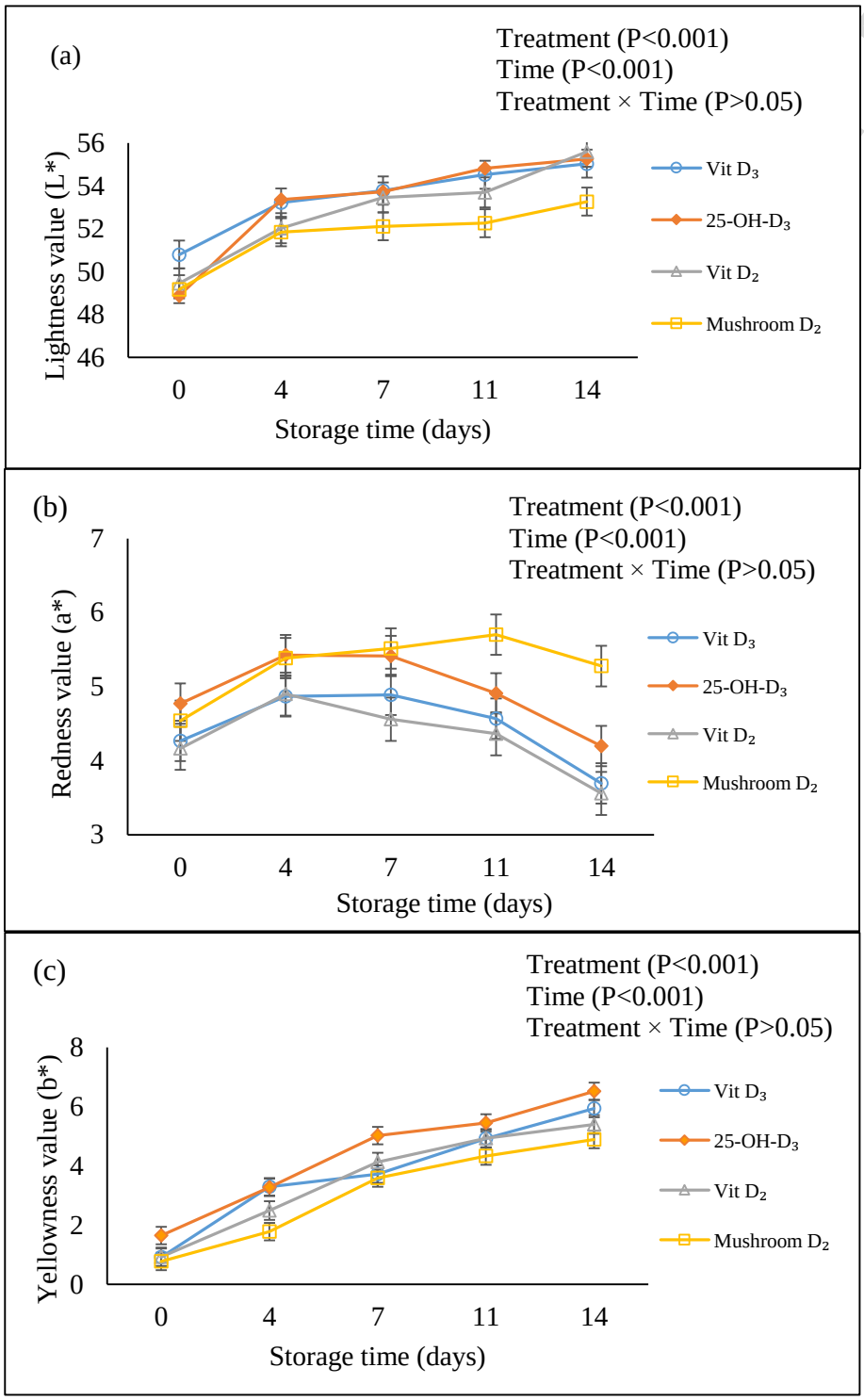

Fig. 1

