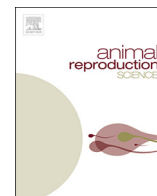




Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



## Supplemental flaxseed modulates ovarian functions of weanling gilts via the action of selected fatty acids

Radoslava Vlčková<sup>a</sup>, Zuzana Andrejčáková<sup>a,\*</sup>, Drahomíra Sopková<sup>a</sup>,  
Zdenka Hertelyová<sup>b</sup>, Katarzyna Koziol<sup>c</sup>, Marek Koziorowski<sup>c</sup>, Soňa Gancarčíková<sup>d</sup>

<sup>a</sup> Institute of Physiology, Department of Anatomy, Histology and Physiology, University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81, Slovak Republic

<sup>b</sup> Department of Experimental Medicine, Faculty of Medicine, Pavol Jozef Šafarik University, Šrobárova 2, 041 80, Košice, Slovak Republic

<sup>c</sup> Department of Physiology and Reproduction of Animals, Faculty of Biotechnology, University of Rzeszow, ul. Pigońia 1, 35-310 Rzeszów, Poland

<sup>d</sup> Institute of Microbiology and Gnotobiology, University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81, Slovak Republic



### ARTICLE INFO

#### Keywords:

Apoptosis  
Fatty acids  
Flaxseed  
Insulin-like growth factor I (IGF-I)  
Pig  
Proliferation

### ABSTRACT

The aim of this study was to examine the influence of dietary flaxseed on the endocrine and ovarian functions of weanling gilts challenged with *E. coli* and *Coronavirus* infections treated with dietary probiotic cheeses and to understand the possible mechanisms of its effects on ovarian function. Probiotics were used as a natural substitution for antibiotics and 10% dietary flaxseed is an effective prebiotic which supports the action of probiotics and has other beneficial effects on the organism. Probiotics with or without flaxseed were fed to weanling gilts starting 10 days before and lasting up until 14 days after weaning. The ovaries were measured and histologically analysed. The blood samples for the levels of steroid hormones and insulin-like growth factor I (IGF-I) were assessed using immunoassays and the levels of fatty acids were assessed using gas chromatography. All samples were collected on the day of weaning and 14 days after weaning. On the day of weaning, increased levels of linoleic acid and IGF-I was associated with higher body weight. The steroid hormones were not affected by the diet. The conversion of alpha-linolenic acid (ALA) to timodonic (EPA) and cervonic (DHA) acids were lower compared to controls, and together with high levels of myristic, palmitic and palmitoleic acids was associated with the higher proliferation and lower apoptosis in the primordial, primary and secondary follicles; although the inhibition of the cell cycle was observed in relation to the low level of eicosadienoic acid. The high levels of ALA, EPA and DHA and the low levels of myristic, palmitic and palmitoleic acids may have been the effect of flaxseed feeding 14 days post-weaning and may have had a reverse effect on the proliferation and apoptosis of ovarian follicles. These data suggest that flaxseed may suppress the follicle development in weanlings via the stimulation of apoptosis and the inhibition of proliferation via the modulation of the metabolism of selected fatty acids.

### 1. Introduction

In the life of piglets, weaning is a highly sensitive period related to the stressful withdrawal from the mother's milk, which results

\* Corresponding author.

E-mail addresses: [radoslava.vlckova@uvlf.sk](mailto:radoslava.vlckova@uvlf.sk) (R. Vlčková), [zuzana.andrejckakova@uvlf.sk](mailto:zuzana.andrejckakova@uvlf.sk) (Z. Andrejčáková), [drahomira.sopkova@uvlf.sk](mailto:drahomira.sopkova@uvlf.sk) (D. Sopková), [zdenka.hertelyova@upjs.sk](mailto:zdenka.hertelyova@upjs.sk) (Z. Hertelyová), [katko@ur.edu.pl](mailto:katko@ur.edu.pl) (K. Koziol), [mkozioro@ur.edu.pl](mailto:mkozioro@ur.edu.pl) (M. Koziorowski), [sona.gancarcikova@uvlf.sk](mailto:sona.gancarcikova@uvlf.sk) (S. Gancarčíková).

<https://doi.org/10.1016/j.anireprosci.2018.04.066>

Received 26 January 2018; Received in revised form 5 April 2018; Accepted 13 April 2018

Available online 18 April 2018

0378-4320/ © 2018 Elsevier B.V. All rights reserved.

in reduced immunity and a high prevalence of digestive infectious diseases. Such diseases are most commonly treated by use of the surface application of commercial antibiotics. Currently, natural substitutes of antibiotics, such as probiotics are preferred to be used for: the elimination of pathogenic/potentially pathogenic microorganisms in the gut (Nemcová et al., 2012; Borovská et al., 2013); the recovery of intestinal microflora, immunostimulation (Chytilová et al., 2013; Kubašová and Mudroňová, 2014); and the promotion of overall human and animal health without negative side effects. The effect of probiotics, for improved effectiveness, may be potentiated by special natural substrates used as prebiotics (Nemcová et al., 2010). Suitable prebiotic substrates are, for example polyunsaturated fatty acids (PUFAs) which are richly represented in flaxseed (Rodríguez-Leyva et al., 2010; Nemcová et al., 2012; Andrejčáková et al., 2016; Sopková et al., 2017). Polyunsaturated fatty acids, such as linoleic (LA, C18:2 n-6) and  $\alpha$ -linolenic (ALA, C18:3 n-3) acids are essential for humans and other animals. Since they are unable to be synthesised in these organisms, essential PUFAs have to be ingested from plants, fish oil, or algae (Tvrzická et al., 2009; Grofová, 2010; Žák et al., 2011). Flaxseed is the richest plant source of n-3 PUFA ALA which, together with its derivatives – eicosapentaenoic (EPA, C20:5 n-3) and docosahexaenoic (DHA, C22:6 n-3) acids are unique for their mostly pronounced cardioprotective (Rodríguez-Leyva et al., 2010), hypocholesterolemic (Sopková et al., 2017), hepatoprotective (Andrejčáková et al., 2016), anti-inflammatory (Liu et al., 2003) and neuroprotective (Contreras, 2012) effects on the organisms. They act via improved production of prostaglandins (PG) of series 3 suppressing the inflammation process (Rahbar et al., 2014). The positive effects of n-3 PUFAs have been substantiated by many researchers on the growth and adherence of lactobacilli in the gut (Nemcová et al., 2012; Borovská et al., 2013; Yu et al., 2014).

The PUFAs may affect the number of medium-sized follicles, as well as the conception and pregnancy rates in cows (Mattos et al., 2000; Petit et al., 2001). The hulls of flax seed are a rich source of lignan precursors (the most secoisolariciresinol diglycoside, SDG; Tourre and Xueming, 2010; Singh et al., 2011), which are converted by the gut bacteria into enterodioid and enterolacton having a similar chemical structure as an oestradiol-17 $\beta$ , thus acting like phytoestrogens. Phytoestrogens can: bind to oestradiol receptors and act as an agonists or antagonists (Saggar et al., 2010; Michel et al., 2013), activate the insulin-like growth factor I (IGF-I) receptors (Bourque et al., 2012), modulate cell cycle and apoptosis influencing the reproduction (Kádasi et al., 2015), and promote the health of humans (Ming et al., 2013; Yanagihara et al., 2014). Owing to their pro-apoptotic and anti-proliferative properties (Rietjens et al., 2013; Kádasi et al., 2015), lignans of flaxseed have been studied as potentially useful agents in the hormone replacement therapy for decreasing menopausal symptoms (Hajirahimkhan et al., 2013) and for the treatment of breast, endometrial and ovarian cancer in women (Riediger et al., 2009). Moreover, flaxseed may directly modulate the release of progesterone and testosterone by prepubertal porcine ovarian granulosa cells (Kádasi et al., 2015).

Although the possible mechanism of action of flaxseed on folliculogenesis and endocrine functions have been attributed to lignan phytoestrogens, there are few studies reporting that phytoestrogens can stimulate the conversion of n-3 PUFAs (Böttner et al., 2013). Thus, there is evidence that the flaxseed effects could be mediated via the modulation of fatty acid metabolism or that they could relate to one another in some way; hence, this hypothesis requires further elucidation. Therefore, the aim of our *in vivo* experiment was to examine the influence of dietary flaxseed on the endocrine and ovarian functions of weanling gilts challenged with *E. coli* and *Coronavirus* infections treated with dietary probiotic cheeses (*L. plantarum* and *L. fermentum* strains) and also to understand the possible mechanisms of its effects to determine whether:

1/ Dietary supplementation of flaxseed can affect live body weight, macromorphometric (weight and volume) and micrometric (folliculogenesis) indexes of ovaries of these weanling gilts; 2 / this influence could be due to changes in ovarian cell proliferation [PCNA, marker of G1/S-phase of the cell cycle (Ulrich and Takahashi, 2013), and cyclin B1, marker of G2/M-phase of the cell cycle (Miyazaki and Arai, 2007)] and cytoplasmic/mitochondrial apoptosis (bax, caspase 3); and 3 / the effect of dietary supplementation of flaxseed on the prepubertal pig ovary can be mediated by changes in the blood levels of reproductive hormones (progesterone, testosterone, oestradiol-17 $\beta$ , and IGF-I) or fatty acids (saturated, monounsaturated and polyunsaturated).

