EFFECT OF DIETARY SUPPLEMENTATION WITH DRIED TUBER OF JERUSALEM ARTICHOKE ON SKATOLE LEVEL IN BACKFAT AND *CYP2E1* mRNA EXPRESSION IN LIVER OF BOARS*

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Abstract

The objective of this study was to investigate the effect of diets containing different levels of dried tuber of Jerusalem artichoke, *Helianthus tuberosus*, on skatole levels in backfat and on the *CYP2E1* mRNA expression in the liver of commercial crossbred pigs. A total of 23 uncastrated male pigs from 10 litters of a commercial crossbred population of Large White × (Landrace × Large White), were used in this study. Boars were randomly divided into four different dietary treatment groups – a control group (K1; 5 boars; without supplementation of Jerusalem artichoke) and three experimental groups (6 boars each) that were fed with the diet containing different levels of dried Jerusalem artichoke (K2 – 4.1%; K3 – 8.2%; K4 – 12.2%) for 14 days before slaughter. Significant effects of diet on skatole levels were observed between the control group and the experimental groups ($P = 0.0078$). The lowest level of skatole was in the K3 group with 8.2% of Jerusalem artichoke. As for *CYP2E1*, a negative correlation was observed between the levels of skatole and *CYP2E1* mRNA expression. Significant effect ($P = 0.0055$) was found in all experimental groups compared to the K1 group, and most pronounced in the K2 and K3 groups. The supplementation with Jerusalem artichoke resulted in lower level of skatole and higher *CYP2E1* mRNA expression. The results suggest that affecting the expression of *CYP2E1* by feed supplements could be an option to effectively reduce the levels of skatole in adipose tissue of entire male pigs.

Key words: pig, boar taint, nutrition, *Helianthus tuberosus*, RT-qPCR

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Fattening entire male pigs, when compared with castrated males and gilts, has a number of advantages including better feed conversion, faster growth rates, lower output of nitrogen in environment and reduction of suffering for the animal (Zadinová et al., 2016). In most countries, however, male pigs are still castrated very soon after being born to avoid the boar taint (Duijvesteijn et al., 2010), which is one of the main problems in pork production with entire male pigs (Wesoly and Weiler, 2012). Boar taint is an offensive urine- and faecal-like odour observed in cooked meat and fat of boars and it is mainly caused by androstenone, skatole and indole (Drag et al., 2018; Kubešová et al., 2019). Threshold average acceptance values are 0.5–1.0 µg/g fat for androstenone and 0.2–0.25 µg/g fat for skatole (Rowe et al., 2014). Surgical castration without anaesthesia represents a problem from an ethical point of view and also endangers the welfare of animals (Gray and Squires, 2013). The EU aims for alternative solutions to the boar taint issue within a few years (Van Son et al., 2017). In addition to feeding strategies and using feed additives, genetic selection towards lower boar taint incidence could help pig farmers to shift towards raising boars (Han et al., 2019). The levels of androstenone are mainly under genetic influence, and skatole and indole are produced by bacteria in colon; their levels in adipose tissue are influenced by numerous factors, the most important being nutritional ones and inhibitory effects of sex steroids on the liver enzymes involved in skatole metabolism (Vhile et al., 2012; Zadinová et al., 2016).

Skatole accumulation in adipose tissue is one of the predominant factors causing boar taint (Bilić-Šobot et al., 2016). Skatole is formed from amino acid L-tryptophan in the large intestine of monogastric animals during the microbial degradation of proteins (Bilić-Šobot et al., 2016; Zadinová et al., 2016). It is metabolized in liver mainly by cytochrome P450 (CYP450) enzymes, of which CYP2E1 (cytochrome P450, family 2, subfamily E, polypeptide 1) plays a major role. *CYP2E1* levels in the liver are negatively correlated with skatole levels in backfat of male pigs (Zamaratskaia and Squires, 2009).

Earlier studies report that feeding pigs with diets supplemented with carbohydrates that are not enzymatically hydrolysed in the small intestine but fermented in the hindgut, can increase *CYP2E1* mRNA expression in the liver, and can reduce skatole levels in the hindgut and skatole accumulation in adipose tissue (Borrišer-Pairó et al., 2015; Wesoly and Weiler, 2012; Zammerini et al., 2012). Inulin-type fructans are found in several fruits and vegetables, but industrial production is mainly based on chicory. Numerous studies demonstrated that feeding chicory or pure inulin influenced the content of skatole in the excrement, blood and adipose tissue (Vhile et al., 2012; Wesoly and Weiler, 2012; Zammerini et al., 2012). Jerusalem artichoke (*Helianthus tuberosus*), with 50% inulin, has been confirmed to have a similar effect as chicory. Offering it one week before slaughter showed a tendency towards a decrease of skatole levels in fat (Vhile et al., 2012). In addition, feeding of inulin to pigs could also have a beneficial influence on growth performance, especially on daily weight gain (Grela et al., 2013; Samolińska et al., 2019).

