Characterizing the influence of gut microbiota on host tryptophan metabolism with germ-free pigs

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ABSTRACT

Intestinal microbes are closely associated with host health, depending on metabolic crosstalk between the microbiota and host. Tryptophan metabolism is one of the best examples of metabolic crosstalk between intestinal microbiota and host; however, our understanding about the influence of intestinal microbiota on host tryptophan metabolism is limited. Thus, we established germ-free (GF) pig models to systematically explore the influence of intestinal microbiota on tryptophan metabolism. Five GF pigs were kept in GF conditions throughout the experiment (GF group). Six GF pigs were transplanted with fecal microbiota from donor sows to act as control pigs. Compared with control pigs, the GF pigs had remarkable alterations in tryptophan metabolism. The differential metabolites (P < 0.05) were mainly found in the liver, circulation system and large intestine. Notably, the alteration of metabolites in tryptophan metabolism varied among organs, especially for the serotonin pathway. In GF pigs, tryptophan and kynurenine in the large intestine and 5-hydroxytryptophan in most organs were increased (P < 0.05), while metabolites in the indole pathway in most organs were decreased (P < 0.05). Collectively, our study reveals changes in tryptophan metabolism in GF pigs, highlighting the critical role of gut microbes in shaping host tryptophan metabolism.

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1. Introduction

Alterations in composition or function of intestinal microbiota may result in alterations of host physiology (Belkaid and Harrison, 2017; Turnbaugh et al., 2006) and may potentially trigger diseases, such as type 2 diabetes mellitus (T2DM) (Song et al., 2021; Qin et al., 2012), inflammatory bowel disease (IBD) (Lavelle and Sokol, 2020), colorectal cancer (Wong et al., 2017; Janney et al., 2020). One of the underlying mechanisms for intestinal microbiota to shape host physiology and pathology depends on the metabolic crosstalk between the microbiota and host (Koh et al., 2016; Cait et al., 2018; Li et al., 2018).

Tryptophan (Trp) metabolism is one of the best examples of metabolic crosstalk between intestinal microbiota and host, and has been implicated in the pathogenesis of various metabolic disorders (Hung et al., 2017; Wrzosek et al., 2021) and inflammatory diseases (Zelante et al., 2013). There are three pathways for Trp metabolism, including the kynurenine (Kyn) and serotonin (5-HT) routes (McCarrville et al., 2020; Yano et al., 2015) in host, and the pathway for production of indole and its derivatives in gut microbes (Roager and Licht, 2018). Recent years have witnessed increasing interest in Trp metabolism due to its association with host health and diseases, for example, the kynurenine pathway shapes immune responses and 5-HT plays important roles in gut motility and gut-brain signaling (Agus et al., 2018). As ligands for the aryl hydrocarbon receptor (AhR) and pregnane X receptor...
some of indoles (e.g., 3-indolepropionic acid [IPA]) are closely related to intestinal barrier function, immune homeostasis and the pathogenesis of intestinal diseases (Natividad et al., 2018; Scott et al., 2020; Nikolaus et al., 2017; Lamas et al., 2018). However, research on microbiota–host interaction in Trp metabolism is still unclear.

To explore the influence of intestinal microbiota on Trp metabolism, metabolomics has been employed to analyze samples from germ-free (GF) mice (Wikoff et al., 2009; Claus et al., 2008). Although these compelling investigations increase our knowledge in Trp metabolism, our understanding about the crosstalk between host and intestinal microbiota on Trp metabolism is still fragmented due to a number of limitations. Firstly, previous studies have focused mainly on single or select metabolites in Trp metabolism, as opposed to all metabolites. Additionally, previous studies have generally used untargeted metabolomic approaches, rather than targeted metabolomics to analyze Trp metabolism. Also, only certain organs and/or biofluids were evaluated in previous reports, which are lacking the systemic profile of Trp metabolism. Furthermore, although GF mice are important models to identify the crosstalk between intestinal microbiota and host (Thion et al., 2018; Lee et al., 2011), GF mice display various differences in physiology, anatomy and behavior to humans.

As pigs have emerged as interesting candidates because of physiological similarities with humans (Lunney et al., 2021), we have thus established GF pig models to explore systemic effects of gut microbiota on Trp metabolism with targeted metabolomics, which may provide a better understanding about the crosstalk between intestinal microbiota and host in Trp metabolism.

