Gut microbial metabolism of dietary fibre protects against high energy feeding induced ovarian follicular atresia in a pig model

Yong Zhuo†, Meng Cao†, Yuechan Gong, Lianchao Tang, Xuemei Jiang, Yang Li, Min Yang, Shengyu Xu, Jian Li, Lianqiang Che, Yan Lin, Bin Feng, Zhengfeng Fang and De Wu*

Key Laboratory for Animal Disease-Resistant Nutrition of the Ministry of Education of China, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu 611130, People’s Republic of China

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Abstract
To investigate the effects of dietary fibre on follicular atresia in pigs fed a high-fat diet, we fed thirty-two prepubescent gilts a basal diet (CON) or a CON diet supplemented with 300 g/d dietary fibre (fibre), 240 g/d soya oil (SO) or both (fibre + SO). At the 19th day of the 4th oestrus cycle, gilts fed the SO diet showed 112 % more atretic follicles and greater expression of the apoptotic markers, Bax and caspase-3, and these effects were reversed by the fibre diet. The abundance of SCFA-producing microbes was decreased by the SO diet, but this effect was reversed by fibre treatment. Concentrations of serotonin and melatonin in the serum and follicular fluid were increased by the fibre diet. Overall, dietary fibre protected against high fat feeding-induced follicular atresia at least partly via gut microbiota-related serotonin–melatonin synthesis. These results provide insight into preventing negative effects on fertility in humans consuming a high-energy diet.

Key words: Dietary fibre: Microbiota: Serotonin: Melatonin: Follicular atresia

Energetic overnutrition results in a series of reproductive health issues in females, although the underlying mechanisms remain to be elucidated(1,2). The number of ovarian follicles plays a critical role in determining the female reproductive span. However, women in Europe and the USA lose 88 % of their oocytes by the age of 30 years, and by the age of 40 years, only 3 % of the oocytes remain in their ovaries(3,4). Obese females have increased ovarian follicle loss and lower oocyte quality, thereby decreasing pregnancy rates and reproductive spans(5–7). In livestock production (e.g. the pig industry), the sustainable production of domestic animals requires a continuous supply of oocytes, and excessive loss of oocytes can result in early termination of the reproductive span and premature removal of domestic animals. Mammalian ovaries lack postnatal germline stem cells, thus causing irreversible loss of the female germ line(8,9). Therefore, the ovarian reserve pool must be strictly regulated to prevent premature exhaustion of the oocytes, which terminates the reproductive process and leads to reproductive ageing. In humans and rodents, one protective mechanism against obesity involves increasing the levels of dietary fibre in high-energy diets(10–13). Interestingly, the addition of high dietary fibre also improves oocyte maturation and prenatal embryonic survival in pigs(14–16). Dietary fibre intake is associated with circulating follicle-stimulating hormone in middle-aged women(17), and one prospective study has reported an association between dietary fibre intake and age at menopause(18). However, it remains unknown whether dietary fibre might exert a protective role against follicle loss induced by overconsumption of dietary energy. In addition, the mechanisms through which dietary fibre influences follicle development remain poorly understood and require further investigation.

High-fat and low-fibre diets have recently been reported to decrease beneficial populations of microbes, including Lactobacilli and Bifidobacteria, and to increase the abundance of harmful bacteria, such as enterotoxigenic Escherichia coli and Shigella(19). Dietary fibre, the main substrate of the intestinal microflora, can significantly alter the intestinal microbiota and their metabolites, including SCFA(20,21). Our previous study showed that the addition of 5 % soya oil (SO) decreases microbiota diversity in sows, whereas the addition of dietary fibre restores this loss and increases the relative abundance of representative SCFA-producing genera(22). However, the mechanisms underlying the effects of the microbial metabolism of dietary fibre on ovarian function remain largely unknown.

Abbreviations: 5-HT, 5-hydroxytryptamine receptor; SO, soya oil; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling.

*Corresponding author: De Wu, fax +86-028-86290922, email wude@sicau.edu.cn
† These authors contributed equally to this work.
SCFA are primary metabolites derived from dietary fibre that have been shown to have important physiological functions in various tissues\(^\text{[20]}\). To our knowledge, the concentrations of SCFA in ovarian follicular fluid cannot reach the threshold required to activate the downstream signalling of their G-protein-coupled receptors. Recently, microbial metabolites have been shown to act on colon epithelial enterochromaffin cells and to promote the production of a common metabolite, serotonin\(^\text{[25]}\). Serotonin is a microbiota-regulated metabolite largely (approximately 95 %) derived from the gut\(^\text{[21]}\). Its receptors, 5-hydroxytryptamine (HTR)\(_{1D}\), 5-HTR\(_2\), and 5-HTR\(_7\), are expressed in mammalian ovarian tissues and have been observed to regulate placental development and maturation\(^\text{[24]}\). Studies in crustaceans have found that injection of serotonin induces ovarian follicle development, maturation and ovulation\(^\text{[25–29]}\). Furthermore, studies of fish have shown that serotonin stimulates the maturation of granulocytes and induces oocyte maturation in a dose-dependent manner\(^\text{[29]}\). Several studies have shown that serotonin plays an important role in cellular proliferation, maturation and apoptosis\(^\text{[30–35]}\). Researchers have also reported that serotonin, via binding the 5-HT\(_{2}\) receptor, activates extracellular signal-regulated kinase (ERK)\(_1/2\), thus inhibiting Bax expression and protecting cardiomyocytes against apoptosis\(^\text{[31]}\). Moreover, serotonin prevents cytochrome c release and caspase-9 and -3 activation via cross-talk between phosphatidylinositol-3 kinase/Akt and the ERK1/2 signalling pathways. In addition, multiple biological pathways, including G-protein signalling, vesicular transport, apoptosis and survival, have been found to be regulated by serotonin in an Illumina platform analysis of global gene expression in megakaryocytes isolated from mice infused with serotonin or saline\(^\text{[56]}\). Additionally, serotonin can be converted to melatonin, a powerful antioxidant hormone, which regulates ovarian apoptosis. However, whether serotonin and melatonin synthesis and their action on ovarian follicle cells might be regulated by dietary energy feeding levels and fibre levels was unknown.

