Identification of sinapine-derived choline from rapeseed diet as a source of serum trimethylamine *N*-oxide in pigs

Hong Chen[†,^,#], Ling Peng[†,&,#], Marta Pérez de Nanclares[§], Michaela P. Trudeau[‡], Dan Yao[†], Zaixing Cheng[†,^], Pedro E. Urriola[‡], Liv Torunn Mydland[§], Gerald C. Shurson[‡], Margareth Overland[§], Chi Chen[†*]

[†]Department of Food Science and Nutrition, University of Minnesota, St. Paul, MN, USA, [^]College of Pharmacy, Fujian University of Traditional Chinese Medicine, Fuzhou, China, [&]Department of Food Science, Yichun University, Yichun, China, [§]Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, Ås, Norway, [‡]Department of Animal Science, University of Minnesota, St. Paul, MN, USA

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[#] These authors have contributed equally to this work

*Corresponding author:

Chi Chen, Department of Food Science and Nutrition, University of Minnesota, 1334 Eckles Avenue, St. Paul, MN 55108, Tel.: 612-624-7704, Fax: 612-625-5272 Email: chichen@umn.edu

ABSTRACT: Choline and its metabolites have diverse and important functions in many physiological processes, especially for anabolic metabolism in growth and reproduction. Besides endogenous biosynthesis and direct choline supplement, choline esters in diet is another source of choline in the body. Phenolic choline esters are a group of unique dietary choline esters rich in the seeds of Brassicaceae plants, among which sinapine is a choline ester of sinapic acid abundant in rapeseed. In this study, 40 nursery pigs were fed with rapeseed-derived feed ingredients (RSF) or soybean meal (SBM) for 3 weeks (20 pigs/diet). The metabolic fate of sinapine-derived choline in RSF was examined by comparing the distribution of choline and its metabolites in digesta, liver, and serum samples by liquid chromatography-mass spectrometry (LC-MS) analysis. The results showed that choline was released from extensive hydrolysis of sinapine in the small intestine. However, sinapine-derived choline did not increase the levels of choline and its major metabolites, including betaine, phosphocholine (PC), and glycerophosphocholine (GPC), in the liver and serum. Instead, RSF feeding increased trimethylamine (TMA), the microbial metabolite of choline, in the large intestine, and further increased trimethylamine *N*-oxide (TMAO), the oxidation metabolite of TMA, in the liver and serum. Overall, these results suggested that sinapine-derived choline from rapeseed feeding had limited influences on the post-absorption choline pool due to its low bioavailability, but may serve as a major source of TMAO through microbial metabolism in nursery pigs. Improving the bioavailability of sinapine-derived choline might have the potential to modify the nutritional values and functionalities of rapeseed meal in swine feeding.

KEY WORDS: Rapeseed, Sinapine, Choline, TMA, TMAO, Pig

INTRODUCTION

Choline is a functional nutrient for membrane integrity, lipid transport and signaling, one-carbon metabolism, and neurotransmission through its roles as the precursor of betaine, acetylcholine, and phospholipids (PLs), including phosphatidylcholine and sphingomyelin.[1] Endogenous metabolism, such as serine and phosphatidylcholine metabolism, can generate choline and its esters,[2-3] but the quantity of choline from these metabolic routes is insufficient for normal physiological needs in humans and animals, especially for pre- and postnatal health.[4] Therefore, consumption of choline-containing food or choline supplementation is required. In general, animal-derived ingredients, including egg yolk, meat, and dairy products, contain more choline than plant-based ingredients, but selective plant-based ingredients are also rich in choline and choline-containing molecules. In the USDA Database for the Choline Content of Common Foods, the total choline content in foods is calculated as the sum of free choline, phosphocholine (PC), glycerophosphocholine (GPC), phosphatidylcholine and sphingomyelin.[5] This approach of calculation should cover the majority of choline content in animal-derived ingredients. However, in some plant species, such as cauliflower and rapeseed in Brassicaceae family, choline also exists in significant quantity in the form of phenolic choline esters,[6-7] such as sinapine (sinapoylcholine) in *Brassica napus* (rapeseed), 4-hydroxybenzoylcholine in *Sinapis alba*,[8] and isoferuloylcholine in *Sibara virginica*.[9]

Rapeseed, as one of the most important oilseed crops in many parts of the world, is marked by its unique phytochemical contents, including glucosinolates, erucic acid, phytate, and phenolics.[10] The breeding efforts aiming to improve the phytochemical and nutritional profile of rapeseed have lowered the levels of antinutrients, especially glucosinolate and erucic acid, in rapeseed cultivars, such as in canola.[11] However, phenolics in new rapeseed cultivars remained at their traditional

level, which is about 30-fold greater than that in soybean.[12-13] Sinapine and sinapic acid are the most abundant esterified and free phenolic acids in rapeseed, respectively, and also distributed in other plants belonging to Brassicaceae family.[7] Rapeseed meal is widely used in feeding non-ruminants, mainly for poultry and swine production.[14] In rapeseed meal, sinapine, as a dominating phenolic choline ester, accounts for about 80% of the total phenolics and 1–2% of dry matter,[7] and the ratio between sinapine and sinapic acid ranges from 10:1 to 20:1.[12] After rapeseed consumption, sinapine can be extensively hydrolyzed in the gastrointestinal tract to form sinapic acid and choline.[15]

In our previous study on the metabolite distribution in the intestinal digesta of pigs fed diets based on rapeseed-derived feed ingredients (RSF) or soybean meal (SBM), sinapine and its hydrolysis product, sinapic acid, were detected in high abundances in the digesta from RSF feeding, while largely absent in SBM feeding.[16] More importantly, choline is the other product of sinapine hydrolysis to sinapic acid, but the metabolic fate of sinapine-derived choline in pigs was largely unknown and rarely investigated. Considering widespread usage of sinapine-rich ingredients, such as rapeseed and canola, in swine feeding practice, this study aimed to investigate the metabolic fate of sinapine-derived choline through determining the distribution of choline and its associated metabolites in swine feeds as well as their distribution in the intestinal tract, liver and serum of pigs.

MATERIALS AND METHODS

Chemicals and reagents. Sodium pyruvate, n-butanol, trimethylamine (TMA), trimethylamine *N*-oxide (TMAO) and phosphocholine (PC) were purchased from Sigma-Aldrich (St. Louis, MO,

USA); LC-MS-grade water, acetonitrile (ACN), formic acid, betaine, and *tert*-butyl bromoacetate from Fisher Scientific (Houston, TX, USA); *p*-chlorol-L-phenylalanine from Alexis Biochemicals (San Diego, CA, USA); sinapine thiocyanate from ChemFaces (Wuhan, China), glycero-3-phosphocholine (GPC) from Chem-IMPEX International (Wood Dale, IL, USA); and d_3-betaine obtained from CDN isotope (Quebec, Canada).