2. Materials and methods

2.1. Animal ethics

This animal study was reviewed and approved by the Laboratory Animal Ethical Commission of the South China Agricultural University.

2.2. Animal husbandry

The GF and control pigs were acquired by uterine strippers and sterile animal transporters, and fed in sterile feeding isolators with

![Fig. 1. Differences in Trp metabolites in the jejunal contents of control and GF pigs. (A) Level of Trp. (B) Changes in 5-HT pathway. (C) Changes in kynurenine pathway. (D) Changes in indole pathway. (E) Graphic figure depicting the changes in Trp metabolites in the jejunal contents of GF pigs. Red circle: increase; black circle: no statistical difference; dotted circle: less than 3 samples were detected. All data are shown as the mean ± SD. Unpaired t-test was used to analyze the variation between the 2 groups. *P < 0.05. Trp = tryptophan; 5-HTP = 5-hydroxytryptophan; 5-HT = 5-hydroxytryptamine; NAS = N-acetylserotonin; MLT = melatonin; 5HIAA = 5-hydroxyindoleacetic acid; Kyn = kynurenine; KNA = kynurenate; 3HKyn = 3-hydroxykynurenine; 3HAA = 3-hydroxyanthranilate; PA = picolinic acid; IAM = indole-3-acetamide; IAA = 3-indoleacetic acid; MIA = 3-methyl-2-indolic acid; IAld = indole-3-aldehyde; TAM = tryptamine; IAAd = indole-3-acetaldehyde; IE = indole ethanol; IPYA = indole-3-pyruvate; ILA = indole-3-lactic acid; IA = 3-indoleacrylic acid; IPA = 3-indolepropionic acid.](image-url)
light and dark alternated every 12 h. From 0 to 21 d of age, pigs were fed with sterilized laboratory milk (Fig. S1) purchased from Anyou Biotechnology (Table S1). Then, from 22 to 45 d of age, pigs were fed with a sterilized basal diet (Fig. S1; Table S2). Milk and feed were vacuum-packed, irradiated by 60Co-γ source (dose 20 kGy), and transferred into a feeding isolator after a microbial test. Drinking water was treated with high-temperature and high-pressure. In this study, pigs were obtained from the Technical Engineering Center for the Development and Utilization of Medical Animal Resources (Chongqing, China).

2.3. GF pigs and control pigs

For production of GF and control pigs (Fig. S1), 4 SPF pregnant Bama miniature pigs were selected as donor sows for aseptic cesarean section to obtain GF piglets. Eleven GF piglets were randomly selected in this experiment, and among them, 5 GF piglets were kept in GF conditions throughout the experiment (GF group). The other 6 GF piglets were transplanted with fecal microbiota from donor sows as to act as control pigs. For fecal microbiota transplantation, fresh feces were collected from the donor sows one month before delivery. Fresh feces and sterilized water were mixed with ratio of 1:5 and then filtered through four layers of sterilized medical gauze to obtain a fecal inoculum suspension. The fecal inoculum suspension was mixed with sterilized glycerol at the volume ratio of 9:1 and stored at −20 °C. For control pigs, 7-day-old GF piglets were orally administered the above suspension (1 mL/d) for 3 consecutive days and kept in the sterile isolator. Microbial detection in GF piglets was analyzed with plating. The composition of intestinal microbiota in control piglets was analyzed with 16S rRNA gene sequencing (Table S3) and the results showed that the intestinal microflora of control pigs was similar to that of ordinary pigs.

2.4. Sample collection

On the last day of the experimental period, after bloodletting, samples were collected on a clean bench to ensure that there was no bacterial contamination during the entire sampling process. The liver, spleen, kidney, heart, urine, bile, jejunum, ileum, cecum,
colon, jejunal contents, cecal contents, colonic contents, rectal contents and feces were collected and stored in a −80 °C refrigerator after liquid nitrogen quick-freezing.

