Livestock Science 224 (2019) 60–68

Contents lists available at ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

Dried fermented post-extraction rapeseed meal given to sows as an alternative protein source for soybean meal during pregnancy improves bone development of their offspring

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ARTICLE INFO

Keywords:
Fermented rapeseed meal
Pregnancy
Pigs and offspring development
Bone properties

ABSTRACT

The experiment was conducted to evaluate the effect of inclusion of dried fermented post-extracted rapeseed meal to the diet of pregnant sows (using 20 pregnant sows in total) on bone development in offspring. In one treatment group (n = 10 sows), the soybean meal in the diet of pregnant sows was partially replaced with dried fermented rapeseed meal (40 g/kg) during the whole pregnancy up to parturition. All newborn boars, a total of 45 in each treatment group, were grown up to the end of starter phase at the age of 77 d. Then, one boar from each sow (n = 10) was selected to be sacrificed. Dried fermented rapeseed meal included in the sow diet resulted in longer (P = 0.006) and heavier (P = 0.036) femur in their offspring. Maternal dietary supplementation of dried fermented rapeseed meal increased relative bone volume (P = 0.024) and trabecular number (P = 0.006) and decreased trabecular space (P = 0.007) in femoral epiphysis as well as increased the thickness of hyaline cartilages increased (P < 0.001). The 3-point bending test showed increase in bone mechanical strength (yield and ultimate load, stiffness, elastic energy, and work to fracture; P < 0.05) in boars maternally supplemented with dried fermented rapeseed meal. However, material properties (Young's modulus, yield, and ultimate strain) of bone did not differ between treatments, which was a result of the changes in bone mid-diaphysis's geometry. Although the quantitative indicators of bone mineralization did not differ between treatments, the maternal diet changed the structure of mineral phase of bone, as X-ray diffraction analysis shown the decrease of the mean size of the hydroxyapatite nanocrystallites (P < 0.001) in boars maternally supplemented with dried fermented rapeseed meal. This could be the effect of ionic substitutions of Ca ions in the hydroxyapatite structure, as the content of macro- and microelements in ashed bone samples was different in treatment groups. Based on the results, it can be concluded that inclusion of dried fermented rapeseed meal to the diet of pregnant sows improved the structure and mechanical properties of compact bone in the mid-diaphysis and microarchitecture of trabecular bone in metaphysis and epiphysis of the examined bones in their offspring at the beginning of the grower period.

1. Introduction

As soya bean, a commonly used primary source of protein in feed, cannot be cultivated in environmental conditions of northern and central Europe, looking for alternative sources of dietary protein is one of the most important direction in European animal science. The post-

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https://doi.org/10.1016/j.livsci.2019.04.009
Received 8 June 2018; Received in revised form 20 March 2019; Accepted 10 April 2019
Available online 11 April 2019
1871-1413/

extracted rapeseed meal (RSM) is a by-product of oil extraction from rapeseed, introduced to the European Union Catalogue of feed materials (EC, 2013) as suitable in animal feed (ruminants, pigs, and poultry). Post-extracted rapeseed meal has a small amount of fat (on average 2.5%) and about 38% of protein. Compared to soybean meal, RSM has a greater (about 17%) amount of sulphur amino acids (Florou-Paneri et al., 2014). A characteristic feature of RSM is greater glucosinolates content (a large group of sulphur-containing secondary plant metabolites), which show anti-nutritional effect by the disturbance of iodine metabolism (Tripathi and Mishra, 2007). Glucosinolates hydrolyzed by a myrosinase enzyme release different breakdown products, e.g. thiocyanates, which limit immune function and antioxidative capacity (Tripathi and Mishra, 2007). However, most of the RSM produced nowadays is from "low-glucosinolate" varieties, with the glucosinolates content up to 50 μMol/g (Bourdon and Aumaître, 1990; Mejicanos et al., 2016). Rapeseed meal contains about 11% of crude fiber (mainly derived from seed hulls), which is needed for proper function of intestinal tract of pigs, but its excess affects negatively the degree of digestibility of feed. Post-extracted rapeseed meal contains relatively greater level of phytate phosphorus, poorly digested by monogastric animals. The nutritive value of RSM for pigs is limited also by greater content of tannins and sinapine, which could impair performance (Li and Guo, 2016; Rundgren, 1983). It is suggested that fermentation could be a promising method for improving nutritional value of RSM by effective reduction of anti-nutritional factors, making a fermented RSM a good protein source in feed (Hu et al., 2016; Van Winsen et al. 2002; Vig and Walia, 2001).

Bone development is determined by genes, but it is impacted by nutritional factors during the prenatal period (Holemans et al., 1998; Smith and Ozanne, 2006; Śliwa et al., 2005; Tatara et al., 2007). Various investigations have verified the hypothesis of the fetal programming of postnatal performance and bone development of pigs (Blicharski et al., 2017; McArdle et al., 2006; Śliwa et al., 2006).

To our knowledge, in literature, there are no data about the effect of inclusion of dried fermented RSM to the diet of pregnant sows on their offspring development. The aim of the study was to determine to what extent a dried fermented RSM (DFRSM) through its nutritional qualities (better absorbed protein and lowered content of anti-nutritional factors), and health-supporting potential (source of antioxidant compounds or microorganisms) could influence bone development in weaned pigs when given to pregnant sows.

2. Materials and methods

2.1. Preparation of DFRSM

The rapeseed meal was first autoclaved at 121 °C for 15 min. Then each kg of RSM was mixed with the same volume of distilled water and inoculated with *Lactobacillus* spp. in incubator for 25 d at 30 °C. Ultimately, fermented RSM was dried for 2 d at the range of temperature from 50 °C to 60 °C. The obtained DFRSM was ground and kept at room temperature until mixed in the diet.

2.2. Feed analysis

Dry matter, crude fiber, and crude protein in the diets were determined according to AOAC (2016) methods (934.01, 978.10, and 954.01, respectively), whereas the content of Ca, Na, Zn, Cu and Fe was determined using the FAAS technique (method 968.08); the total phosphorus was determined colorimetrically (method 965.17) and the content of selected of amino acids in DFRSM was determined by chromatography method (method 999.13). Total glucosinolates content in DFRSM was accomplished by a gas chromatography method (Daun and McGregor, 1981).