Animals, dietary treatments, and sample collection. Ingredients and chemical composition of SBM-based diet and RSF-based diet are listed (Table S1 and S2). Rapeseed ingredients in RSF-based diet are 20% coarse fraction of hexane-extracted rapeseed meal and 4% rapessed hull. The design and procedures of animal feeding as well as animal growth performance and health status have been reported previously.[17] Briefly, 40 Norwegian Landrace castrated male pigs (average age of 56 days) were assigned to SBM-based diet and RSF-based diet, respectively (20 pigs/diet), at the experimental farm of the Norwegian University of Life Sciences. The two dietary treatments were conducted in 2 batches with 10 pigs per treatment per batch. Pigs were fed twice daily with their respective experimental diets in the amount equivalent to 3.5% of body weight. In the second batch of feeding, the pigs underwent an outbreak of a mild to moderate diarrhea and then recovered after receiving a probiotic treatment (ZooLac Propaste; VESO AS, Oslo, Norway) in the last week of feeding following veterinary recommendations.[17] After three weeks of feeding, pigs received a normal morning meal 2.5–3 h before slaughter to ensure the presence of digesta along the gastrointestinal tract. Digesta samples from five different sites along the intestinal tract, including the 25 cm sections of the duodenum (25 cm from the pyloric sphincter); mid-jejunum; ileum (20 cm anterior to the ileocecal valve); cecal apex; and the central flexure of the spiral colon, along

with serum and liver samples, were collected, snap frozen, and stored at -80 °C for metabolite analysis.

Sample preparation. Feed sample (SBM, RSF, rapeseed hull, and coarse fraction of rapeseed meal) and digesta samples (duodenum, jejunum, ileum, cecum, and colon) were prepared by mixing with 50% aqueous ACN in 1:10 (w/v) ratio and then centrifuged at 18,000 × g for 10 min to obtain extract supernatants. For serum samples, deproteinization was conducted by mixing one volume of serum with 19 volumes of 66% aqueous ACN and then centrifuging at 18,000 × *g* for 10 min to obtain the supernatants. Liver tissue samples were partitioned using a modified Bligh and Dyer method.[18] Briefly, 100 mg of liver sample were homogenized in 0.5 mL of methanol and then mixed with 0.5 mL of chloroform and 0.4 mL of deionized water. After 10 min centrifugation at 18,000 × g, the upper aqueous fraction was used for analyzing choline and its metabolites.

Quantitative analysis of choline, betaine, PC, GPC and TMAO. Prepared feed, digesta, liver and serum extracts as well as individual standard solutions were mixed with an ACN solution containing 5 μM *d_3-betaine* (internal standard) in 1:1 (v/v) ratio, and then centrifuged at 18,000 × *g* for 10 min and the supernatant was transferred into a sample vial for LC-MS analysis.

Quantitative analysis of TMA. The derivatization reaction of TMA was conducted as described by Johnson.[19] Briefly, after sample (including digesta, liver and serum), and standard solutions were acidified by adding water containing 0.1% formic acid in 1:1 (v/v) ratio, 25 μL sample or standard solution was mixed with 25 μL *d_3*-betaine (internal standard) solution and adding 75 μL ACN solution containing 50 mM *tert*-butyl bromoacetate as well as 10 μL 70% ammonium

hydroxide. The mixture was incubated at ambient temperature for 30 min, and then added 50 μL ACN containing 1% formic acid. After centrifugation at 18,000 × *g* for 10 min, the supernatant was transferred into a sample vial for LC-MS analysis.

Conditions of LC-MS analysis. A 5 μL aliquot was injected into an ultraperformance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOFMS) system (Waters, Milford, MA) and separated by a BEH amide 1.7 μm, 2.1 × 100 mm column (Waters) with a gradient of mobile phase over a 10-min run at the flow rate of 0.5 mL/min. The gradient was 0.5% A for 2 min, to 40% A in 3 min, to 50% A in 5 min, to 50% A for 2 min, to 0.5% A in 7 min, 0.5% A for 1 min, where A was 10% ACN/90% water with 10 mM ammonium formate (pH=5) and B was 95% ACN/5% A. Capillary voltage and cone voltage for electrospray ionization were maintained at 3 kV and 30 V for positive mode detection, respectively. Source temperature and desolvation temperature were set at 120 °C and 350 °C, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (600 L/h), and argon was used as collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution (range *m/z* 50-1000) and monitored by the intermittent injection of the lock mass leucine enkephalin ($[M + H]^+$ = 556.2771 *m/z*) in real time. Mass chromatograms and mass spectral data were acquired and processed by MassLynx^{TM} software (Waters) in centroided format. Individual metabolite concentrations were determined by fitting the ratio between the peak area of each metabolite and the peak area of the internal standard with a standard curve using QuanLynx software (Waters). Representative chromatograms of sinapine, sinapic acid, choline, and choline-associated metabolites and their detection limits in LC-MS analysis are enlisted in Figure S1.

158 *Statistical analysis.* Statistical analysis was performed as two-tailed Student's *t*-tests for unpaired
159 data. Results are presented as mean ± standard deviation (SD). Differences between dietary
160 treatments were considered significant if $P < 0.05$.
161
162 **RESULTS**
163 **Distribution of sinapine, sinapic acid, and choline metabolites in SBM and RSF feeds.** As
164 expected, sinapine and sinapic acid were present in RSF diet, but not in SBM diet (Figure 1A-B).
165 Sinapine was much more abundant than sinapic acid in RSF diet (Figure 1A-B). Further analysis
166 of rapeseed hull and coarse fraction of rapeseed meal, two rapeseed ingredients of RSF diet,
167 indicated that coarse fraction was the main source of sinapine and sinapic acid in the RSF diet
168 (Figure S2A-B). The concentrations of choline were comparable between SBM and RSF diets
169 (Figure 1C). Other major choline-related compounds, including betaine, glycerophosphocholine
170 (GPC), and phosphocholine (PC), were also present in comparable levels in these two diets (Figure
171 1D-F).
172
173 **Distribution of choline in the intestinal tract after RSF feeding.** Distribution of sinapine and
174 sinapic acid in the intestinal tract of pigs has been profiled in a previous study on the metabolic
175 effects of RSF feeding.[16] Sinapine was highly abundant in the duodenal digesta of RSF-fed pigs,
176 with the concentrations up to 1300 μg/g in individual pigs (Figure S3A). From jejunum to colon,
177 the concentrations of sinapine decreased gradually, while the concentrations of sinapic acid were
178 relatively stable (Figure S3B), potentially due to continuous hydrolysis of sinapine to form sinapic
179 acid. To determine whether the conversion of sinapine to sinapic acid affected choline in the
180 intestinal tract, targeted analysis of choline in the same digesta samples was conducted in this

study. The results showed that choline concentration gradually decreased in the small intestine of SBM-fed pigs (Figure 2 and S4). The choline concentration in the duodenal digesta of RSF-fed pigs was comparable to that of SBM-fed pigs. However, its concentration increased dramatically in the jejunal digesta of RSF-fed pigs, and remained higher in the ileum, cecum, and colon than that of SBM-fed pigs (Figure 2 and S4).

Influences of RSF feeding on the choline metabolites from post-absorption metabolism. To determine whether the extra RSF-derived choline detected in the intestinal digesta could affect choline and its metabolites inside the body, the concentrations of choline, betaine, PC, and GPC in the liver and serum of the pigs fed SBM and RSF diets were compared. The results showed that RSF feeding did not significantly affect the hepatic and serum concentrations of choline, betaine, and GPC (Figure 3 and S5). Surprisingly, the concentration of PC in the liver of RSF-fed pigs was lower than that of SBM-fed pigs ($P < 0.01$) (Figure 3B and S5B).

