Effect of dose of thymol and supplemental flavours or camphor on palatability in a choice feeding study with piglets

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ABSTRACT: Thymol's antimicrobial properties urged researchers to study its effect on animal performance and intestinal health in pigs. However, thymol has the characteristic sharp odor of thyme and a bitter, aromatic, and sometimes burning sensation which may elicit feeding aversions. The objectives in the current study were: (1) to determine the effect of dose of thymol and camphor on palatability and (2) to test the hypothesis that supplemental flavours or camphor, the latter as a known Transient Receptor Potential A1 blocker, could mitigate feed avoidance caused by thymol. Two analogous choice-feeding trials were conducted. Feed intake of the test diet was expressed as proportion of the total intake and tested by means of a one-sample Student's *t*-test against a set value of 50%. The preference for feed supplemented with 125, 500, 1250 and 2000 mg/kg thymol was 53.7 ± 6.0% ($P > 0.05$), 47.5 ± 5.1% ($P > 0.05$), 36.8 ± 4.9% ($P = 0.022$), and 3.9 ± 7.9% ($P = 0.005$) respectively. When feed containing 2000 mg/kg thymol with either flavour A (containing intense sweeteners) or flavour B (containing the same intense sweeteners and a caramel aroma) was opposed against a control diet, the relative intake of the test diets was 19.9 ± 5.8% and 14.0 ± 4.9% (both $P < 0.05$) respectively. When animals were offered one of these test diets and a reference diet with 2000 mg/kg thymol, animals exhibited a preference for the feed with 2000 mg/kg thymol + flavour A, but not for the feed with 2000 mg/kg thymol + flavour B. Thus, supplemental flavours containing intense sweeteners partially overcame feed avoidance caused by thymol which was less pronounced when the caramel aroma was present. Exposure to camphor (50 and 200 mg/kg) did not improve feed preference for a diet containing 1250 mg/kg thymol. Thymol's bitter taste might be largely responsible for the recorded feed refusal at high inclusion rates.

Keywords: thymol; camphor; flavour; aroma; TRPA1; palatability; feed intake; pig

Thymol (2-isopropyl-5-methylphenol) is a predominant component of several essential oils derived from plant species belonging to the Lamiaceae family. Its antimicrobial properties (e.g. Didry et al., 1993) urged researchers to study its ability to improve animal performance and intestinal health in pigs (Si et al., 2006; Trevisi et al., 2007; Michiels et al., 2009, 2010). This compound has shown to possess spasmolytic (Beer et al., 2007) and antioxidant (Youdim and Deans, 2000; Luna et al., 2010) properties and to affect the immune response by inhibition of the release of elastase (Braga et al., 2006) and cyclo-oxygenase activity (Marsik et al., 2005) which could be part of a growth-promoting claim. However, thymol, like other essential oils and their components, has a distinctive, marked flavour. Essential oils are traditionally used in pig feeds as flavour or aroma (e.g. Laitat et al., 2004 and list of Sensory additives – flavouring compounds in Community Register of Feed Additives Pursuant

to Regulation (EC) No. 1831/2003 – Revision 106 released December 17th, 2010; accessed December 2010, http://ec.europa.eu/food/food/animalnutrition/feedadditives).