Table 1
Ingredients and nutrients content of control (as-fed basis) and experimental diets for pregnant (Phase I and II) and lactating (Phase III) sows.

Item	Phase I[a]		Phase II[b]		Phase III[c]
	Control	DFRSM	Control	DFRSM	All sows
Ingredient, %					
Wheat middlings			36.0	36.0	36.0
Triticale middlings	30.0	30.0			
Barley middlings	30.0	29.0	37.7	36.7	37.7
Oat	29.32	29.32	4.0	4.0	4.0
Soybean meal	6.0	3.0	16.0	13.0	16.0
Rapeseed oil	1.0	1.0	2.0	2.0	2.0
Supplementing mixture[d]	0.12	0.12	0.24	0.24	0.24
Fodder salt	0.4	0.4	0.4	0.4	0.4
Limestone	0.7	0.7	0.7	0.7	0.7
Dicalcium phosphate	0.26	0.26	0.26	0.26	0.26
L-Lys-HCl (78%)	0.12	0.12	0.12	0.12	0.12
DL-Met (99%)	0.08	0.08	0.08	0.08	0.08
Vitamin-mineral premix[e]	2.0	2.0	2.5	2.5	2.5
DFRSM[f]	–	4.0	–	4.0	–
Analyzed composition					
Dry matter, g/kg	883	882	889	888	889
Crude protein, g/kg	150.3	150.1	170.4	171.2	171.4
Crude fiber, g/kg	73.5	74.4	48.5	50.2	48.5
Crude fat, g/kg	32.2	32.3	27.2	27.3	27.2
Ash, g/kg	50.6	49.8	52.4	51.9	52.4
Ca, g/kg	7.45	7.43	8.62	8.63	8.25
Total P, g/kg	5.23	5.19	5.63	5.67	5.63
Na, g/kg	2.04	2.03	1.98	1.97	1.98
Zn, mg/kg	142.5	144.3	148.7	149.2	148.7
Cu, mg/kg	18.6	18.4	20.3	20.4	20.3
Fe, mg/kg	145.8	149.2	165.3	166.2	165.3
Calculated composition					
ME, MJ/kg	12.3	12.3	12.6	12.6	12.6
Lys, %	1.02	1.02	1.02	1.02	1.02
Met + Cys, %	0.58	0.58	0.58	0.58	0.58

[a] From the confirmation of pregnancy to the 84th day of pregnancy.
[b] From 85th to 114th day of pregnancy.
[c] Up to 27th day of lactation.
[d] Supplementing mixture per kilogram of diet: wheat bran; Ca, 145,000 mg; Na, 10,000 mg; P, 5500 mg; crude protein 2.8%; vegetable oil and crude fat 0.4%; crude fiber 1.6%; crude ash 80%; vitamin A, 1,350,000 IU; vitamin B1, 200 mg; vitamin B2, 800 mg; vitamin B5, 1200 mg; vitamin B6, 500 mg; vitamin B9, 800 mg; vitamin B12, 55 mg; vitamin C, 10,000 mg; vitamin D3, 1,000,000 IU; vitamin E, 8.940 IU; vitamin H, 300 mg; vitamin K3, 270 mg; vitamin PP, 2000 mg; choline 3500 mg; Fe, 4000 mg; Cu, 500 mg; Zn, 4000 mg; Mn, 2000 mg; I, 35 mg; Se, 5 mg.
[e] Vitamin–mineral premix per kilogram of diet: vitamin A, 520,000 IU; vitamin D3, 80,000 IU; vitamin E, 1,192 IU; vitamin K3, 100 mg; vitamin B1, 80 mg; vitamin B2, 280 mg; vitamin B6, 200 mg; vitamin B9, 60 mg; vitamin B12, 1000 μg; vitamin H, 10,000 μg; vitamin PP, 800 mg; vitamin B5, 600 mg; choline 15,000 mg; Fe, 4000 mg; Cu, 800 mg; Zn, 4000 mg; Mn, 2000 mg; Se, 10 mg; I, 30 mg; L-Lys 30,000 mg; Met 10,000 mg; Thr 10,000 mg.
[f] DFRSM—dried fermented rapeseed meal with *Lactobacillus* spp.

2.3. The analyzed nutrient levels of DFRSM

The content of nutritional and bioactive components (per 1 kg of DFRSM) was as follows: dry matter, 882.7 g; crude ash, 78.9 g; total protein, 291.9 g; crude fat, 31.7 g; crude fiber, 91.5 g; total P, 9.09 g; phosphorus from phytate, 5.73 g; Ca, 8.05 g; Met, 5.55 g; Lys, 13.5 g. The content of total glucosinolates was 45.42 μMol/g.

2.4. Animals, breeding and experimental design

The experiment was approved by The Local Ethics Committee on Animal Experimentation in Lublin, Poland. The experiment was carried out on an industrial pig farm. The study was performed on 90 boars born by 20 crossbred Polish Large White × Polish Landrace sows. Sows were sired by the same boars (Polish Landrace). Sows were clinically healthy and singly housed in separated cages under standard rearing

conditions (controlled temperature, humidity and 12:12 h light/dark cycle) with free access to fresh water and fed twice a day (at 08:00 a.m. and 04:00 p.m.; 2.3 kg/d during pregnancy) with properly balanced standard commercial diet for pregnant sows supplied in equal doses for all sows (Table 1). During lactation the amount of given fodder depended on the size of litter (Table 1). All diets were formulated to meet or exceed the nutritional requirement (NRC, 2012).

The sows were randomly assigned into two weight and age-matched groups (10 sows in each treatment group): control, fed standard diet, and experimental group fed the addition of DFRSM. The soybean meal in the diet of pregnant sows was partially replaced with DFRSM at the level of 4% during the whole pregnancy up to parturition. The dose of DFRSM was determined 1) on the basis of available literature, and 2) from a preliminary study, where the body weight of newborn piglets was assessed. The recent study performed by Yun et al. (2018) has showed that RSM can be included in pigs' diets at the level of 4% without negative effects on growth performance or nutrient digestibility, while Opałka et al. (2001) have showed that RSM given at the level of 5% does not alter the reproductive performance of sows. Also Kaczmarek et al. (2016), basing on the results of several studies, has concluded that the inclusion level of rapeseed-based products in sow diets should not exceed 5%.