## 2. Materials and methods

### 2.1. Animals, housing, and diets

The experiments on prepubertal gilts (weanlings) were performed at the Institute of Microbiology and Gnotobiology (IMG), University of Veterinary Medicine and Pharmacy (UVMP) in Košice, Slovak Republic. The investigation was approved by the State Veterinary and Food Administration of the Slovak Republic (Approval No. 2519/10–221) and the animals were handled in accordance with the guidelines established by the relevant commission. The experiments were carried out on 24 piglets at the age of 28 days using Slovak white  $\times$  Landrace cross-breed (KOAN s.r.o., Krásnovce, Slovak Republic) divided into two groups after being transported to the experimental housing at the IMG, UVMP in Košice, where the dietary treatments were initiated. Animals were housed in mobile stainless steel cages fitted with a slatted floor strewn with  $\frac{3}{4}$  insulating rubber. The ambient temperature was 20–26 °C and the relative humidity was 50–70%. Piglets were selected from 12 litters with the application of probiotic cheeses as follows: from each litter 2 female siblings were randomly selected so that one treatment group included 6 piglets from 2 litters for each sampling day (Day 10, n = 6; Day 24, n = 6).

Before the experiments a diagnostic analysis was carried out on the herd of the farmer. The samples of biological material were collected for laboratory sampling (bacteriological examination of rectal swabs, virological and parasitological examination of faeces). These findings showed the presence of enterotoxigenic *E. coli* and *Coronavirus*. We decided to use probiotics instead of antibiotics due to the reduced negative side effects of antibiotics. Pigs were treated with probiotics 10 days before weaning (suckling period) and it lasted up until 14 days post-weaning to ensure their gut health. During the 14-day post-weaning period, all piglets were fed diets mixed for the early weaning of piglets (OŠ-02; Spišské Vlachy, Slovak Republic; Table 1) and had *ad libitum* access to water. The

**Table 1**  
Ingredients (%) and chemical composition (g/kg dry matter) of the basal diet for early weaned pigs OŠ-02.

Ingredient	OŠ - 02	Chemical composition	OŠ - 02	Chemical composition	OŠ - 02
Wheat	27.6	CP (g)	187.9	Vitamin B12 (µg)	26.4
Extracted soybean meal	22.0	ME (MJ)	12.8	Ca (g)	07.5
Maize (8.4% CP)	19.7	Fibre (g)	38.3	P (g)	06.2
Barley	17.0	Lysine (g)	11.6	Na (g)	01.9
Oat (11.2% CP)	5.0	Methionine and Cysteine (g)	06.4	Cu (mg)	10
Calcium carbonate	01.1	Threonine (g)	07.6	Fe (mg)	163.4
Monocalcium phosphate	1.0	Tryptophan (g)	02.3	Zn (mg)	125.8
TKP OS – M var.B 0.5% Vitamin/Mineral premix	0.4	Choline (mg)	1352	Mn (mg)	72.7
Sodium chloride	0.4	Vitamin A (IU)	11530		
L-Lysine-HCl	0.35	Vitamin D3 (IU)	1500		
L-hreonine	0.35	Vitamin E (mg)	68.7		
DL-Methionine	0.1	Vitamin B2 (mg)	07.1		

piglets were fed in groups of 6 and the daily feed intake of the standard feed (OŠ-02) was 300 g per piglet per day divided into 2 doses (150 g at 8:00 a.m. and 150 g at 3:00 p.m.). The diet of the control group of animals (C; n = 18) then consisted of: 10 days before weaning – mother's milk + probiotic cheeses and 14 days after weaning – OŠ-02 + probiotic cheeses. The diet of the experimental group (F; n = 18) consisted of: 10 days before weaning – mother's milk + probiotic cheeses + 10% crushed flax seed (cultivar Flanders, Agritec, Czech Republic) and 14 days after weaning – OŠ-02 + probiotic cheeses + 10% crushed flax seed. The piglets of both groups were supplied probiotic cheeses at a dose of 4 g/animal/day for each cheese. The fatty acid (FA) composition (in percentage) of flaxseed was as follows: lipids (dry matter basis) – 45.78; palmitic FA (C16:0) – 5.1; stearic FA (C18:0) – 3.7; oleic FA (C18:1) – 18.4; linoleic FA (C18:2) – 16.1; and linolenic FA (C18:3) – 56.8.

## 2.2. Probiotic bacteria and Cheddar cheese preparation

The *Lactobacillus* probiotic strains were isolated in the laboratory of the IMG, UVMP in Košice. The *Lactobacillus plantarum* – Bioceno<sup>TM</sup> LP96 (CCM 7512) strain was isolated from the gut contents of healthy suckling piglets (Nemcová et al., 1997). The *Lactobacillus fermentum* – Bioceno<sup>TM</sup> LF99 (CCM 7514) was isolated from the gastrointestinal tract of adult chickens (Nemcová et al., 2003).

Cheddar cheese (basal nutritional composition /1 kg/: proteins 23.8%, sugars 2.8%, lipids 30.1%, metabolisable energy 1.62 MJ) was used as a vehicle for the administration of the probiotic strains. The cheeses containing probiotic strains (each cheese contained one strain) at  $1 \times 10^9$  CFU/g of cheese, were referred to as the probiotic cheeses. The probiotic bacteria were added to the cheese milk together with 2% starter culture (*Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*) during typical Cheddar cheese production.

## 2.3. Blood sampling, necropsy and morphometric analysis

Blood samples from all female piglets were taken from the *plexus venosus suborbitalis* on Day 10 (day of weaning at the age of 28 days; n = 6 for each group) and 24 (14 days after weaning; n = 6 for each group) of supplementation. The blood serum was separated by centrifugation at 4 °C and  $958 \times g$  for 10 min and stored at –70 °C to await the immunoassays and gas chromatography. On sampling days, the piglets were humanely euthanized using T61 a.u.v. (*ad usum veterinarium*; embutramidum 200 mg/ml, mebezoniiodidum 50 mg/ml, tetracainihydrochloridum 5 mg/ml; Intervet International BV, Boxmeer, Netherlands) intracardiac administration of 1 ml/kg to collect their ovaries. The ovaries were carefully pulled into an abdominal incision and cut for other processing. Ovaries were weighed with a sensitivity of 0.01 g, measured for volume calculation using a manual caliper and then fixed in 4% paraformaldehyde for 24 h, dehydrated, embedded in paraffin and cut into 5 µm thick serial sections by use of Leica RM2255 microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany) for routine histology (haematoxylin-eosin staining, H-E) and immunohistochemistry.

## 2.4. Immunoassay

The concentrations of hormones were determined in 25–100 µl of blood serum using immunoassay according to the manufacturer's instructions in duplicate.

The progesterone (P4) concentration in the blood serum was assessed using RIA (Architect Progesterone, Abbott Ireland Diagnostics Division, Lisnamuck, Longford, Ireland). The analytical sensitivity (limit of detection) of progesterone was 0.1 ng/ml and the intra- and inter-assay coefficients of variation were  $\leq 7\%$  and  $\leq 10\%$ , respectively. The measurements were expressed in ng/ml.

The testosterone (T) concentration in the blood serum was measured using the chemiluminescent microparticle immunoassay (Architect Testosterone, Abbott Ireland Diagnostics Division, Lisnamuck, Longford, Ireland). The analytical sensitivity (limit of detection) of testosterone was 0.08 ng/ml and the intra- and inter-assay coefficients of variation were  $\leq 4.0\%$  and  $\leq 6.6\%$ , respectively. The measurements were expressed in ng/ml.

The oestradiol-17 $\beta$  (E2) concentration in the blood serum was assessed using the RIA (E2-RIA-CT Kit KIP0629; DIALsourceImmunoAssays S.A., Nivelles, Belgium). The analytical sensitivity was 2 pg/ml and the intra- and inter-assay coefficients of variation were  $\leq 5.9\%$  and  $\leq 10.1\%$ , respectively. The measurements were expressed in pg/ml.

The IGF-I concentration in the blood serum was assessed using an immunoradiometric assay (IRMA IGF-I A15729; Immunotech s.r.o., Prague, Czech Republic). The analytical sensitivity was 2 ng/ml and the intra- and inter-assay coefficients of variation were  $\leq 6.3\%$  and  $\leq 6.8\%$ , respectively. The measurements were expressed in ng/ml.

## 2.5. Histological analysis

Stained sections of the ovaries were embedded in canadian balsam and evaluated using the basic software for ProgRes® CapturePro ver. 2.8.0 WIN (Jenoptik, Jena, Germany) motion-picture camera and microscope a Nikon Eclipse E200 microscope (Japan). Every 20th section was evaluated ( $n = 6/\text{ovary}$ ) for the number and diameter of primordial (PF), primary (PRF), secondary follicles (SF) and the diameters of their oocytes and germinal vesicles (GV) expressed in  $\mu\text{m}$ . These diameters were calculated from two perpendicular measurements. Since there were no significant macrometric differences between the right and left ovaries, they were evaluated together. The number of follicles were recorded as per ovary section. Primordial follicles were identified as the follicles surrounded by a single layer of flattened follicular cells; primary follicles with a single or a double layer of cuboidal follicular cells (including growing primary follicles); and secondary follicles with a stratified layer of cuboidal cells surrounding the follicular epithelium. All follicles had an oocyte. Follicles without germinal vesicle were excluded from the selection process.