The sensorial qualities of a feed are mainly defined by its aroma, texture and flavour (Laitat et al., 2004) and are important pre-ingestive determinants for feed acceptance. The sum of these characteristics is often called palatability. Aroma is appreciated by olfactive (smell) and nasal sensations, while flavour perception can be ascribed to taste and olfactive and thermo-mechanical stimulation. According to published evidences pigs have a well developed olfactory system with extremely high sensitivity compared to most mammals including humans. Thermo-mechanical sensations are collectively referred to as somatosensing and are linked to the trigeminal nerve covering all the oral and nasal cavities. Sensory neurons of the trigeminal nerve are part of the pain pathway and are involved in the detection of noxious stimuli such as low or high temperatures and pungent substances (e.g. acids, spices) (Roura et al., 2008). Transmembrane ion channel receptors of the transient receptor potential (TRP) family seem to be responsible for the detection of high (TRPVs) and low (TRPMs) temperatures and pungency or noxious cold (TRPAs) (e.g. review by Pedersen et al., 2005). Heavy trigeminal stimulation may lead to an alarm response characterized by feed avoidance, a strong stimulation of digestive secretions and an increase in the intestinal motility aimed at protecting the digestive epithelium (Platel and Srinivasan, 2004). Pigs, aside with most mammals, are highly responsive to several spices. For example moderate levels of cinnamaldehyde (500 mg/kg), carvacrol (30 mg/kg) and capsicum oleoresin resulted in decreased feed intake post-weaning (Bikker et al., 2003). In general, it has been shown that pigs have prefe-rence for specific flavours, and which, when added to feed, were able to enhance feed intake in the first weak post-weaning (not in the following weeks) (McLaughlin et al., 1983). Hence, the addition of thymol to pig feeds might affect the palatability in one way or the other and have impact on meal initiation and size. For humans, thymol has the characteristic, sharp odor of thyme and a bitter, aromatic and sometimes burning sensation. The bitter taste may elicit pig behavioral aversions to feeds containing thymol. Thymol and its isomer carvacrol were found to be strong activators of TRPA1 channels (concentration-dependent, reversible and rapidly desensitized) providing them their rejectable pungency (Xu et al., 2006; Karashima et al., 2007; Lee et al., 2008). TRPA1 is a Ca^{2+}-permeable non-selective cation channel that depolarizes the plasma membrane and causes Ca^{2+}-influx. Interestingly, Lee et al. (2008) showed that pre-treatment with camphor, a known TRPA1 blocker (Xu et al., 2005; Macpherson et al., 2006) inhibits strongly TRPA1 activation by thymol (30 μmol/l) in transfected HEK293 cells (IC_{50} = 400 μmol/l). They suggested that a TRPA1 antagonist could improve the taste and consumer acceptance of thymol-containing care products.

Taken together, thymol may act as a flavour and enhance feed intake or its bitter and chemo-receptor activation may depress feed preference. The latter is especially relevant because when antimicrobial effects are pursued high inclusion levels might be needed (Michiels et al., 2010). Windisch et al. (2008) stated that choice-feeding studies are to be compiled to test palatability. In a choice-feeding study, animals have free access to two or more feeds and their preference for one feed can be determined. It is assumed that, when animals can choose between diets that differ only in flavour, their preferred feed reflects the most palatable feed. The objectives in the current choice-feeding study were: (1) to determine the effect of dose of thymol and camphor on palatability and (2) to test the hypothesis that added flavours or camphor, as TRPA1 blocker, could mitigate the aversive effects of high thymol inclusion rates on palatability.

MATERIAL AND METHODS

Animals and housing

The choice-feeding study consisted of two trials (A and B) conducted with 24 newly weaned male castrated piglets (24 days; Belgian Landrace × Large White; live weight 6.91 ± 0.71 kg) and 24 newly weaned piglets (28 days; Hypor hybrid × Piétrain; live weight 7.40 ± 0.87 kg) for trial A and B respectively. In each trial, piglets were divided into 8 groups of 3 animals with a similar average live weight. Each group of 3 animals was housed in a slatted floor pen (1.30 × 1.25 m) equipped with two identical troughs. The width of each trough was 60 cm. During the first 10 days post-weaning animals were offered the basal diet *ad libitum* in both troughs and were habituated to feed from both troughs equally. At 10 days post-weaning the experimental treatments were started in two consecutive periods (I and II) of two weeks

Table 1. Ingredient and calculated nutrient composition of the basal diets in the two choice-feeding trials with weaned piglets

