Chia (*Salvia hispanica*) harvest residue induces cytokine expression in rabbits

El residuo de la cosecha de chia (*Salvia hispanica*) induce la expresión de citocinas en conejos

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ABSTRACT. The seed of chia plant (*Salvia hispanica*) is characterized by a high content of polyunsaturated fatty acids, which is an important source of α-linolenic acid for human and animal consumption. During the harvest of the seed, waste is generated that contains remnants of leaves, stems and some seeds, which is generally discarded. The objective of the present work was to evaluate the effect of chia seed residue (CSR) on protein expression of the rabbit immune system, when added as a supplement in the diets of animals deliberately induced to an intestinal infection. Twenty rabbits (New Zealand $\times$ California) were randomly distributed in five treatments and fed five diets with different percentages of CSR inclusion (0, 10, 20, 30 and 40%). Quantification of the relative expression of the anti-inflammatory Interleukin-10 (IL-10) and Tumor Necrosis Factor (TNF-α) genes showed that as CSR inclusion levels increased up to 40%, mRNA expression of IL-10 increased significantly (22.4-fold) with respect to the control, while TNF-α mRNA expression was inversely proportional, where a significant increase (7.47-fold) in mRNA expression was found in the control group. It is concluded that the indirect inclusion of α-linolenic acid through the consumption of CSR induces a positive response in the immune system of the rabbits and represents an alternative for the formulation of rations with nutraceutical effects.
Key words: Feeding, α-linolenic acid, IL-10, TNF-α, Relative expression.

RESUMEN. La semilla de la planta de la chía (*Salvia hispánica*), se caracteriza por su alto contenido de ácidos grasos poliinsaturados, por lo que es una fuente importante de ácido α-linolénico para consumo humano y animal. Durante la cosecha de la semilla, generan residuos que contiene remanentes de hojas, tallos y semillas, que son desechadas. El objetivo del presente trabajo fue evaluar el efecto del residuo de la cosecha de semilla de chía (RCS) sobre la expresion de proteínas del sistema inmune de conejos, al añadirlo como complemento en las dietas de animales inducidos con infección intestinal. Se distribuyeron al azar veinte conejos (Nueva Zelanda $\times$ California) en cinco tratamientos y se alimentaron con cinco dietas con porcentajes de inclusión de RCS de 0, 10, 20, 30 y 40%. La cuantificación de la expresión relativa de los genes interleuquina-10 (IL-10) y factor de necrosis tumoral (TNF-α) mostraron que conforme aumentaron los niveles de inclusión RCS hasta 40%, también aumentó de forma significativa la expresión relativa de IL-10 hasta 22.4 veces con respecto al control, mientras que la expresión relativa de la TNF-α fue inversamente proporcional, presentando la mayor expresión relativa el grupo control con 7.47, por lo que la inclusión indirecta de ácido α-linolénico a través del consumo de RCS inducen una respuesta positiva en el sistema inmune de los conejos y representa una alternativa para la formulación de raciones con efectos nutracéuticos.
Palabras clave: Alimentación, Ácido α-linolénico, IL-10, TNF-α, Expresión relativa.

INTRODUCTION

There has been growing interest in recent years in the study of oleaginous plants such as chia (*Salvia hispanica*) due to the properties of its seed, which is a rich source of polyunsaturated fatty acids (Heuer *et al*. 2002) and contains compounds such as myricetin, quercetin, kaempferol and caffeic acid with high antioxidant content (Capitani *et al*. 2012). This seed has already been used in diets for poultry (Komprda 2013), to enrich the production of goat's milk (Martínez 2013) and in rabbit diets (Peiretti and Meineri 2007).

Chia seed residue (CSR) is the by-product generated during the harvest of the seed, usually composed of leaves, stems and seeds that escaped the sieve. There are no studies that provide knowledge on the use of CSR as a nutritional source (Ramírez *et al*. 2012), but there is evidence of the use of seed for animal consumption because of its high fiber and fatty acid contents, especially α-linolenic acid (Coates *et al*. 1996).