2.5. Standard solution and sample preparation

The metabolites in Trp metabolism that we detected included the 5-HT pathway (5-hydroxytryptophan [5-HTP], 5-HT, N-acetylsertotonin [NAS], melatonin [MLT] and 5-hydroxyindoleacetic acid [5HIAA]), kynurenine pathway (Kyn, kynurenate [KNA], 3-hydroxyanthranilate [3HAA], and picolinic acid [PA]) and indole pathway (indolepyruvate [IPYA], indole-3-lactic acid [ILA], indole-3-acrylic acid [IA], indole-3-acetic acid [IAM], indole aldehyde [IAlD] and indole) (Table S4). A standard amount of 1.0 mg of each metabolite was accurately weighed, and then 10 mL methanol-water (50:50, vol/vol) was added to dissolve the metabolites to produce a 100 μg/mL stock solution. Then the standard solution was diluted in gradient to 1,000, 500, 100, 10, 1, 0.5, 0.1, 0.01, 0.001 ng/mL. Quantification of metabolites used an external standard method.

After accurately weighing tissue samples (40.0 mg) in a centrifuge tube, 400 μL ultrapure water was added and then the sample was vortexed for 4.5 min. Then, 200 μL of homogenate was added to 800 μL of methanol-acetonitrile solution (50:50, vol/vol). For samples of serum, urine and bile, 200 μL of samples were directly added to 800 μL of methanol-acetonitrile solution. The mixture was vortexed and sonicated in ice water at 4 °C for 10 min, and then centrifuged at 19,000 × g for 15 min at 4 °C. The supernatant was taken and vacuum-dried at 60 °C for 90 min, then dried with nitrogen to obtain the dry substance. The dry substance was dissolved with 200 μL methanol-water solution (50:50, vol/vol), and sonicated in ice water at 4 °C for 10 min before centrifugation at 19,000 × g for 15 min at 4 °C. The supernatant was filtered through a 0.22-μm membrane filter, and the stock solution was stored at −20 °C prior to UPLC-Orbitrap-MS/MS analysis.

2.6. UPLC-Orbitrap-MS/MS conditions

Thermo Fisher Scientific UPLC system (Dionex UltiMate 3000) was used to separate Trp metabolites with a C18 Hypersil Gold

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**Fig. 3.** Differences in Trp metabolites in the feces of control and GF pigs. (A) Level of Trp. (B) Changes in 5-HT pathway. (C) Changes in kynurenine pathway. (D) Changes in indole pathway. (E) Graphic figure depicting the changes in Trp metabolites in the feces of GF pigs. Red circle: increase; blue circle: decrease; black circle: no statistical difference; dotted circle: less than 3 samples were detected. All data are shown as the mean ± SD. Unpaired t-test was used to analyze the variation between the 2 groups. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Trp = tryptophan; 5-HTP = 5-hydroxytryptophan; 5-HT = 5-hydroxytryptamine; NAS = N-acetylsertotonin; MLT = melatonin; 5HIAA = 5-hydroxyindoleacetic acid; Kyn = kynurenine; KNA = kynurenate; 3HKyn = 3-hydroxykynurenine; 3HAA = 3-hydroxyanthranilate; PA = picolinic acid; IAM = indole-3-acetamide; IAA = 3-indoleacetic acid; MIA = 3-methyl-2-indolic acid; IAlD = indole-3-aldehyde; TAM = tryptamine; IAlD = indole-3-acetaldehyde; IE = indole ethanol; IPYA = indole-3-pyruvate; IIA = indole-3-lactic acid; IIA = 3-indoleacrylic acid; IPA = 3-indolepropionic acid.
column (1.9 μm, 100 mm × 2.1 mm; Thermo Scientific). The main parameters included the following: mobile phase A consisting of 0.1% formic acid solution, mobile phase B consisting of 100% acetonitrile, flow rate 0.2 mL/min, column temperature 35 °C and injection volume 2 μL.

The gradient elution program was applied as follows: 5% to 7% B increased linearly for 3 min and then increased to 13% linearly for 2 min, then to 50% linearly for 10 min and decreased linearly to the initial composition for 1 min, and finally held for 2 min with 5%.

A mass spectrometer (Q-Exactive Focus) was used to obtain mass spectrometry data via electrospray ionization (ESI) in the positive ion mode (spray voltage: +3.5 kV, heater temperature: 300 °C and capillary temperature: 320 °C). The MS scanning mode was set as follows: full MS scan at 70 to 1,000 m/z, resolution at 35,000 and in-source collision induced dissociation (in-source CID) at 0 eV. The MS/MS scanning mode was set as follows: data dependent ms² scan resolution with 17,000 and high collision induced dissociation (HCD) in 10, 30 and 50 eV.