2.5. Piglets and their diet

All piglets were born by physiological partum and had no congenital changes. There were no stillbirths and mummified piglets. Only males were used in the study. Each treatment group consisted of 45 boars. Boars born by sows fed the control diet belonged to the control group and boars born by DFPRM-treated sows belonged to the DFRSM group. At birth, unsuckled piglets were weighed and held with their own mothers, and not translocated between sows. After weaning at the age of 28 d of life, boars from the same litter were held in the same box, and were not translocated between litters. All piglets received prestarter (Table 2) from 7th up to 28th d of life and were fed naturally with sows milk. After weaning in 29th d of life up to 42nd d piglets were fed the prestarter. Piglets received additives per 1 kg of prestarter: vitamin A, 416,250 IU; vitamin D3, 80,000 IU; vitamin E, 298 IU; Fe, 332 mg; I, 2.20 mg: Cu, 165 mg; Mn, 65 mg; Zn, 2600 mg: Se, 0.35 mg; artificial sweeters: sodium saccharin, 48.9 mg; neohesperidin dihydrochalcone 0.09, 162 mg; phytase (20,000 FTU/kg); xylanase (2625 LXU/kg); *Saccharomyces cerevisiae*.

Since 43rd d of life, the piglets were fed twice a day a commercial starter in accordance with the stage of the production cycle (Table 2), and kept under standard rearing conditions with constant access to fresh water.

All diets were formulated to meet or exceed the nutritional requirement (NRC, 2012). Boars were grown up to the end of starter phase at the age of 77 d. One randomly chosen boar from each litter/box was weighed and euthanized by intravenous injections of lethal doses of pentobarbitalum natrium (Morbital, Biowet, Puławy, Poland). The total number of boars used to bone analysis was 20 (n = 10 per each treatment group).

Table 2
Composition of prestarter and starter diets for piglets.

Item	Prestarter[a]	Starter[b]
Analyzed composition		
Dry matter, g/kg	921	890
Crude protein, g/kg	195	184
Crude fiber, g/kg	27.0	40.4
Crude fat, g/kg	45.0	50.3
Ash, g/kg	56.0	50.4
Ca, g/kg	7.0	7.4
Total P, g/kg	6.5	6.8
Calculated composition		
ME, MJ/kg	14.6	13.2
Lys, %	1.43	1.20
Met + Cys, %	0.82	0.83

[a] Form 7th to 42nd day.
[b] From 43rd to 77th day.

Post-weaning period is a critical time in swine production for the general growth and bone development, as it is associated with up-regulation of several intestinal inflammatory cytokines involved in the process of bone growth, which in pigs usually normalizes at the age of 77 d (Leonard et al., 2011; Pié et al., 2004; Sugiharto et al., 2014; Suoza and Lerner, 2013).

Immediately after euthanasia, the femora were dissected, cleaned from adherent tissues, weighted, wrapped in gauze soaked in isotonic saline, and frozen at −26 °C until further analyses. In subsequent stages of analyses, the right femur was subjected to densitometry, strength test and geometric measurements, while left femora were earmarked for histomorphometry and differential scanning calorimetry analysis.

2.6. Bone analysis

Bone metabolism was assessed by determining the whole bone mineral content (BMC) and bone mineral density (BMD) with the densitometric method (dual-energy X-ray absorptiometry, DXA), using a Norland XR 43 densitometer (Norland, Fort Atkinson, WI, USA) calibrated before measurements with bone phantoms of known BMD.

A 3-point bending test was performed to estimate the mechanical properties of femur mid-diaphysis using a Zwick Z010 universal testing machine (Zwick GmbH & Co. KG, Ulm, Germany). Prior the analysis, bones were thawed for 4 h at room temperature. The load was applied with a displacement rate of 10 mm/min until the bone fracture (Śliwa et al., 2010; Tomaszewska et al., 2012a). During the test, the load-deflection curves were registered continuously. Next, the external and internal diameters of the mid-diaphysis cross-section were measured in horizontal (medial-lateral) and vertical (cranial-caudal) planes with a digital caliper. On the basis of the measured diameters, the following geometric parameters were calculated: cortical cross-sectional area, cortical index, vertical cortical index, mean relative wall thickness, midshaft volume, radius of gyration and cross-sectional moment of inertia about medial-lateral axis (Muszyński et al., 2017; Tomaszewska et al., 2017a). On the basis of recorded load-deformation curves and calculated geometrical parameters, the structural (yield load, ultimate load, stiffness, elastic energy, work to fracture, toughness modulus and bending moment) and material properties (Young's modulus, yield strain, ultimate strain, yield stress and ultimate stress) of femora were determined using standard engineering beam-theory equations (Muszyński et al., 2017, 2018a; Tomaszewska et al., 2017b) by means of the Origin software (OriginLab, Northampton, MA, USA).

The bones were wet machined. The mid-diaphysis of femur was washed and defatted, dried at 105 °C to constant mass, and bone tissue density (BTD) was measured using a AccuPyc 1330 gas pycnometer (Micromeritics, Norcross, GA, USA) as described previously (Tomaszewska et al., 2015a). Finally, bone fragments were mineralized in a muffle furnace at 500 °C, and crude ash percentage with bone ash to volume ratio were calculated (Muszyński et al., 2018a).

2.7. The content of bone macro- and microelements

The composition of bone mineral phase was determined using ICP-OES spectrometry (iCAP Series 6500, Thermo Scientific, Waltham, MA, USA) in ashed bone mid-diaphysis samples. Prior to analysis of mineral elements, the mineralization of the samples was conducted in a Microwave Digestion System (Berghof Speedwave, Eningen, Germany) (Kitowski et al., 2017). The TraceCERT multi-element stock solution (Sigma-Aldrich, St. Louis, MO, USA) was used to prepare reference standards. The content of macro- and microelements was expressed in mg or g in 1 g of crude ash.