The benefits provided by the inclusion of α-linolenic acids in the human diet are documented; they strengthen the immune system by means of decreasing the production of mediators and regulators (PGE_2, LTB4, TXA2, IL-1β, IL-6, and TNF-α) of inflammatory processes (Jeong 2010). The effect of α-linolenic fatty acids on inflammatory processes occurs through their incorporation into cell membranes, serving as substrates for the metabolism of eicosanoids, which causes the production of metabolites such as EPA and DHA, with anti-inflammatory properties (Kalupahana *et al*. 2011).

Cytokines are extracellular and water-soluble polypeptides that range in size from 8 to 30 kDa. Although they are redundant in their activities, they are usually linked to specific receptors that trigger cascade signals and activate intracellular messengers that regulate gene transcription (Jun-Ming and Jianxiong 2007). By influencing cytokines in cellular processes such as differentiation, proliferation and longevity of the cell, they also regulate the production and activity of other cytokines, which can increase or attenuate an inflammatory response (Raeburn *et al*. 2002). Some cytokines may have pro-(Th1) or anti-inflammatory (Th2) actions, in tune with the microenvironment in which they are found. Among those considered proinflammatory, we have the interleukins (IL) 1, 2, 6, 7 and TNF (Tumor Necrosis Factor), and the anti-inflammatory agents IL-4, IL-10, IL-13 and TGF-β (Transforming Growth Factor β) (Zhang and An 2007). Due to the above, the aim of this research was to evaluate CSR potential as an animal feed alternative and the effect of its α-linoleic fatty acid content on animal welfare, through the immune system response of rabbits and the analysis of the expression of cytokine genes IL-10 and TNF-α, after an acute infection induced by acute colitis.

MATERIALS AND METHODS

The experiment was carried out in february 2015 at the San Miguel rabbit farm in the municipality of Seyé, Yucatán, Mexico, located at 20°53'03.1" N, 89°22'36.0" W.

Experimental design

The experimental lot consisted of 20 recently-weaned rabbits (New Zealand × California), with weights of 500 ± 10 g. Four experimental treatments with replicates of 4 animals and a control with 0% CSR inclusion were used.

Diet formulation

The rabbits were fed diets prepared with CSR in the form of pellets following the specifications of Rodríguez-Abello *et al*. (2016), at 10%, 20%, 30% and 40% inclusion levels. The diets were formulated in such a way that they were isoenergetic and isoproteic, according to the requirements described for fattening rabbits in intensive production systems (De Blas and Mateos 2010) (Table 1). The diets were subjected to bromatological analysis to determine if they meet the nutritional requirements for rabbit development. The control diet was a PURINA®-brand commercial rabbit feed, without CSR inclusion. During the 10 weeks of evaluation, the rabbits were weighed individually at

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Table 1. Chemical composition of diets containing 10, 20, 30 and 40% chia seed residue.

Ingredient	Control	Diets			
		Ch10	Ch20	Ch30	Ch40
Maize	8.000	8.000	8.000	7.380	8.000
Sorghum	0.380	5.000	5.000	5.000	5.000
Dehydrated Alfalfa	30.960	10.860	0	0	0
Soybean paste	3.990	8.460	10.430	9.720	10.650
Canola pasta	4.000	4.000	4.000	4.000	4.000
Soybean husk	10.000	10.000	8.610	0	0
Wheat bran	35.000	35.000	35.000	35.000	24.05
Chia seed residue	0	10.000	20.000	30.000	40.000
Molasses	4.000	5.000	5.000	5.000	5.000
Acidified fatty acids	1.720	0.500	0.500	0.500	0.770
Calcium carbonate	0.700	1.990	2.320	2.270	1.610
Sodium chloride	0.5000	0.700	0.700	0.700	0.500
Calcium orthophosphate	0.150	0	0	0	0
Methionine 84%	0.100	0.059	0.023	0	0
Lysine 70%	0.079	0.123	0	0	0
Choline	0.030	0.030	0.030	0.030	0.030
Vitamin premix	0.100	0.100	0.100	0.100	0.100
Mineral premix	0.055	0.055	0.055	0.055	0.055
Compactant	0.100	0.100	0.100	0.100	0.100
Antioxidant	0.100	0.100	0.100	0.100	0.100
Antifungal	0.010	0.010	0.010	0.010	0.010
Coccidiostat	0.020	0.020	0.020	0.020	0.020

the beginning and after the experiment at weekly intervals, with a digital scale (Ohaus® Model CKW6R55) of 6 kg with error $\pm$ 10 g. Each rabbit was given 150 g d^{-1} of feed and water with *ad libitum* access, recording daily feed consumption and rejection.

