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DIETARY INCLUSION OF *SACCHAROMYCES CEREVISIAE* FERMENTED RAPESEED MEAL MODULATED IMMUNE, OXIDANT AND ANTIOXIDANT INDICES IN PIGLETS AFTER WEANING

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Abstract

Fermented rapeseed meal could be an attractive feed source for piglets after weaning due to its high level of protein with special amino acids and bioactive compounds (polyphenols, PUFA, vitamins B, minerals, fiber) known for their antimicrobial, antioxidative and immunostimulatory effect and lower antinutrients. Lactic acid bacteria were mainly used for rapeseed meal fermentation while yeasts have been less used. The present study evaluated the effect of a diet including 10% rapeseed meal fermented with Saccharomyces cerevisiae on inflammation, oxidative and antioxidative response in spleen of pigs after weaning. The fermentation reduced efficiently glucosinolates level by half in fermented compared to unfermented rapeseed meal and enriched the rapeseed meal in several bioactive compounds such iron, zinc, manganese, n-6 and n-3 unsaturated fatty acids and fiber. Fermented diet reduced the concentration of two important mediators of inflammation, interleukine-1β (-10.28%) and interleukine-6 (-10.92%) and increased the concentration of IFN-γ (+15.36%), a cytokine involved in the antiviral and antibacterial response. An increase in antioxidant enzymes activity and a reduction in lipid peroxidation was also found in spleen of piglets fed fermented diet.

Key words*: antioxidant status, inflammation, fermentation, pig, rapeseed meal.*

INTRODUCTION

During weaning period, piglets have to defy many challenges such as the replacement of breast milk with solid feed, adaptation to another environment, insufficient development of the immune and enzymatic system etc. In general, these events have been overcome by the use of antibiotics at sub therapeutic level (Magnoli et al., 2022). Their interdiction (2006) has led to the necessity to find new alternatives of antimicrobial compounds and this has opened up many opportunities for animal nutrition research. Rapeseed meal could be such an alternative source rich in protein with important particularities due to its content in amino acids like arginine, cysteine and methionine as well as bioactive compounds such as polyphenols, unsaturated fatty acids, minerals, vitamins etc. (Chen et al., 2019) (Wickramasuriya et al., 2015). In animal nutrition rape seed meal was manly an important source of protein. In young pigs, it has been used sparingly due to anti-nutrients that it contains (e. g. glucosinolates) which could affect piglets' performance, nutrient digestibility (Chen et al., 2019) especially during the sensitive period of weaning. (Pérez de Nanclares et al., 2019) showed that the replacement of soybean meal by expeller rapeseed meal with up to 30% (10%, 20%, 30%) had no effect on growth performance and nitrogen metabolism but affect the apparent total tract digestibility.

The research from recent years has focused on the reduction of anti-nutrients from rapeseed meal by different procedures. Fermentation is the most widely used biotechnological process for this purpose resulting in an enrich in protein levels and a decrease in the concentration of glucosinolates, phytic acid, NDF, etc. For all these reasons fermented rapeseed meal can be an attractive source of feed for piglets. Until now the fermentation has been done manly with lactic acid bacteria (different species of *Lactobaccilus* sp., *Aspergilus* sp.) or a mix with yeasts (Shi et al., 2016) (Plaipetch & Yakupitiyage, 2011; 2013). The fermentation enhanced also rapeseed meal in bioactive

