EXPLORING THE CAPACITY OF AGRO-INDUSTRIAL WASTE TO COUNTERACT THE TOXIC EFFECTS OF MYCOTOXINS

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Abstract

Mycotoxins are fungal secondary metabolites, which threaten the lives of both animals and humans. The decontamination of cereal crops and fruits is almost impossible due to their physical and chemical stability. Zearalenone (ZEA) is a fusariotoxin that can cause hormonal, digestive, neurological or immune dysfunctions, pigs being one of the most affected species. Agro-industrial waste could be a viable solution to counteract the mycotoxins toxic effects as they are rich in bioactive compounds (polyphenols, polyunsaturated fatty acids etc) with anti-inflammatory, antioxidant and anti-microbial properties. Our results have shown that rapeseed meal could alleviate the proinflammatory effects induced by ZEA at the gut level. However, the mechanisms involved in this process are not known, therefore the aim of our ex vivo study was to investigate the signaling pathways responsible for the ability of rapeseed meal (unfermented-RSM or fermented with Saccharomyces cerevisiae-FRSM) to counteract the inflammatory response triggered by ZEA in porcine jejunum explants. The explants collected from the jejunum of healthy piglets were incubated in DMEM/F12 HAM medium and treated or not with ZEA 100µg/mL and with methanolic extracts of RSM/FRSM for 4 hours. Gene expression of a couple of signaling molecules involved in the innate immunity (TLR2, TLR4, TLR5, TLR9, MyD88, MD2) and inflammatory response (Nrf2, IRAK1, TRAF6, TAK1, TGFβ2, AP1, AKT), also of MAPKs (ERK1/2, JNK1/2, p38α) which regulates the activity of pro-inflammatory cytokines were evaluated. Our results showed that 100µg/mL of ZEA increased the gene expression of TAK1, TGFβ2, NF-κB, JNK2 and p38α MAPK. The main mechanism involved seems to be the activation of TLR4/MyD88/MD2 signaling pathway due to the up regulation of these gene expressions by ZEA. The addition of RSM/FRSM extracts was able to significantly reduce the effects of ZEA on the analyzed signaling molecules, to the control levels, mainly through the inhibition of the TLR4/MyD88/MD2 signaling pathway.

Keywords: *zearalenone, rapeseed meal, inflammatory response, signaling pathways, jejunum porcine explants*

1. INTRODUCTION

Secondary metabolites known as fusariotoxins are produced by the fungus *Fusarium* and *Gibberella* species, which together generated over 140 varieties of mycotoxins. Among these, zearalenone, fumonisins, and trichothecenes are the most common mycotoxins having well-demonstrated toxic effects. Mycotoxin strategies for the mitigation of mycotoxins effects, crops being inevitably exposed to them, and extremely few decontamination methods are available.

Zearalenone (ZEA) shares structural similarities with β-estradiol and binds to estrogen receptors, making it a fusariotoxin that falls within the xenoestrogen class (Jia et al., 2020). It has been shown that ZEA, particularly in pigs, promotes hormonal and reproductive abnormalities as well as other reproductive disorders. Additionally, ZEA affects the immunological, neurological, and digestive systems, resulting in symptoms like leukopenia, vomiting, diarrhea, and decreased appetite (Grosu et al., 2023; Reddy et al., 2018; Rogowska et al., 2019).

ZEA is quickly absorbed by mammals (pigs, humans, rabbits, and rats 80–85%) upon oral contamination (Zinedine et al., 2007), the digestive system being organism`s first physical and immunological barrier. According to recent studies, ZEA targets the gastrointestinal tract and causes pathological intestinal alterations (Lahjouji et al., 2020). The natural anatomic structure of the intestine may be affected by

ZEA, depending on many parameters such as specie, sex, dose, and duration of exposure (Lewczuk et al., 2016). In several organs, including the intestines, exposure to ZEA has been linked to decreased cell viability, apoptosis, and necrosis (Johansson and Hansson, 2013). However, regarding the intestinal inflammatory response is unclear how ZEA affects it, most likely due to different experimental models and ZEA doses that have been used in different studies (Bulgaru et al., 2021).

The European Commission through the Recommendation EC/2006/576 states that the maximum allowable levels of ZEA in feed are 0.100 mg ZEA/kg feed for piglets and 0.250 mg ZEA/kg feed for mature pigs (COMMISSION RECOMMENDATION (EU) 576/2006, 2006). More toxicological data are required to establish the maximum level of ZEA that can be admitted in the swine feed. However, although the establishment of norms is essential for the health of pigs, it is also very important to find ways to alleviate the toxic effects of ZEA. A solution could be the inclusion in feed of some agro-industrial wastes rich in bioactive compounds like polyphenols, vitamins, polyunsaturated fatty acids etc. There are studies that have demonstrated the capacity of some industrial by-products such as grape seed meal (Taranu et al., 2020), banana peel (Zavala-Franco et al., 2018), sea buckthorn meal (Popescu et al., 2023) or olives (Sousa et al., 2018) to alleviate the negative effects of mycotoxins.

A popular source of protein for animal feed, rapeseed meal also contains fiber, carbohydrates, phenolic compounds, flavonoids, and minerals that could supplement the nutritional value of the diets (Sharaf eldin et al., 2018). According to several research, rapeseed meal has a high quantity of polyphenolic compounds with strong antioxidant and detoxifying activities (Di Lena et al., 2021). Within the phenolic compounds, free phenolic acids (gallic, cinnamic, gallic, caffeic, chlorogenic, ferulic, p-coumaric, syringic, ellagic, vanillic etc.) were found in rapeseed extracts (Szydłowska-Czerniak et al., 2010). These acids are natural antioxidants that are important for managing and preventing a variety of chronic disorders (Yates et al., 2019). Pig growth performance, blood profiles, and nutritional digestibility are all generally improved by rapeseed meal (Choi et al., 2015). However, anti-nutritional factors like glucosinolates or tannins impair the nutrient digestibility and valorization of rapeseed meal (Tripathi and Mishra, 2007). This problem can be resolved by fermenting the rapeseed meal, this process resulting in a reduction in the quantities of glucosinolates, tannin, phytic acid and isothiocyanates (Hu et al., 2016).