## 2.6. Immunohistochemical analysis (IHC)

The paraffin sections of ovaries were deparaffinized and rehydrated. For detection, antigen retrieval was performed by boiling the slides in 10-mM citrate buffer (pH 6.0) for 2 min. To block endogenous peroxidase activity, the slides were incubated in TBS (0.05 M Tris-HCl plus 0.15 M NaCl, pH 7.6) with 0.3% H<sub>2</sub>O<sub>2</sub> addition for 20 min. To block non-specific binding, the sections were incubated for one hour with 8% bovine serum albumin (BSA). In addition, monoclonal mouse anti-PCNA (dilution 1:250), anti-caspase-3 (dilution 1:100), anti-cyclin B1 (dilution 1:100), and anti-bax (dilution 1:100) antibodies (all from Santa Cruz Biotechnology Inc., Dallas, USA) were applied and incubated overnight at 4 °C. After rinsing with TBST (TBS containing 0.1% Tween20), the sections were incubated with goat anti-mouse secondary antibodies (Dako REAL™ EnVision™/HRP, Rabbit/Mouse (ENV), ready-to-use, Dako, Denmark) for 2 h. Following the incubation, the specimens were rinsed in TBST and then in TBS. A colour reaction was conducted with diaminobenzidine as a chromogen (Dako REAL™ DAB + Chromogen, Dako, Denmark). The stained sections were rinsed with distilled water, counterstained with haematoxylin for stereological assessment, dehydrated and immersed in DPX (Distyrene Plasticiser and Xylene; Buchs, Switzerland). To prepare a negative control for each sample, the primary antibody was omitted. Photographic documentation was obtained using an optical microscope (Olympus BX43, Olympus Corporation, Tokyo, Japan) coupled to a camera (Olympus UC30, Olympus Corporation, Tokyo, Japan) and computer.

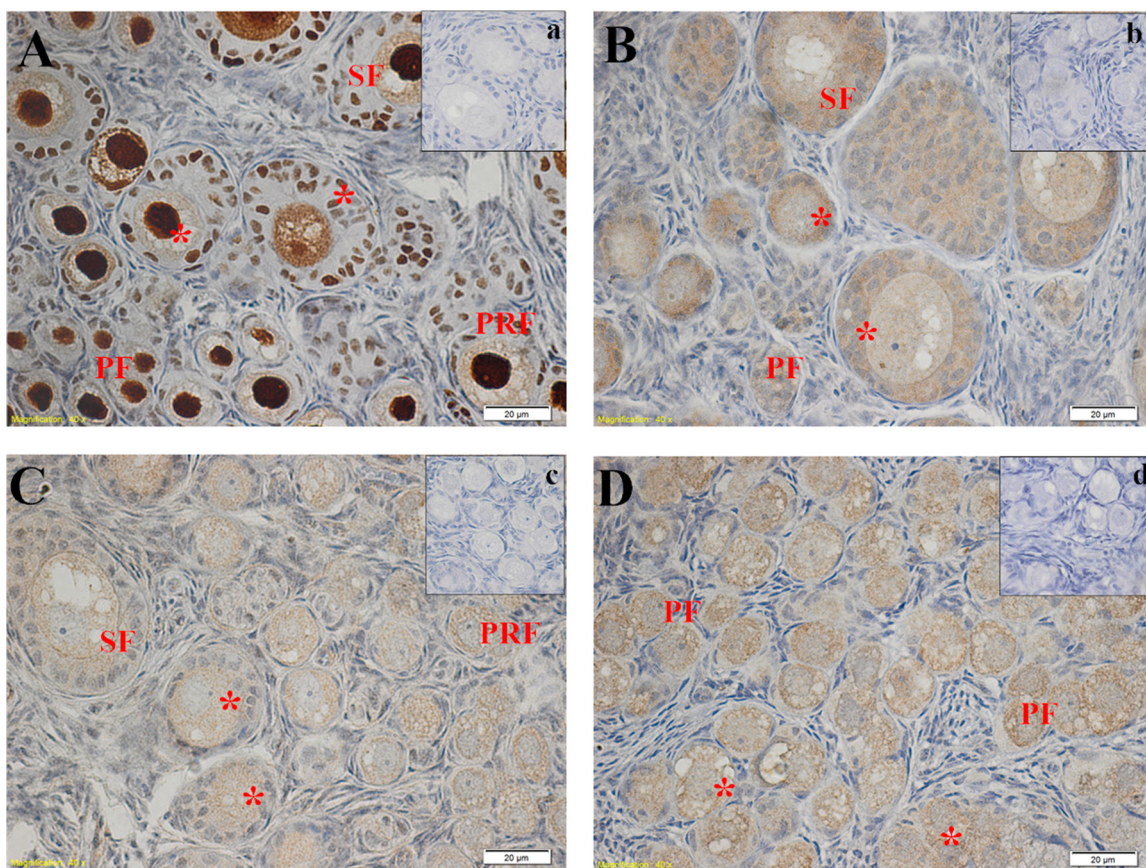
To evaluate the intensity of the immunohistochemical reaction quantitatively, approximately 6 images from sections of each examined animal ( $n = 6$  for each group) were analysed by using the public-domain ImageJ software (National Institutes of Health, Bethesda, MD, USA). The outlines of all cells which demonstrated an immunopositive signal in the ovaries were marked manually and then the grey level (GL) of the marked areas were measured. The intensity of the IHC reaction was expressed as the relative optical density (ROD) of the diaminobenzidine brown reaction products and was calculated using the formula described by Smolen (1990), where GL is the grey level of the stained area (specimen) and unstained area (background) and blank is the GL measured after the slide was removed from the light path.

$$ROD = \frac{OD_{\text{specimen}}}{OD_{\text{background}}} = \frac{\log\left(\frac{GL_{\text{blank}}}{GL_{\text{specimen}}}\right)}{\log\left(\frac{GL_{\text{blank}}}{GL_{\text{background}}}\right)}$$

The diaminobenzidine brown reaction was a sign of a positive reaction in all evaluated parameters and in the comparison with the “negative” control reaction. Specific immunostaining was determined by the comparison with the control reaction (incorporated photos in Fig. 1). All slides were counterstained with hematoxylin to visualise different structures. Histological cuts revealed the presence of PFs, PRFs and SFs. The marker PCNA was concentrated in the nucleus of an oocyte and in the layer of the follicular cells. Therefore, the results of ROD were evaluated separately in the oocytes and follicular cells of all follicle types. The intensity of the colour reaction of the cyclin B1 was largely concentrated in the layer of the follicular cells.

## 2.7. Gas chromatography

The serum fatty acid extraction and gas chromatography fatty acid extraction were done according to the Folch et al., 1957. Extracted lipids were transesterified to fatty acid methyl esters (FAME) with sodium methanolate. The profile of fatty acid methyl esters was established by the use of a gas chromatograph (Shimadzu GC 17, Shimadzu, Japan) with a flame ionization detector. The measurements were expressed as mol%.



**Fig. 1.** Immunohistochemical detection of proliferation (A/PCNA and B/cyclin B1) and apoptotic (C/caspase 3 and D/bax) markers in ovaries of gilts fed and not fed with flaxseed. PCNA, Proliferating cell nuclear antigen; PF, primordial follicle; PRF, primary follicle; SF, secondary follicle; \*, positive reaction in the oocyte and layer of follicular cells; scale bar 25  $\mu$ m; magnification 400 $\times$ ; a, b, c, d are the images of negative reactions (without primary antibody).

### 2.8. Statistical analysis

The weight of the piglets and ovaries, ovary volume, follicular parameters, ROD values as well as the concentrations of hormones and fatty acids were expressed as the mean  $\pm$  SEM. The significant differences were compared using an unpaired t-test (GraphPad Prism 5.0 for Windows, GraphPad Software, San Diego, CA, USA). The differences from the controls or between the sampling days at  $P < 0.05$ ;  $P < 0.01$ ;  $P < 0.001$  were considered significant and marked with superscript letters.

## 3. Results

The live body weight of piglets, volumes and weights of ovaries, mean numbers and diameters of ovarian follicles, their oocytes and germinal vesicles after 10- and 24-day (D) period of feeding and not feeding with flaxseed are shown in Table 2. Flaxseed affected the weight of the piglets positively on both days ( $P < 0.05$ ) of the supplementation when compared to the control animals. The weight ( $P < 0.05$ ) and volume ( $P < 0.001$ ) of the ovaries were higher on D24 of the supplementation compared to the controls, but they were not influenced by flaxseed supplemented milk diet (a shorter supplementation period). The flaxseed increased body weight, weight and volume of the ovaries on D24 of supplementation compared to D10 ( $P < 0.05$ ;  $P < 0.001$ ;  $P < 0.001$ , respectively).

The ovaries of the juvenile piglets looked smooth with no visible follicles on their surface, even though the ovaries on D24 of the supplementation were the largest of all. The primordial, PRF and SF were found in the ovarian cortex. Ten-day flaxseed supplementation (flaxseed supplemented milk diet) in the experimental group of animals led to an increase in the average diameters of PRFs ( $P < 0.05$ ) and SFs ( $P < 0.01$ ), although their oocytes and germinal vesicles were not affected significantly. On D24 of the supplementation, an increased ( $P < 0.001$ ) in size of the PFs and PRFs were recorded relative to the control group.

The feeding of the flaxseed modulated the proliferation and apoptosis in the ovaries of problematic gilts treated with probiotics. Fig. 1 shows the photographs of representative ovary sections with IHC localisation of markers of proliferation (PCNA, cyclin B1) and apoptosis (caspase 3, bax). The theca cells were not positively stained. The colour development of the antigen PCNA was more pronounced in the control group of piglets compared to the group with the addition of flaxseed. The proliferating cell nuclear antigen

**Table 2**

Effect of feeding with and without supplemental flaxseed on body weights, ovary weights and volumes and follicular development on the ovaries of probiotic-treated weanlings.