nutrients (peptides, polyphenols, PUFA, vitamins B, minerals, fiber etc) known for their antimicrobial, antioxidative and immunostimulatory effect. For example, fermentation of rapeseed meal with *Bacillus subtilis* resulted in an increase in peptides with antioxidant activity which demonstrated *in vitro* inhibitory activity on lipid peroxidation and iron ion chelator (Wang et al., 2003). This fermentation product has a high nutritional value, having a high content of essential amino acids: Histidine, Tyrosine, Methionine, and Cysteine (11.59% of the total amino acids). Studies with fermented rapeseed meal in different farm animal species (chicken, turkey, pig) have shown an improve in growth performance, nutrients digestibility, gut morphology and microbiota as well as in antioxidant and health indices (Chen et al., 2019; Drazbo et al., 2018; Hu et al., 2016; Satessa et al., 2020). Fermentation with *Saccharomyces* sp. has been less used. Utilization of yeasts for the fermentation of by-products could be of interest as the cell wall of yeasts contain polysaccharides manly ß-glucan and mannan with antioxidant properties due to their polymeric structure which can trap free radicals through their capacity to encapsulate toxins like lipopolysaccharide endotoxin (LPS) reducing then its negative effect into the cells (Chuang et al., 2021). The use of rapeseed meal fermented with yeasts as a source of biologically active molecules which could support the transitional post-weaning period has been scarcely investigated in pig. That is why the present study evaluated the effect of a diet including 10% rapeseed meal fermented with *Saccharomyces cerevisiae* on several indices of immune, oxidant and antioxidant response in spleen of pig after weaning. The analysis at the spleen level is relevant as it is one of the most secondary important immune organs where the immune response is built.

MATERIALS AND METHODS

Ethical statement

The experimental protocol was approved by the Ethical Committee (no. 118/2019) of the National Institute of Research and Development for Biology and Animal Nutrition, Balotesti, Romania. Animals were carried on the basis of European and Romanian legislation (The EU Council Directive EC/63/2010 and the Romanian Law 43/2014) for handling and protection of animals used for experimental purposes.

Diets

The three groups of piglets were assigned to three experimental diets as followed: 1) control diet: a starter diet based on corn-soybean meal; 2) experimental diet 1: control diet with 10% unfermented rapeseed meal (RSM diet); 3) experimental diet 2: control diet with 10% fermented rapeseed meal (FRSM diet). Rapeseed meal replace 10% soybean meal (Tables 1 and 2). Diets were formulated to meet the NRC (2012) requirements for pigs after weaning.

Table 1. Ingredients and calculated nutrient content of experimental diets

Ingredients (%)	**Weaned phase[1]**		
	Control diet	RSM diet	FRSM Diet
Corn	65.76	64.96	64.96
Soybean meal	24.00	15.00	15.00
Rapeseed meal	-	10.00	-
Fermented rapeseed meal	-	-	10.00
Corn gluten	1.5	1.5	1.5
Monocalcium phosphate	0.83	0.48	0.48
Limestone	1.40	1.45	1.45
NaCl	0.10	0.10	0.10
DL-Metionină	0.07	0.06	0.06
L-Lisine	0.23	0.34	0.34
Choline premix	0.10	0.10	0.10
Mineral vitamin-premix[2]	1.00	1.00	1.00
TOTAL	**100.00**	**100.00**	**100.00**

Table 2. Calculated nutrients content of experimental diets

Calculated Nutrient content	**Weaned phase**		
	Control diet	RSM diet	FRSM diet
Crude Protein (%)	19.00	18.95	18.95
Digestible protein (%)	12.07	11.94	11.94
Crude Fat (%)	2.97	2.95	2.95
Crude fiber (%)	3.33	3.70	3.70
ME (Kcal/kg)	3294	3228	3228
Lysine (%)	1.20	1.20	1.20
Digestible Lysine (%)	1.01	1.01	1.01
Met + Cys (%)	0.72	0.72	0.72
Calcium (%)	0.90	0.90	0.90
Phosphorus (%)	0.69	0.69	0.69

Rapeseed meal was fermented with commercial dry yeast *Saccharomyces cerevisiae* using the protocol described by (Plaipetch & Yakupitiyage, 2011; 2013) for 24 hours. The fermentation time (24 h) of rapeseed was chosen after performing two-time tests (24 and 72 hours). No changes in chemical composition were observed.