Starting from these data, the aim of our *ex-vivo* study was to investigate the signaling pathways responsible for rapeseed meal's (unfermented-RSM or fermented with *Saccharomyces cerevisiae*-FRSM) ability to counteract the inflammatory response triggered by ZEA in porcine jejunum explants. This was carried out in considering the need to clarify the effect of ZEA at the intestinal level as well as the need to find a way to mitigate its toxicity. The chemical composition of the two types of rape seed used in this experiment was described by Vlasssa et. al. (Vlassa et al., 2022), who showed that fermentation led to a significant decrease in anti-nutritional factors such as glucosinolates and 3-butyl isothiocyanate. Moreover, this process increased the amount of compounds that may be great for the animals' health, with higher levels of proteins, zinc, copper and polyphenolic compounds such as gallic acids and synaptic acid being reported compared to the unfermented rape seed. Explants collected from the jejunum of healthy piglets were cultured in DMEM/F12 HAM media and treated or not with ZEA 100µg/mL and methanolic extracts of RSM/FRSM for four hours. Gene expression of some junctional proteins specific to the intestinal epithelium's integrity (*Claudin 4, Occludin, Zonula 1*), innate immunity (*TLR 2, TLR 4, TLR 5, TLR 9, MyD 88, MD 2*), pro-inflammatory cytokines (*TNF-α, IFN γ, IL-1β, IL-6, IL-8*), antioxidant enzymes (*CAT, SOD, GPx*), also of signaling molecules involved in oxidative and inflammatory responses (*NF-kB, Nrf2, IRAK1, TRAF6, TAK1, TGFβ2, AP1, AKT*), as well as MAPKs (*ERK1/2, JNK1/2, p38α*) that control the production of pro-inflammatory cytokines, were assessed by real-time PCR.

2. MATERIALS AND METHODS

2.1. Explant collection

The explants were collected from three weaned piglets with respect to Romanian Law 206/2004 and also with EU Council Directive 98/58/EC. After slaughter, the intestinal contents were emptied and washed, the explants being cut from the jejunal passage using 8 mm biopsy punches. The explants were placed in 6-well culture plates with 2 ml of DMEM/F12 HAM (Sigma) medium supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin (Sigma), 1% L-glutamine (Gibco BRL) and 1% ITS (Insulin/Transferin/Selenium, Gibco BRL). Each experiment lasted 4 hours, the treated tissues were cultivated at 37° C, in an 5% CO_2 atmosphere, under continuous stirring.

2.2. Reagents and experimental design

The explants were treated or not with ZEA 100 µg/mL and with methanolic extracts of RSM/ FRSM. ZEA (FERMENTEK, Jerusalem, Israel) was diluted in DMSO according to the manufacturer's instructions and diluted in medium to the final concentration of 100 ug/ml, and the methanolic extracts of RSM/FRSM were prepared according to the protocol described by (Vlassa et al., 2022), the final ratio used as treatment being 1/10. The resulting experimental groups were: 1) Control, 2) RSM, 3) FRSM, 4) ZEA 100 ug/ml, 5) ZEA 100 ug/ml+ RSM and 4) ZEA 100 ug/ml+ FRSM.

2.3. The quantification of gene expression

The real-time quantitative PCR (qPCR) method was used to measure the expression of gene coding for several molecules involved in the innate immunity and inflammatory response. After the incubation, the explants were collected and homogenized in 700µl QIAzol® Lysis Reagent (QIAGEN, GmbH, Germany). Following the manufacturer's instructions, the miRNeasy Mini kit (QIAGEN, GmbH, Germany) was used to extract the total RNA. The amount of isolated RNA was determined using the NanoDrop spectrophotometer. The following step involved was the use of the M-MLV Reverse Transcriptase Kit (Life Technologies, Carlsbad, USA) to reverse transcribe RNA into complementary DNA (cDNA). To achieve a final volume of 26 µL, 500 ng of RNA was combined with 0.5 µg/mL of Oligo(dT)12-18, 0.5 µg/mL of dNTP, and nuclease-free water. The mixture was incubated at 65ºC for five minutes, and then at 4ºC for another five minutes. Subsequently, each tube was filled with 12 µL of a mix (2:1 of 5X reaction buffer: DTT solution) and incubated for 2 minutes at 37 ºC. The final step was adding 2 µL of M-MLV Reverse Transcriptase Enzyme, incubating for 50 minutes at 37ºC, inactivating the enzyme for 15 minutes at 70ºC, and cooling the mixture to 4°C. With the Rotor-Gene-Q system (QIAGEN GmbH), gene expression of junctional proteins specific to the integrity of the intestinal epithelium (*Claudin 4, Occludin, Zonula 1*), pro-inflammatory cytokines (*TNF-α, IFN γ, IL-1β, IL-6, IL-8*), antioxidant enzymes (*CAT, SOD, GPx*), signaling molecules involved in the innate immunity (*TLR 2, TLR 4, TLR 5, TLR 9, MyD 88, MD 2*) and inflammatory response (*Nrf 2, IRAK 1, TRAF6, TAK1, TGFβ2, AP1, AKT*), also of MAPKs (*ERK1/2, JNK1/2, p38α*) which regulates the activity of pro-inflammatory cytokines were assessed. Using 10 ng of cDNA sample, 0.3 µM primers for the genes of interest, 10 µl SYBRGreen qPCR Master Mix (Life Technologies, CA, USA), and nuclease-free water, a reaction volume of 20 µL was achieved. After two minutes at 50°C, ten minutes at 95°C, forty-five cycles of 60°C and fifteen seconds at 95°C, and an elongation at 72°C, the incubation process was completed. Using the Normfinder software we choose two reference genes from a set of six to normalize the data. The results were presented to the control group and expressed as Fold Change (FC), using the $2^{(-\Delta\Delta C)t}$ calculating method.

2.4. Statistical analysis

The obtained data were presented as mean ± SEM. Utilizing GraphPad Prism (9.3.0) and StatView software, the one-way Anova test and Fisher's test were used to evaluate the statistical differences between the experimental groups. P values less than 0.05 were deemed to be statistically significant, whereas those less than 0.1 indicated a trend.