2.8. X-ray diffraction

Ashed bone mid-diaphysis samples were ground in the mortar in the room temperature. Obtained bone powder was analyzed by X-ray diffraction with Empyrean diffractometer (PANalytical). A wave length of CuKα radiation (λ = 1.5406 Å) was used. All samples were measured over a 2θ range from 2° to 90° with step size of 0.013° with counting time of 1 s/step. The mean size of the nanocrystallites in the bulk material was calculated according to the Scherrer equation with the Scherrer shape constant of 0.9 (Scherrer, 1918). The FWHM (*full width at half maximum*) was calculated from the fits of the Voight function to every peak with use of the Origin 9.0 software. For each sample within the treatment group, the mean size of the nanocrystallites for one piglet was estimated from the size calculated for the first 10 not overlapping peaks. Finally, these 10 means from 10 animals were expressed as a mean in the treatment group.

2.9. Histomorphometry, collagen content and hyaline cartilage analysis

Samples of cartilage and bone were taken from the same anatomical position in the knee joint, i.e. from the middle of the lateral femoral condyle (containing epiphysis and metaphysis). Sagittal 25-mm thick sections of cartilage and bone were cut perpendicular to the articular surface, next they were formaldehyde-fixed, decalcified in EDTA solution and subjected to standard histological procedures (Blicharski et al., 2017; González-Chávez et al., 2013). Briefly, four cross sections (with the thickness of 4 μm, and with 10 μm interval after each two-slice section) were cut with a microtome (Microm HM 360, Microm, Walldorf, Germany) from every bone sample. Each of two semi-serial cuts were placed on one microscopic slide. Masson's trichrome staining was used to assess the morphology of growth plate cartilage, while Picrosirius red (PSR) staining was employed to assess the morphology of articular cartilage as well as to evaluate the distribution of thick and thin collagen fibers in articular cartilage and trabecular bone in epiphysis and metaphysis (Muszyński et al., 2018b). All microscopic images were collected using an Olympus BX63 microscope (Olympus, Tokyo, Japan). The analysis of the collected images was performed with the use of Olympus CellSens graphical analysis software (Olympus, Tokyo, Japan). The thickness of the following zones in the growth plate cartilage: reserve (I; cells occurs singly or in pairs separated by an abundant extracellular matrix), proliferation (II; flattened chondrocytes are arranged in longitudinal columns, enlarge, and divide), hypertrophy (III; cell size rapidly increases and columnar arrangement is less regular), and ossification (IV; region where the transition from cartilage to bone occurs with degeneration and death of chondrocytes) were measured in following manner: from each slice 5 measurements of each variable were made. They gave 20 measurements from every studied animal (as described above, four slices from every piglet were analyzed). An average of these 20 measurements was expressed as the mean for each piglet. Finally, these 10 mean measurements from 10 animals were expressed as a mean for each treatment group. Similarly, the thickness of the three main zones of articular cartilage were measured: horizontal zone (superficial surface, the zone I, small and flattened chondrocytes are arranged parallel to the surface), transitional (II; chondrocytes are large and round, occur singly or in isogenous groups) and radial (III; spherical chondrocytes lie in columns) (Tomaszewska et al., 2016a) were analyzed in the manner as described above.

The bone volume (BV) and tissue volume (TV) were measured in the microscopic images of trabecular bone (epiphysis and metaphysis) sections using the pixel count, and the relative bone volume (BV/TV) was assessed. Other calculated morphometric parameters included: mean trabecular thickness (Tb.Th mean), maximal trabecular thickness (Tb.Th max), mean trabecular separation (Tb.Sp mean), maximal trabecular separation (Tb.Sp max), and trabecular number (Tb.N). The trabecular bone morphometry was measured using the ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA). Measurements were performed on four not overlapping sections for each of four slices and these 16 values were used to calculate a mean of each listed above parameter for one piglet. Finally, these 10 means from 10 animals were expressed as a mean in one treatment group.

2.10. Differential scanning calorimetry (DSC) analysis

After the dissection, articular cartilage samples obtained from femoral condyle were cleaned from other tissues remnants, put into distilled water and stored at 7 °C, no longer than 24 h, before the DSC measurements. Trabecular bone samples were wet machined from the lateral condyle and decalcified in EDTA solution (González-Chávez et al., 2013). In the next step, they were dehydrated under vacuum conditions at room temperature for 24 h. Both samples from articular cartilage and decalcified trabecular bone were trimmed with a razor blade to fill the 40 μl aluminium DSC pan, and sealed to prevent moisture loss. An analysis was performed using a DSC-1 calorimeter (Mettler-Toledo GmbH, Greifensee, Switzerland) and run against an empty pan for reference. Heating was carried out at the heating rate 10 °C/min from 20 °C to 85 °C and 5 °C/min from 20 °C to 200 °C for articular cartilage and trabecular bone samples, respectively (Blicharski et al., 2017). For each endothermal process recorded on the thermograms, the initial (T_{on}) temperature, peak denaturation temperature (T_{max}), and net denaturation enthalpy (ΔH) were determined using a software integrated with the calorimeter. After the analysis, the pans were punctured, next dried in an oven at 105 °C for 24 h for determination samples moisture content. Finally, denaturation enthalpies were normalized to the samples dry weights (Muszyński et al., 2018c).

2.11. Blood plasma biochemical analyses

Blood samples were collected into 9 ml Vacutainer heparinized tubes (Vacutest, Arzergrande, Italy). Tubes containing the blood were placed on ice and then centrifuged at 3000x*g* for 15 min at 4 °C for plasma separation. The separated plasma was stored in 2.0 ml Eppendorf test tubes at −80 °C. The concentrations of Ca, P, Zn, Cu, and Fe were determined colorimetrically using a Metrolab automated analyzer (2300 GL, Metrolab SA, Buenos Aires, Argentina), and sets of ready-made biochemical reagents kits (Hydrex Diagnostics, Warsaw, Poland; BioMaxima, Lublin, Poland) according to the manufacturers' protocols (Muszyński et al., 2018a).