Fatty acid analysis of diets

For the extraction of total lipids, we used 100 g of feed, which were dried and subjected to extraction by the Soxhlet method, with a chloroform/methanol mixture (1:2 v/v) (Halim *et al.* 2012). The fatty acids were derivatized to methyl esters with 2 M of an ethanolic solution of potassium hydroxide. The methyl esters were quantified by gas chromatography with a Perkin Elmer (Autosystem) unit, equipped with a flame ionization detector and Innowax capillary column (Thomas Scientific©) (30 m x 0.320 mm), with nitrogen (N_2) as carrier gas. The temperature of the injector was 250 °C and of the detector 300 °C. The oven temperature program was 4 min at 150 °C with a ramp of 5 °C per minute up to 190 °C with a ramp of 2 °C per minute up to 250 °C. The injection volume was 2 μL per sample. The fatty acids were quantified as percentages of total fatty acids. The identification of the fatty acid profile was made with external standards (Supelco® 37 Component FAME Mix).

Colitis induction

Based on previous reports by Best *et al.* (2012) and Hutton *et al.* (2013), when the animals reached week 11, acute colitis was induced in them for three days by giving them a 28 mg oral dose of the antibiotic Clindamycin (Pet Health®). On the fourth day, all the animals of the four treatments plus the control group presented the symptomatology of acute colitis caused by *Clostridium sp.*

Slaughter and sample preservation

In the order in which the animals were presenting symptoms of colitis, they were sedated with an intramuscular dose of ketamine (50 mg kg^{-1}), and then slaughtered by decapitation, after supervision of an ethics committee following the official Mexican standard (NOM-033-SAG/ZOO-2014). Subsequently, the colon's intermediate re-

Table 2. Oligonucleotides used for real-time PCR analysis.

Cytokines	Size (bp)	Oligonucleotide sequences	Ta (°C)	GenBank Accession no.
HPRT	265	F: 5´- TGATAGATCCATTCCTA -3´ R: 5´- GGTCCGTTTTCACCAGCAG -3´	60	M31642
IL-10	100	F: 5´- AGAACCACAGTCCAGC -3´ R: 5´- GGGAGAAATCGGTGACAT -3´	60	M12845
TNF-α	100	F: 5´- GAGCAGCAACTCCAGA-3 R: 5´- GGTGCGTGAGAGAGGAAG -3´	58	AF 169170

bp: base pairs, Ta: annealing temperature

gion was dissected and identified; then, using a scalpel, approximately 0.5-cm tissue sections were cut and placed in sterile tubes with 0.2 ml of RNA*later* (Invitrogen™). Samples were stored at 4 °C for 24 h, and subsequently at -80 °C until analysis. All processes used in animals of this experiment were approved by a graduate scientific ethics committee at the Instituto Tecnológico de Conkal.

Total RNA extraction and cDNA synthesis

Total RNA was isolated with the TRIzol method (Invitrogen™), according to the manufacturer's instructions. The concentration and quality of the RNA in each sample was determined between A260/A280 and A260/A230 nm in a NanoDrop Lite (Thermo Scientific®). The integrity of the RNA was confirmed with electrophoresis in 2% agarose gels for 40 min at 90 V, with 2X RNA Loading Dye (Thermo Scientific®). Prior to treatment with the DNAse enzyme, all samples were homogenized at 500 ng in a volume of 20 μL. To eliminate any contamination of genomic DNA, approximately 2000 ng of RNA was treated with the DNase I enzyme (TURBO DNA-free™, Ambion®), according to the manufacturer's instructions. For cDNA synthesis, 10 μL of DNase-treated RNA was used with the RevertAid First Strand® kit (Thermo Scientific®). The reaction was carried out in a thermal cycler (Techne®), incubating at 65 °C for 5 min, 25 °C for 5 min, 42 °C for 60 min and finally 70 °C for 5 min to terminate the reaction.