3. RESULTS

3.1. The effects of ex vivo treatments on the integrity of the intestinal epithelium

Three junction proteins' gene expressions were measured in order to assess the impact of ZEA, RSM, FRSM, and their combination on the integrity of the intestinal epithelium: *Zonula 1 (ZO 1)*, *Occludin* and *Claudin 4*. As can be seen in Figure 1, the exposure of the explants to ZEA did not produce significant changes, neither did the two rape seed extracts RSM and FRSM respectively. However, co-exposure to ZEA and unfermented rape seed extract significantly increased the mRNA level of all studied junction proteins. However, co-exposure to ZEA and non-fermented rapeseed significantly increased the mRNA level of all studied tight junction proteins, while co-exposure to ZEA and fermented rapeseed did not produce significant changes (Figure 1).

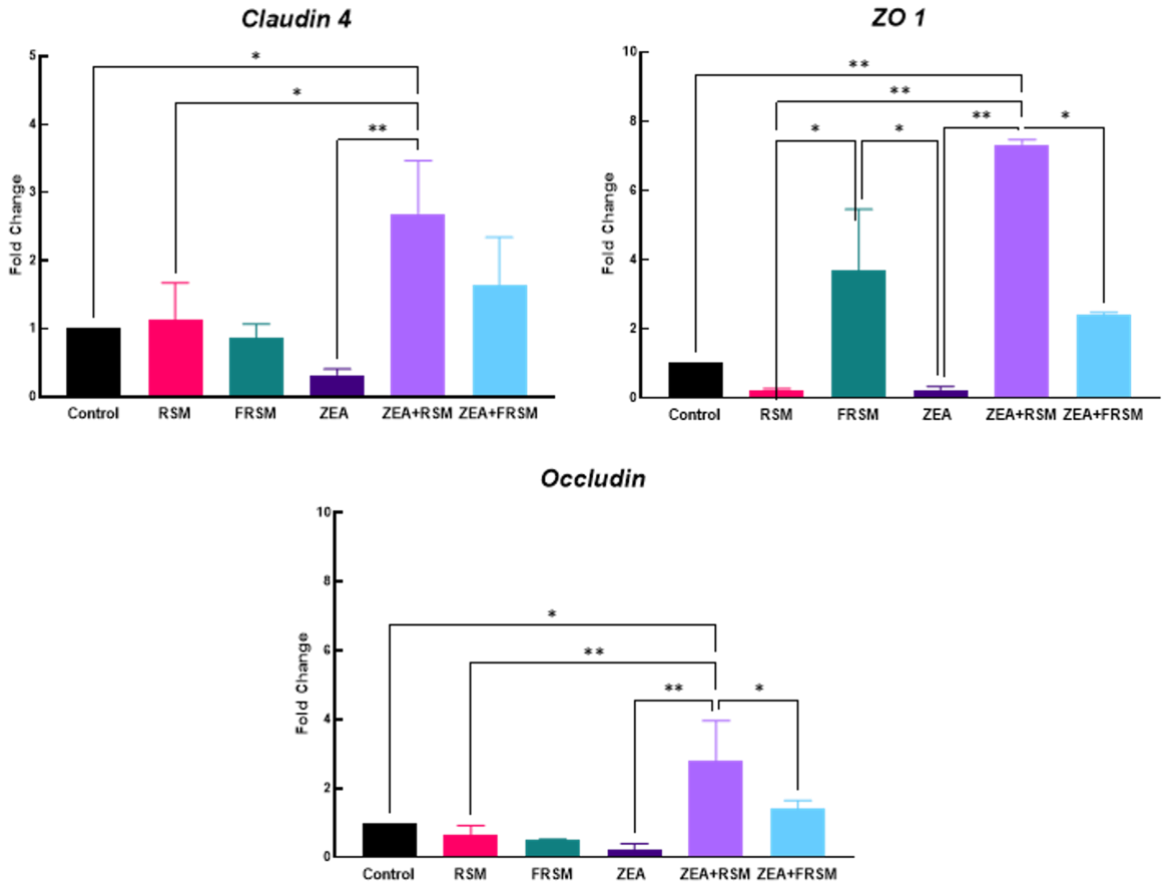

Figure 1. The effects of experimental treatments on junction proteins Claudin4, Zonula1 (ZO 1) and Occludin

3.2. The effects of ex vivo treatments on the innate immunity markers

Examining the effects of ZEA, RSM, FRSM and their combination on the innate immune response-related gene expression of several TLRs, we found that ZEA significantly increased the expression of TLRs 2 and 4, but not TLRs 5 or 9 (Figure 2). The addition of both RSM and FRSM was able to attenuate the effects of ZEA, in both cases a decrease in the gene expression of TLR2 and TLR 4 was observed, at levels toward to the control (Figure 2). Furthermore, ZEA was shown to significantly increase not only the gene expression of TLRs 2 and 4, but also the expression of MYD88 and MD2, indicating that the primary mechanism of ZEA toxicity is the activation of the TLR4/MYD88 signaling pathway. Similarly,

the addition of RSM/FRSM reduced the effects of ZEA, decreasing gene expression of both MYD88 and MD 2 (Figure 2).