The objective of this study was to investigate the effect of different levels of Jerusalem artichoke in the diet on skatole level in backfat and *CYP2E1* mRNA expression levels in liver of crossbred male pigs. The hypothesis was that the supplementation of Jerusalem artichoke to pig diet should increase the *CYP2E1* mRNA expression in boar livers and at the same time decrease the content of skatole in backfat.

Material and methods

All experimental procedures were approved by the Ethics Committee of the Central Commission for Animal Welfare at the Ministry of Agriculture of the Czech Republic (Prague, Czech Republic) and was carried out in accordance with Directive 2010/63/EU for animal experiments and Local Ethics Commission, case number 10/2015; the experiment was conducted in testing station at the Czech University of Life Sciences Prague (CZ21038206). All pigs were slaughtered according to the protocols for certified Czech slaughterhouses under the supervision of an independent veterinarian.

Animals, diets and experimental design

Twenty-three uncastrated male pigs from 10 litters of a commercial crossbred population of Large White $\times$ (Landrace $\times$ Large White), were used. The pigs were maintained in a testing station at the Czech University of Life Sciences Prague, Czech Republic (two pigs per pen – 2.895×1.125 m) under the standard conditions. At the start of the experiment, the pigs were 87 days old and had an average live weight of 46.3 ± 4.68 kg. They were fed *ad libitum* with basal complete feed mixtures, P1 and P2 (containing barley, wheat, soybean meal and premix of vitamins and minerals; Table 1), the compositions of which were continually adjusted with respect to the age and weight of the pigs (Šimeček et al., 2000). Water was available *ad libitum*. From 111 days of age the P1 diet was switched to the P2 diet with different content of grinded dried Jerusalem artichoke tubers, depending on the experimental group. Boars were randomly divided into four different dietary treatment groups – a control group (K1; 5 boars; without Jerusalem artichoke, *Helianthus tuberosus*) and three experimental groups (6 boars each) that were fed with the diet containing different levels of dried Jerusalem artichoke tuber (K2 – 4.1%; K3 – 8.2%; K4 – 12.2%) in feed rations, which were fed for 14 days before slaughter (Table 1). The content of inulin (0.5 g/g Jerusalem artichoke) was determined based on the analysis of dried Jerusalem artichoke. The contents of dry matter and crude fibre were similar to those in the basal complete feed mixture. In the P2 diets for different experimental groups, with differing Jerusalem artichoke content, similar nutrient composition was achieved by adjusting the content of barley, wheat and soybean meal (calculated according to Šimeček et al., 2000 and While et al., 2012; Table 1). The pigs aged 154 days at average live weight of 112 ± 8.16 kg, were slaughtered in the slaughterhouse, stunned using a captive bolt gun and killed by exsanguination.

Table 1. Ingredients and chemical composition of the boar diets

Feed mixture	P1 ¹	P2 ²	P2+4.1% ³	P2+8.2% ³	P2+12.2% ³
Experimental group	All	K1	K2	K3	K4
Span of live weight (kg)	46–66	67–112	93–112	93–112	93–112
Age of pigs (days)	87–110	111–153	140–153	140–153	140–153
Components (g/kg)					
barley	500	390	395	400	410
wheat	313	490	439	388	333
soybean meal	150	90	95	100	105
premix of vitamins and minerals ⁴	30	30	30	30	30
monocalcium phosphate	7	–	–	–	–
Jerusalem artichoke	–	–	41	82	122
Calculated chemical composition					
dry matter (%)	90.95	87.3	87.3	87.3	87.3
crude protein (%)	16.4	14.8	14.8	14.8	14.8
crude fat (%)	1.06	1.06	1.06	1.06	1.06
crude fibre (%)	3.7	3.63	3.63	3.63	3.63
starch (%)	45.4	50.8	50.8	50.8	50.8
ME ⁵ (MJ/kg)	13.2	13.6	13.6	13.6	13.6
lysine/ME	0.73	0.60	0.60	0.60	0.60
Amino acids (g/kg)					
lysine	9.64	8.07	8.07	8.07	8.07
methionine	2.95	2.81	2.81	2.81	2.81
threonine	6.24	5.39	5.39	5.39	5.39
tryptophan	2.06	1.74	1.74	1.74	1.74

¹P1 – complete feed mixture for the 1st phase of fattening.

²P2 – complete feed mixture for the 2nd phase of fattening.