Oligonucleotide design and validation for RT-qPCR

Oligonucleotides were designed for the IL-10 and TNF-α genes with the Fast-PCR program, taking as reference the sequences for these rabbit genes reported in GenBank (D84217 and M12845). The pair of oligonucleotides for the housekeeping gene HPRT (hypoxanthine phosphoribosyl transferase) was designed by Godornes *et al.* (2007) (Table 2). For the validation of the designed oligonucleotides, a standard curve was generated to determine the difference in the predicted amount between a test sample and a calibrator sample. For this, the cDNA of five representative samples of the treatments (200 ng concentration) was used to prepare the calibration curves for each of the genes. For the real-time PCR reaction, a calibration curve was generated from a series of seven 1/10 dilutions of the cDNA (initial concentration of 200 ng) in triplicate. PCR efficiency was calculated using the equation: $E\% = (101/\text{slope}^{-1})$ x 10 (Radonic *et al.* 2004) (Table 3). Data were analyzed with iQ5 optical system software ver.2 (Bio-Rad).

Table 3. Data of the calibration curves for IL-10, TNF-α and the housekeeping gene, HPRT.

Cytokines	R^2 values	PCR efficiency	Slope	Mean E^2
IL-10	0.998	1.000	-3.322	0.492168
TNF-α	0.994	1.082	-3.139	0.791156
HPRT	0.987	1.061	-3.184	0.107973

Analysis by RT-qPCR

RT-qPCR analysis was performed in triplicate with the Maximum SYBR® Green/Fluorescein qPCR kit (Thermo Scientific®) according to the manufacturer's instructions. The reaction of each sample had a final volume of 25 μL, constituted by 12.5 μL of SYBR Green/Fluorescein (2X), 0.3 μM of each oligonucleotide and 2000 ng of RNA. The samples were completed with ultrapure water to

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Table 4. Total composition of fatty acids in diets containing 10, 20, 30 and 40% chia seed residue.

Fatty acids	Control	Ch10	Ch20	Ch30	Ch40
Total saturated	56.29 ± 0.13	10.06 ± 2.10	11.2 ± 2.09	12.19 ± 2.03	13.09 ± 2.08
Total monounsaturated	43.71 ± 0.06	5.09 ± 2.03	6.29 ± 2.08	7.18 ± 2.07	7.68 ± 2.09
18:2w6$^{(omega-6)}$	Traces	9.92 ± 0.08	10.16 ± 0.06	11.33 ± 0.03	12.74 ± 0.04
18:3w6	Traces	0.10 ± 0.03	0.12 ± 0.05	0.13 ± 0.02	0.15 ± 0.06
18:3w3 Cis$^{(omega-3)}$	Traces	46.52 ± 2.03	49.42 ± 1.09	52.33 ± 1.06	53.82 ± 1.05
Total Polyunsaturated	Traces	67.81 ± 0.10	71.17 ± 0.38	75.67 ± 0.10	79.03 ± 0.29