Influences of RSF feeding on microbial metabolites of choline. Because significant amounts of choline were available in the ileal and cecal digesta of RSF-fed pigs for further microbial metabolism in the large intestine (Figure 2), the concentrations of TMA and TMAO, two major microbial metabolites of choline, in the large intestine, liver, and serum were determined. TMA was detected in cecum, colon, and liver samples, but not in serum samples (Figure 4A-D). In contract, TMAO was almost undetectable in cecum and colon sample, but present in liver and serum samples (Figure 4A-D). Significant increase of TMA after RSF feeding was only observed in the colon ($P < 0.01$), but not in the cecum and liver due to great variances of TMA concentrations

within the same feeding groups (Figure 4A-C). An interesting observation was that TMA was absent in most cecal and colonic digesta samples from the pigs in the 2nd batch of feeding (Figure S6A-B). Considering these pigs went through an outbreak of diarrhea and then a probiotic treatment (detailed in Materials and Methods), a separate statistical analysis excluding the samples from this batch of treatment was conducted. Higher levels of TMA were detected in both cecal and colonic digesta from the RSF-fed pigs in batch 1 feeding (Figure S6A-B). More importantly, RSF feeding significantly increased the concentrations of TMAO in the liver ($P < 0.05$) and serum ($P < 0.001$) (Figure 4C-D).

DISCUSSION

The role of sinapine as a dietary choline donor has been extensively examined in poultry nutrition research, mainly due to the occurrences of fishy-odor egg taint after feeding rapeseed meal to laying hens.[20-21] The egg taint is caused by the deposition of TMA in egg yolk, which is jointly contributed by the formation of TMA from microbial metabolism of sinapine-derived choline and the impairment of flavin monooxygenase 3 (FMO3)-mediated conversion of malodorous TMA into odorless TMAO in the liver of egg-laying hens.[22-25] In contrast to comprehensive knowledge on the biotransformation of sinapine and TMA in laying hens, the metabolic fates of sinapine-derived choline in pigs or other monogastric animals are rarely investigated. In the current study, through measuring the concentrations of choline and its metabolites in the small intestine, large intestine, liver, and blood of nursery pigs, the metabolic events in these physiological sites of choline formation, absorption, metabolism, and distribution were defined and implicated (Figure 5), and the following conclusions are drawn and discussed accordingly.

227 *On the production of sinapine-derived choline*. More extensive hydrolysis of sinapine to form
228 sinapic acid and choline occurred in the jejunum than that in the duodenumof pigs based on two
229 major evidences. Firstly, the choline concentrations were comparable in the duodenal digesta of
230 SBM- and RSF-fed pigs, but became dramatically different in the jejunal digesta due to the
231 increase in RSF-fed pig and the decrease in the SBM-fed pigs (Figure 2). Secondly, average
232 concentrations of sinapine and sinapic acid in the duodenal digesta were 857 and 48 μg/g digesta,
233 respectively. This ratio of 17:1 between sinapine and sinapic acid in duodenal digesta was within
234 the range of reported ratios (10-20:1) between sinapine and sinapic acid in rapeseed meal,[12]
235 suggesting that the major breakdown of sinapine to sinapic acid that could affect the ratio between
236 them did not occur before the site of sample collection in the duodenum. The hydrolytic digestion
237 of sinapine can be conducted chemically through acid or base-mediated reactions, or enzymatically
238 through esterase-mediated reactions in the intestinal lumen. This feature of jejunum as a more
239 favorite site of sinapine hydrolysis than duodenum is likely contributed by the basic environment
240 in the duodenum (pH 7-9) because chemical hydrolysis of sinapine is more preferred in alkaline
241 conditions than with weak acids, and the enzymatic activity of sinapine esterases has been reported
242 to peak around pH 8.5.[26]
243
244 *On the bioavailability of sinapine-derived choline*: Despite dramatic increase of free choline in the
245 jejunum and ileum of RSF pigs, the levels of choline and its major functional metabolites,
246 including betaine, PC and GPC, in the liver and serum were not increased by RSF feeding (Figure
247 3). In fact, the hepatic concentration of PC was unexpectedly decreased by RSF feeding. This lack
248 of coordination between the choline level in the small intestine and the choline pools in the liver
249 and serum implicates low bioavailability of sinapine-derived choline in nursery pigs. This

conclusion is consistent with previous observations in chickens, which showed that the utilization of choline from rapeseed meal (24%) was lower compared to that from soybean lecithin (100%), SBM (83%), and peanut meal (76%) in chickens.[27-28] Bioavailability is largely determined by absorption and post-absorption metabolism in the intestine and liver. Regarding the absorption of choline in the intestine, it occur in duodenum, jejunum, ileum, and colon through saturable choline transport systems with K_m ranging from 0.2 to 150 μM.[29-30] Since the concentrations of choline in the small intestine digesta of RSF pigs were well above this range, it is likely that choline absorption in these pigs has been saturated by extra choline from sinapine. Considering the nutritional value of choline, it is reasonable to suggest that improving the bioavailability of sinapine-derived choline may positively affect the nutritional value of rapeseed to pigs through decreasing the need of choline supplementation from other sources, including fish meal and SBM. A potential approach to achieve this goal is to release free choline from sinapine by processing rapeseed meal, such as using the fungi containing feruloyl esterase to break down sinapine.[31] In fact, besides providing greater access to free choline in the alimentary tract than the hydrolysis of sinapine inside the small intestine, this type of pre-feeding processing may also improve the palatability of rapeseed by reducing the bitterness caused by sinapine,[32] and also provide a better supply of sinapic acid, which is a better antioxidant than sinapine.[33]

On the microbial metabolism of choline: Sinapine-derived choline was the main cause behind the increase of TMA in the large intestine and also the increases of TMAO in the liver and serum of pigs fed RSF (Figure 4 and S6). This observation is consistent with the existing knowledge on the microbial metabolism of choline in chickens.[15] A prominent observation from the quantitative analysis of TMA and TMAO was the absence of TMA in serum samples of both treatment groups,

which suggested a complete conversion of TMA to TMAO in the liver of nursery pigs in this study. Another interesting observation is the absence of TMA in many cecal and colonic digesta samples from the 2nd batch of feeding in spite of the presence of choline in the same samples (Figure S4 and S6). It is plausible that diarrhea and prebiotic treatment occurred in the 2nd batch of feeding might eliminate specific bacteria and enzymes in the choline-TMA conversion, a process requiring multiple enzymes in different bacteria,[34-35] or extend microbial metabolism of TMA to its downstream metabolites, such as dimethylamine.[36] Targeted genetic assays have revealed that the bacteria in Clostridia class, especially *Clostridium* and *Eubacterium* strains contain TMA-forming enzymes.[37] Our recent microbiomic analysis on the digesta samples from this feeding trial has also shown that the abundance of the *Clostridium* population was lower in the cecum of RSF-fed pigs compared to SBM-fed pigs.[38] Therefore, further analysis of microbial genes involving in TMA synthesis and degradation might provide additional insights on the variance of TMA production among individual pigs. TMAO, as a terminal metabolite of sinapine-derived choline in this study, has been identified recently as a markers of cardiovascular disease in human.[39] Considering the short growth period in pig production, this observation may carry little relevance to the performance of pigs. However, TMAO is a common component in seafood and fish meal. When used as an additive in swine feed, TMAO has been shown to improve the apparent overall digestibility of crude fat and increase carcass lean mass of growing-finishing pigs.[40] Whether sinapine-derived TMAO could achieve similar effects requires further studies.