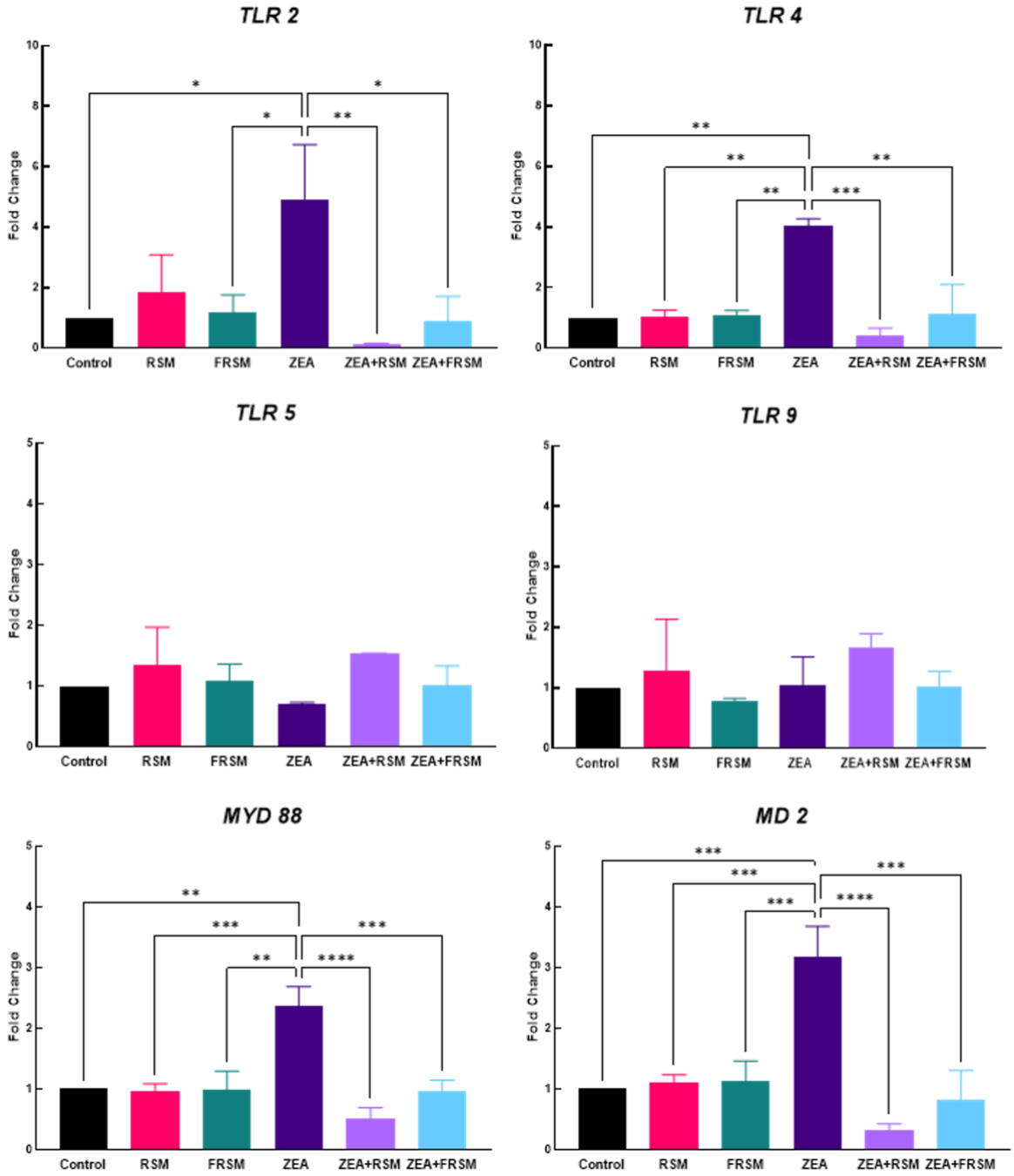

Figure 2. The effects of experimental treatments on gene expression of innate immunity markers

3.3. The effects of ex vivo treatments on the antioxidant response markers

Since the imbalance between antioxidants and oxidants greatly affects the oxidative state, we examined how ZEA affected the expression of the genes encoding the antioxidant enzymes glutathione peroxidase (*GPx*), catalase (*CAT*), and superoxide dismutase (SOD), but also the ability of the two rapeseed meal extracts to alleviate the toxic effects of ZEA. The exposure of intestinal explants to ZEA did not lead to

a significant increase in GPx, CAT or *SOD* gene expression (Figure 3). Similar results being obtained for the co-exposure to ZEA+RSM and ZEA+FRSM (Figure 3). The only significant change observed is an increase in *CAT* in the case of exposure of explants to FRSM.

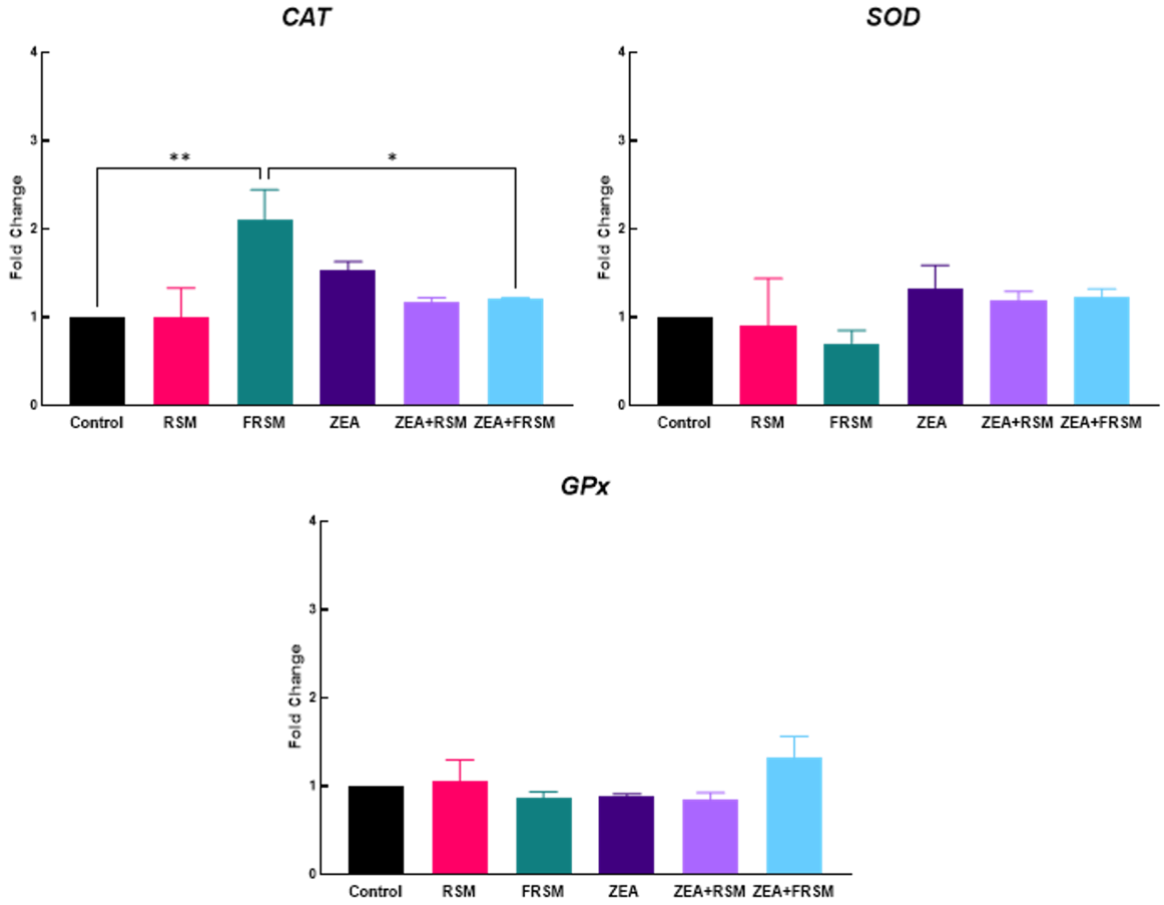

Figure 3. The effects of experimental treatments on gene expression of antioxidant enzymes CAT, SOD and GPx