³P2 – feed mixtures with Jerusalem artichoke. From 111 to 139 days and live weight 67–92 kg the boars were fed with the P2 diet without Jerusalem artichoke.

⁴Premix of micro- and macrominerals, essential amino acids, and vitamins – content in 1 kg of premix: retinol 400 000 IU, cholecalciferol 66 000 IU, α -tocopherol 3 600 mg, menadione 100 mg, thiamine 60 mg, riboflavin 150 mg, niacin 800 mg, Ca pantothenate 375 mg, vitamin B₆ 100 mg, vitamin B₁₂ 1 mg, choline Cl 15 000 mg, folic acid 15 mg, Fe 3 500 mg as FeSO₄·H₂O, Zn 3 600 mg as ZnO, Mn 3 100 mg as MnO, Cu 330 mg as CuSO₄·5H₂O, I 175 mg as Ca(IO₃)₂, Co 15 mg as 2CoCO₃·3Co(OH)₂·H₂O, Se 13 mg as Na₂SeO₃, 6-phytase (EC 3.1.3.26) 25 000 FTU, Ca 220 g, P 20 g, Na 50 g, Mg 10 g, lysine 85 g, methionine 15 g, threonine 15 g.

⁵ME – metabolisable energy.

Growth performance and carcass traits

Boars were weighed individually in weekly intervals and feed intake was monitored daily. Based on the obtained values, live weight, daily feed intake, feed conversion ratio, and average daily gain were calculated. The above traits were calculated

over the entire experimental period. Immediately after slaughtering, the following indicators were measured in the individual pigs: carcass weight, carcass lean meat, fat thickness as described by Čítek et al. (2015). Before dissection, the carcasses were stored at +2°C for 24 h. Carcass dissection was performed according to Walstra and Merkus (1995). From the qualitative parameters, the pH value (45 min *post mortem*) electrical conductivity (50 min *post mortem*) and intramuscular fat content (IMF) were measured.

Tissue samples collection

Liver samples (150 mg) for CYP2E1 mRNA expression analysis were collected at the slaughter within 30 min of boar sacrifice. All samples were cut out from the left lateral lobe of the liver and immediately submerged in RNeasy Lysis Solution (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and stored at -20°C until processing. Samples of subcutaneous fat, for determination of skatole, indole and androstenone levels, were taken in a neck region between cervical vertebrae 1 and 3, 24 hours after slaughter, vacuum packed and frozen at -80°C until processing. The tissue samples for analysis of intramuscular fat content were collected from chop (*musculus longissimus lumborum et thoracis*), shoulder (*musculus serratus ventralis*), neck (*musculus cleidocephalicus*) and ham (*musculus semimembranosus*) 24 hours after slaughter, vacuum packed and frozen at -80°C. IMF was determined using gravimetric method following extraction with petroleum ether by the Soxhlet method.

Determination of skatole, indole and androstenone levels

The skatole, indole and androstenone levels in subcutaneous fat-samples were measured by high-performance liquid chromatography (LC-2000Plus HPLC system; Jasco, Tokyo, Japan) according to the method of Hansen-Møller (1994) as modified by Okrouhlá et al. (2016). The records were evaluated using the programme ChromNAV (Jasco) and quantitation was performed on the basis of the retention times of the standards of androstanone and 2-methylindole. To determine skatole and indole levels, a Kinetex C18 100A column (5 µm, 50 × 4.60 mm ID) operated at 40°C was used. For determination of androstenone, an Agilent Eclipse XDB C18 column (5 µm, 150 × 4.60 mm ID) operated at 40°C was employed. More details are presented in Zadinová et al. (2017).

Primer design, RNA extraction and reverse transcription reaction

Specific transcript variant independent exon-spanning PCR primers for CYP2E1 were designed using the Primer-Blast program (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) based on DNA sequences in the Pig (Sscrofa10.2) Ensembl database (http://www.ensembl.org/Sus_scrofa/; gene ENSSCG00000010780) and the GenBank mRNA sequence NM_214421.1. Based upon previous research, *ACTB*, *B2M*, *HPRT1* and *YWHAZ* were selected as candidate reference genes (Erkens et al., 2006). All primers were ordered from Integrated DNA Technologies (IDT, Leuven, Belgium). The primers and amplicon information are presented in Table 2.