Animals and samples collection

The nutritional trial was performed on the IBNA experimental farm on 24 weaned TOPIG hybrid [(Landrace × Large White) × (Duroc × Pietrain) NORVEGIAN] piglets (8 piglets/group/two replicates per group and 4 pigs per replicate) with an average initial weight of 9.04 ± 0.19 kg for 21 days. After weaning (at 35 days) piglets housed in pens were acclimatized for one week before being used in the experimental protocol. They were individually eartaged and divided in three groups according with their body weight. During the experimental period feed and water was given *ad libitum* to the animals. At the end of experimentally 21 days pigs were euthanized and samples of organs were collected. Spleen samples were perfused with ice-cold physiological serum to remove blood and stored at -80^{0}C until analysed.

Feed chemical analysis

Chemical composition consisting in: dry matter, crude protein, crude fat, crude cellulose and ash, trace elements (calcium, sodium, potassium, magnesium, iron, zinc manganese, copper) of unfermented and fermented rapeseed meal as well as of the complete feed used in the experiment (control, RSM and FRSM) was analysed according to the International Standard Organization methods (SR ISO 6496/2001, Standardized Bulletin 2010, www.asro.ro), flame atomic absorption spectrophotometry with Zeeman background correction and graphite furnace (Pye Unicam, Thermo Electron, Solaar M6, Cambridge, UK). Fatty acids (SFA-PUFA) was determined by gas chromatography (Perkin Elmer, Clarus 500 USA) as described by (Taranu et al., 2018) and (Untea et al., 2012). Polyphenols were extracted in acetone 80% and methanol. Total polyphenols concentration was detected by using Folin-Ciocalteu method (Taranu et al., 2018).

Anti-nutrients such as intact glucosinolates and 3-butenyl isothiocyanate were extracted with water. After ultrasonication and centrifugation sample supernatants were diluted with water and injected into HPLC (Agilent Technologies 1200 Series, Morge, Switzerland) with G1315D DAD detector and G1316B TCC SL column thermostat. The chromatographic data were collected and processed using ChemStation software (version B.04.01, Waldbronn, Germany). The results were expressed as mg sinigrin/g dry sample. Sinigrin is a glucosinolate which was used as external standard.

Measurement of molecular mediators of immune response

Several important molecular mediators of immune response such as interleukine-1 beta (IL-1ß), interferon gamma (IFN-γ), interleukine-6 (IL-6), interleukine-8 (IL-8), tumour necrosis factor alpha (TNF-α) was measured in splenic lysates by ELISA using commercially kits (R & D Systems, Minneapolis, MN 55413, USA), according to the manufacturer's instructions. The lysates were prepared by homogenizing frozen spleen samples in buffer phosphate containing 1% IGEPAL, 0·5% sodium deoxycholate, 0.1% SDS and complete protease inhibitor cocktail tablets (EDTA-free). The supernatant obtained by centrifuging the homogenates was used to measure the total protein concentration with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). For the detection of interleukines in undiluted lysate supernatant, primary capture antibodies (anti-swine cytokines IL-1ß, IFN-γ, IL-6, IL-8, TNF-α) in conjunction with anti-swine biotinylated secondary antibodies, streptavidin-HRP (Biosource, Camarillo, USA) and TMB (tetramethylbenzidine, Sigma-Aldrich, S Louis, MO, USA) were used. Recombinant swine standard protein for IL-1ß, IFN-γ, IL-6, IL-8, TNF-α) diluted according to the manufacturer's instructions was used for standard curve generation. Results were expressed as picograms of cytokine/mL. The absorbance was measured using a Tecan microplate reader (Tecan, SunRise, Austria).