Next, we analyzed the gene expression of *Nrf2*, the nuclear receptor involved in modulating the antioxidant response, but also of other signaling molecules such as *HO 1*, *NQO 1* and *KEAP1*, significant markers of the primary regulatory KEAP1/Nrf2 pathway, which initiates the intracellular defense mechanism against oxidative damage. The results presented in Figure 4 showed that the exposure of porcine jejunal explants to ZEA led to a significant decrease in *Nrf2* gene expression, without significant changes in the other signaling molecules *HO 1*, *NQO 1* and *KEAP1 mRNAs* (Figure 4). Fermented rapeseed methanol extract did not produce significant changes either alone or together with ZEA, but unfermented rapeseed extract resulted in a significant increase in *Nrf2 gene expression*, and RSM+ZEA co-exposure resulted in a significant increase in the levels of *HO1*, *NQO 1* and *KEAP1 mRNAs* (Figure 4).

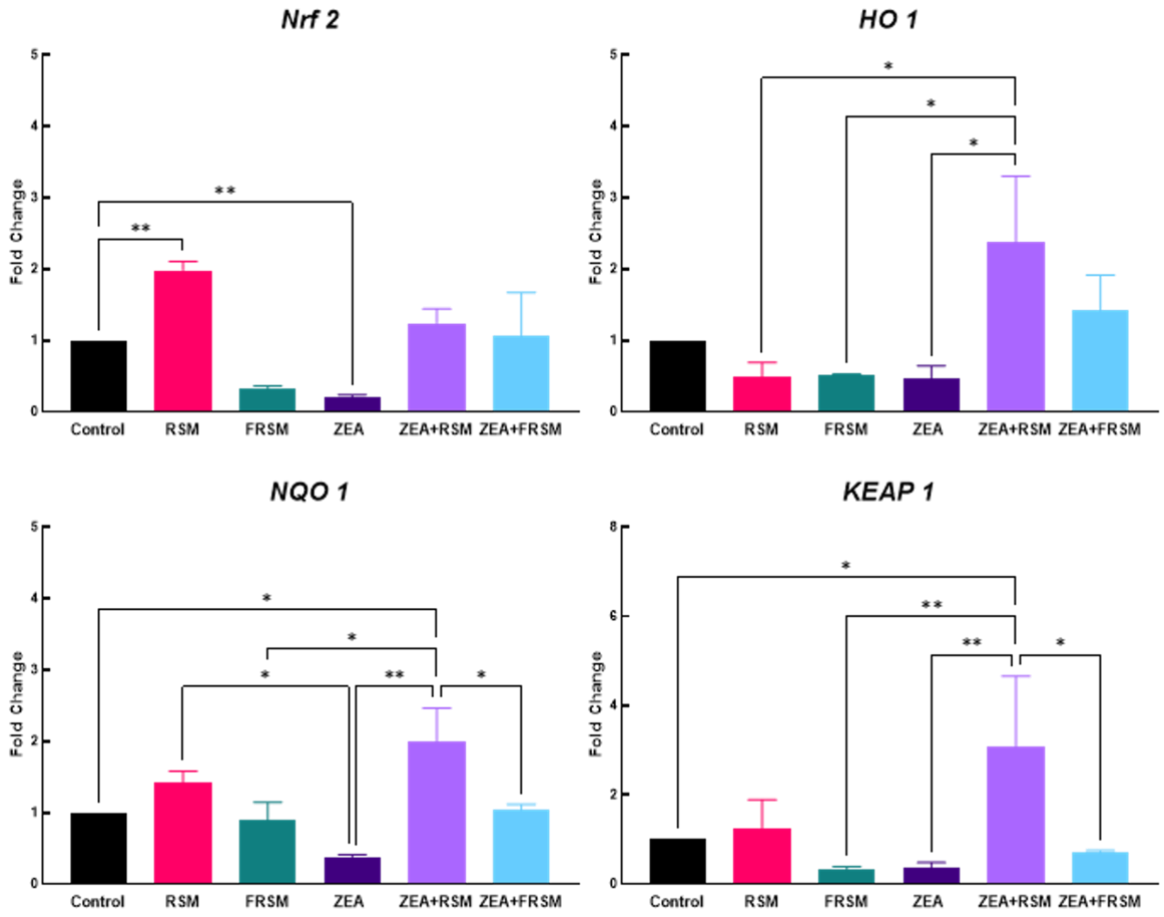

Figure 4. The effects of experimental treatments on gene expression of signaling molecules involved in antioxidant response

3.4. The effects of ex vivo treatments on markers of inflammatory response

The results obtained by qPCR showed that ZEA was able to induce a significant increase in the gene expression of *TNFα*, *IFN γ*, *IL 1β*, *IL 6*, *IL 8* pro-inflammatory cytokines, but also in the gene expression of the nuclear receptor *NF-kB*, nuclear receptor involved in the regulation of oxidative damage and inflammation (Figure 5). Both RSM and FRSM were able to reduce the gene expression of the inflammatory molecules in jejunal explants co-treated with ZEA and RSM/FRSM (Figure 5);