Table 2. Primers sequences and qPCR assay details for *CYP2E1* and reference genes in pigs

Gene ¹	Primers	Primer sequences (5'-3')	Amplicon (bp)	T _a ² (°C)	R ^{2,3}	PCR efficiency (%)	Reference
<i>CYP2E1</i>	F	AACAGGGCAATGAGCAGCGGA	147	64	0.999	101.4	ENSSSCG00000010780
	R	AGTGC'TGGCGGAAGAGGATGTC					
<i>YWHAZ</i>	F	ATGCAACCAACACATCCTATC	178	60	0.997	98.4	Erkens et al. (2006)
	R	GCATTATTAGCGTGTCTCTT					
<i>ACTB</i>	F	TCTGGCACCCACACCTTCT	114	60	0.998	103.2	Erkens et al. (2006)
	R	TGATCTGGGTCACTTCTCAC					
<i>B2M</i>	F	AAACGGAAAGCCAAATTACC	178	60	0.992	102.0	Erkens et al. (2006)
	R	ATCCACAGCGTTAGGAGTGA					
<i>HPRT1</i>	F	CCGAGGATTGGAAAGGT	181	60	0.995	96.4	Erkens et al. (2006)
	R	CTATTCTGTTCAGTCTTGATGT					

¹*CYP2E1*: cytochrome P450 family 2 subfamily E member 1; *YWHAZ*: tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein zeta; *ACTB*: actin beta; *B2M*: beta-2-microglobulin; *HPRT1*: hypoxanthine phosphoribosyltransferase 1.

²T_a – annealing temperature.

³R² – coefficient of determination.

The samples of the liver were removed from RNA later and rinsed several times in PCR ultra water. The 100 mg of liver sample was homogenized in Purezol (Qiagen, Hilden, Germany) and total RNA was isolated using the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad Laboratories Inc., Hercules, California, USA) according to the manufacturer's protocol which included an efficient on-column DNase treatment. Finally, 30 µl elution buffer was added to elute the RNA. Quantity and purity of the RNA samples were analysed with the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). RNA concentration was in the range 790–1696 ng/µl. Additionally, RNA integrity was verified by visualization of the 28S and 18S ribosomal bands on a 2% agarose gel, and minus RT control was performed with the *CYP2E1* primers on 2 µl RNA to confirm the absence of any DNA. Approximately 1 µg of high quality RNA was reverse-transcribed to cDNA with the Improm-II cDNA synthesis kit (Promega, Madison, WI, USA). RNA was mixed with 400 ng oligo (dT) and 400 ng random hexamers. The mix was heated for 5 min at 70°C, followed by cooling on ice for 5 min. Then, 4 µl of Improm-II 5x reaction buffer, 2.4 µl of MgCl₂ (25 mM), 1 µl of dNTP Mix (10 mM each), and 1 µl of Improm-II reverse transcriptase were added. This mix was incubated for 5 min at 25°C (annealing), 60 min at 42°C (synthesis of cDNA), and 15 min at 72°C (reverse transcriptase inactivation).

Quantitative polymerase chain reaction (qPCR)

qPCR was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Inc.) in 10 µl reaction volumes using 5 µl of KAPA SYBR FAST qPCR master mix (KAPA Biosystems, MA), 1 µl primer mix (5 µM each) and 2 µl cDNA (10-fold diluted). The qPCR profile was 95°C for 3 min 40 sec, followed by 40 cycles of 20 s at 95°C and 40 s at the optimal annealing/elongation temperature (Table 2) with detection of fluorescence. A melting curve was generated by heating the samples from 70 to 95°C in steps of 0.5°C for 5 s with fluorescence detection. In each run, a serial dilution (6-points, 4-fold dilution) of a cDNA sample and a negative control sample were included to determine the efficiency, the correlation coefficient, the dynamic range and potential contamination (Table 2). All reactions were performed at least two times. The quantification cycle values of the target gene were converted to raw data based on the efficiency of the qPCR. The relative expression was formulated as a ratio of the target gene to the geometric mean of 3 reference genes (*ACTB*, *HPRT1* and *B2M*), selected from the 4 preselected candidate reference genes using the geNorm algorithm (the pairwise variation (V2/3) of the genes was 0.190; (Vandesompele et al., 2002). The whole RT-qPCR experiment was performed according to the MIQE guidelines (Bustin et al., 2009).

Statistical analyses

Statistical analyses were performed using SAS version 9.4 (Statistical Analysis System, Inst. Version 9.4, 2012, SAS Institute, Cary, NC, USA). The original data of the gene expression study was log-transformed prior to statistical analysis. These data are normally distributed for all nutrition groups. Normality of variables was checked using Kolmogorov-Smirnov test. The data was analysed with one-way

analysis of variance (ANOVA). The results are presented as the least squares means (LSM) and root mean square error (RMSE). Differences between LSM were determined by Duncan's test ($P < 0.05$ and $P < 0.01$). The statistical model was:

$$Y_{ij} = \mu + g_i + e_{ij}$$

where:

Y_{ij} is value of the traits,

μ is overall mean,

g_i is the effect of diet group ($i = K1, K2, K3, K4$),

e_{ij} is random residual error.