	Trial A	Trial B
Ingredient composition (g/kg as fed)		
Corn	666	159[6]
Barley		350
Wheat		150
Soya meal 44/7	282	100
Potato protein		5.00
Full-fat soybeans		100
Soybean flour 200 µm		15.0
Whey powder		37.5
Lactose powder		15.0
Soybean oil	10.3	15.0
Sodium chloride	6.52	4.60
Monocalcium-phosphate	12.5	5.50
Limestone	14.0	6.00
DL-methionine	1.36	
L-lysine HCl	3.63	
L-threonine	0.80	
L-tryptophane	0.39	
Premix vitamins pigs[1]	1.30	
Premix trace elements pigs[2]	1.20	
Closed formula premix starter[3]		37.4
Calculated nutrient composition[4]		
NEv97 (MJ/kg)[5]	9.86	9.75
CP (g/kg)	176.7	169.5
dLYS (g/kg)	10.30	10.87
dMET + CYS (g/kg)	6.10	6.33
dTHR (g/kg)	5.90	6.89
dTRY (g/kg)	1.90	2.16

[1]vit. A, 17 355 IE/kg as fed; vit. D_3, 2314 IE/kg; vit. E, 55 250 µg per kg; vit. K_3, 1888 µg/kg; vit. B_1, 1739 µg/kg; vit. B_2, 6240 µg per kg; vit. B_3, 20 768 µg/kg; vit. B_6, 3380 µg/kg; vit. B_{12}, 496 µg per kg and antioxidants (BHT and ethoxyquin), 23 400 µg/kg
[2]Fe, 122 400 µg/kg as fed; Cu, 10 008 µg/kg; Zn, 99 960 µg per kg; Mn, 80 040 µg/kg; I, 967 µg/kg; Co, 1008 µg/kg and Se, 350 µg/kg
[3]containing supplemental vitamins, minerals, antioxidants and phytase, this formula was free of flavourings
[4]CVB Table (1997), Centraal Veevoederbureau, Lelystad, the Netherlands
[5]net energy for pigs, CVB (1997)
[6]heat-treated

(see further). Water was provided *ad libitum* by two nipple drinkers at the opposite side of the troughs. Pens were cleaned daily to avoid faecal contamination of the pen floor. Average live weight at the start of period I and II in trial A was 8.04 ± 1.31 kg and 13.06 ± 2.36 kg respectively and in trial B it was 8.60 ± 0.63 kg and 14.23 ± 2.66 kg respectively.

Feeds

The composition of the basal diets is given in Table 1. The basal diets were given to all animals until start of period I of the respective trial. The basal diet was used to prepare the experimental diets as shown in Tables 2 and 3. For period I of trial A the following diets were prepared: a basal diet (CO1), and the basal diet supplemented with thymol at 125 mg/kg (THY125), at 500 mg/kg (THY500) and at 2000 mg/kg (THY2000A). Thymol was added to the feed after adsorption on a cellulose carrier (Alphacel non-nutritive bulk; No. 900453; MP Biochemicals, LLC, Brussels, Belgium). First 100 g of thymol (T0501; Sigma-Aldrich NV/SA, Bornem, Belgium) was dissolved in 250 ml methanol. Thereafter, approximately 100 g of the carrier was added to the mixture and further blended for 1 h. The mixture was dried in a rotavapor apparatus (water bath, 30°C) followed by exposure to the air until a dry preparation was obtained. Finally, it was ground and passed through a sieve with 500 µm pores. The preparation of thymol on alphacel for trial A contained 48.8% thymol in the final product. The preparation was analyzed according to Michiels et al. (2008). Alphacel was then added to the diets in order to obtain equal amounts of the basal diet in all experimental diets. In period II, the diets used were: a basal diet (CO2), and the basal diet supplemented with 2000 mg/kg thymol (THY2000B), with 2000 mg/kg thymol and 4000 mg/kg flavour A (THY2000 + FA) and with 2000 mg/kg thymol and 40 000 mg/kg flavour B (THY2000 + FB). Flavour A contained a mixture of intense sweeteners (high-intensity artificial sweeteners for humans) and was intended to compensate for the pungent bitter taste of thymol. It was provided by Scentarom n.v. (Merchtem, Belgium). Dextrose was the carrier for the active ingredients of this flavour. Flavour B contained the same mixture of intense sweeteners as flavour A and in addition it had a caramel aroma (Scentarom n.v., Merchtem, Belgium). It was designed to mask both the taste and smell of thymol.

