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mRNA Expression of *CYP2E1, CYP2A19, CYP1A2, HSD3B, SULT1A1* and *SULT2A1* genes in surgically castrated, immunologically castrated, entire male and female pigs and correlation with androstenone, skatole, indole and Improvac-specific antibody levels

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Supplementary Online Material (SOM)

Material S1. Detection of Improvac-specific antibodies (hereinafter Improvac_Ab)

After clotting, serum was obtained by centrifugation from blood samples. The complex of GnRH with diphtheria toxin contained in Improvac (Zoetis Belgium SA) at the concentration 1.0 μg/ml in a coating solution (carbonate-bicarbonate buffer, 0.05M and pH 9.6) was used as antigen. It was dispensed into each well of 96 well-microplates (Maxisorp, NUNC). The plates were stored overnight at temperature 4°C. The next day, the plates were washed five times in a solution of PBS with 0.05% Tween, using a Biotek ELX 50 automated washer (BioTec Instruments, USA).

After being washed, a solution of PBS with 0.5% Tween, casein and 10% saccharose was pipetted into the wells and left for 60 min to block unspecific binding sites. After a subsequent wash step, the examined serum at dilutions of 300× (PBS, Tween at 0.025% and casein at 0.5%) were pipetted in volume 100 μl/well and left for 60 min at room temperature, after which the plates were washed five times.

One hundred microlitres of horseradish peroxidase-conjugated polyclonal goat anti-pig IgG secondary antibody (Bethyl), 30 000× diluted (PBS, 0.05% Tween, casein and 2% goat serum) were pipetted into each well. The plates were left for 30 min at room temperature and then washed five times. TMB substrate (TestLine, Czech Republic) was added at a volume of 100 μl to each well for a period of 30 min as a visualizing reagent, and this was followed by the addition of 50 μl of 2M H_2SO_4 to stop the reaction. The intensity of the reaction product was measured spectrophotometrically at 450 nm using a Biotek Synergy H1 Hybrid ELISA microplate reader (BioTec Instruments). The results are shown as absorbance.

The ELISA setup was validated in terms of homogeneity coating of plates (SD = 0.0324; coefficient of variability (CV) = 8.19% and repeatability – intra assay (SD = 0.0047; CV = 9.64%) and inter assay (SD = 0.4339, CV = 8.9%)

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Material S2. Determination of boar taint compounds by high-resolution LC-MS/MS

The method was modified according to Hansen-Moller (1994). Individual pig backfat samples (500 μg) were homogenized in a high-speed blender (Ultra-turrax T-18 basic, IKA, Germany) at 14 000 rpm and transferred to a 2 ml centrifuge tube. Then 50 μl of internal standard (50 ng/ml solution of d_2-testosterone in acetonitrile) was added, followed by extraction in 100% methanol (8 ml). Samples were shaken vigorously for 3 min. Samples with the extraction agent were centrifuged (14 000 rpm, 10 min, 4°C). After centrifugation, the sample was cooled to –20°C and the organic phase (6 ml) was taken up in a conical bottom evaporator flask. The sample was evaporated to dryness with a gentle stream of nitrogen at 30°C. The resulting sample was reconstituted with a solution (250 μl) of methanol : water (50 : 50, v/v) and transferred to the chromatographic vial with the insert. A 10 μl sample was injected into the LC-MS/MS system. The LC system was an Accela 1200 (Thermo Fisher Scientific, USA) equipped with a binary pump with vacuum degassing and an autosampler with a temperature-controlled tray and column. Chromatographic separation was carried out on a C18 Luna Omega (100 × 2.1 mm, 1.6 μm) column in combination with a C18 Luna Omega (10 × 2.1 mm) guard column, both from Phenomenex (Torrance, USA). The temperatures of the column oven and autosampler tray were set at 35°C. A gradient LC system was operated using 0.1% formic acid in water : methanol (95 : 5, v/v, Mobile Phase A was a 0.1% formic acid solution) and 0.1% formic acid in water : methanol (5 : 95, v/v, Mobile Phase B) at a flow of 300 μl/min. A gradient elution was performed: 0–2 min (95% A, 5% B), 6 min (linear gradient to 90% B), 6–8 min (10% A, 90% B), 8–10 min (linear gradient to 95% A), and 10–11 min (95% A, 5% B). The total runtime of the method was 11 min.

