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Feed additives can differentially modulate NF-κB (RelA/p65), IGF-1, GLUT2, and SGLT1 gene expression in porcine jejunal explants

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ABSTRACT - The intestinal gene expression of RelA/p65 (NF-κB), insulin growth factor 1 (IGF-1), glucose transporter 2 (GLUT2), and Na+/dependent glucose transporter 1 (SGLT1) were evaluated in response to benzoic acid, yeast culture, L-glutamine, and oregano essential oil, using an *ex vivo* model. Six piglets weighing approximately 20 kg each were sacrificed, and their jejunum was collected and segmented into five 2-cm explants. Each explant was immersed in cell culture medium according to one of the following treatments: control (without additive), 0.5% benzoic acid, 1% yeast culture, 1% L-glutamine, and 0.015% oregano oil. Gene expression was evaluated using RT-PCR. Yeast culture up-regulated the gene expression of RelA/p65, IGF-1, GLUT2, and SGLT1 in comparison with control. In addition, jejunal exposure to L-glutamine and oregano oil increased mRNA levels of GLUT2 compared with the control treatment. Exposure to oregano oil increased intestinal SGLT1 gene expression, while benzoic acid reduced SGLT1 expression compared with the control. Feed additives can differently modulate the gene expression of immune response, gut development, and glucose absorption in jejunal explants. These findings can contribute for a better understanding of the trophic action of these feed additives into the diets to optimize animal performance.

Key Words: chemosensors, glucose, piglet, RT-PCR, swine, trophic effects

Introduction

The early weaning of piglets is a common practice to increase sow productivity and reduce costs. However, early weaning can lead to intestinal inflammation and decreased intestinal integrity, which compromises nutrient digestion and absorption until the adaptation to a solid diet (Lackeyram et al., 2010). Avoiding impaired intestinal integrity is crucial to prevent perturbation of brush-border enzyme activity, loss of protein synthesis, and reduced gene expression of mucosal DNA and RNA, which cause lower gene expression resulting in reduced nutrient absorption due to decreased villus height and increased crypt depth (Khan and Islam, 2012) caused by the lower production of digestive enzymes and lower contact area with the feed. The ingestion of feed additives that efficiently re-establish intestinal health, especially during early weaning period, has been target of some investigations (Bahar et al., 2012; Tucci et al., 2014; Boroojeni et al., 2018), especially after the ban of antibiotics use as growth promoters in the Europe Union in 2006.

The focus on past feed additive research was to identify products with antimicrobial effects to replace antibiotics used as growth promoters (Kommera et al., 2006; Michiels et al., 2009). The emphasis of recent research has been to understand the other effects of feed additives, including the functional balance of immune response, development of gut, and glucose absorption into intestinal tract (Shen et al., 2009; Song et al., 2010; Wu et al., 2011; Gao et al., 2012; Melo et al., 2016; Waititu et al., 2016).

Glutamine is an amino acid that plays important roles in regulating gene expression, cell signalling, antioxidative responses, neurotransmission, and immunity; additionally, it is a major metabolic fuel for the small intestine to maintain its digestive function and to protect the integrity of the intestinal mucosa (Wu et al., 2011).

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Dietary supplementation with yeast culture has a positive effect on performance and health in weaned piglets through stimulating the immune system and maintaining a favourable intestinal environment (Heugten et al., 2003). According to Tan et al. (2015) the use of oregano essential oil improved performance of their piglets through reducing oxidative stress. Benzoic acid has the capacity to increase the digestibility of the diet and, consequently, improve the performance of weaned piglets (Zhang et al., 2016). In this way, all these additives have beneficial effects over the performance of piglets; however, the mode of action should be better understood.

Ex vivo techniques have been recently used to evaluate the effect of feed additives on immune response in swine tissues challenged by lipopolysaccharides (Smith et al., 2011; Bahar et al., 2012; Leonard et al., 2012). These techniques, in which intestine explants are exposed to different treatments, are a rapid, less expensive, specific, and reliable experimental model to determine the trophic effects of feed additives on intestinal mucosa (Basso et al., 2013; Bortoluzzi et al., 2016; Melo et al., 2016).