Measurement of oxidative and antioxidative response

Activity of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GpX) was measured by

using Cayman kits (Michigan, USA) as described by Taranu et al., (2022). Spleen samples (0.2g) were homogenized in specific chilled phosphate buffer indicated by the instruction of each Cayman kit, centrifuged and resulted supernatants were used for enzyme activity measurement. The absorbance was read at 540 nm (CAT), 440-460 nm (SOD) and 340 nm (GPx) using a microplate reader (Tecan Infinite M200, Salzburg, Austria). Lipid peroxidation was assessed by the determination of TBARS-MDA (thiobarbituric acid-reactive substances-malondialdehide). Briefly, spleen sample (0.2 g) homogenized with phosphate buffer was incubated at 95°C for 15 min to form TBARS adducts whose fluorescence was measured with a Tecan Sunrise, Austria and expressed as nmol/g spleen.

Statistical analyses

The results are presented as mean ± standard error of the mean (SEM). One-way ANOVA and *t-test* analysis (SAS Analytics, USA) followed by Fisher's procedure of the least square difference was used to measure the statistical differences between treatments. Differences were considered significant at P value <0.05 and were considered a trend at the P value between 0.05 and 0.1.

RESULTS AND DISCUSSIONS

Chemical composition of rapeseed meal

Rape seed meal is a rich source of protein and other nutrients such as unsaturated lipids, fiber and phytochemicals (e.g., polyphenols, organic acids, vitamin E, vitamin complex B, minerals (Chen et al., 2019; Wickramasuriya et al., 2015). It is considered as the second source of protein in the world after soybean meal (Xue et al., 2009). In our study, the chemical analysis detected a content of 33.51% crude protein in unfermented rapeseed meal. It contained also fiber (8.38%) and is rich in bioactive compounds: minerals (iron, 208.03 ppm, zinc, 495.91 ppm, manganese, 174.86 ppm), polyphenols (85.38 mg GAE/g total polyphenols), unsaturated fatty acids (oleic acid, 44.78 g/100g of FAME, linoleic acid, 31.37 g/100 g of FAME). The fermentation with *Saccharomyces cerevisiae* enriched the rapeseed meal in crude protein (35.08% fermented *vs* 33.51% unfermented, +4.7%), fiber (11.89% fermented vs 8.38% unfermented, +41.9%), minerals: iron (266.38 ppm fermented *vs* 208.03 ppm unfermented, +28.1%), zinc (536.52 ppm fermented *vs* 495.91 ppm unfermented, +8.2%) and manganese (226.42 ppm fermented *vs* 174.86 ppm unfermented, +29.5%) as described by (I. Taranu, Marin, D.E., Pistol, G.C., Untea, A., Vlassa, M., Filip, M., Gras, M., Rotar, C., Anghel, A.C., 2022). The most important improvement that yeast fermentation brought in the composition of rapeseed meal was the decrease level of anti-nutrients such as glucosinolates. Elevated level of glucosinolates was one of the reasons for which rapeseed meal was less used in animal feed in the past (Mejicanos et al., 2016). Many studies have shown a reduction in animals' performance, an impairment in the immune response and internal organ functionality because of their toxicity (Drazbo et al., 2018; Drażbo et al. 2020; Hu et al., 2016; Onarman Umu et al., 2018). The new varieties of rapeseed (e.g. canola) or various biotechnological treatments (e. g. fermentation) have led to a decrease in the concentration of glucosinolates and their negative effects on animal health and performance. Our results showed a significant reduction in glucosinolates concentration (1.70 mg *vs* 3.70 mg sinigrin/g) and one of their most abundant classes, 3-butenil- glucosinolate (4.10 mg *vs* 7.99 mg/g) in *S. cerevisiae* fermented rapeseed meal. Similarly, 50% reduction in glucosinolates content and one third in the content of 4-hydroxy-glucobrassicin was reported by (Maribo, 2012) after fermentation of rapeseed cake and canola with a mix of bran, soy molasses, lactic acid bacteria potato peel and water (Maribo, 2012) resulting in an increase of lactic acid by 5.6% compared to unfermented rapeseed. Moreover, Plaipetch & Yakupitiyage (2013) found 100% reduction of total glucosinolates and 17.5% of phytic acid after fermentation of canola meal with *Saccharomyces cerevisiae.* Yeast fermentation enhanced crude protein (+9.2%), fiber (+9.2%) and several minerals (iron, +26.2%, zinc, +32.1%, Copper, 25.4%).