Treatment	None (control)		Flaxseed	
	10	24	10	24
Length of supplementation (days)				
<b>Parameter</b>				
Body weight (kg)	4.82 ± 0.80 <sup>a,x</sup>	6.88 ± 1.35 <sup>a,x</sup>	6.82 ± 1.40 <sup>a,x</sup>	8.81 ± 0.77 <sup>a,x</sup>
Ovary weight (g)	0.05 ± 0.00 <sup>c</sup>	0.05 ± 0.00 <sup>c,z</sup>	0.05 ± 0.00 <sup>c</sup>	0.07 ± 0.00 <sup>c,z</sup>
Ovary volume (cm <sup>3</sup> )	0.07 ± 0.00 <sup>b</sup>	0.08 ± 0.00 <sup>b,z</sup>	0.08 ± 0.00 <sup>c</sup>	0.14 ± 0.01 <sup>c,z</sup>
Primordial follicles	n = 72	n = 50	n = 84	n = 97
Mean number	16.10 ± 0.82	10.70 ± 0.84 <sup>y</sup>	14.98 ± 0.95	14.72 ± 0.91 <sup>y</sup>
Diameter (µm)	4.54 ± 0.00	4.26 ± 0.00 <sup>z</sup>	4.75 ± 0.00	4.86 ± 0.00 <sup>z</sup>
Oocyte (µm)	3.53 ± 0.00	3.16 ± 0.00 <sup>z</sup>	3.62 ± 0.00	3.63 ± 0.00 <sup>z</sup>
Germinal vesicle (µm)	1.39 ± 0.00	1.22 ± 0.00 <sup>z</sup>	1.58 ± 0.00 <sup>x</sup>	1.60 ± 0.00 <sup>z</sup>
Primary follicles	n = 69	n = 45	n = 69	n = 83
Mean number	7.82 ± 0.44 <sup>z</sup>	5.22 ± 0.41 <sup>x</sup>	4.51 ± 0.33 <sup>z</sup>	4.33 ± 0.25 <sup>x</sup>
Diameter (µm)	7.62 ± 0.00 <sup>x</sup>	7.44 ± 0.01 <sup>z</sup>	7.88 ± 0.00 <sup>x</sup>	0.80 ± 0.00 <sup>z</sup>
Oocyte (µm)	4.26 ± 0.00	4.16 ± 0.00	4.28 ± 0.00	4.33 ± 0.00
Germinal vesicle (µm)	1.65 ± 0.00	1.47 ± 0.00	1.57 ± 0.00	1.64 ± 0.00
Secondary follicles	n = 48	n = 11	n = 20	n = 38
Mean number	2.65 ± 0.25	2.00 ± 0.23	1.85 ± 0.27	2.82 ± 0.28
Diameter (µm)	10.92 ± 0.02 <sup>y</sup>	11.27 ± 0.04	11.7 ± 0.02 <sup>y</sup>	11.79 ± 0.02
Oocyte (µm)	4.81 ± 0.01	4.91 ± 0.03	4.65 ± 0.01	4.95 ± 0.00
Germinal vesicle (µm)	1.65 ± 0.00	1.73 ± 0.01	1.70 ± 0.01	1.84 ± 0.00

Values are means ± SEM; <sup>a,b,c,x,y,z</sup> Mean values in rows with the same superscript letters differ significantly (<sup>a,x</sup>*P* < 0.05; <sup>b,y</sup>*P* < 0.01; <sup>c,z</sup>*P* < 0.001). Superscript letters <sup>a,b,c</sup> show differences on Day 24 compared to Day 10 and <sup>x,y,z</sup> show differences between the groups.

was detected in both follicular cells and oocytes of all follicle types. The intensity of the IHC reaction (ROD) of the proliferation markers is summarised in Table 3. In the control ovaries (no flaxseed), the ROD of PCNA was higher on D24 in the follicular cells of all follicle types (*P* < 0.05), as well as in the oocytes of the PRFs (*P* < 0.001). Cyclin B1 was detected in the follicular cells of all follicle types. The ROD of cyclin B1 antigens was not changed significantly in the controls. The flaxseed decreased the ROD of the PCNA with the length of its supplementation from D10 to D24 in the follicular cells of PFs (*P* < 0.01) and their oocytes (*P* < 0.01), PRFs (*P* < 0.01) and SFs (*P* < 0.05). The ROD of cyclin B1 in the PRFs was affected negatively in a time-dependent manner (*P* < 0.001, D24 vs D10). When compared to the control animals, flaxseed supplementation increased the ROD of PCNA in all follicle types on D10 (*P* < 0.001, PFs; *P* < 0.05, PRFs; *P* < 0.01, SFs). The ROD of cyclin B1 in all follicle types showed lower intensity in the flaxseed group on both observed days (*P* < 0.05).

The intensity of the ROD of apoptotic markers is summarized in Table 4. In the control ovaries, the ROD of bax in the SFs decreased from D10 to D24 (*P* < 0.05), although in other follicle types it was not changed significantly. The ROD of caspase 3 in all follicle types decreased from D10 to D24 (*P* < 0.01, PRFs and SFs; *P* < 0.001, PFs). The flaxseed increased the ROD of bax in PFs and PRFs (*P* < 0.001, D24 vs D10) with the length of supplementation, but not in SFs. The ROD of caspase 3 in all types of follicles was affected positively in a time-dependent manner (*P* < 0.001, PFs; *P* < 0.05, PRFs; *P* < 0.01, SFs). When compared to the control animals, dietary flaxseed decreased the ROD of bax in PFs and PRFs on D10 (*P* < 0.01), but not in the SFs; however it was higher in all follicle types on D24 of feeding (*P* < 0.05). Flaxseed feeding decreased the ROD of caspase 3 in all follicle types on D10 (*P* < 0.05, PFs and PRFs; *P* < 0.01, SFs) and increased on D24 in PFs (*P* < 0.01) and SFs (*P* < 0.05), but not in the PRFs.

The mean concentrations of steroid (progesterone, testosterone, and oestradiol-17β) and peptide (IGF-I) hormones are

**Table 3**

Intensity of immunohistochemical reaction of proliferation markers in gilt's ovaries.

ROD			None (control)	Flaxseed		
			Day 10	Day 24	Day 10	Day 24
PCNA	PF		35.96 ± 0.83 <sup>a,z</sup>	43.32 ± 1.92 <sup>a</sup>	53.65 ± 1.41 <sup>b,z</sup>	38.29 ± 3.93 <sup>b</sup>
		Oocyte	35.02 ± 1.77	37.71 ± 1.67 <sup>y</sup>	42.66 ± 2.88 <sup>b</sup>	28.06 ± 1.81 <sup>b,y</sup>
	PRF	Follicular cells	3.17 ± 0.39 <sup>a,x</sup>	4.46 ± 0.31 <sup>a,y</sup>	4.58 ± 0.28 <sup>b,x</sup>	3.44 ± 0.17 <sup>b,y</sup>
			25.67 ± 1.25 <sup>c</sup>	36.94 ± 0.67 <sup>c,z</sup>	27.92 ± 1.9	24.79 ± 1.99 <sup>z</sup>
SF	Oocyte	3.9 ± 0.22 <sup>a,y</sup>	5.16 ± 0.3 <sup>a,x</sup>	5.1 ± 0.18 <sup>a,y</sup>	4.32 ± 0.17 <sup>a,x</sup>	
	Follicular cells	5.59 ± 0.23 <sup>z</sup>	6.46 ± 0.93 <sup>z</sup>	2.27 ± 0.11 <sup>z</sup>	1.81 ± 0.15 <sup>z</sup>	
Cyclin B1	PF		4.8 ± 0.09 <sup>z</sup>	5.29 ± 0.27 <sup>z</sup>	2.7 ± 0.3 <sup>c,z</sup>	1.72 ± 0.1 <sup>c,z</sup>
	PRF		6.19 ± 0.17 <sup>z</sup>	6.77 ± 0.54 <sup>z</sup>	2.6 ± 0.47 <sup>z</sup>	1.9 ± 0.08 <sup>z</sup>
	SF					

ROD, relative optical density expresses intensity of immunohistochemical reaction; PF, primordial follicles; PRF, primary follicles; SF, secondary follicles; Day 10–24, sampling days; Values are means ± SEM; <sup>a,b,c,x,y,z</sup> Mean values within columns with the same superscript letters differ significantly (<sup>a,x</sup>*P* < 0.05; <sup>b,y</sup>*P* < 0.01; <sup>c,z</sup>*P* < 0.001). Superscript letters <sup>a,b,c</sup> show differences on Day 24 compared to Day 10 and <sup>x,y,z</sup> show differences between the groups.