Dextrose was the main carrier for the active ingredients of flavour B. At the inclusion level applied, flavours A and B provided the same amount of intense sweeteners per kg feed as fed. Both flavours were added at an impractical high level as was recommended by Scentarom n.v., but for the purpose of the experiment it was appropriate.

A basal diet (CO3), and the basal diet supplemented with either thymol at 1250 mg/kg (THY1250A) or camphor at 50 mg/kg (CAM50), at 200 mg/kg (CAM200) and at 800 mg/kg (CAM800) were prepared for period I of trial B. Thymol and camphor (racemic, 14807-5; Sigma-Aldrich NV/SA, Bornem, Belgium) were added to the feed after

Table 2. Composition of the experimental diets (g/kg) in the choice-feeding trial A

Experimental diet	Basal diet	Thymol preparation and carrier[1]		Flavour and carrier			
		thymol preparation	alphacel	flavour A	flavour B	carrier A	carrier B
Period I							
CO1	995.90		4.10				
THY125	995.90	0.25	3.85				
THY500	995.90	1.00	3.10				
THY2000A	995.90	4.10					
Period II							
CO2	951.90		4.10			4.00	40.00
THY2000B	951.90	4.10				4.00	40.00
THY2000 + FA	951.90	4.10		4.00			40.00
THY2000 + FB	951.90	4.10			40.00	4.00	

[1]thymol preparation contained 48.8% thymol and alphacel was used as carrier

Table 3. Composition of the experimental diets (g/kg) in the choice-feeding trial B

Experimental diet	Basal diet	Thymol preparation[1]	Camphor preparation[2]	Alphacel
Period I				
CO3	997.49			2.51
THY1250A	997.49	2.51		
CAM50	997.49		0.09	2.42
CAM200	997.49		0.35	2.16
CAM800	997.49		1.39	1.12
Period II				
CO4	997.15			2.86
THY1250B	997.15	2.51		0.35
THY1250 + CAM50	997.15	2.51	0.09	0.26
THY1250 + CAM200	997.15	2.51	0.35	

[1]thymol preparation contained 49.8% thymol and alphacel was used as carrier
[2]camphor preparation contained 57.4% camphor and alphacel was used as carrier

adsorption on a cellulose carrier in a similar way as described above. The thymol and camphor preparation contained 49.8 and 57.4% of the active ingredient in the final product respectively. The preparations were analyzed according to Michiels et al. (2008). Alphacel was then added to the diets in order to obtain equal amounts of the basal diet in all experimental diets. Diets used in period II were: a basal diet (CO4), and the basal diet supplemented with 1250 mg/kg thymol (THY1250B), with 1250 mg/kg thymol and 50 mg/kg camphor (THY1250 + CAM50) and with 1250 mg/kg thymol and 200 mg/kg camphor (THY1250 + CAM200). All experimental diets were prepared in the week before starting the respective experimental period.

Choice-feeding experiments

As a rule, a reference diet was offered in one trough and a test diet was offered in the second trough. Piglets had free access to both troughs and could therefore choose between the two diets offered. Trial A, period I was started at day 10 post-weaning and aimed at testing the palatability of feed supplemented with increasing levels of thymol. Four treatments (test diet vs. reference diet) were included: CO1 vs. CO1, THY125 vs. CO1, THY500 vs. CO1 and THY2000A vs. CO1. Each treatment was assigned to two pens in both weeks (Table 4). In each week, the test diets were given for 4 consecutive days, preceded by 3 days offering the CO1 diet to all pens in both troughs. At 7.30 a.m. the weighed portions of both diets were offered and 24 h later the residues from both the troughs were weighed. Immediately thereafter, new portions were offered. To avoid bias due to possible local preferences for the left or right through, the diets in both troughs were changed every day. The 24-h intake of the test diet was related to the total feed intake. Feed troughs were designed to minimize feed moistening; throughout the whole study moistening was at minimum and not taken into account in further data processing. In case of contamination with urine or faeces, feed intake for the respective pen and day was withdrawn from the dataset. Feed intake (kg/day per animal) given in the results section refers to total fresh feed intake. Each treatment was thus tested in 4 out of 8 pens during a 4-day period.