According to previous findings, we hypothesised that elevated loss of ovarian follicles induced by a high-energy diet might be prevented by dietary fibre, via regulation of the granulosa cell apoptosis induced by serotonin and melatonin synthesis. Pigs are widely used as a model for human pathologies because of the similarities between pigs and humans in terms of gastrointestinal anatomy and physiology, nutrient digestibility and metabolic physiology\(^\text{[37]}\). Additionally, the ovarian follicle size and number, the wave of follicle activation and maturation, and the manner of follicle loss are similar between pigs and humans\(^\text{[38,39]}\). Therefore, pigs were used as an experimental model in the present study.

**Materials and methods**

**Animals, diets and design**

Experimental procedures were approved by the Animal Care and Use Committee of Sichuan Agricultural University (No. SICAU-2015-034) and were in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals.

Thirty-two prepubescent Landrace × Yorkshire crossbred gilts with similar body weight (91.7 ± 3.7 kg) and age (161.5 ± 5.0 days) were randomly allocated to one of four groups according to a 2 × 2 factorial experimental design. Each dietary group contained eight replicates (pen) with one gilt per replicate. Gilts in the control (CON) group were fed a 2.4 kg/d maize–soya bean oil basal diet providing 33.14 MJ/d digestible energy and 297.6 g/d of dietary fibre, a high-SO regimen including an additional 240 g/d SO, a high-fibre (fibre) regimen including an additional 300 g/d inulin and cellulose at a ratio of 1:4, or a combination of 240 g/d SO and 300 g/d inulin and cellulose (fibre + SO). Owing to the additional SO and dietary fibre intake, the SO and fibre + SO gilts consumed 26.5 % more digestible energy (41.92 v. 33.14 MJ/d) than the CON gilts, and gilts in the fibre and fibre + SO groups were fed 100 % more dietary fibre (597.6 v. 297.6 g/d) than the CON gilts (online Supplementary Table S1). The daily intake of other nutrients, including lysine, tryptophan, minerals and vitamins, remained similar among groups (online Supplementary Table S1). The CON diet was formulated to ensure an average daily gain of 897 g/d, according to the nutrient requirements for swine (2012), and was sufficient to ensure the growth potential of the gilts. The additional intake of 240 g/d SO resulted in excess energy intake during this phase. In the present study, dietary fibre concentrations in the diet were measured through an enzymatic-gravimetric method AOAC 991.43, which differs from that for crude fibre or neutral-detergent fibre. The dietary fibres used in this trial were inulin and cellulose (Guangxi Shangda Tech Co., Ltd). Inulin is water soluble and is easily fermented in the gut by the microbiota. Cellulose is insoluble to water and therefore is difficult for the gut microbiota to ferment. Inulin and cellulose were added at a ratio of 1:4. The day at the beginning of the experiment was designed as day 1, and experiments were terminated at day 19 of the fourth oestrus. Gilts were housed individually (2.0 m × 0.8 m) in a breeding facility and fed twice daily at 08.00 and 14.30 hours. Drinking water was provided ad libitum.

The environmental temperature was controlled to 20–24°C. Artificial lights were provided from 07.00 to 19.00 hours.

**Measurement of pubertal data and growth traits**

The onset of oestrus was detected by one experienced stockperson on the basis of the behavioural and vulvar characteristics of gilts, as previously described\(^\text{[40]}\). The first day of standing heat was considered pubertal oestrus and was defined as day 1 of the first oestrus cycle. The body weight and backfat thickness of the gilts were measured at the beginning of the experiment, at puberty and at day 19 of the fourth oestrus. The backfat thickness was measured at 65 mm on both sides of the dorsal midline at the last rib (P\(_2\)) with an ultrasound scanner (Renco LeanMeater). Measurements for both sides were averaged to obtain a final value.

**Collection of faecal samples, blood samples, and colonic and ovarian tissue**

On day 18 of the fourth oestrus cycle, serial blood samples were collected from all pigs at 07.00, 09.00, 10.00, 12.00 and 14.00 hours to measure serotonin concentrations.
Faecal samples were collected after 12 weeks of feeding. To collect freshly excreted faeces, defecation was promoted by rectal stimulation. The excreted faeces were transferred into sterile tubes with a sterile cotton swab pre-wetted with ice-cold sterile PBS. Faeces were stored in liquid N₂ immediately and were then transferred to a freezer at –80°C. Any contacts with faeces were kept sterile during the entire sampling procedure. All gilts were euthanised through intravenous injection of xylazine (0.02 ml/kg body weight) in the morning of day 19 of the fourth oestrus cycle at 2 h after feeding. The proximal colonic contents were quickly transferred to 1·5 ml sterile tubes. Proximal colonic tissues were washed with ice-cold PBS three times and dried with sterile tissue paper. Both colonic tissues and contents were stored in N₂ immediately and then transferred to a freezer at –80°C. The ovaries were isolated, washed with ice-cold PBS, dried with tissue paper and weighed. Follicular fluids were aspirated from large follicles more than 3 mm in diameter with a 10-ml syringe equipped with a 12-gauge needle and then filtered with a 300-Mesh cell strainer to remove foreign tissues. The ovarian granulosa cells were collected from antral follicles with diameters larger than 1 mm, as previously described. The right ovary was fixed in 4 % paraformaldehyde in 100 mmol/l phosphate buffer, pH 7·4. The left ovaries were cut into pieces with scissors, snap frozen with N₂ and stored at –80°C.