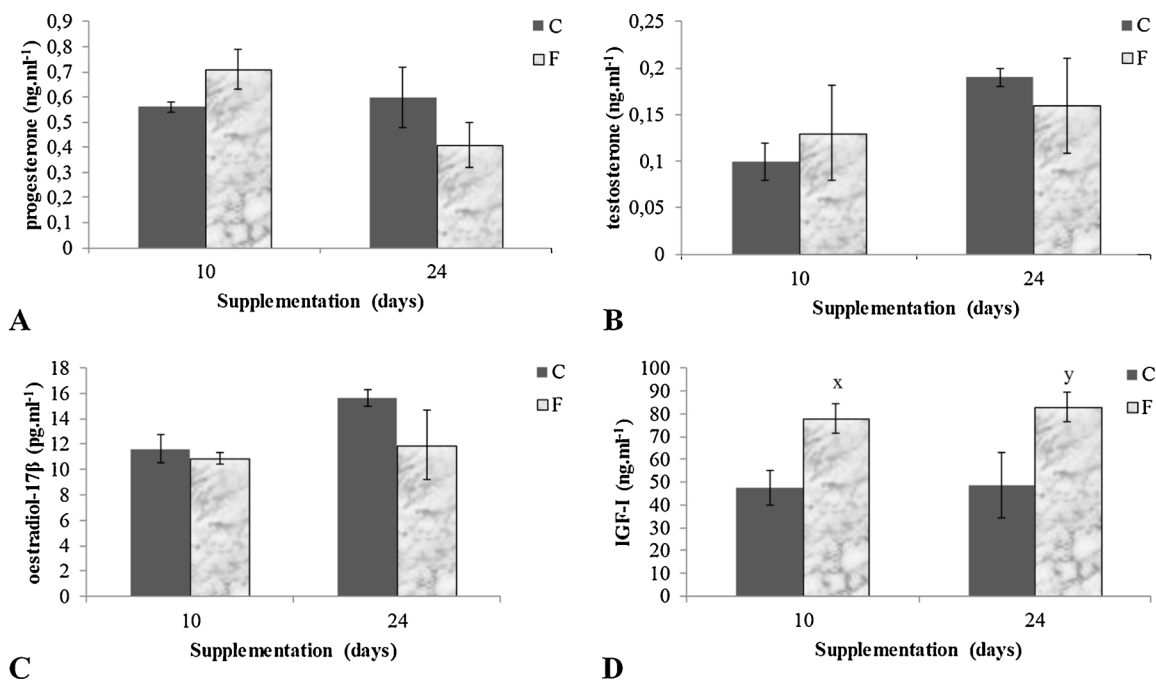
**Table 4**  
Intensity of immunohistochemical reaction of apoptotic markers in gilt's ovaries.

ROD		None (control)		Flaxseed	
		Day 10	Day 24	Day 10	Day 24
<b>Bax</b>	<b>PF</b>	8.6 ± 0.69 <sup>y</sup>	6.55 ± 0.47 <sup>y</sup>	5.15 ± 0.41 <sup>c,y</sup>	11.07 ± 1.02 <sup>c,y</sup>
	<b>PRF</b>	8.85 ± 0.5 <sup>y</sup>	6.66 ± 0.4 <sup>x</sup>	6.11 ± 0.71 <sup>c,y</sup>	11.23 ± 1.07 <sup>c,x</sup>
	<b>SF</b>	8.72 ± 0.52 <sup>b</sup>	5.63 ± 0.28 <sup>b,x</sup>	7.37 ± 1.05	8.44 ± 0.92 <sup>x</sup>
<b>Caspase 3</b>	<b>PF</b>	5.33 ± 0.26 <sup>c,z</sup>	2.49 ± 0.18 <sup>c,y</sup>	1.99 ± 0.1 <sup>c,z</sup>	4.5 ± 0.33 <sup>c,y</sup>
	<b>PRF</b>	4.23 ± 0.28 <sup>b,z</sup>	2.06 ± 0.04 <sup>b</sup>	1.78 ± 0.16 <sup>a,z</sup>	3.9 ± 0.59 <sup>a</sup>
	<b>SF</b>	4.66 ± 0.67 <sup>b,y</sup>	1.82 ± 0.17 <sup>b,z</sup>	1.58 ± 0.08 <sup>b,y</sup>	3.87 ± 0.23 <sup>b,z</sup>

ROD, relative optical density expresses intensity of immunohistochemical reaction; PF, primordial follicles; PRF, primary follicles; SF, secondary follicles; Day 10–24, sampling days; Values are means ± SEM; <sup>a,b,c,x,y,z</sup> Mean values within columns with the same superscript letters differ significantly (<sup>a,x</sup>*P* < 0.05; <sup>b,y</sup>*P* < 0.01; <sup>c,z</sup>*P* < 0.001). Superscript letters <sup>a,b,c</sup> show differences on Day 24 compared to Day 10 and <sup>x,y,z</sup> show differences between the groups.

summarized in Fig. 2. Serum steroid hormone levels in the control animals and animals fed flaxseed were more or less similar, thus no significant differences were observed. We measured a positive correlation in the serum level of IGF-I in the flaxseed group on D10 (*P* < 0.05) and D24 (*P* < 0.01) compared to the control group; although no significant effect between the groups were demonstrated.

The concentrations of fatty acids (FA) in the blood serum of the controls and flaxseed fed animals are shown in Table 5. In the control group there was a significant decrease in the level of eicosapentaenoic acid (EPA; *P* < 0.001) and docosahexaenoic acid (DHA; *P* < 0.05) and total n-3 PUFAs concentration (*P* < 0.01) on D24 compared to D10. The ratio of n-6/n-3 PUFAs increased from D10 to D24 almost by 1.5 times (*P* < 0.01). Other FAs levels in the blood of the control piglets were not changed significantly. In the group of piglets fed with flaxseed, overall levels of saturated FAs (SFA) decreased, although the levels of the unsaturated FAs (USFA) (*P* < 0.001) and PUFAs (*P* < 0.001) increased. The monounsaturated FAs (MUFA) were decreased on D24 (*P* < 0.01). Particularly, levels of myristic (C14:0) and myristoleic (C14:1 n-5) acid decreased and arachidic acid (C20:0) increased from D10 to D24 (*P* < 0.05). The length of dietary supplementation by the flaxseed affected the serum levels of the following FAs: palmitic (C16:0), palmitoleic (C16:1 n-7) and osbond (C22:5 n-6) acids decreased, although ALA (*P* < 0.01), arachidic (*P* < 0.05), EPA (*P* < 0.001), clupanodonic (C22:5 n-3; *P* < 0.001) and DHA (*P* < 0.01) increased from D10 to D24. When compared to the controls, the flaxseed supplemented standard starting diet increased the concentration of myristic (*P* < 0.01), palmitic (*P* < 0.01), palmitoleic (*P* < 0.001), LA (*P* < 0.05), total SFA (*P* < 0.05) and n-6/n-3 ratio (*P* < 0.01), although arachidic (*P* < 0.001), eikosadienoic (*P* < 0.01), DGLA (*P* < 0.05), AA (*P* < 0.001), EPA (*P* < 0.001), adrenic (*P* < 0.05), osbond (*P* < 0.01), DHA



**Fig. 2.** Effect of flaxseed on the serum levels of progesterone (A), testosterone (B), oestradiol-17 $\beta$  (C) and IGF-I (D) in probiotic-treated weanling gilts. Values are means ± SEM. <sup>x</sup>*P* < 0.05; <sup>y</sup>*P* < 0.01 indicating significant differences with the control; C – control group (without flaxseed), F – supplemental flaxseed group; IGF-I, insulin-like growth factor I.



**Table 5**  
Effect of feeding with and without supplemental flaxseed on the level of fatty acids in the blood serum of weanlings.

Fatty acids (mol%)	None (control)		Flaxseed	
	Length of supplementation (days)			
	10	24	10	24
14:00	0.56 ± 0.05 <sup>y</sup>	0.89 ± 0.11 <sup>x</sup>	1.06 ± 0.13 <sup>b,y</sup>	0.61 ± 0.04 <sup>b,x</sup>
14:1n-5	0.03 ± 0.01	0.06 ± 0.02 <sup>x</sup>	0.04 ± 0.01 <sup>b</sup>	0.01 ± 0.00 <sup>b,x</sup>
16:00	20.64 ± 0.95 <sup>y</sup>	20.48 ± 1.15 <sup>x</sup>	26.86 ± 1.39 <sup>c,y</sup>	17.31 ± 0.38 <sup>c,x</sup>
16:1n-7	4.72 ± 0.72 <sup>x</sup>	3.97 ± 0.56 <sup>y</sup>	6.01 ± 0.34 <sup>c,x</sup>	1.91 ± 0.25 <sup>c,y</sup>
18:2 n-6	17.05 ± 1.17 <sup>x</sup>	20.37 ± 0.83 <sup>z</sup>	21.35 ± 1.23 <sup>x</sup>	24.82 ± 0.43 <sup>z</sup>
18:3 n-6	0.53 ± 0.09	0.46 ± 0.05	0.37 ± 0.02	0.37 ± 0.03
18:3 n-3	0.46 ± 0.03	0.63 ± 0.06 <sup>z</sup>	0.56 ± 0.04 <sup>b</sup>	5.99 ± 0.50 <sup>b,z</sup>
20:2 n-6	0.51 ± 0.03 <sup>y</sup>	0.61 ± 0.05 <sup>y</sup>	0.37 ± 0.03 <sup>y</sup>	0.44 ± 0.02 <sup>y</sup>
20:3 n-6	0.44 ± 0.05 <sup>x</sup>	0.33 ± 0.02 <sup>x</sup>	0.31 ± 0.03 <sup>x</sup>	0.27 ± 0.01 <sup>x</sup>
20:4 n-6	10.35 ± 0.29 <sup>z</sup>	8.48 ± 0.75 <sup>x</sup>	7.30 ± 0.45 <sup>z</sup>	6.12 ± 0.56 <sup>x</sup>
20:5 n-3	0.57 ± 0.02 <sup>c,z</sup>	0.16 ± 0.01 <sup>c,z</sup>	0.18 ± 0.01 <sup>c,z</sup>	2.92 ± 0.16 <sup>c,z</sup>
22:4 n-6	0.72 ± 0.03 <sup>x</sup>	0.68 ± 0.06 <sup>z</sup>	0.60 ± 0.04 <sup>b,x</sup>	0.28 ± 0.03 <sup>b,z</sup>
22:5 n-6	0.38 ± 0.03 <sup>y</sup>	0.41 ± 0.06 <sup>z</sup>	0.23 ± 0.02 <sup>c,y</sup>	0.08 ± 0.02 <sup>c,z</sup>
22:5 n-3	1.26 ± 0.12	0.96 ± 0.08 <sup>z</sup>	1.02 ± 0.12 <sup>c</sup>	2.32 ± 0.10 <sup>c,z</sup>
22:6 n-3	1.93 ± 0.12 <sup>a,y</sup>	1.29 ± 0.19 <sup>a,x</sup>	1.13 ± 0.14 <sup>b,y</sup>	2.05 ± 0.15 <sup>b,x</sup>
SFA	33.71 ± 1.00 <sup>x</sup>	33.83 ± 4.58	36.81 ± 2.64 <sup>c,x</sup>	30.77 ± 0.87 <sup>c</sup>
USFA	66.29 ± 1.00 <sup>x</sup>	66.17 ± 4.58	63.20 ± 2.64 <sup>c,x</sup>	69.23 ± 0.87 <sup>c</sup>
MUFA	32.09 ± 2.21	32.72 ± 5.40 <sup>y</sup>	29.76 ± 4.09 <sup>a</sup>	23.07 ± 1.96 <sup>a,y</sup>
PUFA	34.20 ± 2.42	33.45 ± 3.63 <sup>z</sup>	33.43 ± 2.73 <sup>c</sup>	46.16 ± 2.05 <sup>c,z</sup>
n-3	4.22 ± 0.40 <sup>b,y</sup>	2.99 ± 0.54 <sup>b,z</sup>	2.90 ± 0.62 <sup>c,y</sup>	13.54 ± 1.58 <sup>c,z</sup>
n-6	29.98 ± 2.56	30.47 ± 3.28	30.53 ± 2.74	32.62 ± 1.55
n-6/n-3	7:1 <sup>b,y</sup>	10:1 <sup>b,z</sup>	11:1 <sup>c,y</sup>	2:1 <sup>c,z</sup>
EPA/AA	1:18 <sup>c,z</sup>	1:54 <sup>c,z</sup>	1:40 <sup>c,z</sup>	1:2 <sup>c,z</sup>