In period II, the hypothesis was tested whether flavours had the ability to alleviate the depressive effect on feed palatability of 2000 mg/kg thymol. Treatments (test diet vs. reference diet) were: THY2000 + FA vs. CO2, THY2000 + FB vs. CO2, THY2000 + FA vs. THY2000B and THY2000 + FB vs. THY2000B. Further protocol details were identical to period I (Table 4).

Camphor is a known TRPA1 inhibitor and hence a candidate to reduce thymol's TRPA1 activation potential. The effect of increasing levels of camphor on feed preference was tested in period I of trial B in order to find a dose that on itself does not affect feed palatability. Doses 50, 200 and 800 mg

Table 4. Study design with four treatments (test diet vs. reference diet) allocated to two pens per week in each period of choice-feeding trial A[1]

Pen	Period I		Period II	
	week 1	week 2	week 3	week 4
1	THY500/CO1	CO1/CO1	THY2000B + FA/THY2000B	THY2000B + FB/CO2
2	THY125 /CO1	THY2000A/CO1	THY2000B + FB/CO2	THY2000B + FA/THY2000B
3	CO1/CO1	THY125/CO1	THY2000B + FA/CO2	THY2000B + FB/THY2000B
4	THY2000A/CO1	THY500/CO1	THY2000B + FB/THY2000B	THY2000B + FA/CO2
5	THY2000A/CO1	THY500/CO1	THY2000B + FB/THY2000B	THY2000B + FA/CO2
6	THY500/CO1	CO1/CO1	THY2000B + FA/THY2000B	THY2000B + FB/CO2
7	CO1/CO1	THY125/CO1	THY2000B + FA/CO2	THY2000B + FB/THY2000B
8	THY125/CO1	THY2000A/CO1	THY2000B + FB/CO2	THY2000B + FA/THY2000B

[1]in each week the test diets were fed for 4 consecutive days, preceded by 3 days offering the respective control diet to all animals

per kg vs. CO3 were tested (Table 5). In addition, feed containing 1250 mg/kg thymol vs. CO3 was elaborated. In period II the hypothesis was tested whether camphor could improve the palatability of feed containing 1250 mg/kg thymol. Treatments (test diet vs. reference diet) were: THY1250 + CAM50 vs. CO4, THY1250 + CAM200 vs. CO4, THY1250 + CAM50 vs. THY1250B and THY1250 + CAM200 vs. THY1250.

Statistical methods

Total feed intake per pen and day were recorded. The intake of the test diet was expressed as proportion of the total feed intake, further referred to as feed preference (%). Data were analysed for normal distribution using the Kolmogorov-Smirnov procedure in the SPSS 15.0 program (SPSS Inc., Chicago, USA). Data of period I of both trials were analysed by the GLM repeated measures procedure whereby data from 4 consecutive days within a week were considered as repeated measures for that pen. The factors week (2) and treatment (4) and their interaction were included in the model. In case the interaction was not significant ($P > 0.05$) it was deleted from the model. Data are presented as adjusted means and compared by the Least Significance Difference test. If animals had no preference for one of the two diets offered, it can be expected that the relative intake of the test diet equals to 50%. Therefore the null hypothesis was that neither of the diets would be preferred and this was tested by means of a one-sample Student's *t*-test (set value = 50%). The same hypothesis was put forward for analysis of data of experimental period II. In this period two treatments had the CO diet as a reference diet. Data were analysed by the GLM repeated measures procedure as described above. The same approach was used for the two treatments whereby THY2000 was considered the reference diet.