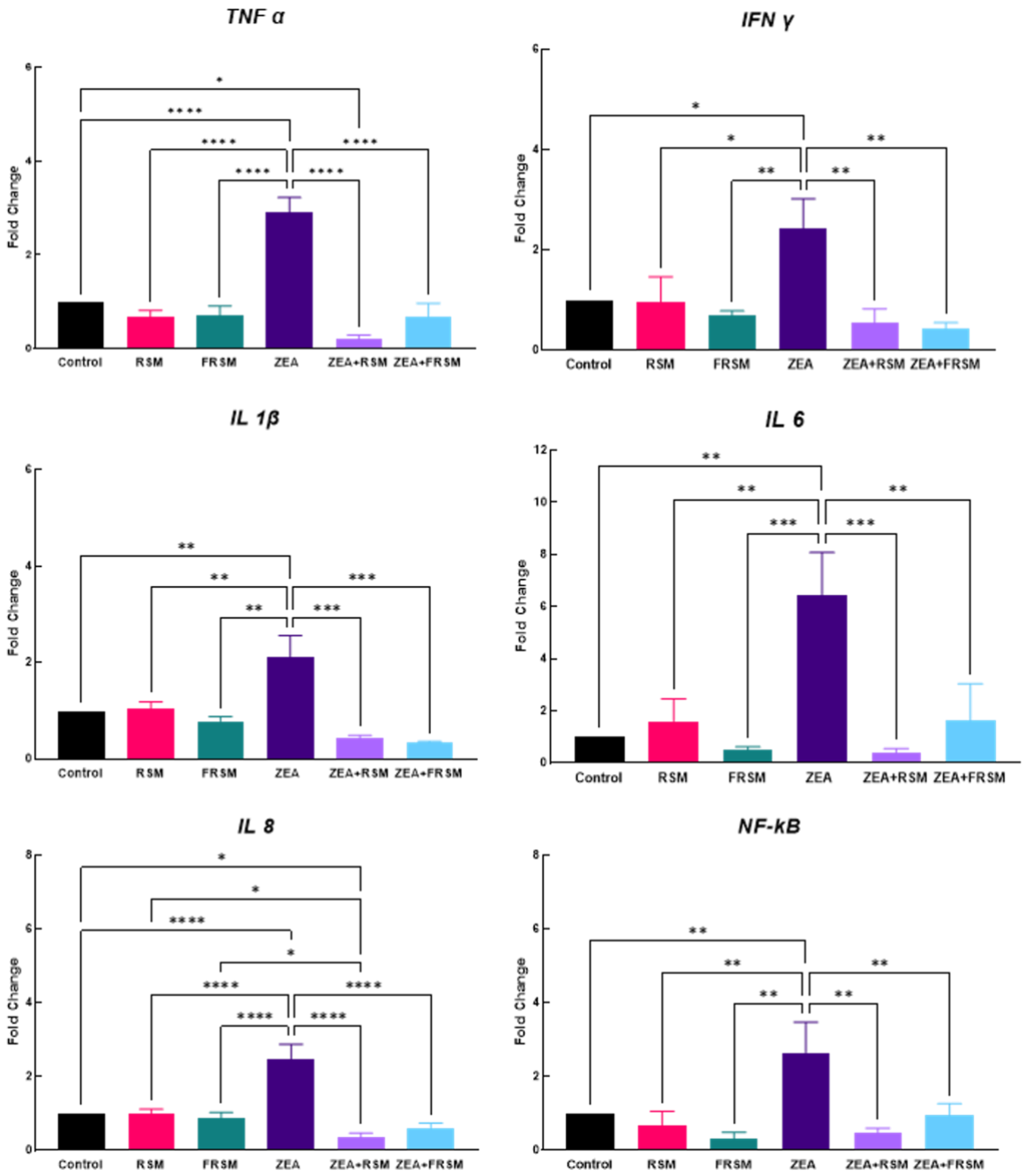

Figure 5. The effects of experimental treatments on gene expression of pro-inflammatory cytokines (TNFα, IFN γ, IL 1β, IL 6, IL 8) and signaling molecule NF-kB (de scris genele in italic)

3.5. The effects of ex vivo treatments on markers of signaling pathways related to inflammation and immune response

Analyzing the gene expression of MAPKs, we observed a significant increase in *p38α* and *JNK 2* under the influence of ZEA, similar increases being observed in the case of other signaling molecules involved in triggering the inflammatory response such as *TAK1* and *TGFβ*. These effects were reverted in ZEA+RSM/FRSM co-exposed jejunal explants (Figure 6). RSM and FRSM alone did not induce significant changes in the expression of genes coding for *ERK1/2*, *p38* and *JNK2* MAPKs, and for *TRAF6*, *TAK1* and *AKT* signaling markers (Figure 6). By contrast, the co-exposure of jejunal explants

to ZEA and RSM/FRSM induced significant increases in the levels of *TRAF6*, *AP1* and *AKT* mRNAs (Figure 7).