2.12. Statistical analysis

Data were analyzed using Statistica 13 software (TIBCO Software Inc., Palo Alto, CA, USA) with each piglet as the experimental unit. The distribution of the variables was tested for normality using the Shapiro–Wilk test. Comparison between normally distributed variables from the control and DFRSM groups was carried out using two-tailed Student's *t* test for independent samples. When variables were not normally distributed comparisons were made using the Mann–Whitney U test. For all tests $P < 0.05$ was considered statistically significant.

3. Results

3.1. Body weight and bone morphology

The loss of fat tissue or too slow body weight and fat tissue gains in the pregnant sows in both treatment groups were not observed. The length of gestation did not differ between sows fed control diet or enriched with DFRSM. Average litter size (14.2 ± 1.3 and 14.4 ± 1.8, in control and DFRSM group, respectively) did not differ between groups ($P > 0.05$). Body weight of newborn piglets (1.39 ± 0.30 kg and 1.41 ± 0.22 kg, for control and DFRSM group, respectively), at the

Table 3
Osteometric characteristics of the femur obtained from piglets at the age of 77 d.

Dependent variable	Group Control[a]	DFRSM[a]	Polled SEM[b]	*P* value[c]
Basal geometric properties				
Bone length, mm	143	156	1	0.006
Bone weight, g	149	156	1	0.036
Midshaft geometrical properties				
Midshaft volume, mm^3	12.2	14.6	0.4	0.046
C-C plane external diameter, mm	19.9	20.7	0.2	0.164
C-C plane internal diameter, mm	9.8	11.0	0.2	0.047
M-L plane external diameter, mm	18.4	20.8	0.3	0.005
M-L plane internal diameter, mm	9.8	12.1	0.2	0.003
Cross-sectional area, cm^2	2.13	2.33	0.06	0.196
Mean relative wall thickness	1.49	1.24	0.04	0.037
Vertical wall thickness, mm	4.30	4.32	0.13	0.964
Cortical index, %	48.9	44.1	0.74	0.031
Vertical cortical index, %	46.8	41.5	0.53	0.003
Cross-sectional moment of inertia, mm^4	5801	8175	301	0.012
Radius of gyration, mm	5.12	5.91	0.07	0.002
Densitometry properties				
Bone mineral content BMC, g	33.3	34.0	0.3	0.620
Bone mineral density BMD, g/cm^2	0.809	0.841	0.014	0.401
Bone tissue density BTD, g/cm^3	1.90	1.89	0.01	0.707
Bone ash, %	65.1	64.8	0.4	0.766
Ash to volume ratio, g/cm^3	1.24	1.22	0.01	0.557

C-C plane, cranial-caudal plane; M-L plane, medial-lateral plane; BMC, bone mineral content; BMD, bone mineral density; BTD, bone tissue density.
[a] Data are presented as mean, $n = 10$ in each treatment group.
[b] Polled SEM—polled standard error of the means.
[c] Statistical significance (*t*—Student test).

weaning (7.58 ± 0.42 kg and 7.61 ± 0.49 kg, for control and DFRSM group, respectively), and at the age of 77 d (33.41 ± 0.66 kg and 33.45 ± 0.71 kg, for control and DFRSM group, respectively) did not differ between treatment groups.

Dried fermented RSM included in the sow diet resulted in longer and heavier femur in their offspring (Table 3). It also changed the bone geometry, as the increase of the values of horizontal (medial-lateral plane) external and internal diameters of the mid-diaphysis cross-section and internal vertical diameter in cranial-caudal plane as well as cross-sectional moment of inertia, and radius of gyration (Table 3). Further, a decrease of the mean relative wall thickness, cortical index, vertical cortical index was noted in the DFRSM group.

3.2. Densitometric analysis and mechanical characteristic of the femur

All determined densitometric parameters (BTD, BMD, and BMC, Table 2) as well as bone ash and ash to volume ratio (Table 3), and bone material properties (Table 4) did not differ between the control animals and pigs born by DFRSM-treated sows. The inclusion of DFRSM to the diet of pregnant sows increased the values of all bone structural properties determined during mechanical testing (except toughness modulus).

3.3. Histomorphometry, collagen content and hyaline cartilage analysis

Dried fermented RSM included in the sow diet resulted in greater relative bone volume and trabecular number in femoral metaphyseal femur in their offspring. On the other hand, mean and maximal trabecular space decreased. The relative bone volume increased also in metaphysis, as a result of the increase of mean trabecular thickness and the decrease of mean trabecular space (Table 5).

The content of thin (immature) collagen in bone trabeculae decreased in both parts of bone (epiphysis and metaphysis). Dried fermented RSM given to sows did not influence the collagen content in articular cartilage of their offspring (Table 6). However, the thickness of the growth plate, and total thickness of the articular cartilage, and zone I and III of the articular cartilage increased (Table 7).

Table 4
Mechanical characteristics of femur obtained from piglets at the age of 77 d.

Dependent variable	Group Control[a]	DFRSM[a]	Polled SEM[b]	*P* value[c]
Structural properties				
Yield load, kN	1.64	1.88	0.03	0.012
Ultimate load, kN	1.91	2.26	0.06	0.038
Stiffness, N/mm	830	1067	34	0.021
Elastic energy, J	1.74	1.81	0.01	0.022
Work to fracture, J	2.87	3.60	0.11	0.032
Toughness modulus, mJ/mm^3	2.31	2.28	0.10	0.906
Bending moment, N m	23.4	29.9	0.6	0.002
Material properties				
Young's modulus, MPa	562	624	56	0.676
Yield strain, %	7.48	6.85	0.38	0.536
Ultimate strain, %	9.51	9.22	0.24	0.656
Yield stress, MPa	40.2	38.3	1.9	0. 629
Ultimate stress, MPa	46.7	45.0	2.1	0.761

[a] Data are presented as mean, $n = 10$ in each treatment group.
[b] Polled SEM—polled standard error of the means.
[c] Statistical significance (*t*—Student test).

Table 5
Trabecular bone morphology in femur obtained from piglets at the age of 77 d.