RESULTS AND DISCUSSION

Effect of increasing dose of thymol and camphor on palatability

Thymol and camphor were prepared on alphacel as an inert carrier. Hereby, the flavour of these compounds was preserved, and hence its effect on palatability could be tested. In period I of trial A, animals had no preference for one of the two CO1 diets in the treatment CO1 vs. CO1 (53.7%, $P = 0.617$; Table 6). This means that feed intake from both troughs was equal which is a prerequisite in a choice-feeding experiment. The supplementation with 125 and 500 mg/kg thymol did not affect the palatability of the diet as is illustrated by *P*-values > 0.05 in the one-sample Student's *t*-test (Table 6). However, animals clearly preferred the CO1 diet to a diet with 2000 mg/kg thymol (feed preference, 3.9%). The animals almost completely refused to eat feed with 2000 mg/kg thymol. Thymol aversion was

Table 5. Study design with four treatments (test diet vs. reference diet) allocated to two pens per week in each period of choice-feeding trial B[1]

Pen	Period I		Period II	
	week 1	week 2	week 3	week 4
1	THY1250A/CO3	CAM200/CO3	THY1250 + CAM50/THY1250B	THY1250 + CAM50/CO4
2	CAM50/CO3	THY1250A/CO3	THY1250 + CAM200/CO4	THY1250 + CAM50/THY1250B
3	CAM200/CO3	CAM800/CO3	THY1250 + CAM50/CO4	THY1250 + CAM200/THY1250B
4	CAM800/CO3	CAM50/CO3	THY1250 + CAM200/THY1250B	THY1250 + CAM200/CO4
5	CAM800/CO3	CAM50/CO3	THY1250 + CAM200/THY1250B	THY1250 + CAM200/CO4
6	THY1250A/CO3	CAM200/CO3	THY1250 + CAM50/THY1250B	THY1250 + CAM50/CO4
7	CAM200/CO3	CAM800/CO3	THY1250 + CAM50/CO4	THY1250 + CAM200/THY1250B
8	CAM50/CO3	THY1250A/CO3	THY1250 + CAM200/CO4	THY1250 + CAM50/THY1250B

[1]in each week the test diets were fed for 4 consecutive days, preceded by 3 days offering the respective control diet to all animals

presumably a combination of smell and taste/somatosensing sensations. A daily preference of 2.1% (minimum value) means that feed was rejected before eating, hence based on smell perception. Taste and somatosensing are perceived while eating and also smell due to mastication, some amount of feed will then be ingested (maximum value was 29.5%). It can be understood that it is difficult for flavours to mask substantially the flavour of thymol at 2000 mg/kg because the animals fully rejected this feed. Therefore it was appropriate to test a lower thymol inclusion level in advance to test other masking substances. In trial B the level of 1250 mg per kg was chosen. The preference for feed containing 1250 mg/kg was 36.8% and thus intermediate with the results of trial A. In previous work (Michiels et al., 2010) the addition of 500 and 2000 mg/kg thymol to diets for freshly weaned piglets in a 12-day trial was investigated. Although the objective in that study was not to evaluate the effect on animal performances, the actual feed intake relative to the control diet was 123 and 99% for the treatments 500 and 2000 mg/kg thymol respectively. It seems that the results of that and the current study do not exactly match. Trevisi et al. (2007) found that inclusion of 1% thymol in the diet reduced feed intake significantly to 70 and 87% at 5 and 25 days post-weaning. Inconsistencies between choice-feeding and no choice-feeding trials have been found frequently (Torrallardona and Solà-Oriol, 2009). Differences in basal diet composition between the two studies (current choice-feeding study vs. Michiels et al., 2010; no choice-feeding) could be involved. In the current study feed choice is certainly driven by the feed flavour (short duration and daily alternating feeder position) in contrast to the 12-day duration of the other study where nutritional, metabolic and physiological factors may become relevant. However, in Michiels et al. (2010) no such arguments were found that could explain the higher feed intake in the no choice-feeding situation. Perhaps, thymol's pungency stimulated digestive secretions and intestinal motility leading to shortening of digesta transit and increased feed consumption as was evidenced with several spices (Platel and Srinivasan, 2001). Finally pigs may have acquired acceptance for these feeds.