Overall, this study revealed that, in comparison to SBM, RSF can provide pigs additional sources of choline and TMAO, which are two common additives in swine feeding. In order to harvest their potentials to benefit pigs in rapeseed feeding, further studies are required to improve the

296 bioavailability of sinapine-derived choline, as well as to achieve better understanding on the
297 physiological and metabolic consequences of supplying additional choline and TMAO.
298

ABBREVIATIONS: ACN, acetonitrile; GPC, glycerophosphocholine; LC-MS, liquid chromatography-mass spectrometry; PC, phosphocholine; PL, phospholipid; RSF, rapeseed-derived feed ingredients; SBM, soybean meal; TMA, trimethylamine; TMAO, trimethylamine *N*-oxide.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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FIGURE LEGENDS

Figure 1. Concentrations of sinpaine, sinapic acid, and choline metabolites in SBM and RSF diets. (n = 3 replicates). ***A***. Sinapine. ***B***. Sinapic acid. ***C***. Choline. ***D***. Betaine. ***E***. GPC. ***D***. PC.

Figure 2. Concentrations of choline in the small and large intestines of the pigs fed with SBM and RSF diets (n = 20/treatment). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

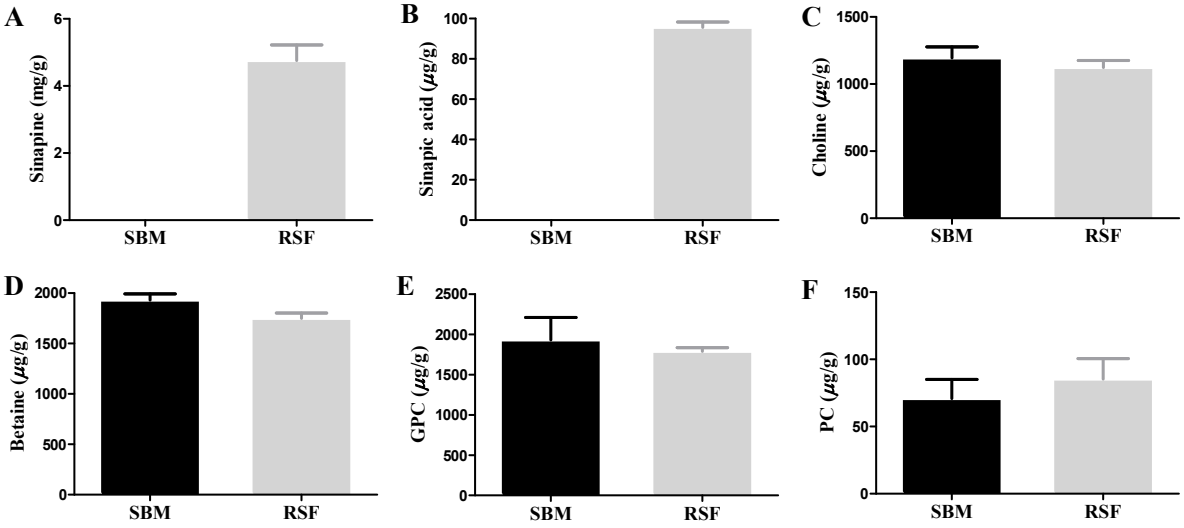
Figure 3. Concentrations of choline and its metabolites in the liver and serum of the pigs fed with SBM and RSF diets (n = 20/treatment). ***A***. Concentrations of hepatic choline and betaine. ***B***. Concentrations of hepatic PC and GPC. ***C***. Concentrations of serum choline and betaine. ***D***. Concentrations of serum PC and GPC. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 4. Concentrations of TMA and TMAO in the large intestine, liver and serum of the pigs fed with SBM and RSF diets (n = 20/treatment). ***A***. Concentrations of TMA and TMAO in cecal digesta samples. ***B***. Concentrations of TMA and TMAO in colonic digesta samples. ***C***. Concentrations of hepatic TMA and TMAO. ***D***. Concentrations of serum TMA and TMAO. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 5. Major metabolic routes of sinapine-derived choline in the pigs after RSF feeding. Sinapine from rapeseed meal undergoes extensive hydrolysis in the small intestine to generate additional choline. Due to low bioavailability, sinapine-derived choline does not increase the choline pools in the liver and serum. Instead, it is mainly degraded by microbial metabolism to

453 form TMA. After the absorption, TMA is completely oxidized by flavin monooxygenases (FMO)
454 in the liver to form TMAO. The metabolites increased by rapeseed feeding are marked in red.
455

456 **Figure 1**



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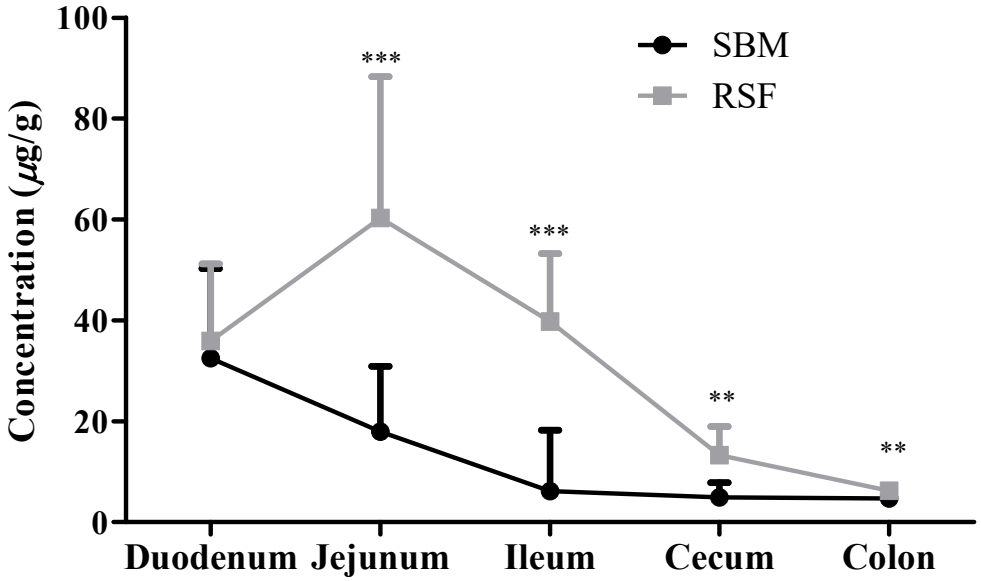
458 **Figure 2**