2.7. Identification of metabolites

Information such as retention time, mass (m/z), peak and fragment ion MS or MS/MS intensity was obtained from raw data (*.raw files) by Xcalibur software (version 3.0, Thermo Fisher, USA). Then, a mass list for each MS scan was generated by peak detection in the raw data file and the chromatogram builder. Corresponding to each mass that was continuously detected in the scan, a chromatogram was built. According to the mass, retention time and tolerance range of each chromatographic peak, the detection peak of the metabolite sample was matched and peak area was calculated for qualitative and quantitative analysis. All retention times of Trp metabolites can be seen in Table S4.

2.8. Data analysis and statistics

Data in instrument specific format (.raw) was converted to a common data format (.XLS) file by Xcalibur software, which displayed information about metabolites including calculated amount,
retention time and peak areas. All data are shown as the mean ± SD. Unpaired t-test was used to analyze the variation between the 2 groups by GraphPad Prism 8.0. All statistical plots were calculated by GraphPad Prism 8.0.

3. Results

3.1. Trp metabolites in the intestinal contents and feces

In this study, we performed a sterile caesarean section to obtain GF pigs and applied fecal microbiota transplantation to obtain control pigs (Fig. S1). The intestinal microbiota in GF pigs and control pigs were confirmed with plating and 16S rRNA gene sequencing (Table S3). Then, we analyzed the concentrations of metabolites in Trp metabolism in different organs and the circulatory system by UPLC (Fig. S1). These metabolites were from 3 pathways: the 5-HT pathway (5-HTP, 5-HT, NAS, MLT and 5HIAA), kynurenine pathway (Kyn, KNA, 3HAA and PA) and indole pathway (IPYA, ILA, IA, IPA, TAM, IE, IAA, MIA, IAld and Indole) (Table S4).

Compared with control pigs, 4 metabolites (5-HTP, TAM, IAA, IE) in the 5-HT and indole pathways increased in the jejunal contents of GF pigs (Fig. 1). In the cecal contents, 2 metabolites (TAM and PA) decreased in GF pigs (Fig. S2). In the colonic contents, 4 metabolites (Trp, 5-HTP, Kyn and MIA) increased, but 8 metabolites (5-HT, NAS, MLT, KNA, PA, TAM, IE and IPA) decreased in GF pigs (Fig. 2). In the rectal contents, 3 metabolites (Trp, 5-HTP and Kyn) increased, while 4 metabolites (KNA, NAS, TAM and PA) decreased in GF pigs (Fig. S3). In the feces, GF pigs had lower levels of NAS, MLT, KNA, PA, IPA, IE, TAM and IAA, but higher levels of Trp, 5-HTP and Kyn (Fig. 3).

3.2. Trp metabolites in the intestine

Compared with control pigs, IAld decreased in the jejunum of GF pigs (Fig. S4). In the ileum, Kyn decreased in GF pigs (Fig. S5). In the cecum (Fig. 4) and colon (Fig. 5), Trp and 5-HTP increased in GF pigs, while 5-HT, NAS, IE and IPA in the cecum as well as IE, IPA, IAA and IAld in the colon decreased.

3.3. Trp metabolites in other organs

In addition to intestinal contents/tissues and feces, we also investigated the alteration of Trp metabolites in extraintestinal organs. In the liver, 4 metabolites (5-HT, Indole, MIA and IAld) increased, but 3 metabolites (Trp, 5-HTP and KNA) decreased in GF pigs (Fig. 6). In the spleen, IAA decreased in GF pigs (Fig. S6). In the kidney, 5-HTP and IPYA increased, but IPA decreased in GF pigs (Fig. S7). In the heart, 5-HT increased in the GF pigs (Fig. S8).

3.4. Trp metabolites in the serum, urine and bile

Considering the importance of intestinal microbiota in guiding metabolite changes in the circulatory system, we also explored the alteration of Trp metabolites in biofluids. In the serum, 3 metabolites (5-HTP, 5HIAA and PA) increased, but 3