Morphological classification of follicles

The right ovary was dissected into two parts along the middle with a scalpel and then embedded in paraffin. Sections (5 μm) were collected at intervals of 50 μm and stained with haematoxylin and eosin. Twenty sections per ovary (ten sections per paraffin block) were analysed. Follicle types in ovarian cross-sections were defined and counted according to a previously described method. Primordial follicles comprised an oocyte with a clear nucleus surrounded by a single layer of small cuboidal granulosa cells. Preantral follicles comprised an oocyte with a clear nucleus surrounded by single or multiple layers of cuboidal granulosa cells. Antral follicles (with diameters below 1 mm) were distinguished by the presence of an antrum within the granulosa cell layers enclosing the oocyte and were counted only if the diameter was less than 1 mm in the sections. Follicles were determined to be atretic if they displayed two or more of the following criteria within a single cross-section: more than two pyknotic nuclei, granulosa cells within the antral cavity, granulosa cells pulling away from the basement membrane or uneven layers of granulosa cells. The ovarian cross-section areas were measured with Image Pro Plus for Windows (version 6.0; Media Cybernetics). The abundance of each type of follicle was normalised to the volume of ovarian tissue in the sections. The images were digitised with a computer coupled to a light microscope with a final magnification of 200× for the primordial and preantral follicles and 100× for the antral follicles. To avoid repeat counting, only follicles that showed the nucleus of the oocyte were counted.

TUNEL assays

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) assays were performed with a Roche In Situ Cell Death Detection Kit (POD, 11684817910). Briefly, ovarian paraffin sections were incubated with TUNEL reaction agents according to the manufacturer’s instructions. Nuclei stained with haematoxylin were blue. The positive apoptotic cells detected with the diaminobenzidine reagent had brown-yellow nuclei. All antral follicles showing clear oocytes and a diameter less than 2 mm in the sections were included in the analysis. Observations were conducted in four different directional areas of each antral follicle. If the positive cells in the four areas of an antral follicle exceeded one-third, the follicle was defined as atretic. The percentage of atretic antral follicles (less than 2 mm in diameter) per section was calculated as the number of atretic antral follicles divided by the total number of antral follicles with clear oocytes and a diameter less than 2 mm.

Microbial and SCFA analysis

The composition of the microbial community in the faeces was assessed with high-throughput pyrosequencing, as recently described (online Supplementary Methods). SCFA including acetate, propionate and butyrate in the colonic contents were analysed as previously described.

Serotonin and melatonin measurements

Serum samples collected at five different time points (07.00, 09.00, 10.00, 12.00 and 14.00 hours) on day 18 of the fourth oestrus cycle, and ground proximal colon, ovary tissues and follicular fluids at slaughter were assessed for serotonin concentrations with an ELISA kit according to the manufacturer’s instructions (DLD Diagnostika GmbH) (online Supplementary Methods). The serotonin content in the proximal colon tissues and ovary tissues was normalised to tissue weight. The serum and follicular fluids at slaughter were assessed for melatonin concentrations with a commercial ELISA kit (IBL #RF54021) (details in online Supplementary Methods).

Gene expression

The total RNA from ovarian granulosa cells and colonic tissue samples was extracted with TRIzol reagent (Ambion). A commercial reverse transcription kit (TaKaRa) was used for the synthesis of cDNA. The mRNA levels were then analysed with a 7900HT Fast Real-Time PCR system (Thermo Fisher Scientific) with SYBR Green Real-Time PCR reagent (RR820A, Takara Bio). Primers are shown in online Supplementary Table S2. The threshold cycle (2–ΔΔCt) method was used to calculate the relative gene expression. β-Actin was used as the housekeeping gene, and the relative gene expression levels are expressed as fold changes relative to the average mRNA levels of genes in gilts in the CON group.

Western blot analysis

Protein expression in ovarian tissues was detected by Western blotting, as previously described. Primary antibodies against Bax (ab32503) and Bcl-2 (ab32124) were obtained from Abcam, and antibodies against caspase-3 (9665S) and β-actin (4970S) were obtained from Cell Signaling Technology. Blots...
were quantified with ImageJ software (National Institutes of Health).

**Statistical analysis**

This experiment used a completely randomised design with a 2 × 2 factorial treatment arrangement.

The data were checked for equal variances and normal distribution of the residuals before parametric analyses. Statistical analyses were performed through the mixed procedure in SAS 9.4 (SAS Institute Inc.). The following statistical model was used: 

\[ Y_{ij} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + e_{ij} \]

where \( Y_{ij} \) is the response variable, \( \mu \) is the overall mean, and \( \alpha_i \) and \( \beta_j \) are the fixed effects of the SO and dietary fibre intake levels, respectively. \( \alpha\beta_{ij} \) is the interaction among fixed effects, and \( e_{ij} \) is the residual error. The least significant difference method was used to compare the group means.