NF-κB is of major importance for the activation of the transcription of a variety of genes related to cytokine production (May and Ghosh, 1998); IGF-1 plays a vital role in the modulation of piglet intestinal post-natal growth (Burrin et al., 1996); the glucose transporters GLUT2 and SGLT1 are crucial for adequate absorption of glucose in the small intestine (Rodriguez et al., 2004). The evaluation of these genes can contribute to understand how the additives improve the performance of piglets.

Due to the similarities in intestinal physiology between pigs and humans, studies that examine the nutritional influences on intestinal health are preferably carried out in pig models (Roura et al., 2016). The objective of this study was to evaluate jejunal gene expression of NF-κB, IGF-1, GLUT2, and SGLT1 in response to yeast culture, benzoic acid, L-glutamine, and oregano essential oil exposure using an *ex vivo* model.

Material and Methods

All experimental procedures were approved by the local Animal Care and Use Committee (case no. 1111000138). The experiment was performed in West Lafayette, Indiana, USA (40°27'19" N and 86°54'42"W).

Jejunal tissues from six piglets (20 kg) were collected 30 cm from the stomach. Five intestinal samples were collected from each pig for assignment to the treatments. The samples were immediately washed post-harvest with phosphate buffer solution (PBS) using *pisset* until there were no more contents. Then, the samples were immersed individually in 50 mL polypropylene tubes containing a buffer solution (50 mM/L mannitol, 2 mM/L tris-HCL, pH 7.4) with antibiotics for 15 min, kept in an insulated box with ice, and transported to laboratory. The buffer solution was made using an ultra-pure water containing an antibiotic mixture of penicillin, neomycin, and streptomycin, 1% (0.1 mL/mL of solution, Sigma Aldrich, St. Louis, MO, EUA) to reduce microbial presence in the samples.

Then the tissues were rinsed with PBS and immersed for 60 min in cell culture media (Dulbecco's Modified Eagle Medium (DMEM) – high glucose HEPES modification, with 4500 mg/L glucose, 25 mM HEPES, and sodium bicarbonate, without L-glutamine and sodium pyruvate, sterile-filtered liquid, suitable for cell culture – DMEM). The treatments evaluated were: control (without additives), 1% L-glutamine (≥ 99%; Sigma Aldrich, St. Louis, MO, EUA), 1% yeast culture (Varied Industries Corporation, Mason, IO, EUA), 0.5% benzoic acid (≥ 99.5%; Sigma Aldrich, St. Louis, MO, EUA), and 0.015% oregano essential oil (100%; NOW Foods, Bloomingdale, IL, EUA). The tissues were incubated at 37 ºC in a closed Falcon tube. Some additives were not soluble in the medium; thus, the plates were placed on a rocking platform to keep the tissue in contact with the additive.

After the incubation, the tissues were rinsed with PBS and stored at −80 ºC in a cryovial tube with 0.5 mL of TRIzol® reagent (Invitrogen, China) for subsequent RNA extraction, cDNA synthesis, and gene expression analyses. All experimental procedures were performed in accordance to the protocol outlined by Bortoluzzi et al. (2016).

Total RNA was isolated from approximately 50 mg jejunum tissue samples using TRIzol® reagent (Invitrogen, China), following the manufacturer's instructions. Precipitated RNA was suspended in 20 µL of RNAse free water and treated with DNAse (Invitrogen, China), then stored at −80 ºC. RNA quantity was assessed by UV spectrophotometer. To synthesize the cDNA, the same RNA quantity per sample was used, which was produced by diluting the higher RNA concentration with RNAse free water. First-strand cDNA was synthesized from 5 µL of total RNA using oligodT primers and Superscript II reverse transcriptase, in accordance with the manufacturer's instructions (Invitrogen, China). Synthesized cDNA was diluted five times with sterile water and stored at −20 ºC before use.