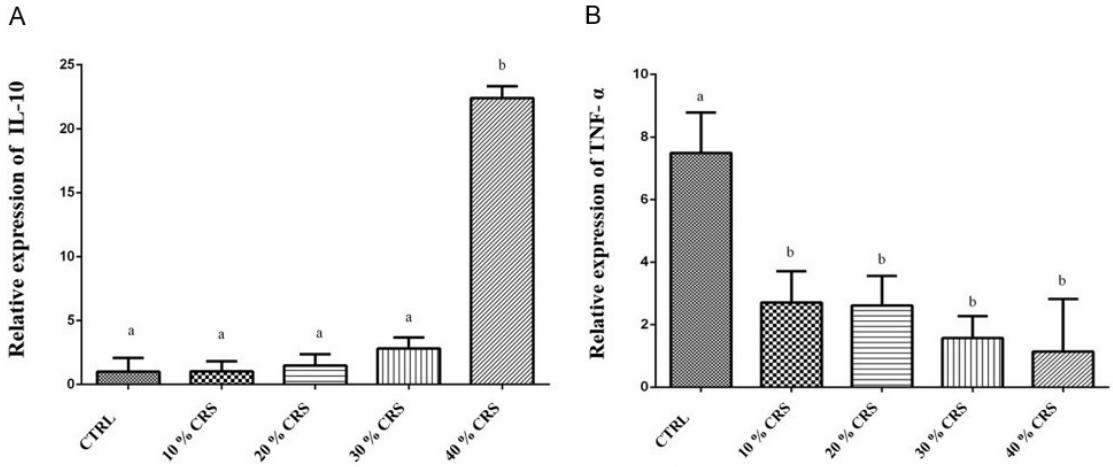

Figure 1. Relative mRNA expression of IL-10(A) and TNF-α(B) genes related to inflammatory process in the middle colon region of rabbits fed chia waste subjected to experimental colitis. CTRL, diet without residues of chia; percentage of chia residue content in diets (10% CRS, 20% CRS, 30% CRS and 40% CRS). Different letters denote significant differences (P < 0.05); values are presented as the mean ± standard deviation.

reach their final volume. The reaction was carried out in an iQ5 Cycler thermal cycler (Bio-Rad), with a standard protocol (initial denaturation at 95 °C for 5 min, 40 cycles of denaturation, annealing and extension at 95 °C for 30 s, 64 °C for HPRT and IL-10, at 62 °C for TNF-α for 1 min and 72 °C for 15 s).

Statistical analysis

The factor of change in the relative mRNA expression of HPRT, IL-10 and TNF-α was calculated using the 2-$\Delta\Delta$Ct method by Livak and Schmittgen (2001). The ΔCt was obtained by subtracting the Ct from the endogenous gene minus the Ct of the genes of interest (IL-10 and TNF-α). The $\Delta\Delta$Ct values were presented as the mean ± the standard error. The significances of the expression levels between the control group and the treatments were determined by means of an ANOVA and then the means were separated with Tukey's range test ($p < 0.05$) using SAS ver 10 software.

RESULTS

Results show that the seed remnants that make up the chia harvest residue contribute between 46.52, 49.42, 52, 33 and 53.82% of Omega-3 fatty acids in the diets with 10, 20, 30 and 40% chia residue content, respectively, while the Omega-6 content is 9.92, 10.16, 11.33 and 12.74% for the same diets (Table 4). In the case of the control diet, only trace levels of this fatty acid were identified.

For the RT-qPCR, standard curves were obtained that comply with the parameters to accept the oligonucleotides designed for the HPRT, IL-10 and TNF-α genes, with minor standard error equal to 0.05 of E^2 value, a slope near -3.32 and PCR efficiency of approximately 2.0 E = (10- 1/curve). The correlation coefficient (R^2) for the standard curves

of the cytokines (IL-10 and TNF-α) and for the housekeeping gene (HPRT) was greater than 0.98, which represents a linear relationship between the crossing point (cP) and the logarithm of the RNA concentration (Table 3).

The analyses of the gene expression of the genes (IL-10 and TNF-α) related to the inflammatory process are shown in Figure 1. These results indicate a significant increase ($p < 0.05$) in the IL-10 gene in the treatments fed with a diet of 30 and 40% chia residue with values of 2.81 and 22.4, with respect to the control, while for the treatments fed with 10 and 20% the increase in the IL- gene 10 was not significantly different from the control treatment with mRNA expression units of 1.02 and 1.49 respectively (Fig. 1A).

The mRNA expression of the TNF-α gene was inversely proportional as the percentage of chia residue in the treatments decreased. Thus, the highest mRNA expression of the TNF-α gene was found in the control diet feed with expression values of 7.49 ($p < 0.05$), while in the diet containing 40% chia residue the expression was significantly lower ($p < 0.05$), but it is enough to add 10% chia residue to the total diet to achieve inhibition of the gene to almost a third of the mRNA expression of the control group where no chia residue was added (Fig. 1B).