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460 **Figure 3**

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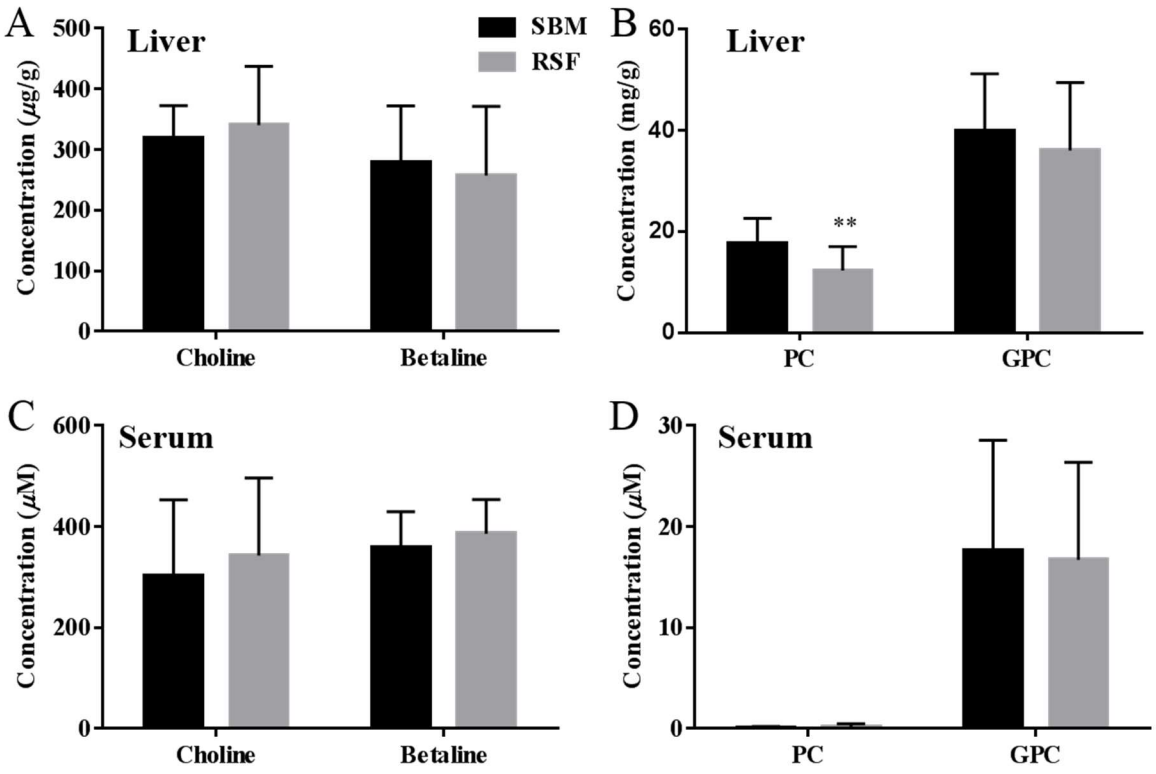
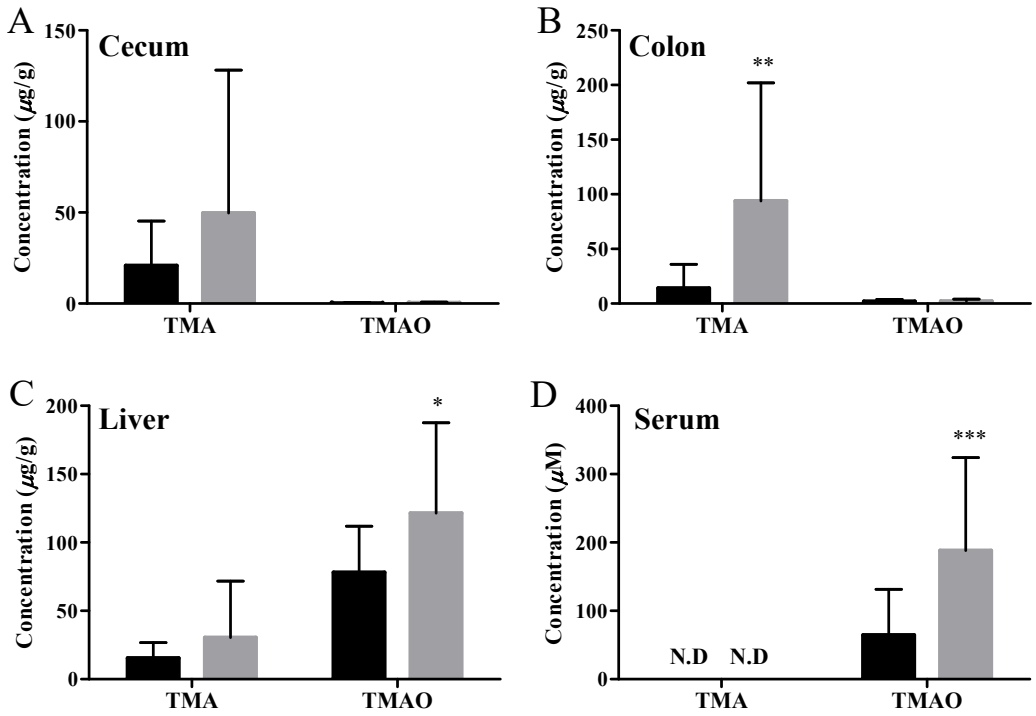


462 **Figure 4**



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464 **Figure 5**

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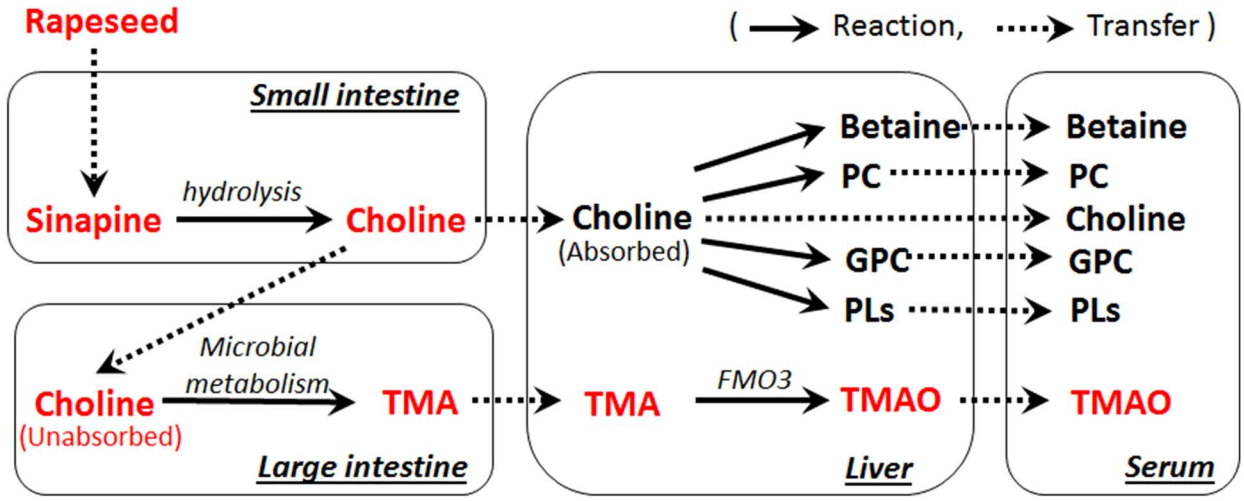

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Supplemental information for "Identification of sinapine-derived choline from rapeseed feeding as a source of serum trimethylamine *N*-oxide in pigs" H. Chen, L. Peng, M. Pérez de Nanclares, M. P. Trudeau, D. Yao, Z. Cheng, P. E. Urriola, L. T. Mydland, G. C. Shurson, M. Overland, C. Chen

Table S1. Dietary composition of experimental diets.

Table S2. Measured concentrations of chemical components in experimental diets.