Effect of FRSM diet on immune response mediators

Taken into consideration the changes produced by fermentation on rapeseed meal we further

investigated the effect of the diets with or without rape seed meal on several mediators of immune response in spleen as one of the most secondary important immune organs where the immune response is built (Jhun et al., 2013). It is the site of lymphocytes and monocytes/ macrophages producing antibodies and cytokines (Jia & Pamer, 2009) which are the effectors and mediators of the immune response and inflammation. Thus, in the study herein we analysed the effect of dietary rapeseed meal fermented or not on several inflammatory mediators such as interleukins in spleen tissue. Our results revealed a significant ($p = 0.05$) reduction in the concentration of interleukine-1 beta (IL-1β) and a decreasing tendency ($p = 0.098$) for IL-6 in spleen of piglets receiving fermented rapeseed meal diet in comparison to control diet (Figure 1). IL-1β and IL-6 secreted mainly by macrophages in different organs are significant mediators of inflammation and of tissue injury (Arend, 2002; Braunstein et al., 2020). IL-1β is one of the most important members of the interleukins family also involved in the modulation of the immune response by activating T and B lymphocytes (Cavaillon, 1996).

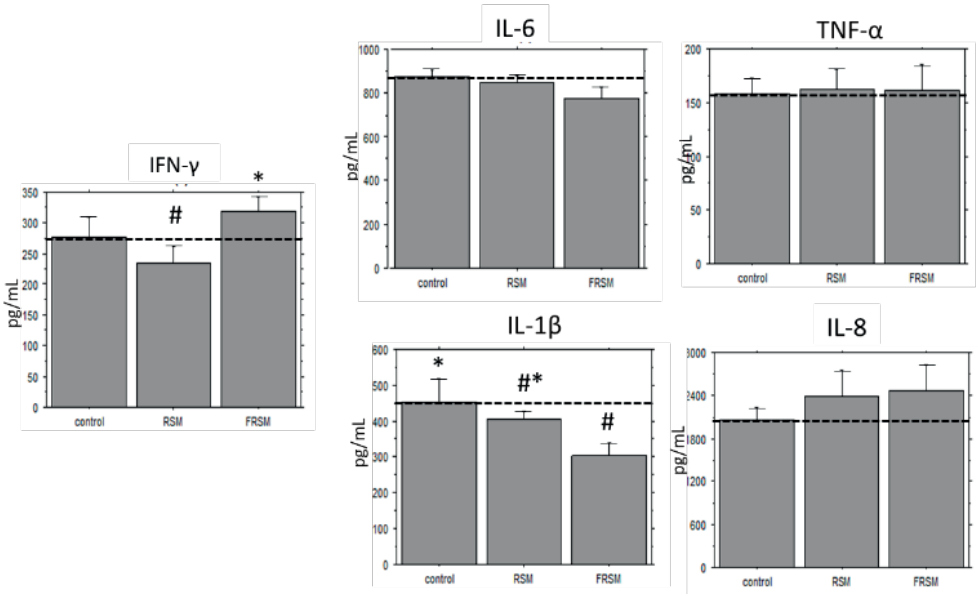

Figure 1. Effect of FRSM diet on inflammatory cytokines in spleen

Pigs received three different dietary treatments: basal diet (control), RSM (unfermented rapeseed meal) and FRSM diet (fermented rapeseed meal). The means value ± SEM were calculated and presented as histogram (n = 8). Statistical analysis was performed using one-way ANOVA followed by Fisher test (#P< 0.05)*