Serum concentrations of serotonin were analysed by repeated-measures ANOVA. Several covariance structures were modelled before compound symmetry was used as the best covariance structure through the mixed procedure in SAS, and comparisons of individual time points were made with two-way ANOVA. The model included SO intake, dietary fibre intake and time as fixed factors. The basal value recorded before the morning meal was used as a covariate. Data on the relative abundance of gut microbiota were analysed with the Glimmix procedure in SAS, and a principal component analysis was performed for downstream analyses (online Supplementary Table S5).

**Results**

**High fibre intake protected against ovarian follicular atresia induced by high soya oil feeding**

The average daily body weight gain and backfat gain (\( P < 0.01 \)) and the age at puberty, were greater in gilts receiving extra SO feeding (\( P < 0.05 \)). The average daily body weight gain, backfat gain and age at puberty were not affected by additional consumption of dietary fibre (online Supplementary Table S3). The fasting circulating concentrations of TAG and total cholesterol were affected by fibre treatment (\( P < 0.05 \)), and NEFA levels (\( P < 0.05 \)) in gilts were influenced by fibre treatment (\( P < 0.01 \), online Supplementary Table S4) and the interaction between fibre and SO (\( P < 0.05 \), online Supplementary Table S4).

The numbers of follicles at each developmental stage are shown in Fig. 1(A)–(D). The number of primordial follicles in gilts showed a tendency to be affected by fibre treatment (\( P = 0.082 \), Fig. 1(A)) and was greater in fibre + SO gilts than SO gilts (\( P = 0.062 \), Fig. 1(A)). The number of preantral follicles in gilts was not affected by dietary treatment (\( P > 0.05 \), Fig. 1(B)). The number of antral follicles in the gilts increased with dietary fibre treatment (\( P < 0.05 \), Fig. 1(C)). The number of atretic follicles was affected by SO, fibre and the interaction between SO and fibre (\( P < 0.01 \), Fig. 1(D)). With extra dietary fibre intake, the increase in atretic follicles induced by SO feeding was restored to levels similar to those in the CON group (\( P < 0.05 \), Fig. 1(D)). The number of total follicles and the sum of follicles at each stage were significantly lower in the SO gilts than the other groups (\( P < 0.05 \), Fig. 1(E)).

TUNEL-stained ovarian sections (Fig. 1(F)–(H)) indicated that SO increased granulosa cell apoptosis and had a tendency to increase the percentage of atretic antral follicles (\( P = 0.086 \), Fig. 1(I)). Fibre treatment significantly decreased granulosa cell apoptosis and the percentage of atretic antral follicles (\( P < 0.01 \), Fig. 1(I)).

**Ovarian apoptosis-related markers in gilts were affected by dietary treatment**

The mRNA expression of apoptosis-related markers in ovarian granulosa cells are shown in Fig. 2(A)–(C). The relative gene expression of Bax (\( P = 0.043 \), Bcl-2 (\( P = 0.005 \)) and caspase-3 (\( P = 0.017 \)) in the ovarian granulosa cells of gilts was affected by fibre treatment (Fig. 2(A)–(C)), and the mRNA expression of caspase-3 (\( P = 0.009 \)) was affected by SO treatment (Fig. 2(C)). The mRNA expression of Bax and caspase-3 (\( P = 0.009 \)) in the granulosa cells of SO gilts was lower than that in fibre + SO gilts (Fig. 2(A)) and (C), \( P < 0.05 \), and Bcl-2 mRNA expression in the granulosa cells of SO gilts was greater than that in fibre + SO gilts (Fig. 2(B), \( P < 0.05 \)). As shown in Fig. 2(D)–(G), the protein expression of Bax and caspase-3 in granulosa cells was significantly up-regulated (Fig. 4(E) and (G), \( P < 0.05 \)), whereas the protein expression of Bcl-2 was significantly down-regulated by SO treatment in gilts (Fig. 4(F), \( P < 0.05 \)). The protein expression of Bax (\( P < 0.05 \)) and caspase-3 (\( P = 0.098 \)) was down-regulated, and the protein expression of Bcl-2 was significantly (\( P < 0.05 \)) up-regulated by fibre treatment (Fig. 4(E)–(G)).

**Microbial community structure was affected by diets**

A total of 2 277 531 effective tags were obtained from all faecal samples, and 40 801 distinct operational taxonomic units (OTU) at a 97% identity level were obtained from all samples and used for downstream analyses (online Supplementary Table S5).

On the basis of Venn diagram analysis, a total of 1447 OTU were shared among the four groups (online Supplementary Fig. S1(A)). \( \alpha \)-Diversity and \( \beta \)-diversity can reflect the richness and diversity of microbial communities. The Shannon index was calculated for \( \alpha \)-diversity (online Supplementary Fig. S1(B)), and a principal component analysis was performed for \( \beta \)-diversity (online Supplementary Fig. 1(C)). The gilts fed the high-energy diet with no extra dietary fibre had lower Shannon index values in faecal samples, and the microbial clusters were clearly separate from those of the other three diet groups in the principal component analysis. SO had an adverse effect on intestinal microbial diversity and reduced microbial abundance. In contrast, the high-dietary fibre diet prevented the loss of gut microbial abundance caused by the SO regimen. In all samples at the phylum and genus levels, the abundance levels of the top nineteen phyla and the top thirty-five genera were selected and used to construct heatmaps. As shown in online Supplementary Fig. 2(A), clear differences in the phylum distribution of the faecal microbiota were found. The proportions of Firmicutes and Planctomycetes bacteria were increased,
whereas the proportions of *Fibrobacteres*, *Spirochaetes* and *Bacteroides* bacteria were lower in the high energy group than the low energy group when no additional dietary fibre was consumed. As shown in online Supplementary Fig. 2(B), clear differences were also found in the genus distribution of faecal microbiota.