Figure S1. Representative chromatograms of sinapine, sinapic acid, choline, and choline-associated metabolites**.**

Figure S2. Concentrations of sinapine and sinapic acid in the hull and coarse fraction ingredients of RSF diet**.**

Figure S3. Concentrations of sinapine and sinapic acid in the intestinal digesta of the pigs fed with SBM and RSF diets.

Figure S4. Concentrations of choline in the intestinal digesta of the pigs fed with SBM and RSF diets.

Figure S5. Concentrations of choline and its metabolites in the liver and serum of individual pigs fed with SBM and RSF diets.

Figure S6. Concentrations of TMA in the large intestine of individual pigs in 2 batches of SBM and RSF feedings.

Table S1. Dietary composition of experimental diets

Ingredient, g/kg as-fed	Control[a]	RSF[a]
Wheat[b]	629.1	506.5
Barley[c]	100.0	100.0
Soybean meal[d]	140.0	30.0
Coarse rapeseed meal[e]	–	200.0
Rapeseed hulls[f]	–	40.0
Fish meal	40.0	40.0
Soybean oil	50.0	50.0
Monocalcium phosphate	16.4	9.1
Limestone	11.3	11.2
L-Lys·HCl	3.4	3.4
DL-Met	0.5	0.5
L-Thr	1.3	1.3
L-Trp	0.2	0.2
Sodium chloride	4.0	4.0
Vitamin and trace mineral premix[g]	3.2	3.2
Attractant[h]	0.5	0.5
Marker (Y_2O_3)	0.1	0.1

[a] Control diet based on wheat and soybean meal; RSF = rapeseed-based feed.
[b] Whole wheat: 86.4% DM, 11.1% CP, 1.6% EE, 58.1% starch, 9.0% NDF, 2.2% ADF, 1.4% ash.
[c] Barley: 86.2% DM, 7.4% CP, 1.3% EE, 53.5% starch, 16.0% NDF, 5.1% ADF, 1.6% ash.
[d] Soybean meal: 89.0% DM, 43.3% CP, 1.4% EE, 1.4% starch, 8.9% NDF, 5.7% ADF, 5.4% ash.
[e] Coarse fraction from an air-classified hexane-extracted rapeseed meal: 90.0% DM, 31.2% CP, 2.5% EE, 26.2% NDF, 18.6% ADF, 6.7% ash.
[f] Rapeseed hulls: 88.8% DM, 13.2% CP, 8.0% EE, 55.1% NDF, 48.6% ADF, 4.4% ash.
[g] Provided per kilogram of diet: 90 mg Zn (ZnO); 90 mg Fe ($FeSO_4$); 45 mg Mn (MnO); 19.5 mg Cu ($CuSO_4$); 0.45 mg I ($Ca(IO_3)_2$); 5700 IU vitamin A; 4500 IU cholecalciferol; 100.7 mg dl-α-tocopheryl acetate; 2.40 mg menadione; 9.0 mg riboflavin; 36.0 mg D-pantothenic acid; 12.0 µg cyanocobalamine; 12.0 mg niacin; 0.24 mg biotin; and 1.8 mg folic acid.
[h] Maxarome; Felleskjøpet, Kambo, Norway.

510 **Table S2**. Measured concentrations of chemical components in experimental diets.

Item, g/kg of DM	Control	Rapeseed based Feed
Gross energy, MJ/kg	17.6	17.8
DM, g/kg	908.4	906.1
CP, %	201.8	201.8
Ether extract, %	79.2	87.7
Starch, %	402.1	370.8
NDF, %	113.0	154.8
ADF, %	41.8	82.3
Ash, %	57.0	60.8
P, %	9.7	8.7
Y, %	0.1	0.1
Amino acid, %		
Ala	8.9	9.1
Arg	11.5	11.1
Asp	17.1	15.3
Cys	3.7	4.5
Glu	43.6	41.3
Gly	9.5	10.1
His	5.0	5.1
Ile	8.4	8.3
Leu	14.7	14.4
Lys	12.8	13.2
Met	4.0	4.3
Phe	9.0	8.2
Pro	14.7	15.0
Ser	10.4	10.0
Thr	9.4	10.4
Trp	2.8	2.7
Tyr	5.1	5.2
Val	9.2	9.8
Total amino acids, %	199.8	198.0
Monosaccharides, %		
Arabinose	13.3	20.2
Fucose	4.2	4.2
Galactose	12.2	11.1
Glucosamine	0.4	0.7
Total glucose	588.9	457.7
Rhamnose	1.1	2.3
Xylose and Mannose	19.7	18.8
Total glucosinolates, mmol/kg	–	1.0

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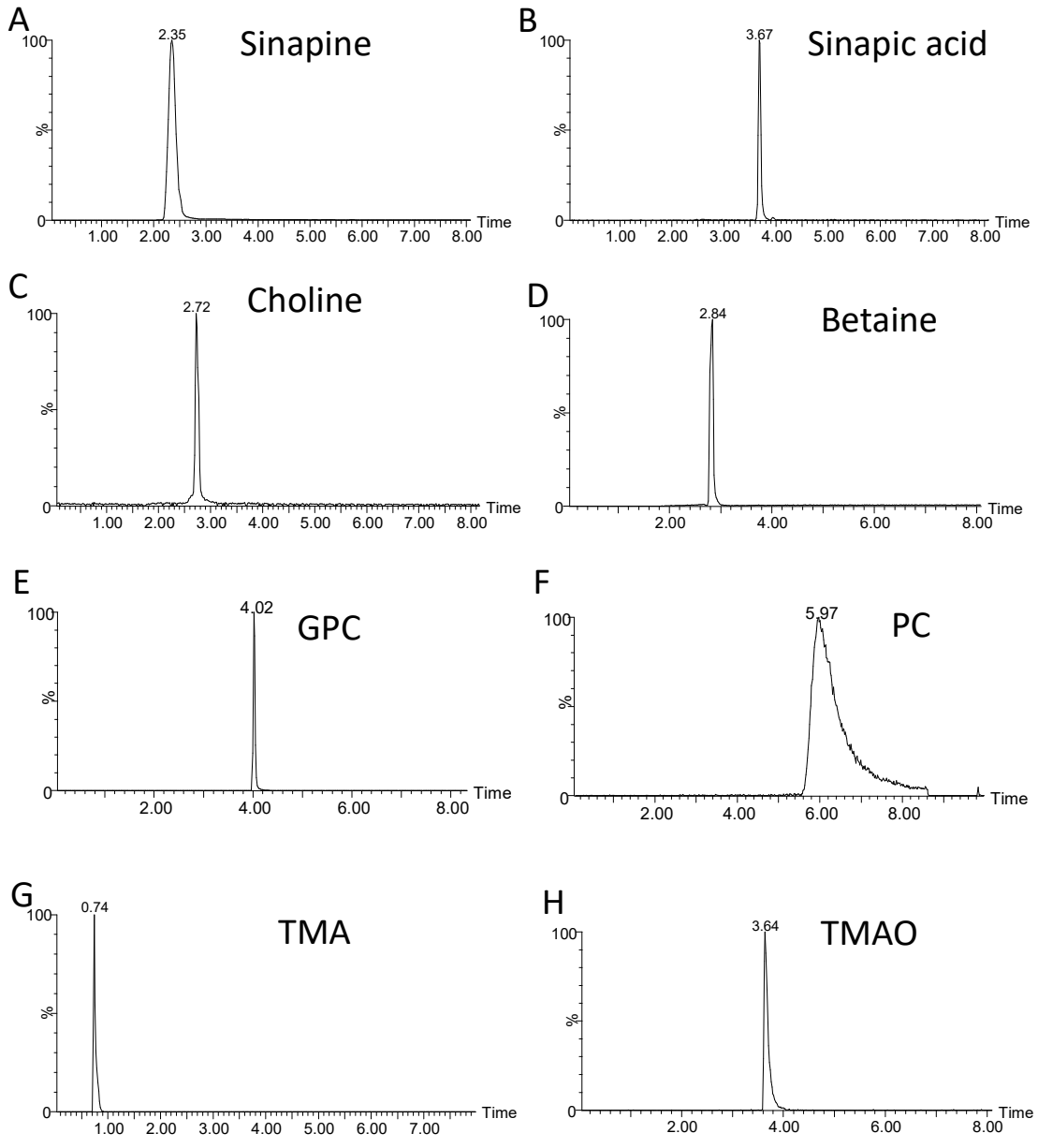
512