The Pearson correlation coefficients between levels of skatole, indole and androstenone, and between levels of boar taint compounds and *CYP2E1* mRNA expression were calculated.

Regression equations were obtained using REG procedure of SAS to determine how the response variable increased/decreased skatole level and *CYP2E1* mRNA expression per unit of Jerusalem artichoke included in the diet.

Results

Effect of feeding Jerusalem artichoke on growth performance, carcass traits and levels of boar taint compounds in backfat

During fattening no significant differences in growth performance and carcass traits were observed between the control and experimental groups (Table 3). The average daily gain was 1024 ± 84.12 g and feed conversion was 2.25 ± 0.15 kg. A significant effect of feeding Jerusalem artichoke on growth performance and carcass traits was not observed.

A significant effect of feeding Jerusalem artichoke on levels of boar taint compounds was shown only for skatole. Significant differences in skatole levels are evident between control group K1 and all experimental groups (Table 4). There were non-significant differences between the individual experimental groups. The lowest levels of skatole were observed in the K3 group with addition of 8.2% dried Jerusalem artichoke. Androstenone and indole levels showed the same trend as skatole. However, there were no significant differences between the groups for both compounds. The relationship between Jerusalem artichoke content as a feeding additive and backfat skatole levels is presented in Table 5. With an increase of Jerusalem artichoke content in feed mixture by 1%, skatole level in backfat decreased by 0.011 $\mu\text{g/g}$.

The Pearson correlation coefficients were 0.40 ($P < 0.05$) for androstenone – skatole, 0.24 ($P < 0.0001$) for androstenone – indole, and 0.77 ($P = 0.19$) for skatole – indole.

Table 3. Effect of feeding Jerusalem artichoke on growth performance and carcass traits

Traits	Group				RMSE ²	P-value
	K1 ¹	K2 ¹	K3 ¹	K4 ¹		
Live weight (kg)	111.41	114.36	112.93	110.52	8.29	0.545
Feed intake (kg)	2.39	2.40	2.39	2.39	0.10	0.994
Feed conversion ratio (kg)	2.25	2.23	2.22	2.32	0.16	0.369
Average daily gain (g)	1026.05	1040.82	1032.82	992.89	84.46	0.463
Carcass weight (kg)	86.3	86.18	86.05	83.07	6.97	0.596
Carcass lean meat (%)	59.76	59.75	59.58	59.48	1.20	0.924
Fat thickness (mm)	13.65	13.51	14.13	13.31	2.52	0.870
pH ₄₅ ³	6.44	6.49	6.45	6.46	0.31	0.094
Electrical conductivity (EC ₅₀) ⁴	3.78	3.80	3.86	3.63	0.46	0.653
IMF ⁵ chop (%)	2.56	2.58	2.13	2.77	0.73	0.179
IMF ⁵ shoulder (%)	2.23	2.01	2.26	1.99	0.58	0.531
IMF ⁵ neck (%)	3.13	2.93	2.98	3.04	0.47	0.740
IMF ⁵ ham (%)	2.65	2.63	2.81	2.96	0.71	0.643

¹the values are presented as least squares means.²RMSE – root mean square error.³pH₄₅ – pH value 45 min *post mortem*.⁴EC₅₀ – electrical conductivity 50 min *post mortem*.⁵IMF – intramuscular fat content.

Table 4. Effect of feeding Jerusalem artichoke on levels of boar taint compounds in backfat of boars

Traits	Group				RMSE ²	P-value
	K1 ¹	K2 ¹	K3 ¹	K4 ¹		
Skatole (µg/g backfat)	0.206 A	0.074 B	0.023 B	0.070 B	0.08	0.0078
Indole (µg/g backfat)	0.085	0.047	0.040	0.036	0.04	0.2593
Androstenone (µg/g backfat)	7.665	4.069	2.305	2.623	5.05	0.319

¹the values are presented as least squares means.

²RMSE – root mean square error.

Values with different letters within the row are highly significantly different at: A, B – P<0.01.