The gene expression analyses were performed to the following gut genes: RelA/p65 (Santos et al., 2007), IGF-1 (Yin et al., 2009), GLUT2, and SGLT1 (Song et al., 2010) with GAPDH (Smith et al., 2011) as endogenous control;

primers are shown in Table 1. Amplification of RT-PCR was performed in 25 µL of reaction mixture containing 5 µL of diluted cDNA, 12.5 µL of SYBR Green PCR Master Mix (Roche, Switzerland), 2.5 µL of each primer, and 3 µL of PCR-grade water. The PCR procedure for RelA/p65 consisted of heating the reaction mixture to 95 ºC for 5 min, followed by 40 cycles of 94 ºC for 30 s, 54 ºC for 30 s, and 72 ºC for 40 s. IGF-1 had a heating reaction of 95 ºC for 5 min followed by 40 cycles of 94 ºC for 30 s, 56 ºC for 30 s, and 72 ºC for 40 s. The GLUT2 heating reaction was 94 ºC for 5 min followed by 40 cycles of 94 ºC for 30 s, 51 ºC for 30 s, and 72 ºC for 40 s. The heating reaction for SGLT1 was 94 ºC for 5 min followed by 40 cycles of 94 ºC for 30 s, 53 ºC for 30 s, and 72 ºC for 40 s.

A dissociation curve was run for each plate to confirm the production of a single product. Data of PCR obtained from the ABI 7500 Real-time PCR System were automatically analysed by Applied Bio systems Software. The relative standard-curve method was used to quantify the mRNA concentrations of each gene in relation to the reference gene (GAPDH). The mRNA relative abundance was calculated as described by Pfaffl (2001). Relative mRNA expression was calculated according to the methods proposed by Livak and Schmittgen (2001). All samples were analysed in quadruplicate.

The analysis of variance was performed according to the statistical model below:

$$Yij = \mu + Gi + \varepsilon ij,$$

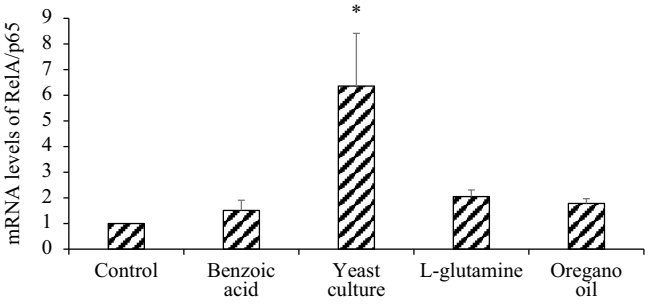
in which Yij = observation of the effect of the treatment i and at replication j, μ = overall mean, Gi = effect of treatment, and εij = random error associated with each observation.

For statistical analysis, each jejunal explant collected from the piglets (five explants/piglet) was considered as one experimental unit (n = six replicates/treatment). Cochran's test was carried out to verify data homogeneity. The effect of treatments on gene expression levels was assessed using the General Linear Model (GLM), following Dunnett's test. Significance was determined to be $P \leq 0.05$.

Results

The addition of 1% yeast culture increased ($P<0.05$) the mRNA levels of RelA/p65 6.36±2.05-fold compared with control treatment (Figure 1). The mRNA levels of RelA/p65 after Benzoic acid, L-glutamine, and oregano essential oil exposure were not different from the control treatment. Only the porcine jejunum explants exposed to 1% of yeast culture had increased levels of mRNA level for IGF-1, 4.58±1.14-fold greater than control (Figure 2).