Figure S1. Representative chromatograms of sinapine, sinapic acid, choline, and choline-associated metabolites. The detection limits of sinapine, sinapic acid, choline, betaine, PC, GPC, TMA and TMAO were 50 nM, 100 nM, 2.5 nM, 50 nM, 500 nM, 5 nM, 500 nM and 250 nM, respectively. The conditions of LC-MS analysis are detailed in Materials and Methods. ***A***. Sinapine. ***B***. Sinapic acid. ***C***. Choline. ***D***. Betaine. ***E***. GPC. ***F***. PC. ***G***. TMA. ***H***. TMAO.



519 **Figure S2**. Concentrations of sinapine and sinapic acid in the hull and coarse fraction ingredients

520 of RSF diet (n = 3 replicates). ***A***. Concentrations of sinapine. ***B***. Concentrations of sinapic acid.

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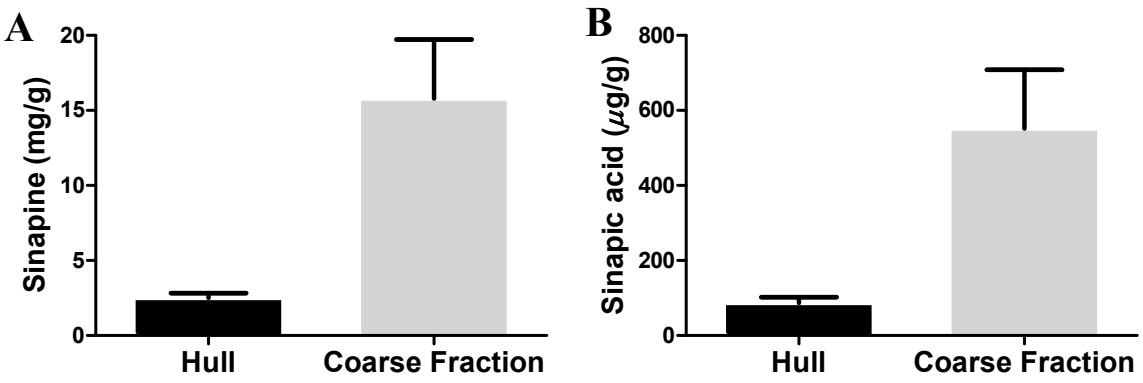

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Figure S3. Concentrations of sinapine and sinapic acid in the intestinal digesta of the pigs fed with SBM and RSF diets (n = 20/treatment). ***A***. Concentrations of sinapine. ***B***. Concentrations of sinapic acid. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

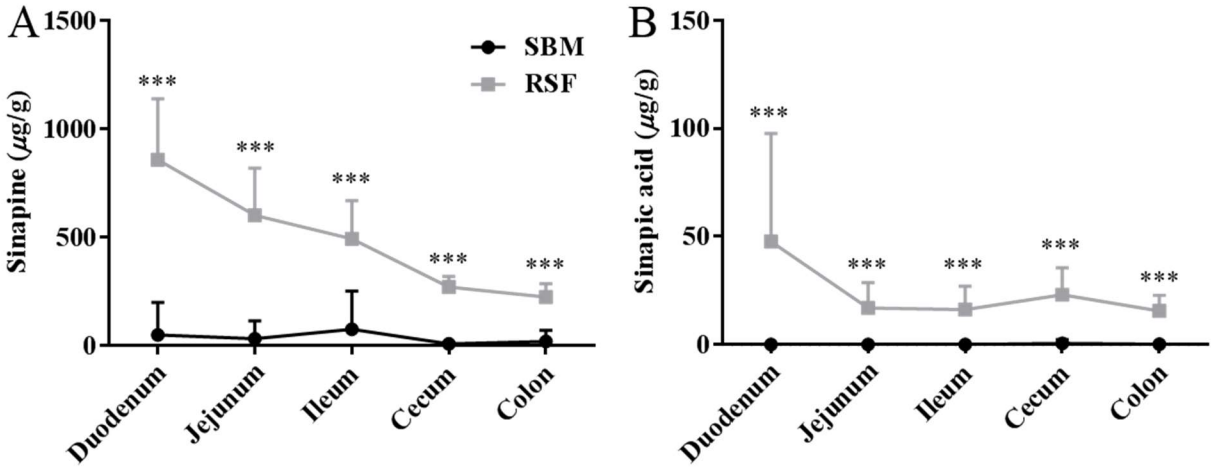


Figure S4. Concentrations of choline in the intestinal digesta of individual pigs fed with SBM and RSF diets (n = 20/treatment). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

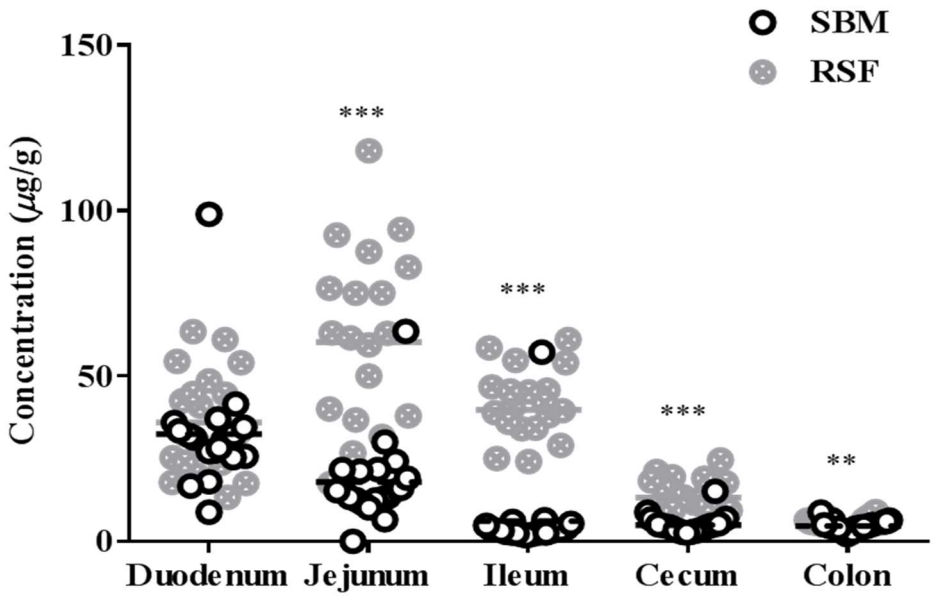


Figure S5. Concentrations of choline and its metabolites in the liver and serum of individual pigs fed with SBM and RSF diets (n = 20/treatment). ***A***. Concentrations of hepatic choline and betaine. ***B***. Concentrations of hepatic PC and GPC. ***C***. Concentrations of serum choline and betaine. ***D***. Concentrations of serum PC and GPC. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