Table 5. Regression coefficients for skatole levels by Jerusalem artichoke content and mRNA expression of *CYP2E1*

Variable	Jerusalem artichoke	Intercept	R ²	P-value
Skatole	– 0.0109	0.1565	0.2439	0.0166
<i>CYP2E1</i>	0.0235	0.5511	0.1182	0.1083
Variable	Skatole	Intercept	R ²	P-value
<i>CYP2E1</i>	– 1.5280	0.8329	0.2435	0.0167

CYP2E1 mRNA expression in boar livers

The specificity of the *CYP2E1* qPCR amplicon was checked by qPCR melt curve analysis, 2% agarose gel electrophoresis and sequencing. The results confirmed the amplification of a single fragment of 147 bp (without non-specific products or primer dimers) and 100% sequence identity with NM_214421.1. The *CYP2E1* mRNA expression was significantly higher in the three experimental groups (with addition of Jerusalem artichoke) in comparison with the control group ($P = 0.0055$; Figure 1). The highest expression was observed in the K2 and K3 feeding groups. Significant differences were not observed between experimental groups K2, K3, and K4. Experimental feeding groups presented significantly lower levels of skatole and higher mRNA expression than control group. The skatole decrease of 1 μg means increase in *CYP2E1* mRNA expression level of 1.53. An increase of Jerusalem artichoke content of 1% increased mRNA expression of 0.023.

The Pearson correlation coefficient was -0.49 ($P = 0.017$) for *CYP2E1* expression – skatole (or indole) level.

Figure 1. Means ($\pm$ SE) of the relative expression of *CYP2E1* in liver from entire male pigs. Boars were divided into four different dietary treatment groups: K1 – control group without Jerusalem artichoke, and experimental groups that were fed with the diet containing different levels of dried Jerusalem artichoke (K2 – 4.1%; K3 – 8.2%; K4 – 12.2%) in feed rations, which were fed for 14 days before slaughter. Data were subjected to statistical analysis using Kolmogorov-Smirnov test. Bars with different letters are significantly different at: a, b – $P < 0.05$; A, B – $P < 0.01$

Discussion

Effect of feeding Jerusalem artichoke on growth performance, carcass traits and on boar taint compounds levels in backfat

Effect of feeding Jerusalem artichoke on growth performance and carcass traits was not demonstrated in this study. The same conclusions were reached by Aluwé et al. (2017), Bee et al. (2017), Bilić-Šobot et al. (2016), Vhile et al. (2012) and Kjos et al. (2010).

A significant effect of the feeding of Jerusalem artichoke on the levels of skatole was now proved. All experimental feeding groups showed significantly lower levels of skatole compared to the control group. The lowest skatole level was observed in K3 group with 8.2% dried Jerusalem artichoke. The effect of inulin, which is contained in Jerusalem artichoke, was also observed by Vhile et al. (2012), who used the same feeding groups as in our experiment. Feeding Jerusalem artichoke one week before slaughter led to a decrease of skatole levels in gut and adipose tissue. However, the differences in adipose tissues between the negative control and experimental groups were non-significant ($P = 0.06$), while in our experiment, with the two-week feeding with Jerusalem artichoke addition, the differences were highly significant ($P < 0.01$). A positive effect of inulin addition was observed in other studies as well. Kjos et al. (2010) analysed chicory inulin in 3 different concentrations (3, 6 and 9% chicory inulin) 4 weeks before slaughter. They observed a significant decrease of skatole levels in groups with 6 and 9% inulin. Aluwé et al. (2017) observed a similar effect of 5% inulin in feed mixture that was fed 3 weeks before slaughter. Non-significant effect of Jerusalem artichoke on androstenone levels was observed in the study of Vhile et al. (2012). Kjos et al. (2010) and Aluwé et al. (2017) did not evaluate the effect of respectively Jerusalem artichoke and inulin on androstenone levels. This is probably due to the fact that androstenone is not primarily affected by diet. On the other hand, Bilić-Šobot et al. (2016) reported a negative effect of tannins on androstenone levels. In all experimental groups the levels of androstenone were higher compared to the control group.

In addition, feeding inulin positively influences the populations of *Lactobacillus* and *Bifidobacterium* in large intestine of piglets (Kozłowska et al., 2016). Therefore, the changes in bacterial microflora in digestive tract of pigs can be associated with the decreased skatole level. The change of microflora in digestive tract could be probably affected not only by the content of Jerusalem artichoke in the feed mixture but also by the duration of feeding. Determination of the optimal level and duration of dietary supplementation with Jerusalem artichoke could positively affect the economy of boars feeding.

CYP2E1 mRNA expression in boar livers

The level of skatole in adipose tissue of male pigs depends on different factors, one of which is the rate of its hepatic metabolism. CYP2E1 is a key enzyme involved in phase I of the skatole metabolism in the liver. The mRNA expression of *CYP2E1* in liver was significantly higher in the experimental groups compared to the control group. Negative correlations between *CYP2E1* mRNA expressions