A total of twenty-three genera (relative abundance > 0.2% in at least one sample) with significant changes ($P < 0.05$) or with a trend to significance ($0.05 < P < 0.1$) at the genus level were affected by the dietary treatments (Table 1). The relative abundance of *Treponema_2*, *Prevotella_9*, *Prevotella_7*, *Sphaerochaeta*, *Anaerovibrio*, *Fibrobacter*, *Ruminiclostridium_6* ($P < 0.05$) and *Oscillospira* ($P = 0.081$) was decreased, whereas the relative abundance of *Eubacterium_coprostanoligenes_group* ($P < 0.05$), *p-1088-a5_gut_group* ($P = 0.089$) and *Leeia* ($P = 0.089$) was increased by high SO intake. The relative abundance of *Rikenellaceae_RC9_gut_group*, *Alloprevotella*, *Sphaerochaeta*, *Bifidobacterium*, *Fibrobacter*, *Cellulosilyticum* ($P < 0.05$), *Oscillospira* ($P = 0.071$) and *Faecalibacterium* ($P = 0.082$) was increased, whereas the relative abundance of *Streptococcus*, *Clostridium_sensu_stricto_1*, *p-1088-a5_gut_group*, *Leeia* ($P < 0.05$) and *Eubacterium_coprostanoligenes_group* ($P = 0.072$) was decreased ($P < 0.05$) by extra dietary fibre intake. Moreover, interactive effects between SO and fibre were found for the genera *Streptococcus*, *Treponema_2*, *Lactobacillus*, *Rikenellaceae_RC9_gut_group*, *Eubacterium_coprostanoligenes_group*, *Escherichia-Shigella*, *p-1088-a5_gut_group*, *Leeia*, *Acidiminococcus*, *Cellulosilyticum*, *Faecalibacterium* ($P < 0.05$), *Terrisporobacter* ($P = 0.096$), *Mitsuokella* ($P = 0.079$) and *Fibrobacter* ($P = 0.076$).

**Production of SCFA was elevated by high fibre intake**

Acetate concentrations in the colonic contents were not affected by dietary treatment ($P > 0.05$). The SO treatment significantly

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**Fig. 1.** Ovarian atretic follicles are affected by dietary energy levels and fibre levels (n 8). (A–E) Numbers of ovarian primordial, preantral, antral, atretic follicles and total number of follicles ($10^2$) per cm$^3$ of ovarian tissue. (F–I) Representative micrographs of the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assays, showing atretic antral follicles in the ovaries, whereas (F) shows atretic antral follicles (black arrow), (G) shows healthy antral follicles (black arrow) and (H) shows apoptotic granulosa cells in atretic antral follicles (black arrow). Magnification: 40× in (A) and (B), 200× in (C). (I) Percentage of atretic follicles per section. CON, control, daily intake of 2·4 kg basal diet; Fibre, CON diet plus 300 g/d inulin and cellulose at a ratio of 1:4; SO, CON plus 240 g/d SO; Fibre + SO, CON diet plus 300 g/d inulin and cellulose (1:4) and 240 g/d SO. a,b Mean values with unlike letters were significantly different ($P < 0.05$; least significant difference method).
Fig. 2. Expression of apoptosis-related markers in ovarian granulosa cells, as affected by dietary energy feeding levels and fibre levels (n=8). (A–C) mRNA expression of Bax, Bcl-2 and caspase-3. (D–G) Western blotting results of the proteins Bax, Bcl-2 and caspase-3 in granulosa cells. CON, control, daily intake of 2·4 kg basal diet; Fibre, CON diet plus 300 g/d inulin and cellulose at a ratio of 1:4; SO, CON plus 240 g/d SO; Fibre + SO, CON diet plus 300 g/d inulin and cellulose (1:4) and 240 g/d SO. a,b,c Mean values with unlike letters were significantly different (P < 0·05; least significant difference method).

Fig. 3. Concentrations of SCFA in the colonic contents of gilts, as affected by dietary energy feeding levels and fibre levels (n=8). CON, control, daily intake of 2·4 kg basal diet; Fibre, CON diet plus 300 g/d inulin and cellulose at a ratio of 1:4; SO, CON plus 240 g/d SO; Fibre + SO, CON diet plus 300 g/d inulin and cellulose (1:4) and 240 g/d SO. a,b Mean values with unlike letters were significantly different (P < 0·05; least significant difference method).
decreased the concentrations of propionate in the colonic contents ($P < 0.05$, Fig. 3(B)). However, the propionate and butyrate concentrations in the colonic contents of gilts fed extra dietary fibre were significantly increased ($P < 0.05$, Fig. 3(C)).