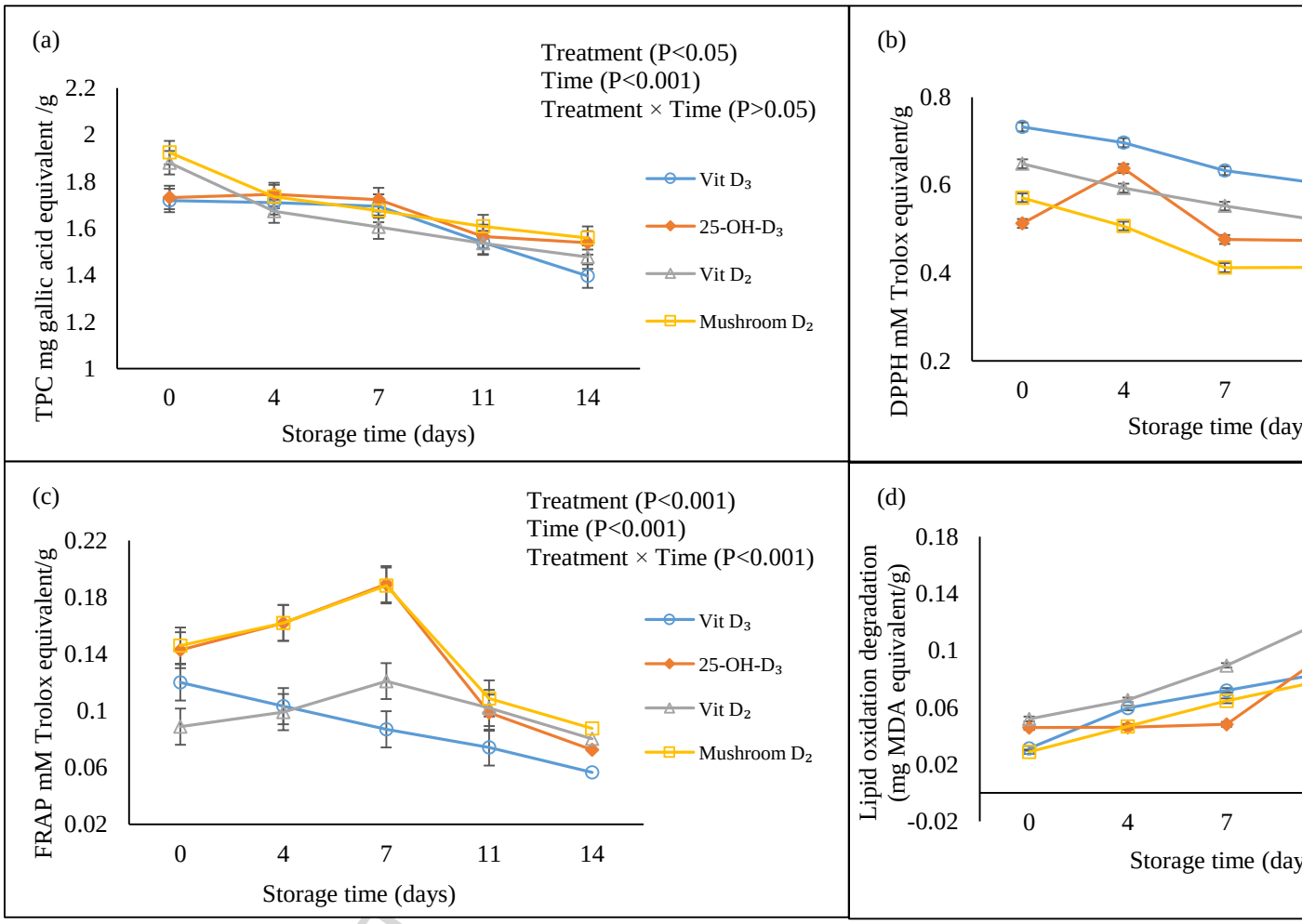

Fig. 2.