Camphor is a known TRPA1 inhibitor and hence a candidate to reduce thymol's TRPA1 activation potential. Firstly, the effect of increasing levels of camphor on feed preference was tested in period I of trial B in order to find a dose that on itself does not affect feed palatability. A dose-dependent decrease of the relative intake was found that be-

Table 6. Feed intake and relative intake of test diets in two choice-feeding trial A

Treatment	Total feed intake (g/day per animal)[1,3]	Intake of test diet[2]	
		relative intake (%)[1,3]	*P*-value[4]
Period I			
CO1/CO1	599 ± 51 (307–987)	53.7[a] ± 5.1 (26.0–85.3)	0.617
THY125/CO1	555 ± 60 (340–780)	53.7[a] ± 6.0 (29.2–87.5)	0.447
THY500/CO1	557 ± 60 (400–920)	47.5[a] ± 5.1 (12.0–76.7)	0.599
THY2000A/CO1	501 ± 60 (280–727)	3.9[b] ± 7.9 (2.1–29.5)	0.005
Period II			
THY2000 + FA/CO2	891 ± 81 (600–1267)	19.9 ± 5.8 (3.4–39.1)	0.003
THY2000 + FB/CO2	866 ± 81 (520–380)	14.0 ± 4.9 (0–44.4)	0.012
THY2000 + FA/THY2000B	876 ± 53 (580–1360)	70.3[a] ± 8.1 (35.8–96.7)	0.078
THY2000 + FB/THY2000B	931 ± 53 (540–1413)	27.5[b] ± 8.1 (14.2–55.9)	0.004

[1]adjusted means ± standard error (min-max)
[2]test diets in period I were CO1, THY125, THY500, and THY2000A; in period II, THY2000 + FA and THY2000 + FB were assigned as test diets
[3]values with different superscripts in the same period and column part represent significant differences, $P < 0.05$
[4]the null hypothesis was that the test diet was not preferred and this was tested by means of a one-sample Student's *t*-test (set value = 50%)

came significant at 200 and 800 mg/kg (Table 7). It remains difficult to clarify the sensorial cues of camphor that might be responsible for this.

Masking effect of flavours and camphor to high thymol containing diets

From period II of trial A it can clearly be observed that the animals preferred the CO2 diet compared to the diets supplemented with 2000 mg/kg thymol and either of the two flavours (Table 6). The preference for THY2000 + FA and THY2000 + FB was 19.9 ± 5.8 and 14.0 ± 4.9% respectively which was considerably higher than the relative intake of THY2000A in period I (3.9 ± 7.9%). So, it is reasonable to state that the flavours were able to partially reduce the negative effect of 2000 mg per kg thymol on palatability. The pigs' preference for diets containing intense sweeteners is yet controversial (e.g. Grinstead et al., 1960; Glaser et al., 2000). Here, the mix of intense sweeteners showed clear benefits when added to diets with 2000 mg/kg thymol. However, an age effect cannot be ruled out since the animals in period II were two weeks older than in period I. It is well known that the susceptibility for flavours declines with age (McLaughlin et al., 1983) which is also seen by Trevisi et al. (2007) in the case of thymol. Less frequent feeding behaviour as animals age might also interact with feed preferences. The relative intake of 70.3 ± 8.1% for THY2000 + FA compared to the reference diet THY2000B confirms the ameliorating effect of flavour A. The opposite was recorded for flavour B, because the animals ate more from the reference diet than from the test diet in treatment THY2000 + FB vs. THY2000B. This might be explained by the fact that most aromas possess a bitter taste (Scentarom N.V., personal communication). Then, the addition of flavour B to a diet with 2000 mg/kg might result in a higher bitterness then the same diet without flavour B. The positive effect of THY2000 + FB when opposed to CON compared to THY2000 vs. CON might reflect a sole effect of the intense sweeteners. At least, it can be assumed that flavour A, which contained only intense sweeteners, was more effective than flavour B in reducing the aversive effects of 2000 mg/kg thymol. Perhaps, thymol avoidance is more projected in taste rather than smell sensation. Exposure to camphor did not improve feed preference for a diet containing 1250 mg/kg thymol (Table 7). Feed preference of diets THY1250 + CAM50 and THY1250 + CAM200 vs.