Intestinal GLUT2 gene expression was up-regulated by 1% of yeast culture (3.47±0.56-fold greater than control), 1% L-glutamine (2.82±0.68-fold greater than control), and 0.015% oregano essential oil (5.62±0.89-fold greater than control) (Figure 3). Furthermore, the addition of 1% of yeast culture and 0.015% oregano oil also increased intestinal SGLT1 gene expression compared with the control (3.58±0.42- and 3.16±0.44-fold, respectively)



SEM - standard error of the mean.
* $P<0.05$ control vs treatment.
There were six replicates per treatment.

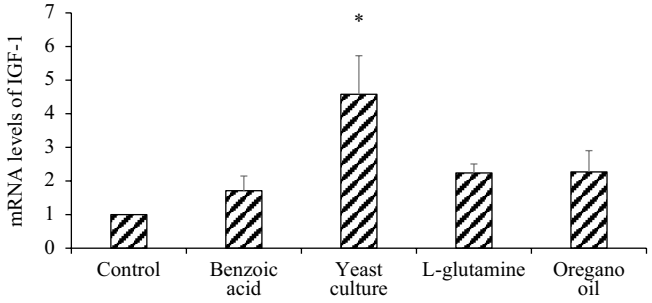
Figure 1 - Gene expression of RelA/p65 in porcine jejunal explants induced by different feed additives determined by qRT-PCR (expressed as fold greater than control based on 2-ΔΔCt, using GAPDH as reference gene) means with ±SEM.

Table 1 - Gut markers and sequence of primers to real time PCR

Gut marker	Primer	Sequence (5'-3')	Reference
GAPDH	Forward	CAGCAATGCCTCCTGTACCA	Smith et al. (2011)
	Reverse	ACGATGCCGAAGTTGTCATG	
GLUT2	Forward	CAGGGGTGCTATTGGTGC	Song et al. (2010)
	Reverse	TTCCTTGCTTTGGCTTCC	
SGLT1	Forward	CATCATCGTCCTGGTCGTC	Song et al. (2010)
	Reverse	TGCCTCCTCTTCCTTGGT	
p65/RelA	Forward	GGAACACGATGGCCACTTG	Santos et al. (2007)
	Reverse	AAGAGGACATCGAGGTGTATTTCAC	
IGF-1	Forward	CTGTAACCATGAGGCTGAGA	Yin et al. (2009)
	Reverse	CTCCATACTTCCTGTACTCC	

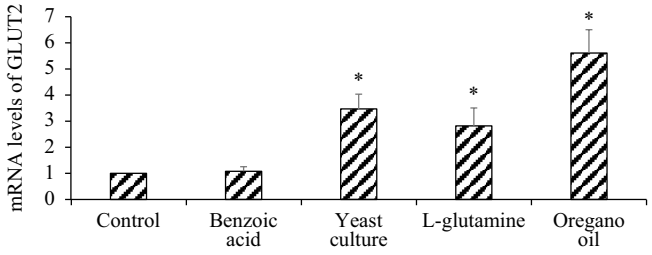
PCR - polymerase chain reaction.

(Figure 4). Benzoic acid was a suppressor of SGLT1 mRNA levels (66% lesser than control), while L-glutamine did not alter the expression of this marker of glucose transport.



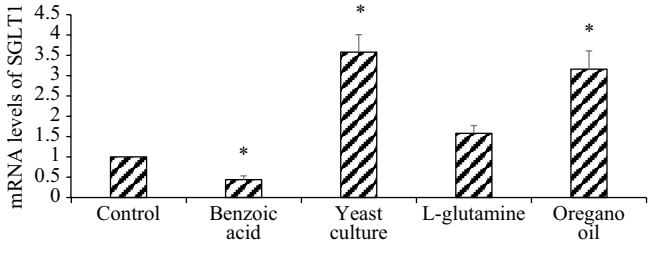
SEM - standard error of the mean.
* P<0.05 control vs treatment.
There were six replicates per treatment.

Figure 2 - Gene expression of IGF-1 in porcine jejunal explants induced by different feed additives determined by qRT-PCR (expressed as fold greater than control based on 2-ΔΔCt, using GAPDH as reference gene) means with ±SEM.