This result suggests that fermented rapeseed meal is able to counteract the transient inflamemation that might occur after weaning when piglets switch from the milk to solid feed and confront pathogenic infections. This might be due to the fact that fermentation modulated the level of different nutritional and anti-nutrients (decreased glucosinolates and increased minerals and n-6 and n-3 fatty acids, etc) with beneficial impact on animal health and could bring to the feed diet ß-glucans from the yeast wall. Municio et al. (2013) reported that differentiated macrophages form serum stimulated with ß-glucan produced low level of IL-1β and TNF-α (Municio et al., 2013). Also, an interesting study of (Liu et al., 2021) showed that phenethyl isothiocyanate, a degradation product of glucosinolates (GSLs) in a nontoxic dose (1.25-5 μM) was able to suppress the increase in pro-inflammatory cytokines (IL-1β, IL-6, IL-18, TNF-α) produced by 4 μM of deoxynivalenol (DON), a fusarium mycotoxin or by co-contamination with DON and *E. coli* -LPS. The study offered a basis for rational use of rapeseed meal in animal feed. Compared with unfermented rapeseed, FRSM diet significantly ($p =$

0.05) increased the concentration of IFN-γ, a cytokine with an essential role in antiviral and antibacterial defence being implicated in ROS production to respond in the case of infection (Awaad et al., 2011) as well as in the modulation of the immune response (Figure 1). An upward trend of IFN-γ level in FRSM group was also observed in comparison to the control, even if the difference was not significant. A slight non significantly increase in interleukin-8 (IL-8) concentration was noticed as well in both groups fed rapeseed meal fermented or not and no effect on TNF-α. Modulatory effects on immune mediators have been reported in other studies that have investigated the impact of feed sources rich in bioactive compounds. For example, active ingredients (e.g. proanthocyanidin) from grape seed and grape skin extract (GSSE) or cocoa extract diminished the Th17 cells and pro-inflammatory interleukin 17 (IL-17) in spleen (Bedhiafi et al., 2018) as well as the production of TNF-α, MCP-1, IL-6 and IL-8 in plasma and whole blood cells (Pérez-Cano et al., 2013). Moreover, grape proanthocyanidin significantly improved the weight and functions of important immune organs such as spleen and thymus and inhibited the growth of Sarcoma 180 tumour cells in mice (Tong et al., 2011).

Effect of FRSM diet on oxidative and antioxidative response

Beside the immune response, the antioxidant system is part of the body's defence which served to counteracts the harmful effect of excessive oxidants and include two main categories: enzymatic and non-enzymatic components (Birben et al., 2012). We further investigated the effect of dietary rapeseed meal either fermented or not on several antioxidant enzymes activity. Our results show a significant enhanced of superoxide dismutase and catalase activity in the spleen derived from piglets fed dietary fermented rapeseed meal compared to piglets fed control diet. The difference is also significant between fermented and unfermented groups (Figure 2). These results suggest that by increasing SOD and CAT activity the diet containing FRSM is able to counteract the accumulation of superoxide anion ($O^{2-\cdot}$ dismutation by SOD) and of hydrogen peroxide (H_2O_2 degradation by CAT), two major components of produced ROS (reactive oxygen species).

Although the activity of glutathione peroxidase, another antioxidant enzyme involved in the reduction of hydrogen peroxide and lipid peroxidation was not influenced by the FRSM diet, TBARS-MDA, a marker of lipid peroxidation decreased significantly in the spleen of piglets receiving FRSM diet. Compared to control this diet has a higher content of bioactive compounds (minerals, zinc and manganese, n-6, n-3 unsaturated fatty acids etc) that support the antioxidant system. Zinc for example is essential for the maintenance of redox homeostasis (Yi et al., 2022). Broiler chickens fed diet supplemented with 80mg/kg hot-melt extrusion (HME) processed zinc sulphate ($ZnSO_4$) had a higher SOD activity in serum and liver as well as reduced malonaldehyde (MDA) concentration compared to control (Lee et al., 2022). Unsaturated fatty acids, n-6 and n-3 are also recognised bioactive compounds with the capacity to modulate antioxidant system. (Avramovic et al., 2012; Lionetti et al., 2012; Taranu et al., 2014), demonstrated that n-3 PUFA supplements derived from camelina cakes, fish oil, or donkey's milk enhanced the activity of SOD, CAT, GPx, GST enzymes in spleen of pigs, brain tissue, and liver of rats and decreased MDA concentration. These studies indicated that the underlying molecular mechanism is based on the activation of nuclear factor 2 erythroid-related factor 2 (Nrf2) pathway and the inhibition of nuclear factor kappa B (La Marca et al., 2013; Lionetti et al., 2012), two important pathways which interfere in controlling the transcription of oxidative stress and inflammatory processes.