DISCUSSION

Polyunsaturated fatty acids found in CSR, even when it is a waste, contain a higher proportion of Omega-3 (ALA: α-linolenic acid, 18:3w3) than Omega-6 (LA: linolenic acid, 18:2w6). Similar studies carried out using chia seed as the main source of fatty acids have found that the seed contains between 25 to 40% fatty acids, of which 60% corresponds to Omega-3 and 20% to Omega-6 polyunsaturated acids (Jiménez *et al.* 2013; Sierra *et al.* 2015). One of the important functions of the Omega-6 and Omega-3 polyunsaturated fatty acids is that both are precursors of molecules called "eicosanoids", which have important functions in the regulation of the inflammation process (Patterson *et al.* 2012). The eicosanoids derived from Omega-6 include prostaglandins and leukotrienes, products of arachidonic acid metabolish, which are potent mediators in various physiological and pathological processes, inducing inflammation (Greene *et al.* 2011). The enzyme families cyclooxygenase (COX) and lipoxygenase (LOX) are responsible for the metabolism of arachidonic acid; these enzymes are the molecular targets of drugs for the treatment of pain, inflammation, asthma, and allergies. On the other hand, the eicosanoids derived from Omega-3 are EPA and DHA, products of eicosapentaenoic acid metabolism. The mode of action for these eicosanoids includes binding to a receptor coupled to a specific G protein, which allows the development of agonists and receptor-specific antagonists; these eicosanoids have important functions in the anti-inflammatory process (Dennis and Norris 2015).

Changes in the n-6:n-3 intake ratio with an increase in n-6 can increase chronic inflammatory diseases such as heart disease, diabetes, obesity, asthma and rheumatoid arthritis, among others (Patterson *et al.* 2012). It is important to point out that a double bond in the structure of omega-3 (α-linolenic acid) is the substantial factor to obtain the anti-inflammatory effect, largely due to its incorporation into cell membranes (Arita *et al.* 2005).

Increasing CSR inclusion levels regulated the expression of the anti-inflammatory protein gene (IL-10) and inhibited the expression of the proinflammatory protein gene (TNF-α) in the colon tissue of rabbits, whereas the expression of the TNF-α gene increased in the control treatment where there is no CSR presence. Inflammation of intestinal tissue is characterized by the infiltration of M1 and M2 type macrophages, with the M1 macrophages being stimulated by INFγ and LPS to express proinflammatory cytokines such as TNF-α IL-6, IL-8, IL-23, IL-1β, and reactive species, whereas M2 macrophages are activated by IL-4 and IL-13 to express anti-inflammatory factors such as IL-10, TGFβ, IL-1 receptor antagonist-α, IL-4 and arginase (Kalupahana *et al.* 2011). The expression of the IL-10 and TNF-α genes in rabbits with induced colitis was because the toxins of the bacteria

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of the species *Clostridium sp*. acted in the cells of the intestinal epithelium, causing tissue detachment and necrosis to break the protective barrier of the mucosa (Solomon *et al*. 2013).

The host must respond quickly to avoid further cell damage and the spread of toxins to the bloodstream. Epithelial cells intoxicated with *Clostridium sp*. produce proinflammatory mediators such as TNF-α and the inflammatory protein of macrophage 2, to initiate an inflammatory response of the intestinal mucosa (Kühl *et al*. 2015). All this underlies the benefits provided by CSR, an agricultural waste that can be used as animal feed, with benefits to the health of domestic animals, and provides a source of omega 3 of plant origin.

CONCLUSIONS

All the diets had high percentages of polyunsaturated fatty acids of the Omega-3 type relative to the Omega-6 type. The diets with 30 and 40% CSR had the highest level of expression of the anti-inflammatory protein (IL-10). This indicates that the CSR contains significant concentrations of α-linoleic acid in the formulated diets, allowing for greater gastrointestinal health in rabbits, by stimulating a greater response of the immune system and conferring greater response to the effect of colitis caused by *Clostridium sp*. The use of CSR is recommended because of its low animal feed production cost and because of its high content of Omega-3 polyunsaturated fatty acids. The designed oligonucleotides (IL-10 and TNF-α) can be used for future studies with this animal model.

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