SEM - standard error of the mean.
* P<0.05 control vs treatment.
There were six replicates per treatment.

Figure 3 - Gene expression of GLUT2 in porcine jejunal explants induced by different feed additives determined by qRT-PCR (expressed as fold greater than control based on 2-ΔΔCt, using GAPDH as reference gene) means with ±SEM.



SEM - standard error of the mean.
* P<0.05 control vs treatment.
There were six replicates per treatment.

Figure 4 - Gene expression of SGLT1 in porcine jejunal explants induced by different feed additives determined by qRT-PCR (expressed as fold greater than control based on 2-ΔΔCt, using GAPDH as reference gene) means with ±SEM.

Discussion

NF-κB is a transcription activator of genes involved in immune response, including the stimulation of T cell activity. An abnormal overstimulation of NF-κB is dangerous to the health of the host, due the potent pro-inflammatory effects of T cells (Baeuerle and Henkel, 1994). The modulation of pro- and anti-inflammatory cytokines must be considered as an important property of feed additives to control immune response (Shen et al., 2009; Gao et al., 2012).

In this study, up-regulation of RelA/p65 (NF-κB) gene expression was produced by yeast culture; similar results were found by Wang et al. (2016), evaluating live yeast over the expression of this gene on the gut of broilers. In the same way, our results agree with Shen et al. (2009), who reported that yeast culture increased IFN-γ, a T helper-1 cytokine of the intestinal mucosa of piglets, probably induced by the NF-κB molecule, as this molecule.

An alternative hypothesis is that, in animal production, this up-regulation could deviate the energy destined for muscular growth to immune response and reduce body weight gain. Positive effects of yeast culture intake have been reported, including improvement of intestinal integrity, feed digestibility, and growth performance (Shen et al., 2009). Wang et al. (2016) evaluating broilers challenged and unchallenged with *E. coli* and receiving supplementation of live yeast, observed a lower expression of NF-κB for animals challenged and an increase in animals unchallenged. It demonstrates that live yeast can affect the expression, modulating the immune response according to the environment, thus causing beneficial effects to the host.

IGF-1 has a wide range of biological actions, including the stimuli to cell proliferation and differentiation in many tissues (Jones and Clemmons, 1995), and plays a vital role in the modulation of piglet intestinal postnatal growth (Burrin et al., 1996). Besides the effects of the intestinal growth, this molecule has a great effect over the growth of the animal during its life (Hellström et al., 2016). Therefore, the absence of IGF-1 could negatively affect animal growth and productivity.

Jiang et al. (2015) also found that supplementation of piglet feed with yeast improve the serum IGF-1 concentration as well as improves animal performance. The beneficial effects could be associated with the composition of cell wall components of yeast, which is a complex polymer and composed of β-glucans, α-mannans, mannoproteins, and a minor component of chitin.

The literature reports that pro-inflammatory cytokines released by NF-κB activation can increase the resistance of hepatic growth hormone receptor and, consequently,

reduce synthesis of IGF-1 in target tissues (Broussard et al., 2001). However, it has also been reported that IGF-1 is an up-regulator of RelA/p65 (Zhao et al., 2011). Probably, a negative feedback system may control the gene expression of IGF-1 and pro-inflammatory cytokines released by NF-κB activation. In our findings, without the induction of inflammatory downstream, the effect of yeast culture increased the expression of RelA/p65 (NF-κB) and IGF-1.

The expression of glucose transporter genes such as GLUT2 and SGLT1 are crucial for adequate absorption of glucose in the small intestine (Rodriguez et al., 2004). Low expression of GLUT2 and SGLT1 are associated to a minor capitation of intestinal glucose in piglets (Song et al., 2010).

GLUT2 acts as a facilitator of glucose diffusion through lipid bilayers in the basolateral membrane, transporting glucose and fructose, providing a common exit pathway to the blood (Breves et al., 2007). The second glucose transporter, SGLT1, mediates Na^+/glucose co-transport function both in kidney and intestine as a secondary active transporter (Breves et al., 2007).