Dependent variable	Group Control[a]	DFRSM[a]	Polled SEM[b]	*P* value[c]
Epiphysis				
BV/TV, %	25	32.2	1.6	0.024
Tb.Th mean, μm	96	97	7	0.956
Tb.Th max, μm	235	228	15	0.800
Tb.Sp mean, μm	221	192	5	0.007
Tb.Sp max, μm	566	447	41	0.015
Tb.N, 1/mm	2.71	3.54	0.16	0.006
Metaphysis				
BV/TV, %	32.1	46.4	1.4	<0.001
Tb.Th mean, μm	58	8622	5	0.014
Tb.Th max, μm	164	303	14	0.131
Tb.Sp mean, μm	129	111	5	0.065
Tb.Sp max, μm	297	273	10	0.201
Tb.N, 1/mm	4.99	5.87	0.32	0.142

BV/TV, relative bone volume; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb.N, trabecular number.
[a] Data are presented as mean, $n = 10$ in each treatment group.
[b] Polled SEM—polled standard error of the means.
[c] Statistical significance (*t*—Student test).

Table 6
Thin (immature) collagen content in bone tissues in femur obtained from piglets at the age of 77 d.

Dependent variable	Group Control[a]	DFRSM[a]	Polled SEM[b]	*P* value[c]
Trabecular bone, epiphysis,%	0.436	0.232	0.056	0.046
Trabecular bone, metaphysis,%	0.380	0.168	0.044	0.013
Articular cartilage,%	0.058	0.082	0.009	0.269

[a] Data are presented as mean, $n = 10$ in each treatment group.
[b] Polled SEM—polled standard error of the means.
[c] Statistical significance (*t*—Student test).

3.4. Blood plasma biochemical analysis, the content of bone minerals, and X-ray diffraction analysis

Inclusion of DFRSM to the diet of pregnant sows resulted in the decrease of the concentration of Ca and P, and the increase of concentration of Cu in the blood plasma of their offspring (Table 8). In that

Table 7
Femoral cartilages morphology in femur obtained from piglets at the age of 77 d.

Dependent variable	Group Control[a]	DFRSM[a]	Polled SEM[b]	*P* value[c]
Growth plate cartilage				
Zone I thickness, μm	213	494	28	<0.001
Zone II thickness, μm	298	365	10	<0.001
Zone III thickness, μm	244	353	10	<0.001
Zone IV thickness, μm	448	697	24	<0.001
Total cartilage thickness, μm	1369	2100	48	<0.001
Articular cartilage				
Zone I thickness, μm	22	52	2	<0.001
Zone II thickness, μm	519	522	16	0.862
Zone III thickness, μm	959	2431	37	<0.001
Total cartilage thickness, μm	2067	3640	24	<0.001

In the growth plate cartilage: zone I, reserve zone; zone II, proliferation zone; zone III, hypertrophy zone; zone IV, ossification zone.
In the articular cartilage: zone I, horizontal zone; zone II, transitional zone; zone III, radial zone.
[a] Data are presented as mean, $n = 10$ in each treatment group.
[b] Polled SEM—polled standard error of the means.
[c] Statistical significance (*t*—Student test).

Table 8
Blood plasma macro- and microelements concentration in piglets at the age of 77 d.

Dependent variable	Group Control[a]	DFRSM[a]	Polled SEM[b]	*P* value[c]
P, mmol/L	3.21	2.96	0.04	0.030
Ca, mmol/L	2.79	2.48	0.02	<0.001
Mg, mmol/L	0.94	1.05	0.07	0.547
Cu, μmol/L	17.3	20.2	0.36	<0.001
Zn, μmol/L	11.5	11.7	0.10	0.462
Fe, μmol/L	48.0	47.6	0.92	0.869

[a] Data are presented as mean, $n = 10$ in each treatment group.
[b] Polled SEM—polled standard error of the means.
[c] Statistical significance (*t*—Student test).

Table 9
Bone macro- and microelements content and size of the bone hydroxyapatite nanocrystallites in femur obtained from piglets at the age of 77 d.

Dependent variable	Group Control[a]	DFRSM[a]	Polled SEM[b]	*P* value[c]
Bone macro- and microelements content				
Ba, mg/kg	8.1	11.2	0.3	<0.001
Ca, g/kg	323	310	3	0.045
Cd, μg/kg	47.9	57.3	2.1	0.039
Cr, mg/kg	5.87	5.80	0.06	0.525
Cu, mg/kg	1.23	0.95	0.07	0.043
Fe, mg/kg	26.9	27.3	0.8	0.792
Li, mg/kg	8.56	8.11	0.13	0.093
Mg, g/kg	5.65	5.76	0.05	0.328
Mn, mg/kg	1.62	2.21	0,02	<0.001
P, g/kg	148	143	1	0.050
Pb, mg/kg	2.39	2.31	0.08	0.640
S, g/kg	2.37	2.31	0.07	0.169
Si, mg/kg	251	251	3	0.957
Sr, mg/kg	143	136	1	0.029
Zn, mg/kg	488	600	5	<0.001
Ca/P, g/g	2.18	2.17	0.01	0.249
Sr/Ca, g/kg	0.443	0.438	0.002	0.216
Bone hydroxyapatite size				
Nanocrystallites size, nm	45.6	38.22	1.0	<0.001

[a] Data are presented as mean, $n = 10$ in each treatment group.
[b] Polled SEM—polled standard error of the means.
[c] Statistical significance (*t*—Student test).

Table 10
Thermal characteristics of collagen in articular cartilage and trabecular bone of femur obtained from piglets at the age of 77 d.