**Serotonin synthesis and signalling were improved by high fibre intake**

As shown in Fig. 4(A), the concentrations of serotonin in the serum significantly increased after feeding ($P < 0.01$), peaked 2 h later and gradually decreased thereafter. The expression of mRNA encoding tryptophan hydroxylase 1, the rate-limiting enzyme controlling the biosynthesis of serotonin, was significantly up-regulated in colonic tissues of pigs fed the fibre diet (Fig. 4(B)). The fibre diet significantly increased the concentrations of serotonin in the serum (Fig. 4(A)), colonic mucosa (Fig. 4(C)), ovarian tissue (Fig. 4(D)) and follicular fluid (Fig. 4(E), $P < 0.01$). The SO treatment did not affect the serotonin concentrations in the colonic mucosa, follicular fluid and serum, with the exception of a tendency towards decrease observed in ovarian tissues.
Dietary fibre protects porcine follicle loss

Table 1. Relative abundances of twenty-three genera in faeces (>0.2% in at least one sample) with significant effects by dietary treatment* (Mean values with their standard errors; n 8 for each treatment)

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>Fibre</th>
<th>SO</th>
<th>Fibre + SO</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
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<tr>
<td>Streptococcus</td>
<td>6.62 ± 0.46</td>
<td>6.77 ± 0.52</td>
<td>8.86 ± 0.90</td>
<td>5.08 ± 0.35</td>
<td>0.982 ± 0.002</td>
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<tr>
<td>Treponema_2</td>
<td>6.01 ± 0.63</td>
<td>4.91 ± 0.74</td>
<td>3.01 ± 0.49</td>
<td>5.34 ± 0.55</td>
<td>0.023 ± 0.155</td>
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<tr>
<td>Prevotella_9</td>
<td>4.02 ± 0.61</td>
<td>5.17 ± 0.82</td>
<td>2.79 ± 0.32</td>
<td>3.11 ± 0.42</td>
<td>0.003 ± 0.194</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>3.09 ± 0.65</td>
<td>2.18 ± 0.16</td>
<td>2.57 ± 0.50</td>
<td>3.55 ± 0.75</td>
<td>0.386 ± 0.980</td>
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<td>Rikenellaceae_RC9_gut_group</td>
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<td>3.96 ± 0.33</td>
<td>3.29 ± 0.30</td>
<td>4.66 ± 0.24</td>
<td>0.885 ± 0.014</td>
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<td>Clostridium_sensu_stricto_1</td>
<td>3.39 ± 0.14</td>
<td>2.85 ± 0.29</td>
<td>3.67 ± 0.27</td>
<td>2.57 ± 0.10</td>
<td>0.838 ± &lt;0.001</td>
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<td>Eubacterium_coprostanoligenes_group</td>
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<td>1.80 ± 0.06</td>
<td>2.53 ± 0.27</td>
<td>1.84 ± 0.14</td>
<td>0.012 ± 0.072</td>
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<td>Oscillibacter</td>
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<td>1.96 ± 0.12</td>
<td>1.49 ± 0.12</td>
<td>1.77 ± 0.16</td>
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<td>Aloiroplovella</td>
<td>1.06 ± 0.09</td>
<td>1.21 ± 0.13</td>
<td>0.89 ± 0.13</td>
<td>1.41 ± 0.12</td>
<td>0.903 ± 0.007</td>
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<tr>
<td>Escherichia-Shigella</td>
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<td>0.41 ± 0.10</td>
<td>0.47 ± 0.20</td>
<td>0.29 ± 0.02</td>
<td>0.734 ± 0.722</td>
</tr>
<tr>
<td>Prevotella_7</td>
<td>0.64 ± 0.11</td>
<td>0.67 ± 0.12</td>
<td>0.40 ± 0.07</td>
<td>0.49 ± 0.08</td>
<td>0.030 ± 0.469</td>
</tr>
<tr>
<td>p-1089-as_gut_group</td>
<td>0.16 ± 0.02</td>
<td>0.15 ± 0.03</td>
<td>0.39 ± 0.15</td>
<td>0.14 ± 0.02</td>
<td>0.089 ± 0.017</td>
</tr>
<tr>
<td>Leaia</td>
<td>0.16 ± 0.02</td>
<td>0.15 ± 0.03</td>
<td>0.39 ± 0.15</td>
<td>0.14 ± 0.02</td>
<td>0.089 ± 0.017</td>
</tr>
<tr>
<td>Acidaminococcaceae</td>
<td>0.08 ± 0.01</td>
<td>0.15 ± 0.03</td>
<td>0.24 ± 0.14</td>
<td>0.07 ± 0.01</td>
<td>0.533 ± 0.209</td>
</tr>
<tr>
<td>Terrisporobacter</td>
<td>0.73 ± 0.08</td>
<td>0.56 ± 0.04</td>
<td>0.60 ± 0.05</td>
<td>0.63 ± 0.08</td>
<td>0.656 ± 0.265</td>
</tr>
<tr>
<td>Sphaerochaeta</td>
<td>0.51 ± 0.05</td>
<td>0.76 ± 0.10</td>
<td>0.32 ± 0.04</td>
<td>0.38 ± 0.04</td>
<td>&lt;0.001 ± 0.014</td>
</tr>
<tr>
<td>Anaerovibrio</td>
<td>0.53 ± 0.07</td>
<td>0.57 ± 0.08</td>
<td>0.42 ± 0.05</td>
<td>0.40 ± 0.05</td>
<td>0.025 ± 0.924</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>0.05 ± 0.01</td>
<td>0.20 ± 0.03</td>
<td>0.06 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>0.372 ± &lt;0.001</td>
</tr>
<tr>
<td>Mitsuokella</td>
<td>0.17 ± 0.05</td>
<td>0.20 ± 0.03</td>
<td>0.27 ± 0.09</td>
<td>0.14 ± 0.04</td>
<td>0.721 ± 0.279</td>
</tr>
<tr>
<td>Fibrobacter</td>
<td>0.12 ± 0.01</td>
<td>0.16 ± 0.03</td>
<td>0.06 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>0.001 ± &lt;0.001</td>
</tr>
<tr>
<td>Cellulosilyticum</td>
<td>0.13 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.18 ± 0.02</td>
<td>0.807 ± 0.001</td>
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<tr>
<td>Ruminoclostridium_6</td>
<td>0.18 ± 0.01</td>
<td>0.19 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.012 ± 0.266</td>
</tr>
<tr>
<td>Faecalibacterium</td>
<td>0.08 ± 0.02</td>
<td>0.08 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.174 ± 0.082</td>
</tr>
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</table>

CON, control, daily intake of 2.4 kg basal diet; Fibre, CON diet plus 300 g/d inulin and cellulose at a ratio of 1:4; SO, CON plus 240 g/d SO; Fibre + SO, CON diet plus 300 g/d inulin and cellulose (1:4) and 240 g/d SO.