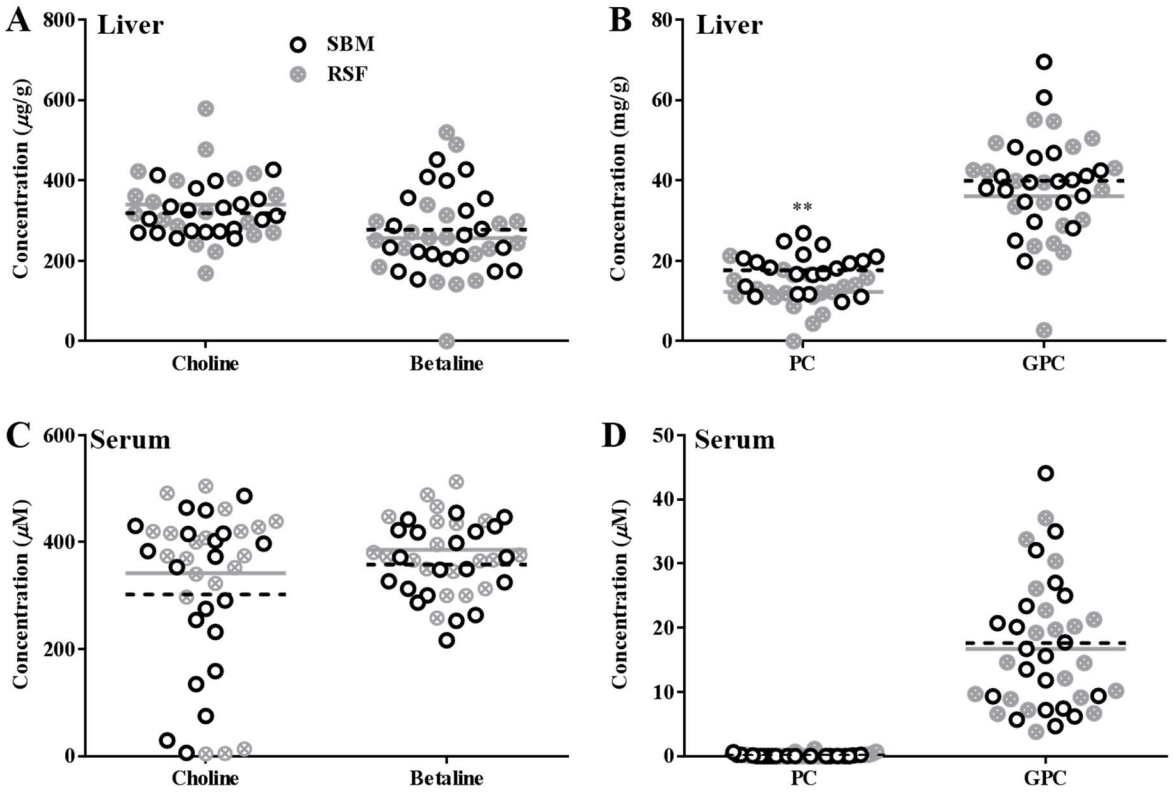
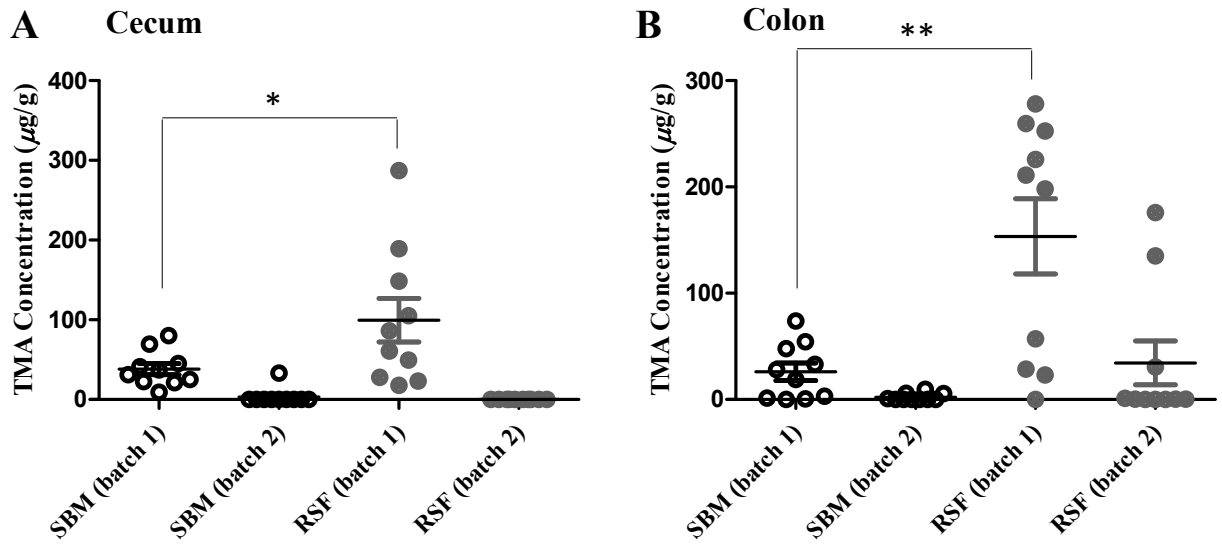


Figure S6. Concentrations of TMA in the large intestine of individual pigs in 2 batches of SBM and RSF feedings (n = 10/batch/treatment). ***A***. Concentrations of TMA in cecal digesta samples from 2 batches of SBM and RSF feeding. ***B***. Concentrations of TMA in colonic digesta samples from 2 batches of SBM and RSF feeding. *, $P < 0.05$; **, $P < 0.01$.



578 **Graphic for table of contents**

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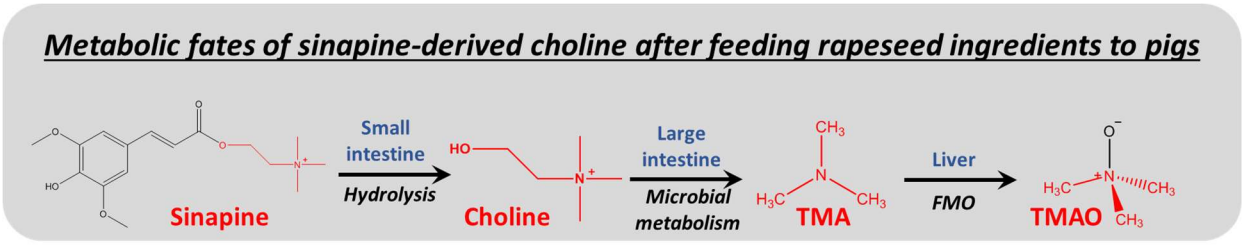

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