![Fig. 5. Differences in Trp metabolites in the colon of control and GF pigs. (A) Level of Trp. (B) Changes in 5-HT pathway. (C) Changes in kynurenine pathway. (D) Changes in indole pathway. (E) Graphic figure representing the changes in Trp metabolites in the colon of GF pigs. Red circle: increase; blue circle: decrease; black circle: no statistical difference; dotted circle: less than 3 samples were detected. All data are shown as the mean ± SD. Unpaired t-test was used to analyze the variation between the 2 groups. *P < 0.05, **P < 0.01, ***P < 0.001. Trp = tryptophan; 5-HTP = 5-hydroxytryptophan; 5-HT = 5-hydroxytryptamine; NAS = N-acetylserotonin; MLT = melatonin; 5HIAA = 5-hydroxyindoleacetic acid; Kyn = kynurenine; KNA = kynurenate; 3HKyn = 3-hydroxykynurenine; 3HAA = 3-hydroxyanthranilate; PA = picolinic acid; IAA = indole-3-acetamide; IAAld = 3-methyl-2-indolic acid; IAld = indole-3-aldehyde; TAM = tryptamine; IAld = indole-3-acetaldehyde; IE = indole ethanol; IPYA = indole-3-pyruvate; IAAld = indole-3-lactic acid; IA = indoleacrylic acid; IPA = indolepropionic acid.]
metabolites (Kyn, TAM and IPA) decreased in GF pigs (Fig. 7). In the urine, MLT increased, while 4 metabolites (5-HT, TAM, IAA and IE) decreased in GF pigs (Fig. 8). However, there were no statistical differences in Trp metabolites in the bile between control and GF pigs (Fig. S9).

3.5. Summary of Trp metabolic alteration in GF pigs

Compared with control pigs, GF pigs had changes in all 3 Trp metabolic pathways. Most of the altered metabolites in Trp metabolism were found in the large intestine, feces, liver, serum and urine (Fig. 9). Trp increased in the large intestine, but decreased in the liver (Fig. 9). The alteration of Trp metabolites varied among organs, especially for the 5-HT pathway. In the 5-HT pathway, most metabolites increased in the jejunal contents, cecal contents, colon, liver, kidney, heart, serum and urine, but decreased in the colonic contents, rectal contents, feces and cecum (Fig. 9). Notably, 5-HP increased in most organs. In the kynurenine pathway, most metabolites increased in the serum, but decreased in the contents of the large intestine, feces, ileum and liver (Fig. 9). Interestingly, Kyn increased in the contents of the large intestine. In the indole pathway, metabolites decreased in most of the samples analyzed, such as the large intestine, serum and urine (Fig. 9).

4. Discussion

With the help of GF mice, we have a fundamental understanding of the crosstalk between the intestinal microbes and host. For example, 5-HT levels in the colon and serum are affected by luminal concentrations of particular microbial metabolites in GF mice (Yano et al., 2015). These results in GF mice are similar with those from our results in GF pigs, which showed lower levels of 5-HT in the colonic contents, cecum and even urine. In addition, GF mice experience significant changes in levels of Trp metabolites in the liver and serum (Claus et al., 2008; Wikoff et al., 2009), suggesting that intestinal microorganisms are vital for Trp metabolism in extraintestinal organs. Similarly, in this study, the
altered metabolites in Trp metabolism were also found in the liver, serum and urine of GF pigs. However, there were differences in the changes in Trp metabolism between GF mice and GF pigs (e.g., higher Trp in GF mouse serum vs. no difference in GF pig serum). Possible explanations for this difference include the animal models (mice vs. pigs) and methods (untargeted metabolomics vs. targeted metabolomics) used to analyze Trp metabolism. For example, pigs and mice have various dissimilarities in physiology, anatomy and behavior. Therefore, differences in their respective Trp metabolism could be a result of different gut microbial compositions.

In this study, the different metabolites in GF pigs were mainly observed in the large intestine, liver and circulation system. Interestingly, in most large intestinal samples, GF pigs had lower levels of metabolites in the indole pathway, but higher levels of those in the 5-HT and Kyn pathways, like Trp, 5-HTP and Kyn. Trp is mainly absorbed in the small intestine (Evenepoel et al., 1999), and the bacteria in the colon are the main players for production of indole and its derivatives from Trp (Roager et al., 2016). For example, many Gram-negative bacteria and Gram-positive bacteria (e.g., Escherichia coli and Clostridium spp.) produce indole from Trp by tryptophanase (TnaA) (Smith and Macfarlane, 1996; Lee and Lee, 2010). Bacteroides, which accounted for the majority of the fecal microbiota in the control pigs, metabolize Trp into indole, IAld and other metabolites (Roager and Licht, 2018). Therefore, the indole pathway was greatly decreased after intestinal microorganism clearance, and most metabolites in this pathway decreased in GF pigs. As higher levels of Trp remained in the large intestine, it is reasonable that the 5-HT and Kyn pathway increased and higher levels of 5-HTP and Kyn were observed in GF pigs. However, the bacterial metabolism of Trp is complex (Brazier et al., 2002; Li and Young, 2013; Nuidate et al., 2016), and the mechanism underlying the change in Trp metabolites in GF pigs needs further investigation.

