Supplementary information

Table S1

Ingredients composition of the basal diet offered to pigs over the experimental period.

Ingredient (g/kg)	(g/kg)*
Wheat	383
Barley	250
Soya bean	170
Maize	150
Soya oil	18.0
Salt	5.00
Monocalcuim phosphate	6.60
Limestone	12.5
Lysine HCl	2.30
L-threonine	0.50
Vitamins and mineral premix$	2.50

*Vitamin D_3 was added to the basal diet in order to obtain 4 levels of dietary 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).
Vitamin D_2-enriched diets was added to the diet at an inclusion level of 0.89 mushroom D_2 g/kg of feed to obtain the inclusion level of 50 µg/kg of feed.

$ The premix provided vitamins and minerals (per kg diet) as follows: 0.01g/kg of retinol acetate, 0.16 g/kg of alpha tocopherol acetate, 0.007 g/kg of menadione, 0.00125 g/kg of thiamine mononitrate, 0.005 g/kg of riboflavin, 0.0025 g/kg of pyridoxine HCl, 0.003 g/kg of cyanocobalamin,0.0229 g/kg of nicotinamide, 0.0138 g/kg of calcium-D-pantohenate, 0.06 g/kg of copper as copper sulphate, 0.4167 g/kg of iron as iron sulphate, 0.0806 g/kg of manganese as manganese oxide, 0.0032 g/kg of iodine as calcium iodate, 0.1389 g/kg of zinc as zinc oxide, 0.0056 g/kg selenium, 0.05 g/kg of phytase, 1.24 g/kg of calcium.

Table S2

The analysed chemical profile of the experimental treatments (g/kg, unless otherwise stated).

	Dietary treatments*			
Item (g/kg)	Vit D_3	25-OH-D_3	Vit D_2	Mushroom D_2
Dry matter	886	886	886	886
Crude protein (N × 6.25)	147	149	148	147
Ash	42.7	41.6	43.4	42.6
Gross energy (MJ/kg)	15.9	16.0	15.6	16.0
Ether extract	24.5	23.5	25.1	24.2
Lysine¥	10.5	10.5	10.5	10.5
Methionine and cysteine¥	6.3	6.3	6.3	6.3
Threonine¥	7.2	7.2	7.2	7.2
Tryptophan¥	1.9	1.9	1.9	1.9
Calcium	6.1	6.0	5.7	5.9
Phosphorous	4.9	4.8	4.8	4.9
Vitamin D (IU)+	2230	2230	2230	2230

*Vitamin D_3 was added to the basal diet in order to obtain 4 levels of dietary 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).

¥ Calculated for the tabulated nutritional composition (Sauvant et al., 2004).

+Calculated for the tabulated nutritional composition (Charlton and Ewing et, 2007).

Pork meat sample preparation and LC-MS/MS analysis of vitamin D_3 and 25(OH)D_3 at UCC

The extraction procedure for the analyses of vitamin D_3 and 25(OH)D_3 in pork meat was modified from a previously published method (Jakobsen, et al., 2004). To 10 g pork meat, 50 µL internal standard solution (1 µg/mL [d_6]-25(OH)D_3 and 1 µg/mL [d_3]-vitamin D_2 in ethanol), 0.2 g sodium ascorbate, 15 mL ethanol, and 10 mL KOH 50% were added. Atmospheric air was replaced by nitrogen and the sample was left for saponification overnight (16–18 h) at ambient temperature.

Water (5 mL) was added followed by extraction with 10 mL 10% ethyl acetate in n-heptane (0.005% BHT) for 1 min. The organic phase was transferred to a clean tube and the water phase was re-extracted twice. The pooled organic phases were washed with 10 mL water. The organic phase was evaporated to dryness using a miVac Quattro Sample Concentrator (Genevac, Suffolk, UK) and reconstituted in 2 mL 1% 2-propanol in n-heptane. The sample was loaded onto a Sep-Pak Vac 6cc (500 mg) silica cartridge (Waters, Milford, MA, USA) conditioned with 5 mL n-heptane. The cartridge was washed with 5 mL 0.5% 2-propanol in n-heptane and the analytes were eluted twice with 4 mL 6% 2-propanol in n-heptane followed by 5 mL 10% 2-propanol in n-heptane. The combined eluates were evaporated to dryness, as described above, and reconstituted in 100 µL 1% 2-propanol in n-heptane.