Table 7. Feed intake and relative intake of test diets in choice-feeding trial B

Treatment	Total feed intake (g/day per animal)[1,3]	Intake of test diet[2]	
		relative intake (%)[1,3]	*P*-value[4]
Period I			
THY1250A/CO3	649 ± 59 (430–913)	36.8^{ab} ± 4.9 (23.5–51.2)	0.022
CAM50/CO3	726 ± 59 (453–1033)	47.9^{a} ± 4.9 (26.6–60.8)	0.256
CAM200/CO3	580 ± 59 (273–893)	32.5^{bc} ± 4.9 (10.2–54.3)	0.044
CAM800/CO3	687 ± 59 (533–990)	22.1^{c} ± 4.9 (6.1–48.4)	0.005
Period II			
THY1250 + CAM50/CO4	1017 ± 72 (657–1257)	37.3 ± 3.0 (21.5–52.2)	0.036
THY1250 + CAM200/CO4	1153 ± 61 (877–1497)	39.4 ± 2.5 (28.7–49.6)	0.014
THY1250 + CAM50/THY1250B	1174 ± 122 (787–1927)	46.2^{a} ± 1.9 (38.2–52.7)	0.037
THY1250 + CAM200/THY1250B	1073 ± 122 (853–1383)	38.0^{b} ± 1.9 (24.4–53.8)	0.016

[1]adjusted means ± standard error (min–max)
[2]test diets in period I were THY1250, CAM50, CAM200, and CAM800; in period II, THY1250 + CAM50 and THY1250 + CAM200 were assigned as test diets
[3]values with different superscripts in the same period and column part represent significant differences, $P < 0.05$
[4]the null hypothesis was that the test diet was not preferred and this was tested by means of a one-sample Student's t-test (set value = 50%)

CO4 and THY1250 scored in all cases below 50% ($P < 0.05$). The dose of camphor was probably not sufficient to inhibit TRPA1 activation. In an *in vitro* study of Lee et al. (2008) 400 µmol/l camphor reduced TRPA1 activation by thymol at 30 µmol/l by 50%. This camphor dose is 13-fold higher than the thymol dose, in contrast to our experiments (e.g. 200 vs. 1250 mg/kg). The masking effect of camphor at doses higher than 200 mg/kg was not tested here because it made the feed unpalatable. However, the data of Lee et al. (2008) cannot be easily translated to our *in vivo* study since the study of Lee et al. (2008) was conducted *in vitro* with isolated cells. Nevertheless, studies showed that pungent principles negatively affecting feed intake, could be masked successfully (e.g. by cherry-honey flavour; Roura et al., 2004).

CONCLUSIONS

When piglets had free choice between a control diet and a diet with thymol they had neither preference for thymol at 125 mg/kg, nor for thymol at 500 mg/kg. Palatability was dramatically affected when 1250 or 2000 mg/kg was included in the diet. Incremental levels of camphor decreased feed preference that became significant at 200 and 800 mg/kg. Supplemental flavours containing intense sweeteners partially overcame feed avoidance caused by thymol. A caramel aroma presumably did not show further benefits. Exposure to camphor (50 and 200 mg/kg) did not improve feed preference for a diet containing 1250 mg/kg thymol. Thymol's bitter taste might be largely responsible for the recorded feed refusal at high inclusion rates.

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