SFA, saturated fatty acids; USFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; EPA, timodonic acid; AA, arachidonic acid. Values are means ± SEM; <sup>a,b,c,x,y,z</sup> Mean values in rows with the same superscript letters differ significantly (<sup>a,x</sup> $P < 0.05$ ; <sup>b,y</sup> $P < 0.01$ ; <sup>c,z</sup> $P < 0.001$ ). Superscript letters <sup>a,b,c</sup> show differences on Day 24 compared to Day 10 and <sup>x,y,z</sup> show differences between the groups.

( $P < 0.01$ ), total USFA ( $P < 0.05$ ), and total n-3 PUFAs ( $P < 0.01$ ) were decreased on D10 (weaning day). The flaxseed supplemented milk diet (D24, 14 days after weaning) increased the levels of LA ( $P < 0.001$ ), ALA ( $P < 0.001$ ), EPA ( $P < 0.001$ ), clupanodonic acid ( $P < 0.001$ ), DHA ( $P < 0.05$ ), total PUFAs ( $P < 0.001$ ) and n-3 PUFAs ( $P < 0.001$ ), while the levels of myristic ( $P < 0.05$ ), myristoleic ( $P < 0.05$ ), palmitic ( $P < 0.05$ ), palmitoleic ( $P < 0.01$ ), eicosadienoic ( $P < 0.01$ ) acids, DGLA ( $P < 0.05$ ), AA ( $P < 0.05$ ), adrenic ( $P < 0.001$ ), osbond ( $P < 0.001$ ) acids, total MUFAs ( $P < 0.01$ ) and n-6/n-3 ratio ( $P < 0.001$ ) were significantly decreased. The delta-6-desaturase activity was calculated as a ratio between EPA and AA (Table 5). In the control animals (no supplemental flaxseed) there was a higher activity of  $\Delta$ -6-desaturase on D10 when compared to D24 ( $P < 0.001$ ). The flaxseed supplementation increased the activity of  $\Delta$ -6-desaturase in a time-dependent manner ( $P < 0.001$ ). When compared to the control animals, its activity was lower on D10 ( $P < 0.001$ ), although higher on D24 ( $P < 0.001$ ).

#### 4. Discussion

The data in our study supported the previous reports regarding the direct influence of flaxseed on the proliferation and apoptosis in prepubertal porcine ovarian cells (Kádasi et al., 2015) and the influence of supplemental flaxseed feeding on the conversion of the high intake of ALA (from flax seed) into its other metabolic n-3 PUFA derivatives, mostly EPA and DHA (Grofová, 2010). Most authors dealing with the effects of flaxseed on the reproduction system suggest that the anti-proliferative and pro-apoptotic effects of flaxseed are the effects of its lignan phytoestrogens acting predominantly via the oestrogen receptors beta (Rietjens et al., 2013; Sirotkin and Harrath, 2014; Kádasi et al., 2015). However, the preferential conversion of n-3 PUFAs instead of n-6 PUFAs (Böttner et al., 2013) could have the same effect or they could relate to one another, although further elucidation of this action mechanism is needed. Whether the effects are the result of phytoestrogens was not studied in this experiment, although it should not be omitted. Our observations are the first demonstration, to our knowledge, that indicate that the feeding of flaxseed have such a positive correlation with the reproductive system of weanling gilts, as shown by ovarian cell apoptosis and proliferation, the metabolism of the fatty acids, as well as the response of the ovaries to such changes regarding the folliculogenesis. Furthermore, our findings suggest that dietary flaxseed supplementation can modify the cell cycle and apoptosis of ovarian cells via the action of highly unsaturated FAs (HUFAs) and SFAs after weaning and that this effect of dietary flaxseed can be inhibited during the suckling period of piglets.

This study showed that the flaxseed supplemented milk diet (short-term flaxseed supplementation period) as well as flaxseed supplemented standard starting diet (long-term dietary supplementation period) have a positive correlation with the body weight of piglets and serum levels of IGF-I, although it did not show an obvious effect on the steroid hormones levels. Similar results were reported in gnotobiotic piglets, in which their weight increased by 70–80% after the application of probiotics and flaxseed oil

(Nemcová et al., 2012). Our observations are not in agreement with some studies (Woodside et al., 2006; Chen et al., 2011) that suggested that the flaxseed added to the diet may lower the circulating levels of IGF-I or may not modulate the basal weight of weanlings and that the levels of circulating IGF-I are affected only by the growth phase (Li et al., 2014). However, our findings are supported by the observations of Dirandeh et al. (2016) who reported that flaxseed or diets rich in n-3 PUFAs fed to postpartum cows, altered the hepatic expression of genes related to somatotrophic axis function resulting in an increased levels of IGF-I in the blood.

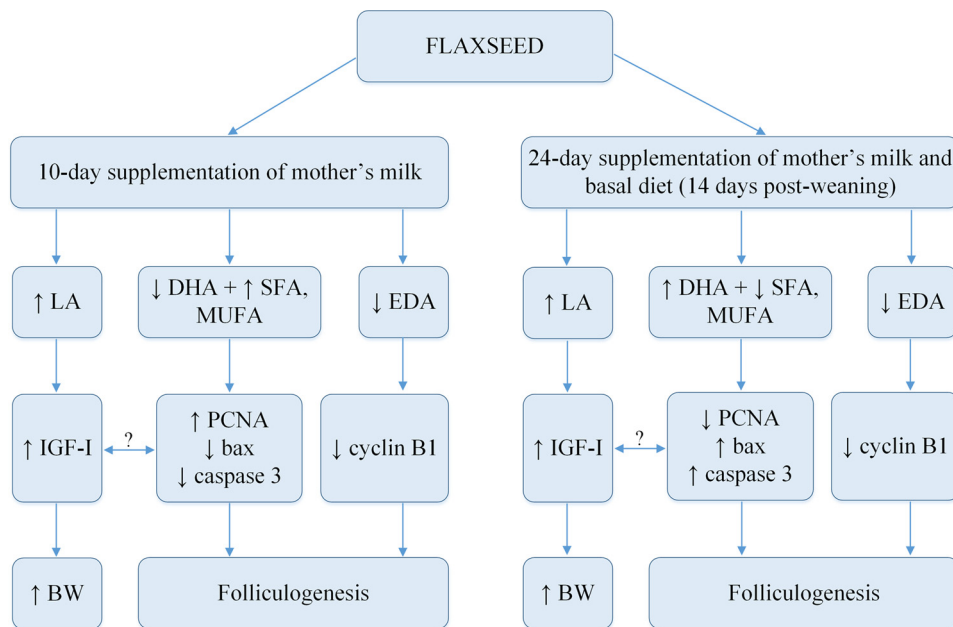
Morphometric analysis found that flaxseed supplemented milk diet (10-day flaxseed supplementation of mother's milk) resulted in larger primary and secondary follicles in piglets' ovaries, but did not affect significantly the size of the primordial follicles and their oocytes. Less primordial follicles were activated to primary follicles after the flaxseed was applied to piglets during this period. Fourteen days after weaning (flaxseed supplemented standard starting diet), less primordial follicles were activated to primary follicles, which were larger. Even though, the size and number of secondary follicles were not affected by the diet although the weight and volume of ovaries were larger. This is the first demonstration, to our knowledge, that flaxseed supplemented standard starting diet (long-term dietary supplementation) by crushed flaxseed delayed the activation of primordial follicles to primary stage in the weaning gilt's ovaries.