in porcine liver and the skatole levels in adipose tissue have earlier been reported (Bee et al., 2017; Bilić-Šobot et al., 2016; Kubešová et al., 2019; Rasmussen et al., 2014). These results suggest a possibility of affecting the overall levels of boar taint by decreasing skatole levels by feeding strategy and by selection of boars with high *CYP2E1* mRNA expression levels. However, in connection with feeding strategy, it is difficult to determine whether increased expression of *CYP2E1* mRNA is caused by a decrease in skatole production in the gastrointestinal tract or whether it is influenced by plant extract effects from Jerusalem artichoke. As suggested by Rasmussen et al. (2014), secondary metabolites from chicory affect mRNA expression of genes of the *CYP* family. The effects of individual Jerusalem artichoke substances on the expression of the *P450* family genes or enzymatic activity could be the subject of a further study. In addition, a significant positive correlation between skatole and indole, and between skatole and androstenone was observed in this study as well. The same result was achieved in the study of Kubešová et al. (2019). Regression analysis suggests a relationship between Jerusalem artichoke addition and skatole levels in backfat as well as between skatole and *CYP2E1* mRNA expression level. The activity or expression of enzymes that can influence the boar taint components may also be affected by genetic variants in the responsible genes (e.g. Rowe et al., 2014; Zadinová et al., 2016, 2017). In our experimental boars, polymorphism in *CYP2E1* was negligible and had no effect on the expression.

Conclusions

We have shown a positive effect of supplementation of boar diet with Jerusalem artichoke during the last 14 days before slaughter on the level of the boar taint component, skatole, in adipose tissue. Skatole was significantly reduced in all three experimental groups with Jerusalem artichoke addition, and the most pronounced effect was in the K3 group with 8.2% of dried Jerusalem artichoke. The supplementation with Jerusalem artichoke affected also the *CYP2E1* mRNA expression in liver. The experimental groups with Jerusalem artichoke (and low levels of skatole) had a higher mRNA expression of *CYP2E1*. The supplementation with Jerusalem artichoke had neither positive nor adverse effect on growth performance and carcass traits. Future studies should be directed to the search for specific feed supplements and their effects on skatole, as well as androstenone and indole levels, and the expression of genes of the *CYP* family.

References

- Aluwé M., Heyrman E., Theis S., Sieland C., Thurman K., Millet S. (2017). Chicory fructans in pig diet reduce skatole in back fat of entire male pigs. *Res. Vet. Sci.*, 115: 340–344.
- Bee G., Silacci P., Ampiero-Kragten S., Čandek-Potokar M., Wealleans A.L., Litten-Brown J., Salminen J.P., Mueller-Harvey I. (2017). Hydrolysable tannin-based diet rich in gallotannins has a minimal impact on pig performance but significantly reduces salivary and bulbourethral gland size. *Animal*, 11: 1617–1625.
- Bilić-Šobot D., Zamaratskaia G., Rasmussen M.K., Čandek-Potokar M., Škrlep M., Prevolnik Povše M., Škorjanc D. (2016). Chestnut wood extract in boar diet reduces intestinal skatole production, a boar taint compound. *Agron. Sustain. Devel.*, 36: 62.