Table 1 Effect of dietary treatment on animal growth performance during the experimental period. (Least-square means ± S.E.M.).

Performance	Dietary treatments[1]					Sex		
	Vit D_3	25-OH-D_3	Vit D_2	Mushroom D_2	SEM[2]	Female	Male	SEM
Final BW (kg)	108[a]	107[a]	110[a]	113[b]	1.254	109	109	0.62
ADG (kg/d)	0.91[a]	0.93[a]	0.95[a]	1.01[b]	0.023	0.91	0.99	0.01
ADFI (kg/d)	2.56	2.75	2.64	2.59	0.055	2.58	2.69	0.03

FCR (kg/kg)	2.87[b]	2.96[b]	2.83[b]	2.61[a]	0.058	2.89	2.74	0.04

[1]Treatments: (1) 50 µg vitamin D_3/kg of feed (Vit D_3); (2) 50 µg of 25-OH-D_3/kg of feed (25-OH-D_3); (3) 50 µg vitamin D_2/kg of feed (Vit D_2); (4) 50 µg vitamin D_2-enriched mushrooms/kg of feed (Mushroom D_2).
[2]SEM = Standard error of the mean.
[a,b] Means within a row with different superscripts differ ($P < 0.05$).
BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio.
[*]There was no Treatment × Time × Sex interaction ($P > 0.05$).

Table 2 Effect of dietary treatment on carcass characteristics. (Least-square means ± S.E.M.).

Item	Dietary treatments[1]					Sex			Significance[*]	
	Vit D_3	25-OH-D_3	Vit D_2	Mushroom D_2	SEM[2]	Female	Male	SEM[2]	Treatment	Sex
Carcass weight (kg)	79.6[a]	79.5[a]	79.9[a]	83.9[b]	1.531	80.0	81.6	1.134	0.050	0.268
Kill out %	74.6	74.2	73.6	74.0	0.598	73.7	74.6	0.425	0.629	0.131
Back-fat depth (mm)	13.4[a]	14.8[ab]	16.5[b]	15.6[b]	0.785	14.9	15.2	0.558	0.057	0.716

Muscle depth (mm)	54.5	55.7	56.5	56.9	1.639	57.3	54.5	1.166	0.770	0.097
Lean meat %	55.7	55.5	54.7	54.7	0.735	55.5	54.8	0.521	0.671	0.390

[1]Treatments: (1) 50 µg vitamin D_3/kg of feed (Vit D_3); (2) 50 µg of 25-OH-D_3/kg of feed (25-OH-D_3); (3) 50 µg vitamin D_2/kg of feed (Vit D_2); (4) 50 µg vitamin D_2-enriched mushrooms/kg of feed (Mushroom D_2).
[2]SEM = Standard error of the mean.
[a,b] Means within a row with different superscripts differ ($P < 0.05$).
[*]There was no Treatment × Sex interactions ($P > 0.05$).

Table 3 Effect of treatments on serum 25-OH-D_3, serum 25-OH-D_2, serum total 25-OH-D and serum P and Ca concentration collected from pigs offered experimental treatments. (Least-square means ± SEM).

Item (nmol/L)[†]	Dietary treatments[1]				SEM[2]	P-value
	Vit D_3	25-OH-D_3	Vit D_2	Mushroom D_2		
Serum 25-OH-D_3	69.3^{c}	147.1^{d}	10.2^{a}	36.4^{b}	8.906	0.001
Serum 25-OH-D_2	0.08^{a}	0.42^{b}	29.62^{d}	17.31^{c}	1.326	0.001
Serum total 25-OH-D	69.4^{b}	147.5^{c}	39.8^{a}	53.7^{ab}	9.154	0.001
Serum P (nmol/L)	4.25^{ab}	3.91^{a}	4.62^{b}	4.36^{b}	0.164	0.037

Serum Ca (nmol/L)	2.85	2.79	2.79	2.79	0.034	0.572

[1]Treatments: (1) 50 µg of vitamin D_3/kg of feed (Vit D_3); (2) 50 µg of 25-OH-D_3/kg of feed (25-OH-D_3); (3) 50 µg of vitamin D_2/kg of feed (Vit D_2); (4) 50 µg of vitamin D_2-enriched mushrooms/kg of feed (Mushroom D_2).
[2]SEM = Standard error of the mean.
[†]n= 7/treatment.
[a,b,c,d] Means within a row with different superscripts differ ($P < 0.05$).
P, phosphorous; Ca, calcium.