Mean values within a row with unlike superscript letters are significantly different between diet groups (P < 0.05).

* At least one sample with a relative abundance greater than 0.2% was analysed at the genus level.

Fig. 5. Melatonin concentrations in the serum and follicular fluid, as affected by dietary energy levels and fibre levels (n=8). (A) Serum melatonin concentration at slaughter. (B) Follicular fluid melatonin concentration at slaughter. (C) Correlation analysis between serum serotonin and melatonin concentrations, where y denotes serum melatonin and x denotes serum serotonin. CON, control, daily intake of 2.4 kg basal diet; Fibre, CON diet plus 300 g/d inulin and cellulose at a ratio of 1:4; SO, CON plus 240 g/d SO; Fibre + SO, CON diet plus 300 g/d inulin and cellulose (1:4) and 240 g/d SO. Mean values with unlike letters were significantly different (P < 0.05; least significant difference method).

(Melatonin concentrations were increased by extra dietary fibre intake)

Synthesis of melatonin requires serotonin as a precursor. The melatonin concentrations in the serum on the day of slaughter and in the follicular fluid were significantly elevated by fibre treatment (Fig. 5(A) and (B)). Correlation analysis revealed that the serum melatonin and serotonin concentrations were positively associated (y = 0.018x + 5.3369, R² = 0.6056, Fig. 5(C)).
Discussion

Follicular development involves maintenance, recruitment, maturation and ovulation\(^{44,45}\). However, the number of ovulated mature oocytes represents only a small population within each ovary, and most follicles undergo follicular atresia, which leads to the death of oocytes and probably to early menopause in women. Many researchers have reported that high-fat diets accelerate the activation of primordial follicles, increase follicular atresia and adversely affect oocyte quality and embryo survival, thus potentially decreasing pregnancy rates and shortening the reproductive span\(^{46-47}\). In the present study, the SO diet led to a smaller primordial follicle reserve and increased antral follicular atresia in the ovaries of gilts. Interestingly, feeding of extra dietary fibre prevented these adverse effects. The metabolism of dietary fibre occurs in the gastrointestinal tract and requires the gut microbiota; therefore, the metabolites released from the gut microbiota represent key signals linking host–microbe mutualism. To date, there is a paucity of data elucidating the mechanisms contributing to the gut–ovary axis in the literature.

In the present study, we aimed to establish a link between the gastrointestinal tract and ovarian follicular atresia and presumed that this link might mediate the effects of microbial metabolism of dietary fibre on host reproductive physiology. We found that the effects of dietary fibre on ovarian development were associated with gut microbial regulation of serotonin and melatonin synthesis.

Alterations in gut microbial diversity are considered the primary response to dietary fibre intake\(^{20}\). In the adult gastrointestinal tract, the number of bacterial cells is several times greater than the number of human cells in the body\(^{46,49}\). Because the microbiota can be considered the largest ‘organ’ in humans, many recent studies have reported that micro-organisms have important effects on the development of host tissues\(^{50}\) and physiological processes including metabolism, immunity, inflammation and disease\(^{20,53-54}\). In the present study, we found that the Shannon index significantly decreased with intake of a diet high in SO. However, the intake of a diet high in dietary fibre prevented this decrease in the Shannon index. This finding is similar to the results of a previous study reporting a significant decrease in the Shannon index in mice fed a high-fat diet, which was restored after the addition of dietary bamboo fibre\(^{55}\). We analysed the relative abundance of the microbiota at the genus level and found that high SO intake decreased the relative abundance of the SCFA-producing genera Prevotella\(_9\), Prevotella\(_7\), Anaerovibrio\(^{57}\) and Fibrobacter and increased the relative abundance of Eubacterium coprostanoligenes\(_{group}\), which has been reported to be highly positively correlated with total TAG and negatively correlated with acetic acid. Consumption of additional dietary fibre increased the relative abundance of the SCFA-producing genera Rikenellaceae\(_{RC9\_gut\_group}\), Alloprevotella, Bifidobacterium\(^{58}\), Fibrobacter and Cellulosilyticum and decreased the relative abundance of Streptococcus and Clostridium sensu\(_{stricto\_1}\). In addition, Clostridium sensu\(_{stricto\_1}\) has been reported to metabolise tryptophan, thus producing skatole\(^{59}\). The microbiota produce metabolites through the decomposition of substrates, thus directly or indirectly regulating physiological processes\(^{60}\). SCFA are the major metabolites formed through the fermentation of dietary fibre in the hindgut\(^{61}\); therefore, high-energy and low-dietary fibre diets generally decrease the concentrations of SCFA in the hindgut\(^{62}\). Our study found similar results, in which a high-SO and low-dietary fibre diet decreased the concentration of propionate in the colonic contents. Most of the SCFA produced by gut fermentation of dietary fibre are absorbed by the intestine and can be taken up by peripheral tissues, where they exert distinct functions\(^{63}\).