Some glucose molecules can be rapidly captured by SGLT1 and GLUT2, which are the only co-transporters able to actively transport glucose against the concentration gradient (Kellett and Brot-Laroche, 2005). Therefore, apical GLUT2 can provide a major route of monosaccharide absorption by which absorptive capacity is rapidly and precisely up-regulated to match the dietary intake of carbohydrates. Apical GLUT2 is not only regulated by long and short-term supply of dietary carbohydrates, but also by local and endocrine hormones, cellular energy status, stress, and diabetes. Regulation occurs through a network of intracellular signalling pathways (Kellett and Brot-Laroche, 2005). *In vitro* IGF-I can stimulate cellular transport processes, including facilitated glucose uptake (Prosser et al., 1987). Alexander and Carey (1999) verified an increase in SGLT1 gene expression and increase in proliferation of enterocytes in IGF-I-treated piglets.

In this study, IGF-1, SGLT1, and GLUT2 gene expression increased in response to the yeast culture, and there may be a relationship between these variables, as a greater IGF-1 gene expression could result in increased gene expression of SGLT1 and GLUT2, as suggested by Prosser et al. (1987) and observed in the present trial for the explants exposed to yeast culture.

Glutamine is an amino acid required for the synthesis of purine and pyrimidine, essential for gene expression, and proliferation of cells, including cells of intestinal mucosa and intraepithelial lymphocytes (Wu et al., 2011; Tucci et al., 2014). Consequently, the use of 1% glutamine to weanling piglets enhances intestinal oxidative-defence capacity, prevents jejunal atrophy, promotes small intestine growth, and body weight gain in weaned piglets (Wu et al., 2011). He et al. (2016) also found that 1% glutamine improves the performance of weaned piglets and disaccharide enzyme activity, which could provide more substrate for the glucose transports; thus, the up-regulation of GLUT2 by glutamine is interesting, which can contribute with intestinal health, increasing the absorption of cell energy substrates.

GLUT2 and SGLT1 gene expressions were up-regulated by the herb mixture of *Cortex Phellodendron*, *Rhizome Atractylodes*, *Agastache rugosa*, and *Gypsum Fibrosum* in the small intestine of pigs subjected to heat stress (Song et al., 2010). In this trial, oregano essential oil was also an up-regulator of GLUT2 and SGLT1 mRNA. The correct use of herb compounds can optimize glucose absorption, in absence of a challenge, as showed in the present study.

Among the feed additives tested, only benzoic acid acted as a down-regulator of glucose co-transporters. The absence of GLUT2 regulation and the down-regulation of SGLT1 gene expression after benzoic acid exposure could be associated to its acidifying effect into the medium and its mucosal stimulus to mucin production. The reduction in pH was observed by changes in the colour of the medium after the addition of benzoic acid. However, additional studies are necessary to understand these results.

The knowledge about mucosal response to feed additives included into the diet has a great importance for piglet production, especially due to the high stress promoted by weaning process associated to glucocorticoid release and reduction of feed intake, which contributes to a reduction in glucose co-transporter gene expression, reduction in the intestinal villus height and crypt depth, and increase in the intestinal inflammatory response.

The gene expression of beneficial intestinal health genes in the mucosa is a complex point of investigation. Several *in vivo* conditions impact gene expression, including the food consumed, established microbiota, intestinal and immune maturity, and environmental conditions. Thus, the modulation of gene expression in response to a feed additive should be investigated without these confounding conditions, to certify if its use can induce gene expression (Melo et al., 2016).

Conclusions

Feed additives differently modulate the gene expression of immune response, gut development, and glucose absorption in jejunal explants. These findings can contribute

for a better understanding of the trophic action of these feed additives into the diets to optimize the animal performance.

Acknowledgments

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