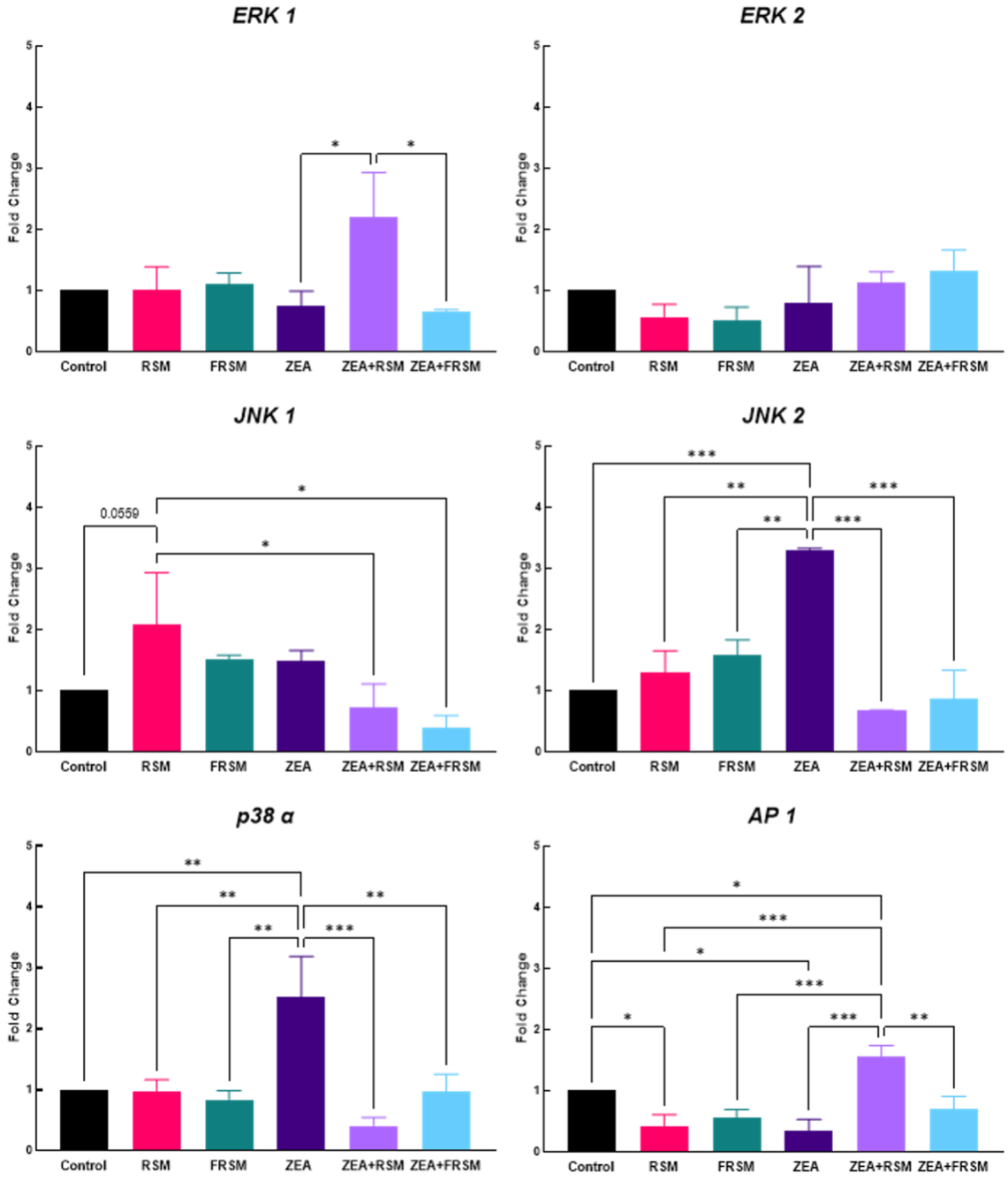

Figure 6. The effects of experimental treatments on gene expression of MAPKs (ERK1/2, JNK1/2, p38α) and molecules involved in signaling pathways (AP 1, IRAK, TRAF 6, TAK 1, AKT, TGFβ 2)

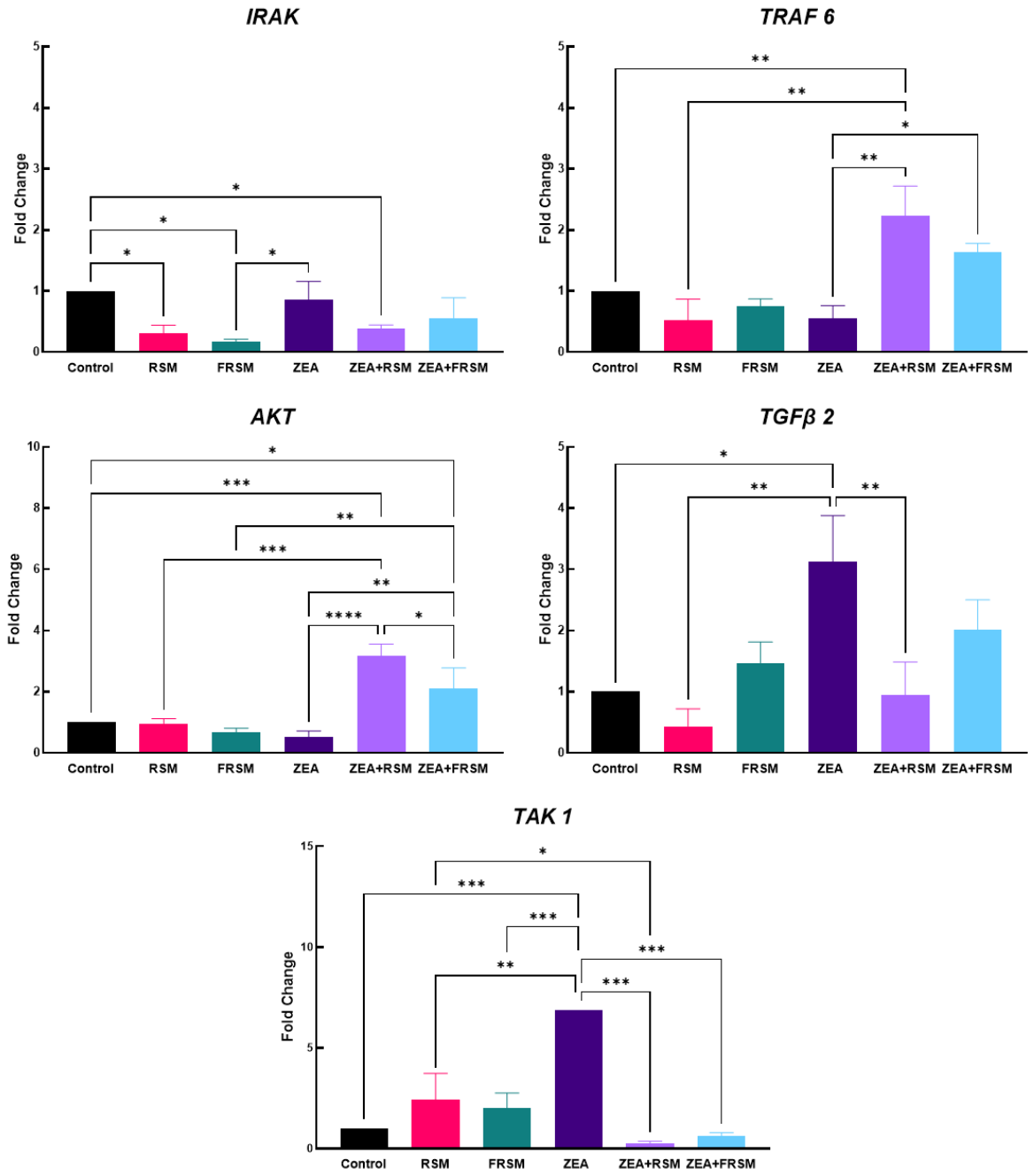

Figure 7. The effects of experimental treatments on gene expression of MAPKs (ERK1/2, JNK1/2, p38α) and molecules involved in signaling pathways (AP 1, IRAK, TRAF 6, TAK 1, AKT, TGFβ 2)