Dependent variable	Group Control[a]	DFRSM[a]	Polled SEM[b]	*P* value[c]
Articular cartilage				
T_{on}, °C	66.93	65.51	0.16	<0.001
T_{max}, °C	69.16	67.90	0.17	<0.001
ΔH, J/g	0.764	0.445	0.02	<0.001
Trabecular bone				
T_{on}, °C	125.3	122.9	0.3	0.001
T_{max}, °C	132.0	129.2	0.4	0.006
ΔH, J/g	12.9	12.3	0.3	0.361

T_{on}, initial collagen denaturation temperature; T_{max}, peak denaturation temperature; ΔH, net denaturation enthalpy.
[a] Data are presented as mean, $n = 10$ in each treatment group.
[b] Polled SEM—polled standard error of the means.
[c] Statistical significance (*t*—Student test).

group, the bone content of Ba, Cd, Mn, and Zn increased, while the content of Ca, Cu, and Sr decreased (Table 9). In all bone samples the main mineral component was a hydroxyapatite. However, the mean size of the nanocrystallites differed. For the DFRSM group, the peaks broadening corresponded to mean crystallite size of 38.22 nm. The mean size of crystallites for control sample was 45.6 nm (Table 9).

3.5. Thermal analysis of articular cartilage and trabecular bone collagen

Results of the thermal analysis of samples from articular cartilage and trabecular bone are presented in Table 10. There was an effect of maternal nutrition on all thermal parameters of collagen in articular cartilage. Similarly, thermal stability of collagen in trabecular bone differed in the control and DFRSM offspring, and onset and peak temperatures were greater in the DFRSM group (Table 10).

4. Discussion

It has been proved that fermentation changes the nutritional characteristic of RSM, increasing crude protein and lowering crude fiber, glucosinolates and tannins (Batal and Karem, 2001; Hu et al., 2016; Rozan et al., 1996). Vig and Walia (2001) have reported that the content of crude fiber and glucosinolates could be reduced during fermentation by 25.5% and 43%, respectively. Thus, microorganisms in the process of fermentation reduce toxic or anti-nutritive factors from RSM, and improve the quality of RSM (Hill, 1979; Lo and Hill, 1971; Rakariyatham and Sakorn, 2002). Moreover, there is a hypothesis that microorganisms can use the carbohydrate from fermented RSM to produce proteins. Further, proteins are degraded into peptides in fermented RSM (Hong et al., 2004; Hölker et al., 2004). Other studies have shown that the fermentation can result in the production of the growth factors, which could increase protease, amylase and probiotic activity. The fermentation increases also the concentration of free amino acids (aspartic acid, glutamate, glutamine, threonine, serine, proline, glycine, alanine, valine, histidine, tryptophan, ornithine, lysine, and arginine) (Ouoba et al., 2003; Shao et al., 2007).

Owing to its intensive growth, pig seems to be an attractive model for the investigation of nutritional and even toxicological factors influencing the growth, maturation and the skeletal development during the prenatal and postnatal life. The genetic selection of livestock animals towards greater body weight gains as well as conditions of commercial intensive production systems predispose them to many disturbances in bone development (Śliwa et al., 1996). Leg deformities, bone weakness and fractures disturb animal welfare, and cause economic losses due to decreased final body weight and lower carcass quality. Yun et al. (2018) have reported that RSM can be included in finishing pigs' diets at the level of 4% without negative effects on

growth performance, nutrient digestibility, blood characteristics and meat quality. Despite numerous studies performed in growing animals, the influence of maternal feeding with RSM or DFRSM on skeletal development is still unknown. In presented study, DFRSM was given to pregnant sows during the whole pregnancy. Our study showed, that although body weight of control offspring and that delivered by sows fed DFRSM did not differ between themselves, maternal feeding with diet enriched with 4% of DFRSM resulted in longer and heavier bones with better geometry and mechanical properties of compact bone in the mid-diaphysis and microarchitecture of trabecular bone in metaphysis and epiphysis. Stronger bones allow for more efficient movement, resulting in proper behavior, which improves the general welfare of pigs. Thus, it could be supposed that maternal diet enriched with 4% of DFRSM improved bone development in offspring thanks to the presence of proteins, peptides, growth factors or probiotics in DFRSM. Moreover, micro- and macroelements could play additional role, as their bioavailability increased through the fermentation. This could explain the increase of the concentration of Cu in the blood plasma in our boars. It should be emphasized, that Cu plays a significant role in bone homeostasis as an essential cofactor required for the activity of lysyl oxidase (Rodríguez et al., 2002), which regulates total enzymatic cross-link formation of collagen in connective tissue and normalizes the deposition of Ca and P in bones (Linder and Hazegh-Azam, l996). Studies on Cu supplementation indicate that Cu insufficiency leads to bone loss (osteopenia or osteoporosis) caused by the decrease of function of osteoblasts (bone tissue forming cells) (Rodríguez et al., 2002), which causes a decrease in mechanical endurance and consequent fractures (Muszyński et al., 2018b; Tomaszewska et al., 2017c). The ICP-OES analysis showed that bone Cu content decreased in the DFRSM group, however, the analysis was performed for cleaned mid-diaphysis fragment of bone containing cortical bone, while the formation of new bone takes place in bone distal parts where intense processes of proliferation and hypertrophy of chondrocytes in the growth plate connected with its elongation is observed.

A large number of metal cations of macro- and microelements primarily present in the bone mineral phase, like Ba, Cd, Co, Cr, Ni, Mg, Mn, Pb, and Sr, can replace Ca ions in crystallite structures, changing the size of hydroxyapatite crystallites (Wopenka and Pasteris, 2005). ICP-OES analysis showed that the content of Ba, Cd, Mn, Zn increased, and Sr decreased in the DFRSM group. It has been shown that Mn and Zn substitution reduces, and Sr substitution increments crystallographic parameters of hydroxyapatite crystallites (Ghadimi et al., 2013). On the other hand, elements like Zn cannot be accurately attributed to specific components in bone structure, as they can be located in both bone organic matrix and mineral phase (Rey et al., 2009). However, Zn cannot only substitute Ca in hydroxyapatite crystals, but it is also closely linked to bone metabolism, especially during the stages of rapid growth, as it plays a crucial role as a catalyst of many enzymes that affect bone development and formation (Seo et al., 2010; Yamaguchi, 2010). High bioavailability of Zn results in greater bone maturity and improvement of its mechanical properties (Muszyński et al., 2018a). Moreover, mineral crystallinity, the degree of mineralization, and ionic substitutions are shown to have a potential effect on bone mechanical parameters (Akkus et al., 2004). The small dimensions of the hydroxyapatite nanocrystallites are favorable for mechanical properties, likely preventing crack propagation (Akkus et al., 2004). In our study, bones obtained from boars delivered by DFRSM-treated sows were characterized by greater stiffness. It is with agreement with other studies, where it has been shown that smaller crystals tend to increase the stiffness of the bone (Jager and Fratzl, 2000; Ruppel et al., 2008). On the other hand, all the quantitative indicators of bone mineralization in the offspring (BMD, BMC, crude ash) were not affected by the maternal diet. However, BMD, BMC, and crude ash content refer to the overall mineral density of bone tissue, while crystallographic size of hydroxyapatite nanocrystallites is a quality parameter of mineral phase and dominates the mechanical functions of bones to a large extent than bone mineral density (Akkus et al., 2004).