The tandem hybrid mass spectrometer Q Exactive (Thermo Fisher Scientific) equipped with a heated electrospray ionisation probe measured in positive mode (H-ESI+). For targeted quantification analysis, the mass spectrometer worked in the parallel reaction monitoring mode with high resolution RP = 17 000 (FWHM). Before the start of each acquisition series, the mass spectrometer was externally calibrated to the mass accuracy with positive ion calibration solution and negative ion calibration solutions (Thermo Fisher Scientific).

Instrument and collision cell (HCD) parameters were optimized by syringe direct infusion of working solutions using 50 ng/ml of each compound with a 5 μl/min flow rate. The mass spectrometer setting was as follows: sheath gas flow rate 25 (unit), aux gas flow rate 4 (unit), spray voltage 4.50 (kV), capillary temperature 220°C, heater temperature 320°C, S-lens RF level 70, AGC target of $6.e^6$ and a maximal inject time of 200 ms.

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Table S1. Primer sequences used for qPCR analysis

Gene name	Primer sequence	Accession number	Size (bp)	T_a (°C)	Authors
CYP2E1	F: CGGAAAGTTCAAGTACAGTGATCATT R: AGATGGCAGCCATGAACAGG	ENSSSCT00000011795	110	60	forward primer: Rasmussen et al. 2011
CYP1A2	F: CCAGCCCTACTCTGCAAAGATT R: CGCTTGTGATGTCCTGGATACA	ENSSSCG00000001901	120	60	reverse primer: Rasmussen et al. 2011
CYP2A19	F: TGGATGAGAACGGGCAGTTT R: AGAGCTCCATTCTAGCCAGACCTT	NM_214417	97	60	Rasmussen et al. 2011
HSD3B	F: GTGGTCATCCACACTGCCTCTA R: GGAGCTGGGTACCTTTCACATT	ENSSSCT00000036493.1	91	60	reverse primer: Chen et al. 2007
SULT1A1	F: ACTGGGGCCACTGGAGAGTT R: AAATCTGGTACCACCTGGGTGA	AY193893.1	83	60	this study
SULT2A1	F: CCATGCGAGACAAGGAGAAC R: CATGACCTGGAAG GAGCTGT	ENSSSCT00000003471.2	155	60	Sinclair et al. 2006
PPIA	F: CTGAGTGGTTGGATGGCAAA R: CCACAGTCAGCAATGGTGATCT	ENSSSCT00000018219	130	60	Svobodova 2011
TOP2B	F: CTAATGATGCTGGTGGCAAAC R: CCGATCACTCCTAGCCCAG	AF222921	100	60	Svobodova et al. 2008
TBP1	F: AACAGTTCAGTAGTTATGAGCCAGA R: AGATGTTCTCAAACGCTTCG	DQ845178	153	60	Nygart et al. 2007
GAPDH	F: CAGCAATGCCTCCTGCACCA R: GATGCCGAAGTGGTCATGGA	AF141959	70	60	Svobodova 2011
HPRT	F: AAGGACCCCTCGAAGTGTTG R: CACAAACATGATTCAAGTCCCTG	ENSSSCT00000030343	122	60	Svobodova et al. 2008

T_a = annealing temperature

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Table S2. Basic statistical parameters

	Indole (ng/g)	Skatole (ng/g)	Androste-none (ng/g)	Improvac_Ab NC (absorbance)	Improvac_Ab SC (absorbance)	Improvac_Ab IM8 (absorbance)	Improvac_Ab IM15 (absorbance)	Improvac_Ab GI (absorbance)
n	40	40	40	40	20	12	8	10
Mean	31.30	47.53	15.17	0.313	0.259	1.893	1.157	0.185
SD	63.84	97.49	31.60	0.197	0.136	0.407	0.369	0.043

Basic statistical parameters for indole, skatole and androstenone in non-castrated boars (NC), data for other groups (SC, IM8, IM15 and GI) were below detection limit (1.14, 1.16 and 0.68 ng/g, respectively) and for Improvac-specific antibodies (Improvac_Ab) in NC, SC, IM8, IM15 and GI groups. Levels of absorbance in detection of Improvac_Ab are at the level of background in groups NC, SC and GI. In contrast, increase of Improvac_Ab was clearly detectable in groups IM8 and IM15

Our data show a high standard deviation (SD), because of the presence of very low levels of metabolites in many animals and asymmetric distribution associated with that, which is typical for this trace analysis of very variable biological material