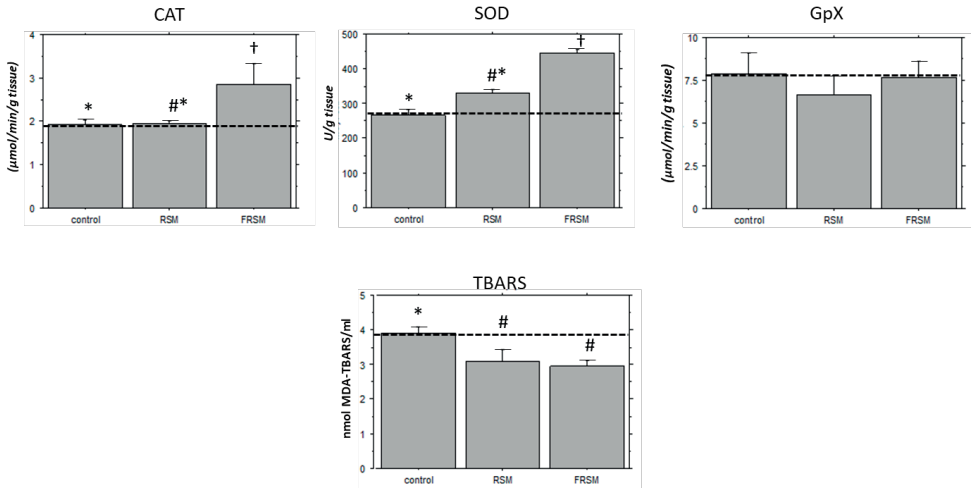

Figure 2. Effect of FRSM diet on oxidant and anti-oxidant markers

Pigs received three different dietary treatments: basal diet (control), RSM (unfermented rapeseed meal) and FRSM diet (fermented rapeseed meal). The means value ± SEM were calculated and presented as histogram (n = 8). Statistical analysis was performed using one-way ANOVA followed by Fisher test (#P< 0.05)*

CONCLUSIONS

In conclusion, our results showed that fermentation is efficacy to reduce the level of anti-nutrients by decreasing the concentration of glucosinolates by half in fermented rapeseed meal compared to unfermented. It also enriched the rapeseed meal in several bioactive compounds such as minerals, (iron, zinc, manganese), unsaturated fatty acids (n-6 and n-3) and fiber. The presence of these compounds in the diet containing 10% rapeseed meal positively impacted the immune and antioxidant response in spleen of piglets after weaning. Our results revealed a significant reduction in the concentration of interleukine-1 beta and IL-6, important mediators of inflammation as well as an increase in the concentration of IFN-γ, a cytokine involved in the antiviral and antibacterial response whose strengthening is crucial during and after weaning when piglets are confronted with many challenges (feed and environmental change, pathogen infection). An increase in antioxidant SOD and CAT activity and a reduction in lipid peroxidation was found in spleen of piglets fed FRSM diet with suggest also the capacity of FRSM diet to counteract the oxidative stress. Further investigations are needed to see the effect of other dietary inclusion rates of fermented rapeseed meal on immune and antioxidant status.

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