- Borrisser-Pairó F, Rasmussen M.K., Ekstrand B., Zamaratskaia G. (2015). Gender-related differences in the formation of skatole metabolites by specific CYP450 in porcine hepatic S9 fractions. *Animal*, 9: 635–642.
- Bustin S.A., Benes V., Garson J.A., Hellemans J., Huggett J., Kubista M., Mueller R., Nolan T., Pfaffl M.W., Shipley G.L., Vandesompele J., Wittwer C.T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.*, 55: 611–622.
- Čítek J., Stupka R., Okrouhlá M., Vehovský K., Brzobohatý L., Šprysl M., Stádník L. (2015). Effects of dietary linseed and corn supplement on the fatty acid content in the pork loin and backfat tissue. *Czech. J. Anim. Sci.*, 60: 319–326.
- Drag M., Hansen M.B., Kadarmideen H.N. (2018). Systems genomics study reveals expression quantitative trait loci, regulator genes and pathways associated with boar taint in pigs. *PLoS One*, 13: 2.
- Duijvesteijn N., Knol E.F., Merks J.W.M., Crooijmans R., Groenen M.A.M., Bovenhuis H., Harlizius B. (2010). A genome-wide association study on androstenone levels in pigs reveals a cluster of candidate genes on chromosome 6. *BMC Genet.*, 11: 42.
- Erkens T., Van Poucke M., Vandesompele J., Goossens K., Van Zeveren A., Peelman L.J. (2006). Development of a new set of reference genes for normalization of real-time RT-PCR data of porcine backfat and *longissimus dorsi* muscle, and evaluation with *PPARGC1A*. *BMC Biotech.*, 6: 41.
- Gray M.A., Squires E.J. (2012). Effects of nuclear receptor transactivation on steroid hormone synthesis and gene expression in porcine Leydig cells. *J. Ster. Bioch. Mol. Biol.*, 133: 93–100.
- Grela E.R., Pietrzak K., Sobolewska S., Witkowski P. (2013). Effect of inulin and garlic supplementation in pig diets. *Ann. Anim. Sci.*, 13: 63–71.
- Han X., Zhou M., Cao X., Du X., Meng F., Bu G., Kong Huang A., Zeng X. (2019). Mechanistic insight into the role of immunocastration on eliminating skatole in boars. *Theriogenology*, 131: 32–40.
- Hansen-Møller J. (1994). Rapid high-performance MDGC/MS – chromatography method for simultaneous determination of androstenone, skatole and indole in back fat from pigs. *J. Chrom. B: Biom. Sci. Appl.*, 661: 219–230.
- Kjos N.P., Overland M., Fauske A.K., Sorum H. (2010). Feeding chicory inulin to entire male pigs during the last period before slaughter reduces skatole in digesta and backfat. *Livest. Sci.*, 134: 1143–1145.
- Kozłowska I., Maré-Pieńkowska J., Bednarczyk M. (2016). Beneficial aspects of inulin supplementation as a fructooligosaccharide prebiotic in monogastric animal nutrition – a review. *Ann. Anim. Sci.*, 16: 315–331.
- Kubešová A., Šťastný K., Faldyna M., Sládek Z., Steinhauserová I., Bořilová G., Knoll A. (2019). 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. *Czech J. Anim. Sci.*, 64: 89–97.
- Okrouhlá M., Stupka R., Čítek J., Urbanová D., Vehovský K., Kouřimská L. (2016). Method for determination of androstenone, skatole and indole in dorsal fat of pigs (in Czech). *Chemické Listy*, 110: 593–597.
- Rasmussen M.K., Klausen C.L., Ekstrand B. (2014). Regulation of cytochrome P450 mRNA expression in primary porcine hepatocytes by selected secondary plant metabolites from chicory (*Cichorium intybus* L.). *Food Chem.*, 146: 255–263.
- Rowe S.J., Karacaören B., De Koning D.J., Lukić B., Hastings-Clark N., Vellander I., Haley C.S., Archibald A.L. (2014). Analysis of the genetics of boar taint reveals both single SNPs and regional effects. *BMC Genom.*, 15: 424.
- Samolińska W., Grela E.R., Kiczorowska B. (2019). Effects of inulin extracts and inulin-containing plants on haematobiochemical responses, plasma mineral concentrations, and carcass traits in growing-finishing pigs. *J. Elem.*, 24: 2711–2726.
- Šimeček K., Zeman L., Heger J. (2000). Nutrient requirements and tables of dietary requirements of feeds for pigs (in Czech). 2nd ed. MZLU, Brno, 124 pp.

- Van Son M., Kent M.P., Growe H., Agarwal R., Hamland H., Lien S., Grindflek E. (2017). Fine mapping of a QTL affecting levels of skatole on pig chromosome 7. *BMC Genet.*, 18: 85.
- Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A., Speleman F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.*, 3: 7.
- Vhile S.G., Kjos N.P., Sorum H., Overland M. (2012). Feeding Jerusalem artichoke reduced skatole level and changed intestinal microbiota in the gut of entire male pigs. *Animal*, 6: 807–814.
- Walstra A.P., Merkus G.S.M. (1995). Procedure for assessment of the lean meat percentage as a consequence of the new EU reference dissection method in pig carcass classification. DLO Research Institute for Animal Science and Health (ID-DLO). The Netherlands, Zeist.
- Wesoly R., Weiler U. (2012). Nutritional influences on skatole formation and skatole metabolism in the pig. *Animals (Basel)*, 2: 221–242.
- Zadinová K., Stupka R., Stratil A., Čítek J., Vehovský K., Urbanová D. (2016). Boar taint – the effect of selected candidate genes associated with androstenone and skatole levels – a review. *Anim. Sci. Pap. Rep.*, 34: 107–128.
- Zadinová K., Stupka R., Stratil A., Čítek J., Vehovský K., Lebedová N., Šprysl M., Okrouhlá M. (2017). Association analysis of SNPs in the porcine CYP2E1 gene with skatole, indole and androstenone levels in backfat of a crossbred pig population. *Meat Sci.*, 131: 68–73.
- Zamaratskaia G., Squires E.J. (2009). Biochemical, nutritional and genetic effects on boar taint in entire male pigs. *Animal*, 3: 1508–1521.
- Zammerini D., Wood J.D., Whittington F.M., Nute G.R., Hughes S.I., Hazzledine M., Matthews K. (2012). Effect of dietary chicory on boar taint. *Meat Sci.*, 91: 396–401.

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