The results of the immunohistochemistry indicated that low n-3 PUFAs conversion in sucklings (flaxseed supplemented milk diet) could promote the accumulation of the proliferation marker PCNA, promoter of the G1/S phase of the cell cycle (Ulrich and Takahashi, 2013), in follicular cells of all types of follicles and resulted in larger diameters of those follicles. The flaxseed supplemented standard starting diet (long-term supplementation) revealed the suppressive effect of flaxseed on all types of follicles. This suppression could be due to the increased activation of apoptosis (accumulation of bax and caspase 3) and weaker proliferation (accumulation of PCNA) in the follicular cells, thus less follicles were recruited for other stages of development. The intensity of pro-apoptotic or anti-proliferative effect of flaxseed seems to depend on the length of flaxseed feeding or other dietary conditions (e.g. mother's milk). Our findings of the pro-apoptotic and anti-proliferative effects of flaxseed are in line with the *in vitro* studies on porcine ovarian healthy (Kádasi et al., 2015) and rat colon cancer cells (Hernández-Salazar et al., 2013), as well as with studies performed in women with reproductive cancers who ate flaxseed (Riediger et al., 2009; McIlwain et al., 2013); therefore flaxseed potentially inhibits ovarian follicle growth and development. In addition, there was significant inhibition of the proliferation marker cyclin B1, the promoter of G2/M phase of the cell cycle (Miyazaki and Arai, 2007) during the overall supplemental period. We also found the decrease in the level of eicosadienoic acid throughout the supplemental period and therefore we suggest, and this is the first report, that the inhibition of elongation of LA to eicosadienoic acid may block the G2/M phase of the cell cycle related to the low level of cyclin B1, but the mechanism is unknown. However, Xiong et al. (2015) found that enterolactone (a lignan metabolite) has an inhibitory effect on the expression of cyclin B1 in breast cancer cells, thus confirming the anti-proliferative effect of the flaxseed. The relations between eicosadienoic acid, cyclin B1 expression and phytoestrogens, as well as the possible mechanism of action should be studied further. Whether the block or activation of apoptosis could be due to the IGF-I action is debatable. IGF-I may mediate cellular survival by acting upstream of the caspase activation and may suppress or delay the initiation of the apoptotic process mainly via the type-I IGF receptor (Willis et al., 1998; Butt et al., 1999). In our study, the serum IGF-I level was increased regardless of the increase or decrease in DHA.

We found that flaxseed supplemented standard starting diet, as well as for flaxseed supplemented milk diet applied to prepubertal gilts resulted in high serum levels of LA but inhibited its conversion down its metabolic pathway (from DGLA to osbond acid). However the level of ALA (from flaxseed) was normal and its conversion to EPA and DHA was decreased on D10 (day of weaning). We suggest that the observed suppression of n-6 PUFAs metabolism and accumulation of LA in the blood at weaning and 14 days post-weaning did not depend on the source of fatty acids (milk or flaxseed) and related to the increase in IGF-I levels in the blood resulting in higher body weight. Moreover,  $\Delta$ -6-desaturase, the enzyme common for the catalysis of both n-6 and n-3 PUFAs (Stoffel et al., 2008) was decreased as well. The low activity of  $\Delta$ -6-desaturase in control sucklings could be due to its weak synthesis or activity as it was found in new-borns and children less than 6 month of age (Grofová, 2010), but may be affected positively by the dietary flaxseed when high intake of n-3 ALA competes for this enzyme with n-6 LA in favor of n-3 PUFAs metabolism thus resulting in higher levels of DHA in the blood (Stoffel et al., 2008; Perini et al., 2011). The gas chromatography also revealed an increase in the levels of some SFAs, mostly myristic and palmitic acids and MUFA (palmitoleic acid) on D10 (day of weaning), although these were decreased on D24 (14 days after weaning). The low levels of DHA together with the high levels of myristic, palmitic and palmitoleic acids could promote proliferation and suppress of the apoptosis of follicular cells. However, the opposite changes in levels of those FAs had the reverse effects, which subsequently blocked the cell cycle and activated the bax-caspase 3 pathway of cell apoptosis (Butt et al., 1999). This is the first report of the level of those SFAs, MUFA and HUFA in the blood, which could affect the proliferation, and apoptosis of follicular cells in primordial, primary and secondary follicles on the ovaries of prepubertal gilts. The possible actions of the flax seed on the folliculogenesis are shown in Fig. 3. The possible mechanisms of action of selected fatty acids on the cell cycle and apoptosis and functional interrelationships between those fatty acids and phytoestrogens from flaxseed require further studies. It is possible that the action of the flaxseed on reproduction depends on the age of the females and the natural steroid production as observed *in vitro* on porcine ovaries of prepubertal 100-day old gilts (Kádasi et al., 2015) or in menopausal women (Hajirahimkhan et al., 2013), the length of flaxseed supplementation and other dietary conditions (e.g. mother's milk), but not on IGF-I plasma level.

## 5. Conclusions

Taken together, the study observations show the effect of fatty acids on porcine ovarian functions during a supplemental flaxseed period; nevertheless their direct effect and mechanism should be elucidated. It was demonstrated that the flaxseed supplemented standard starting diet (long-term supplementation period) with high intake of ALA from the flaxseed lowered the n-6/n-3 PUFAs ratio



**Fig. 3.** Targets of 10-day and 24-day flaxseed supplementation actions on probiotic-treated weaning gilts ovaries and body weight. LA, linoleic acid (C18:2 n-6); DHA, cervonic acid (C22:6 n-3); SFA, saturated fatty acids; MUFA, monounsaturated fatty acid; EDA, eicosadienoic acid (C20:2 n-6); IGF-I, insulin-like growth factor I; PCNA, proliferating cell nuclear antigen; BW, body weight.

and promoted the  $\Delta$ -6-desaturase activity for the metabolic pathway of n-3 PUFAs rather than for n-6 PUFAs, thus increasing the serum levels of DHA which together with low levels of selected SFA (myristic and palmitic acids) and MUFA (palmitoleic acid) activated apoptosis and suppressed the proliferation of all ovarian follicles. The inhibition of the elongation of LA to eicosadienoic acid decreased the cyclin B1 intensity in follicles, thus blocking the follicular cells in the G2/M phase of the cell cycle regardless of the type of PUFAs intake (n-6 or n-3). All these effects resulted in the suppression of follicular development on the ovaries of problematic weaning gilts. Flaxseed supplemented milk diet (short-term flaxseed feeding period) had the reverse effects, except for cyclin B1. Moreover, the accumulation of LA have probably positive correlation to the IGF-I synthesis in the liver and related with the growth phase of piglets thus resulting in higher body weight. The potential anti-gonadal action must be taken into account when consuming the flaxseed for longer periods.

### Conflicts of interest

The authors state that they have no conflict of interest to declare.

### Acknowledgements

We would like to thank Dr. Jana Kaľatová for technical collaboration, to RIA laboratory in Košice, Slovak Republik for the hormone analysis and to assoc. prof. Stewart Odendhal, DVM, PhD. for English correction. This work was financially supported by the projects of the Ministry of Education, Science, Research and Sport of the Slovak Republik No. 1/0476/16 and of the Internal Grant Agency of the University of Veterinary Medicine and Pharmacy in Košice No. 15/2016.

### References

- Andrejčáková, Z., Sopková, D., Vlčková, R., Kulichová, L., Gancarčíková, S., Almášiová, V., Holovská, K., Petrilla, V., Krešáková, L., 2016. Synbiotics suppress the release of lactate dehydrogenase, promote non-specific immunity and integrity of jejunum mucosa in piglets. *Anim. Sci. J.* 87, 1157–1166.
- Borovská, D., Nemcová, R., Gancarčíková, S., Koščová, J., 2013. The synbiotic effect of lactobacilli and flaxseed on selected intestinal microflora and organic acid levels in weaned piglets. *Microbiology* 2, 82–86.
- Böttner, M., Thelen, P., Jarry, H., 2013. Estrogen receptor beta: tissue distribution and the still largely enigmatic physiological function. *J. Steroid Biochem. Mol. Biol.* 139, 245–251.
- Bourque, M., Dluzen, D.E., di Paolo, T., 2012. Signaling pathways mediating the neuroprotective effects of sex steroids and SERMs in Parkinson's disease. *Front. Neuroendocrinol.* 33, 169–178.
- Butt, A.J., Firth, S.M., Baxter, R.C., 1999. The IGF axis and programmed cell death. *Immunol. Cell Biol.* 77, 256–262.
- Chen, J., Saggari, J.K., Corey, P., Thomson, L.U., 2011. Flaxseed cotyledon fraction reduces tumour growth and sensitises tamoxifen treatment of human breast cancer xenograft (MCF-7) in athymic mice. *Br. J. Nutr.* 105, 339–347.
- Chytilová, M., Mudroňová, D., Nemcová, R., Gancarčíková, S., Buleca, V., Koščová, J., Tkáčiková, L., 2013. Anti-inflammatory and immunoregulatory effects of flaxseed oil and *Lactobacillus plantarum* – Biocenol™ LP96 in gnotobiotic pigs challenged with enterotoxigenic *Escherichia coli*. *Res. Vet. Sci.* 95, 103–109.
- Contreras, M.C., 2012. Fatty acids and emotional behavior. *Neuroscience - Dealing With Frontiers*. (In Tech: Rijeka.). pp. 1–440.