4. DISCUSSIONS

The ZEA fusariotoxin is a frequent contaminant of cereal crops and products generated from them. Because of its molecular resemblance to 17β-estradiol, ZEA is regarded as a xenoestrogenic molecule in addition to providing stability and temperature tolerance (Frizzell et al., 2011). ZEA primarily targets the reproductive system because of its affinity for estrogen receptors, although adverse effects have also been noted in the hepatic, neurological, and digestive systems. Since oral ingestion is the primary method of exposure for piglets to ZEA, the effects it has on the intestines are crucial. Moreover, due to the role of the jejunum in the absorption of nutrients from food, but also of water, the study of the effects and mechanisms of action of ZEA at this level, as well as the discovery of potential solutions to

overcome its toxicity, are important, especially in piglets, which among the animals from the farm are exposed daily to mycotoxin contamination (Obremski et al., 2008). The intestinal explants, containing all wall structure (the whole intestinal mucosa and submucosa layers), are a valuable model for studying the effects of nutrients and contaminants in intestinal environment, due to the preservation of the interaction between different cells and compartments in this type of *ex vivo* system. That is why in the present study we aimed to investigate the effects of nutrients (RSM and FRSM) and of contaminants (ZEA) using porcine jejunal explants.

Inflammation is closely linked to any disruption of the innate immune response, which is in charge of identifying and eliminating any foreign microorganisms (Koenderman et al., 2014). Glycoproteins called toll-like receptors (TLRs), which are found on the surface of cells or in intracellular vesicles, are crucial for identifying and attaching the pathogens. They also trigger the body's defense systems, including the production of chemokines or cytokines (Medzhitov, 2001). Also, TLRs form complexes with membrane proteins like MYD88 and MD2, leading to the activation of TLR4/MYD88 signaling pathway. Our study showed that in porcine jejunal explants ZEA produces a significant increase in *TLR 2*, *TLR 4* gene expression, but also in the expression of the signaling molecules *MYD 88* and *MD2* mRNAs. Both RSM and FRSM were able to reduce these effects, but values closer to those of the control were observed in the case of fermented rapeseed meal. There are several studies that indicate the toxicity of ZEA through the TLR 4/MYD88 pathway, similar results to this study being observed in *in vivo* studies. A significant increase in *TLR 4* and *MYD88* gene expressions was observed in the kidneys of rats (Jia et al., 2014) and the colon of piglets (Bulgaru et al., 2023).

When TLRs are activated, adaptor molecules like MyD88, TRAF 6, and IRAK are involved in a signaling cascade regulated by MAPKs (p38α, JNK1/2, ERK1/2), which is triggered by the activation of the IRAK or MYD88-dependent signaling pathway. This process activates transcription factors like AP-1 and NF-*k*B, which are important modulators of cytokine production (Kumar et al., 2009). After analyzing the gene expression of MAPKs, we found that ZEA significantly increased the expression of *p38α* and *JNK 2*. Other signaling molecules that are important in inducing the immunological response, like *TAK1* and *TGFβ2*, also showed significant increases in ZEA-treated jejunal explants. The addition of fermented rape seed meal was able to reduce the effects of ZEA.

The activation of the TLR4/MYD88 pathway could be correlated with the induction of oxidative stress. Analyzing the effects on the gene expression of antioxidant enzymes, we did not observed ZEA induced effects. Although some results obtained on the liver of piglets show that ZEA could induce an increase in the antioxidant enzymes *CAT* and *GPx* (Marin et al., 2013), there are contradictory data that show that ZEA can also induce a decrease in the activity of the antioxidant enzymes in ileum, duodenum, and jejunum of piglets (Liu et al., 2020). Additionally, we examined the gene expression of several signaling molecules, including *HO 1*, *NQO 1*, and *KEAP1*, which are important indicators of the primary regulatory KEAP1/Nrf2 pathway, involved in the intracellular defense mechanism against oxidative damage. Porcine jejunal explants exposed to ZEA showed a substantial reduction in *Nrf2*, but not in the other signaling molecules. RSM+ZEA co-exposure significantly increased *HO1*, *NQO 1*, and *KEAP* gene expression, showing again that RSM could have a toxic effect synergic with ZEA.

The inflammatory response has a strong connection to oxidative stress. The effects of ZEA on some inflammatory markers such as cytokines are intensely debated in specialized literature. While some studies indicate an anti-inflammatory character of ZEA (Marin et al., 2011), there are other studies that show that ZEA can produce a significant increase in the levels of TNF-α, IL-1α, IL-1β, IL-6, IL8, IL10 (Benthem de Grave et al., 2021; Marin et al., 2015). Through qPCR, our results demonstrated that ZEA could not only significantly increase the expression of the nuclear receptor *NF-kB*, a crucial marker of the signaling pathway linked to oxidative damage and inflammation, but also the gene expression of pro-inflammatory cytokines *TNFα, IFN γ, IL 1β, IL 6,* and *IL 8*. Both RSM and FRSM were effective in lowering the gene expression of the inflammatory molecules; however, the gene expression observed in explants exposed to ZEA+FRSM was lower than that of the control, suggesting that FRSM would be a more effective strategy to counteract the inflammatory effects of ZEA.

The difference between the effects of FRSM and RSM in reducing the toxic effects of ZEA could be due to the different chemical composition of the two rapeseed meals. It was observed that the fermentation led to a significant decrease in glucosinolates concentration (antinutritional factors), but also in the concentration of organic acids such as citric, tartaric, malic acid. Moreover, the fermentation led to an increase in the concentration of succinic and fumaric acid, but also of flavonoids: epicatechin and ferulic acid, compounds with proven beneficial activity.

5. CONCLUSIONS

The issue of contamination with mycotoxins continues to be topical, especially since neither their toxic effects nor their mechanisms of action are fully known. Zearalenone represents a major problem especially in the case of pigs that are exposed daily to contaminated feed. This fusariotoxin does not only target the endocrine system, but is able to affect the digestive, neurological and immune systems as well. Currently, there is a need to clarify its effects and mechanisms of action, but also to find ways to mitigate the effects of ZEA exposure. The solution proposed in the present paper is the use of rape seed meal, which, due to its composition rich in bioactive compounds, could counteract the effects of ZEA.

Our results showed that in the case of porcine jejunal explants, both unfermented rapeseed meal and rapeseed meal fermented with *Saccharomyces cerevisiae* were able to reduce the toxic effects of ZEA, their effects being exerted in jejunal explants more specifically at the level of TLRs and their related signaling pathways, but also at the level of pro-inflammatory cytokines. However, additional studies are needed to confirm the results obtained by qPCR.

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