The concentrations of SCFA that we detected in the ovarian follicular fluid were very low and seemed to be below the threshold required to activate the downstream signalling G-protein-coupled receptor\(^{20}\). Thus, secondary signalling induced by SCFA or other microbial metabolites was considered. Most microbial metabolites, particularly SCFA, have been observed to stimulate a common metabolic signal serotonin in the enterochromaffin cells\(^{23}\), and in turn, gut serotonin affects gastrointestinal motility and haemostasis. Interestingly, gut serotonin accounts for approximately 95 % of circulating serotonin and plays an important role in peripheral tissue metabolism\(^{21}\). SCFA have been observed to act as signalling molecules that are sensed by enterochromaffin cells and promote tryptophan hydroxylase 1 transcription, a rate-limiting enzyme controlling serotonin synthesis and production\(^{25,64}\). In the present study, high-dietary fibre diets significantly increased the gene expression of tryptophan hydroxylase 1 in the colon, as well as the concentrations of serotonin in the colon mucosa, serum, ovarian tissue and follicular fluids, thus indicating that gut-derived serotonin can be considered a metabolic signal regulated by dietary fibre.

Most follicles undergo atresia at the early antral stage\(^{65}\). Follicular atresia is mediated by granulosa apoptosis, followed by apoptosis of the theca cells\(^{66,67}\). The mechanism of apoptosis signal transduction is complex but might be associated with three major signalling pathways\(^{68}\). The first is high levels of cAMP, induced by growth factors via granzyme B; the second affects mitochondrial function via Bcl2 family member activation and the third uses TNFa, Fas ligand (FasL)-Fas and other death receptors; all three pathways result in caspase-induced DNA fragmentation\(^{68}\). Mitochondrial apoptosis, the primary pathway in programmed cell death, participates in most regulatory processes in apoptosis. The apoptosis signal stimulates the release of cytochrome c from mitochondria to the cytoplasm. Cytochrome c binds and activates Apaf-1, which in turn activates caspase-9, thus resulting in activation of caspase-3, an enzyme that cleaves key substrates during the apoptotic process\(^{69,70}\). The Bcl-2 gene family has been shown to be the primary regulator of the mitochondrial apoptosis pathway. Some members such as Bcl-2 and Bcl-XL inhibit apoptosis, whereas others such as Bax, Bad and Bak accelerate apoptosis by altering mitochondrial membrane permeability, thereby inducing cytochrome c release\(^{71}\). Caspase-3, one of the most important members of the cysteine protease family, is a key effector of apoptosis and the ultimate enforcer of apoptotic death. In the present study, the mRNA and protein expression of Bax and caspase-3 was up-regulated in the ovaries of gilts fed a high-fat and low-dietary fibre diet. However, additional dietary fibre consumption resulted in greater mRNA and...
protein expression of Bcl-2. Serotonin receptor signalling may be involved in regulating the ovarian granulosa via the Bcl-2 gene family. Serotonin binding to the 5-HT2B-receptor activates ERK kinases, which inhibit Bax expression induced by serum deprivation and prevent cytochrome c release and caspase-9 and -3 activation via cross-talk between phosphatidylinositol-3 kinase/Akt and ERK1/2 signalling pathways. In addition, recent studies have found that serotonin induces pulmonary artery smooth muscle cell proliferation and inhibits apoptosis in these cells in a dose-dependent manner, thereby protecting placental cells against apoptosis and promoting uterine leiomyoma cell survival. In the present study, high dietary fibre intake significantly increased the concentrations of serotonin in the follicular fluid and ovarian tissues and induced up-regulation of serotonin receptor transcripts for 5-HT1D, 5-HT2B and 5-HT7 in ovarian tissue. These effects may be associated with decreased apoptosis of the granulosa cells caused by high SO intake. However, few studies have reported the effects of serotonin on the development of mammalian ovaries. Moreover, serotonin can further be converted to melatonin by the action of the rate-limiting enzyme arylalkylamine-N-acetyltransferase. Butyrate has been found to stimulate the mRNA expression of arylalkylamine-N-acetyltransferase and melatonin synthesis in an intestinal cell line. In line with this evidence, the melatonin concentrations in the serum and follicular fluid were enhanced by dietary fibre consumption in the present study. Melatonin is a powerful antioxidant that exerts broad beneficial effects on ovarian follicle survival and oocyte maturation. According to our findings and those from other studies, gut-derived serotonin and melatonin may be at least partly responsible for the beneficial effects of dietary fibre on ovarian follicle survival.

Conclusion

High dietary fibre consumption can counteract the excessive antral follicular atresia induced by high SO intake, and the beneficial effects of dietary fibre can be ascribed, at least partly, to the gut-microbiota increases in serotonin and melatonin. Global consumption of lipids per capita is rising, and rates of infertility are becoming a serious health problem. According to the results of the present study, intake of sufficient dietary fibre can be considered an important nutritional solution to prevent reproductive disorder under a high-energy diet.

Study limitations

Although the current findings revealed that serotonin and melatonin are dietary fibre-regulated metabolites with antioxidant properties, we cannot rule out the possibility that other metabolites or functional signals might also confer the effects of dietary fibre on follicle loss. Second, despite the similarities between humans and pigs in the digestive process, follicle number and size, and folliculogenesis, the effects of dietary fibre on follicular atresia in humans still require further investigation. Finally, only inulin and cellulose were used as dietary fibre in the present study, but many different types of dietary fibre exist and might act differently on ovarian follicle development. Thus, the optimal composition of different dietary fibre types is uncertain.

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The authors declare that there are no conflicts of interest.

Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0007114520002378

References