- Dirandeh, E., Towhidi, A., Ansari, Z., Zeinoaldini, S., Ganjkanlou, M., 2016. Effects of dietary supplementation with different polyunsaturated fatty acids on expression of genes related to somatotrophic axis function in the liver, selected blood indicators, milk yield and fatty acids profile in dairy cows. *Ann. Anim. Sci.* 16, 1045–1058.
- Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226, 497–509.
- Grofová, Z., 2010. Mastné kyseliny (In Czech). *Medicína pro praxi*, vol. 7. pp. 388–390.
- Hajirahimkhan, A., Dietz, B.-M., Bolton, J.L., 2013. Botanical modulation of menopausal symptoms: mechanisms of action? *Planta Med.* 79, 538–553.
- Hernández-Salazar, M., Guevara-González, R.G., Cruz-Hernández, A., Guevara-Olvera, L., Bello-Pérez, L.A., Castaño-Tostado, E., Loarca-Piña, G., 2013. Flaxseed (*Linum usitatissimum* L.) and its total non-digestible fraction influence the expression of genes involved in azoxymethane-induced colon cancer in rats. *Plant Foods Hum. Nutr.* 68, 259–267.
- Kádasi, A., Štochmalová, A., Maruniaková, N., Kolesárová, A., Grossman, R., Sirotkin, A.V., 2015. Effect of natural plant extracts on porcine ovarian functions. *J. Microbiol. Biotechnol. Food Sci.* 4, 45–48.
- Kubašová, I., Mudroňová, D., 2014. The influence of probiotic Lactobacilli and flaxseed on the immune response of weaned piglets experimentally infected with enterotoxigenic *E. coli*. *Folia Vet.* 58, 122–126.
- Lí, Q., Brendemuhl, J.H., Jeong, K.C., Badinga, L., 2014. Effects of dietary omega-3 polyunsaturated fatty acids on growth and immune response of weanling pigs. *J. Anim. Sci. Technol.* 56, 7.
- Liu, Y.L., Li, D.F., Gong, L.M., Yi, G.F., Gaines, A.M., Carroll, J.A., 2003. Effects of fish oil supplementation on the performance and immunological, adrenal, and somatotrophic responses of weaned pigs after *Escherichia coli* lipopolysaccharide challenge. *J. Anim. Sci.* 81, 2758–2765.
- Mattos, R., Staples, C.R., Thatcher, W.W., 2000. Effects of dietary fatty acids on reproduction in ruminants. *Rev. Reprod.* 5, 38–45.
- McIlwain, D.R., Berger, T., Mak, T.W., 2013. Caspase functions in cell death and disease. *Cold Spring Harb. Perspect. Biol.* 5, 1–29.
- Michel, T., Halabalaki, M., Skatsounis, A.L., 2013. New concepts, experimental approaches, and dereplication strategies for the discovery of novel phytoestrogens from natural sources. *Planta Med.* 79, 514–532.
- Ming, L.G., Chen, K.M., Xian, C.J., 2013. Functions and action mechanisms of flavonoids genistein and icariin in regulating bone remodelling. *J. Cell. Physiol.* 228, 513–521.
- Miyazaki, T., Arai, S., 2007. Two distinct controls of mitotic cdk1/cyclin B1 activity requisite for cell growth prior to cell division. *Cell Cycle* 6, 1419–1425.
- Nemcová, R., Lauková, A., Gancarčíková, S., Kaštel, R., 1997. In vitro studies of porcine lactobacilli for possible probiotic use. *Berliner und Munchener Tierärztliche Wochenschrift* 110, 413–417.
- Nemcová, R., Guba, P., Gancarčíková, S., Bomba, A., Lauková, A., 2003. Štúdium probiotických vlastností laktobacilov u hydiny (In Slovak). *Agriculture* 49, 75–80.
- Nemcová, R., Gancarčíková, S., Mudroňová, D., Koščová, J., Buleca, V. (Eds.), 2010. Probiotiká a naturálne látky v chove hospodárskych zvierat (In Slovak). University of Veterinary Medicine and Pharmacy in Košice, pp. 77.
- Nemcová, R., Borovská, D., Koščová, J., Gancarčíková, S., Mudroňová, D., Buleca, V., Pisl, J., 2012. The effect of supplementation of flax-seed oil on interaction of *Lactobacillus plantarum* – Biocenol™ LP96 and *Escherichia coli* O8:K88ab:H9 in the gut of germ-free piglets. *Res. Vet. Sci.* 93, 39–41.
- Perini, J.A.L., Stevanato, F.B., Visentainer, J.E.L., Sargi, S.C., Oliveira, M.M., Souza, N.E., Matsushita, M., Visentainer, J.V., 2011. Incorporation of n-3 fatty acids by the liver of mice fed linseed oil as a function of feeding duration. *Braz. Arch. Biol. Technol.* 54, 307–313.
- Petit, H.V., Dewhurst, R.J., Proulx, J.G., Khalid, M., Haresign, W., Twagiramungu, H., 2001. Milk production, milk composition, and reproductive function of dairy cows fed different fats. *J. Anim. Sci.* 81, 263–271.
- Rahbar, B., Safdar, A.H.A., Kor, N.M., 2014. Mechanisms through which fat supplementation could enhance reproduction in farm animals. *Eur. J. Exp. Biol.* 4, 340–348.
- Riediger, N.D., Othman, R.A., Suh, M., Moghadasian, M.H., 2009. A systemic review of the roles of n-3 fatty acids in health and disease. *J. Am. Diet. Assoc.* 109, 668–679.
- Rietjens, I.M., Sotoca, A.M., Vervoort, I., Lousse, J., 2013. Mechanisms underlying the dualistic mode of action of major soy isoflavones in relation to cell proliferation and cancer risks. *Mol. Nutr. Food Res.* 57, 100–113.
- Rodríguez-Leyva, D., Dupasquier, C.M., McCullough, R., Pierce, G.N., 2010. The cardiovascular effects of flaxseed and its omega-3 fatty acid, alpha-linolenic acid. *Can. J. Cardiol.* 26, 489–496.
- Saggari, J.K., Chen, J., Corey, P., Thompson, L.U., 2010. Dietary flaxseed lignan or oil combined with tamoxifen treatment affects MCF-7 tumor growth through estrogen receptor- and growth factor- signaling pathways. *Mol. Nutr. Food Res.* 54, 415–425.
- Singh, K.K., Mridula, D., Rehal, J., Barnwal, P., 2011. Flaxseed: a potential source of food, feed and fiber. *Crit. Rev. Food Sci. Nutr.* 51, 210–222.
- Sirotkin, A.V., Harrath, A.-H., 2014. Phytoestrogens and their effects. *Eur. J. Pharmacol.* 741, 230–236.
- Smolen, A.J., 1990. Image analytic techniques for quantification of immunocytochemical staining in the nervous system. In: Conn, P.M. (Ed.), *Methods in Neurosciences*. Academic Press, New York, pp. 208–229.
- Sopková, D., Hertelyová, Z., Andrejčáková, Z., Vlčková, R., Gancarčíková, S., Petrilla, V., Ondrašovičová, S., Krešáková, L., 2017. The application of probiotics and flaxseed promotes metabolism of n-3 polyunsaturated fatty acids in pigs. *J. Appl. Anim. Res.* 45, 93–98.
- Stoffel, W., Holz, B., Jenke, B., Binczek, E., Gunter, R.H., Kiss, C., Karakesiosoglou, I., Thevis, M., Weber, A.A., Armhold, S., Addicks, K., 2008. Delta6-desaturase (FADS2) deficiency unveils the role of omega 3- and omega 6-polyunsaturated fatty acids. *EMBO J.* 27, 2281–2292.
- Tourre, A., Xueming, X., 2010. Flaxseed lignans: source, biosynthesis, metabolism, antioxidant activity, bio-active components, and health benefits. *Comp. Rev. Food Sci. Food Saf.* 9, 261–269.
- Tvrzická, E., Stanková, B., Vecka, M., Žák, A., 2009. Fatty acids – 1. Occurrence and biological significance. *Cas Lek Cesk* 148, 16–24.
- Ulrich, H.D., Takahashi, T., 2013. Readers of PCNA modifications. *Chromosoma* 122, 259–274.
- Willis, D.S., Mason, H.D., Watson, H., Franks, S., 1998. Developmentally regulated responses of human granulosa cells to insulin-like growth factors (IGFs): IGF-I and IGF-II action mediated via the type-I IGF receptor. *J. Clin. Endocrinol. Metab.* 83, 1256–1259.
- Woodside, J.V., Campbell, M.J., Denholm, E.E., Newton, L., Honour, J.W., Morton, M.S., Young, I.S., Leatham, A.J., 2006. Short-term phytoestrogen supplementation alters insulin-like growth factor profile but not lipid or antioxidant status. *J. Nutr. Biochem.* 17, 211–215.
- Xiong, X.Y., Hu, X.J., Li, Y., Liu, C.M., 2015. Inhibitory effects of enterolactone on growth and metastasis in human breast cancer. *Nutr. Cancer* 67, 1324–1332.
- Yanagihara, N., Zhang, H., Toyohira, Y., Takahashi, K., Ueno, S., Tsuitsui, M., Takahashi, K., 2014. New insights into the pharmacological potential of plant flavonoids in the catecholamine system. *J. Pharmacol. Sci.* 124, 123–128.
- Yu, H.N., Zhu, J., Pan, W.S., Shen, S.R., Shan, W.G., Das, U.N., 2014. Effects of fish oil with a high content of n-3 polyunsaturated fatty acids on mouse gut microbiota. *Arch. Med. Res.* 45, 195–202.
- Žák, A., et al., 2011. Ateroskleróza: Nové Pohledy (In Czech). Praha: Grada, Czech Republic. pp. 183.