There is a recent study suggesting that dietary additives can influence hydroxyapatites crystals. Dietary xylooligosaccharides, via carbonate substitution of phosphate in hydroxyapatite crystals, improve the bone mineral crystallinity in swine femur, as evidenced by ICP analysis and Raman spectroscopy studies (Wang et al., 2017). In our previous study, we used X-ray diffraction analysis to show that source dietary microelements ($CuCO_3$ or Cu nanoparticles) can influence the structure of bone mineral phase (Tomaszewska et al., 2017b). However, present study is the first showing that the quality of bone mineral phase in offspring could be moderated by the modulations of maternal diet.

The subsequent effect of maternal DFRSM feeding was the improvement of structural properties of the articular cartilage, where doubling of the thickness of the zone I (horizontal zone—superficial surface) was observed in offspring at the age of 77 d. The zone I of the articular cartilage is a crucial component of the mature tissue, because it provides resistance to shear forces in the joint (Tomaszewska et al., 2016a,b). This type of hyaline cartilage forms a low-friction, shock-absorbing layer at the ends of bones, and plays important role in facilitating joint motion and weight bearing. Similarly, collagen fibers and proteoglycans present in the articular cartilage ensure stability and a low coefficient of friction (Tomaszewska et al., 2015b). Present study provided additional information concerning the thick and thin collagen fibers determining structural properties of the articular cartilage. This study showed that maternal DFRSM feeding resulted in more abundant mature (thin) collagen fibers content in the articular cartilage. It gave further information about collagen synthesis and bone formation. Collagen is a major component of the extra-cellular matrix in many tissues. The thermal stability of the articular cartilage and trabecular bone was also analyzed in this study. The differences in the calorimetric peak temperatures could be caused by the different collagen fibril density in their structure as a result of maternal DFRSM feeding. Our other study with maternal β-hydroxy-β-methylbutyrate (HMB) supplementation showed that the peak of the structural phase transformation occurs at lower temperature for cartilages with morphologically less compacted collagen bundles, as less energy is required to disintegrate their structure (Blicharski et al., 2017).

All results described above are consistent with the hypothesis of the existence of the gut-bone axis, showing that digestive tract supports bone formation in different ways (Śliwa, 2010; Tomaszewska et al., 2012b, 2016a, 2018a,b,c). The existence of the gut-bone axis has been proved in several others experiments performed on mammals during pregnancy (Blicharski et al., 2017; Śliwa et al., 2005, 2006; Świetlicka et al., 2016; Tomaszewska et al., 2015a, 2016b). It has been shown that fermented RSM increases the absorptive area of the intestinal epithelium (Hu et al., 2016). Moreover, DFRSM given to pregnant sows probably provided a variety of bioavailable and well absorbed nutrients, which directly influence the total metabolism of the pregnant, and indirectly the development of their fetuses. Intestinal cells utilize some part of DFRSM, while some part is used as a “fuel” by microbe. An interaction between bacteria present in the gastrointestinal tract and the host exerts an additional impact on general metabolism. Well-balanced gut microbiome determines the maintenance of the gut-barrier function. What is more, it is not known, which part of this renewed “fuel” could cross the placenta, and in which form it is available to fetal cells. Moreover, as it was mentioned above, the fermentation process increases the concentration of free amino acids, such as glutamate or proline (Ouoba et al., 2003; Shao et al., 2007). Our earlier studies have shown that prenatal administration of a precursor of glutamine improves protein synthesis in the skeletal muscle and bones, increases the length and weight of the examined bones, and negatively influences bone mechanical properties (Śliwa et al., 2006, 2005). Opposite effect of the action of precursor of glutamine on bone mechanical properties is observed when it is given in postnatal time (Śliwa, 2010). The metabolic pathway of glutamine is associated with the synthesis of proline and hydroxyproline. Both proline and hydroxyproline are qualitatively

and quantitatively the most abundant amino acids in type I collagen (Boucher and Cynober, 1998). It could partially explain our present results. It should be remembered, that densitometric analysis showed that there was no difference in bone mineralization, and better mechanical parameters in the DFRSM group were caused by the improvements in bone mid-diaphysis geometry. Maternal DFRSM feeding at the concentration of 4% can, through altered bone development during prenatal time, triggered better postnatal bone development.

The results are new, and for the first time the influence of maternal DFRSM feeding on bone tissue development in offspring has been shown. To the best of our knowledge, there is no published study about the prenatal nutritional programming of the bone hydroxyapatite nanocrystallites. Moreover, the effects of dried fermented RSM were greater than it have been expected. However, this study did not provide a complete explanation of mechanism of the action of the DFRSM inclusion to maternal diet on long-term effects observed in their offspring at the age of 77 d These effects may be mediated by DRFSM utilization in digestive tract of pregnant sows, which could lead to improved synthesis of amino acids important for collagen formation in bones (glutamine, proline); or DFRSM may increase absorption of other metabolites, which influence the processes of growth and development of the skeletal system in fetuses. However, it can be only speculated, as the concentration of hormones, free amino acids or other growing factors were not determined. Further studies are needed to explain mechanism of the maternal inclusion of DFRSM on the development of the skeletal system of their offspring during its postnatal life.

Conflict of interest

The authors do not have any conflict of interest and this submission has been done upon agreement of all the co-authors.

Acknowledgment

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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