In addition to the large intestine, the liver and circulatory system of GF pigs also showed considerable differences in Trp metabolism. Notably, the metabolites in the 5-HT pathway increased in the serum, while metabolites in the indole pathway decreased in the serum and urine. The possible reason is that intestinal Trp metabolites can be absorbed into the bloodstream through intestinal epithelial cells and transported to the liver as precursors in other metabolic pathways (Lin et al., 2015; Santana et al., 2018). Thus, it is

**Fig. 7.** Differences in Trp metabolites in the serum of control and GF pigs. (A) Level of Trp. (B) Changes in 5-HT pathway. (C) Changes in kynurenine pathway. (D) Changes in indole pathway. (E) Graphic figure representing the changes in Trp metabolites in the serum of GF pigs. Red circle: increase; blue circle: decrease; black circle: no statistical difference; dotted circle: less than 3 samples were detected. All data are shown as the mean ± SD. Unpaired t-test was used to analyze the variation between the 2 groups. *P < 0.05, **P < 0.01. Trp = tryptophan; 5-HTP = 5-hydroxytryptophan; 5-HT = 5-hydroxytryptamine; NAS = N-acetylserotonin; MLT = melatonin; SHAA = 5-hydroxyindoleacetic acid; Kyn = kynurenine; KNA = kynurenic acid; 3HKyn = 3-hydroxykynurenine; 3HAA = 3-hydroxyanthranilate; PA = picolinic acid; IAM = indole-3-acetamide; IAA = 3-indoleacetic acid; MIA = 3-methyl-2-indolic acid; IAld = indole-3-aldehyde; TAM = tryptamine; IAld = indole-3-acetaldehyde; IE = indole ethanol; IPYA = indole-3-pyruvate; ILA = indole-3-lactic acid; IA = 3-indoleacrylic acid; IPA = 3-indolepropionic acid.
understandable that these organs in GF pigs displayed obvious differences in Trp metabolism. Interestingly, the change in metabolites in Trp metabolism varied among organs, and even metabolites in the same metabolic pathway varied within the same organ, indicating that Trp metabolism has organ heterogeneity. Indeed, Trp is mainly catabolized to produce NAD$^+$ in the liver,
while it is mainly involved in the Kyn pathway outside the liver (Platten et al., 2019).

Pigs are large animals, whose physiological states are not easy to control, resulting in high variation within the group. Thus, it would be better to verify these findings with an expanded sample size in each group. Given that GF pigs in this study were piglets, there were limitations to exploring whether the intestinal microbiota had an age-stage effect on host Trp metabolism. Moreover, the expressions and activities of Trp-related metabolic enzymes were not analyzed in this study. Notably, the immune system and anatomy characteristics of pigs are similar to those of humans (Iqbal et al., 2019; Lunney et al., 2021), so results from this study may provide transferable points for humans taking antibiotic drugs causing alterations in intestinal flora.

5. Conclusion

Compared to control pigs, GF pigs displayed changes in all 3 Trp metabolic pathways, especially in the large intestine, feces, liver, serum and urine. In different organs, the alterations in Trp metabolites differed, especially for the 5-HT pathway. Notably, metabolites in the indole pathway decreased in most of samples analyzed.

Author contributions

Wenkai Ren designed the experiment. Dongming Yu, Jing Sun and Liangpeng Ge prepared the germ-free pigs and control pigs. Bingnian Liu, Zhongquan Xin, and Baichuan Deng performed the targeted metabolomics. Lijuan Fan, Xiaoyan Wu and Jian Fu helped the sample preparation. Bingnian Liu, Xiaoyan Wu and Jian Fu prepared the figures. Bingnian Liu, Lijuan Fan and Jian Fu drafted the manuscript. Wenkai Ren revised and approved the final manuscript. All authors contributed to the article and approved the submitted version.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2022.07.005.

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