The extract was further purified by semi-preparative HPLC using a Shimadzu Nexera X2 system (incorporating a FRC-10A fraction collector) (Shimadzu, Marlborough, MA) equipped with a Lichrospher Si-60 (5µm, 250mm x 4.6mm) column in series with a Lichrospher Amino (5µm, 150 x 4.6mm) column in which 50 µL extract was injected. The vitamin D_3 and 25(OH)D_3 fractions were collected separately. Both fractions were evaporated by a miVac Quattro Sample Concentrator (Genevac, Suffolk, UK) and reconstituted in 70 µL 1% 2-

propanol in n-heptane. A second semi-preparative HPLC step was conducted using a Supelcosil LC-CN (5µm, 150mm x 4.6mm) (SUPELCO, Bellefonte, PA, USA) column in which 50 µL extract was injected. The collected fractions were concentrated and stored up to two days at -20C until liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

Vitamin D_3 and 25(OH)D_3 fractions were reconstituted in 100 µL of 68:32 methanol:water and injected onto a Waters Acquity UPLC™ with triple quadrupole mass detector (TQD) using electrospray ionization (ES^+), capillary 2.6 kV. The sample vials were maintained at 10 °C, and the sample was injected using full loop mode with a 20 µL loop and overfill factor of 3. A nitrogen generator provided the desolvation gas, while argon was the collision gas. The column and chromatography conditions are described in **Table S3**, and the multiple reaction monitoring parameters are listed in **Table S4**.

Table S3

LC-MS/MS chromatography

Column	Supelco Ascentis Express F5, 100 x 2.1 mm, 2.7 µm with 5 mm guard column
Column Temp	35^0C
Mobile Phase A	0.1% formic acid, 2 mM NH_4OAc in water
Mobile Phase B	0.1% formic acid, 2 mM NH_4OAc in methanol
Flow rate	0.45 mL/min
Gradient	Initial 28% A, 72% B to 4.0 min.; gradient to 20% A, 80% B, 10.0 min; 2% A, 98% B to 11.15 min; revert to initial conditions, run time 12.0 min

Table S4

Multiple reaction monitoring parameters

Compound	Transition*	Cone (V)	Collision (eV)
Vitamin D_3	385.5 → 273.1	22	12
	385.5 → 367.2	27	8
***d_3*-Vitamin D_2**	400.6 → 271.1	24	14
25(OH)D_3	401.3 → 159.0	24	28
	401.3 → 365.4	22	11
***d_6*-25(OH)D_3**	407.4 → 159.1	20	25
	All dwell times are 0.2 s		

*Quantifying transition listed first

Table S5

Comparison of vitamin D_3 and 25(OH)D_3 content estimates in 6 pork samples analysed by LC-MS/MS method in University College Cork (UCC) and Technical University of Denmark (DTU).

	Vitamin D_3 (μg/100 g)			25(OH)D_3 (μg/100 g)		
Sample No.	DTU	UCC	DTU as a % of UCC	DTU	UCC	DTU as a % of UCC
1	0.103	0.130	79	0.26	0.31	83
2	0.067	0.074	91	0.21	0.23	94
3	0.081	0.103	79	0.19	0.21	92
4	0.049	0.089	55	0.19	0.21	92
5	0.084	0.092	91	0.21	0.22	97
6	0.098	0.128	77	0.27	0.32	83
Mean	0.080	0.103	79	0.22	0.25	90

Reference

Jakobsen, J., Clausen, I., Leth, T. & Ovesen, L. (2004). A new method for the determination of vitamin D_3 and 25-hydroxyvitamin D_3 in meat. *Journal of Food Composition and Analysis*, 17, 777-787.

Sauvant, D., Perez, J. M., Tran, G. 2004. Table of composition and nutritional value of feed materials. Pigs, poultry, cattle, sheep, goats, rabbits, horses, fish. Wageningen Academic publishers, Netherlands

Charlton, S.J., Ewing, W. N. (2007) The Vitamins Directory 2nd edition. Context Products LTD, England.