Table 4 Effect of treatments on LT vitamin D_3, LT 25-OH-D_3, LT vitamin D_2, LT 25-OH-D_2 and LT total vitamin D concentration collected from pigs offered experimental treatments (µg/100g). (Least-square means ± SEM).

Item (µg/100g)[2†]	Dietary treatments[1]					
	Vit D_3	25-OH-D_3	Vit D_2	Mushroom D_2	SEM[3]	P-value
LT vitamin D_3	0.14^{b}	0.03^{a}	0.03^{a}	0.03^{a}	0.015	0.001
LT 25-OH-D_3	0.13^{b}	0.26^{c}	0.02^{a}	0.06^{a}	0.016	0.001
LT vitamin D_2	$<0.01^{\Psi}$	$-^{\Psi}$	0.13^{b}	0.03^{a}	0.009	0.001
LT 25-OH-D_2	$<0.01^{\Psi}$	$-^{\Psi}$	0.05^{c}	0.03^{b}	0.003	0.001
LT total vitamin D activity[4]	0.79^{b}	1.33^{c}	0.51^{a}	0.48^{a}	0.088	0.001

[1]Treatments: (1) 50 µg of vitamin D_3/kg of feed (Vit D_3); (2) 50 µg of 25-OH-D_3/kg of feed (25-OH-D_3) (3) 50 µg of vitamin D_2/kg of feed (Vit D_2); (4) 50 µg of vitamin D_2-enriched mushrooms/kg of feed (Mushroom D_2).
[2]1µg is equivalent to 40 IU.
†n= 5-7/treatment.
[3]SEM = Standard error of the mean.
[4]Total vitamin D activity is calculated as vitamin D_3 + 25-OH-D_3 (× 5) + vitamin D_2 + 25-OH-D_2 (× 5) (Cashman et al, 2012).
[a,b,c] Means within a row with different superscripts differ ($P < 0.05$).
LT = *Longissimus thoracis*.
[Ψ]No values for vitamin D_2 or 25-OH-D_2 included in estimation of LT total vitamin D activity as less than limit of quantification (<0.01) or not measured for this reason.

Table 5 The effect of dietary treatment on pork quality parameters including cook loss %, water holding capacity and WBSF on day 0. (Least-square means ± S.E.M.).

	Dietary treatments[1]					
Item	Vit D_3	25-OH-D_3	Vit D_2	Mushroom D_2	SEM[2]	P-value
Cook loss (%)	28.7	27.6	28.3	28.9	1.081	0.833
Water holding capacity (%)	10.2	11.7	9.6	11.9	0.819	0.153
WBSF* (N)	45.2	44.8	45.1	45.0	1.360	0.807

[1]Treatments: (1) 50 µg of vitamin D_3/kg of feed (Vit D_3); (2) 50 µg of 25-OH-D_3/kg of feed (25-OH-D_3) (3) 50 µg of vitamin D_2/kg of feed (Vit D_2); (4) 50 µg of vitamin D_2-enriched mushrooms/kg of feed (Mushroom D_2).
[2]SEM = Standard error of the mean.
*WBSF, Warner Bratzler Shear Force.

Highlights

- 25-hydroxyvitamin D_3 supplementation resulted in the highest pork vitamin D content
- Vitamin D_2-enriched mushrooms improved pig growth performance and carcass weight
- 25-hydroxyvitamin D3 enhanced antioxidant status
- Vitamin D_2-enriched mushrooms enhanced pork antioxidant status and colour stability