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Pig models reveal the interplay between fatty acids and cytokines in skeletal muscle

Fernanda Nery Ciconello[1], Christopher Keith Tuggle[2], Julia Dezen Gomes[1], Bruna Pereira Martins da Silva[1], Mariah de Castro Durval[3], Camila Sabino de Oliveira[1], Lucas Echevarria Nascimento[1], Luciana Sales Freitas[5], James Eugene Koltes[2] & Aline Silva Mello Cesar[4]✉

Fatty acids, particularly those derived from plant and animal oils, play roles in physiological functions and metabolic regulation in pigs. Imbalances in the ratio of polyunsaturated fatty acids (PUFAs) may influence inflammatory responses, including the modulation of pro-inflammatory cytokines. This study aimed to investigate the gene co-expression profiles in the skeletal muscle of pigs fed diets supplemented with 3.0% soybean, canola, or fish oils, correlating these profiles with cytokine abundance and identifying hub genes associated with immune-related pathways using a systems biology approach. Skeletal muscle samples from 27 pigs were subjected to RNA sequencing and weighted gene co-expression network analysis (WGCNA) to construct gene co-expression networks. The concentrations of six cytokines (IL-10, IFN-γ, IL-1β, IL-6, IL-18, and TNF-α) were quantified in muscle tissue using ELISA. Functional enrichment analysis and hub gene identification revealed several key genes involved in immune function and fatty acid metabolism. WGCNA uncovered distinct co-expression modules associated with specific dietary oil treatments. These findings provide new insights into the immunomodulatory effects of soybean, canola, and fish oils, highlighting the relevance of nutrigenomics in understanding gene–diet interactions in pigs.

Keywords ELISA, Gene expression, Inflammation, Lipids, Swine, WGCNA

Pigs are regarded as excellent biomedical and genetic models for metabolic and neurological diseases, drug studies, xenotransplantation, and certain types of cancer[1,2]. Given the significant similarities between pigs and humans, pigs are used as a model in human nutritional studies. This allows for a more detailed understanding of physiological and metabolic responses, ultimately contributing to advancements in nutrition and the development of strategies for improving human health.

The inclusion of fatty acids in the pig diet, in addition to providing energy, has been associated with enhancements in the body's function and physiological processes[3]. Additionally, investigating fatty acids with meat quality and health is especially important, considering pork is one of the most widely consumed meats worldwide. Among the main sources of dietary fatty acids in animal and human nutrition from plants, remarkable are the oils extracted from soybean, canola, sunflower, corn, and flaxseed[4]. These oils are rich sources of unsaturated fatty acids, such as monounsaturated fatty acid (MUFA), which has just one double bond in the chain, and polyunsaturated fatty acid (PUFA), which has multiple double bonds in the chain[3]. Fatty acids play a fundamental role in health, as essential components of cellular membranes and participate in various metabolic processes[4]. In the skeletal muscle, fatty acids can play a role in the fluidity and stability of membrane structures, helping transport, cell signaling, response to oxidative damage, and apoptosis processes besides being influenced by phospholipids and triacylglycerols due to diets[5].

In addition to these plant-based sources, fish oil also represents a rich source of fatty acids. Fish oil is an n-3 PUFA primarily composed of eicosapentaenoic acid (C20:5 n-3, EPA) and docosahexaenoic acid (C22:6 n-3, DHA), offering various health benefits, improving either cardiovascular system protection or regulating

[1]Department of Animal Science, "Luiz de Queiroz" College of Agriculture, Av. Pádua Dias, 11, Piracicaba, São Paulo 13418-900, Brazil. [2]Department of Animal Science, Iowa State University, Kildee Hall, 806 Stange Road, Ames, IA 50011-3150, USA. [3]Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering, R. Duque de Caxias, Pirassununga, São Paulo 225, 13635-900, Brazil. [4]Department of Food Science and Technology, "Luiz de Queiroz" College of Agriculture, Av. Pádua Dias, 11, Piracicaba, São Paulo 13418-900, Brazil. [5]DB Genética Company, Av. JK, 2094, Patos de Minas, Minas Gerais 38706-000, Brazil. ✉email: alinecesar@usp.br

gene expression[6]. Supplementation with fish oil in pig nutrition emerges as a promising strategy to optimize levels of conditionally essential fatty acids, particularly EPA and DHA, in pigs. This practice not only enriches the composition of pork but also provides health benefits for the consumer, exerting positive effects on brain function and the immune system[6,7]. A high ratio of n-6/n-3 PUFAs can lead to adverse effects on both animal and human health, attributed to an increase in the production of eicosanoid metabolites derived from EPA[8]. Some metabolites have been associated with an inflammatory state and a higher aggregation propensity, compared to eicosanoids derived from EPA[7,9]. Moreover, supplementation with fish oil in the pig's diet can modulate the inflammatory response by reducing the expression of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α)[10,11].

The TNF-α and other adipokines play a key role in altering the insulin sensitivity in muscle by impairing fatty acid metabolism and causing lipid accumulation in mice[12]. TNF-α exerts several effects promoting catabolism, inhibiting contraction, and modulating myogenesis[13]. Similarly, IL-6 is a pro-inflammatory adipocytokine, secreted by adipose tissue, skeletal muscle, and macrophages, contributing to metabolic dysregulation. Notably, the single nucleotide polymorphism (SNP) in the *IL-6* gene has been linked to obesity, elevated plasma triglycerides, very-low-density lipoprotein (VLDL), fasting plasma lipid, and post-glucose load free fatty acid concentrations in Caucasian populations[14,15].

Previously, our group identified interesting findings in the same group of animals, with differentially expressed genes (DEGs) involved in lipid metabolism and mainly inflammation between oil groups, in addition to changes in blood parameters[7]. Therefore, a better understanding of the specific cytokine responses between diets is fundamental, and gene co-expression analysis is essential to gain insight into the regulatory mechanisms that differentiate these diets.

The role of fatty acids as an immune response modulator is well-established, but the specific influence of these fatty acids on cytokine profiles within the skeletal muscle of healthy pigs remains largely unexplored. This study aimed to address this knowledge gap by investigating the impact of cytokine abundance in the skeletal muscle of pigs. For that, we employed a systems biology approach to integrate cytokine data with the muscle transcriptomic profile, revealing gene co-expression clusters associated with these key inflammatory mediators. This integrative analysis provides a comprehensive understanding of the interplay between dietary fats, cytokine signaling, and muscle gene expression, paving the way for the development of targeted nutritional strategies to optimize pig health and productivity.

Results

In this study, we analyzed RNA-Seq data from 27 male pigs (9 per treatment) fed diets supplemented with 3.0% soybean oil (as reference), 3.0% canola oil, or 3.0% fish oil to identify key gene co-expression patterns that may indicate differential responses due to immunomodulatory mechanisms by the inclusion of different oil sources in the diet.

Cytokine abundance in the skeletal muscle by ELISA

The abundance of six cytokines was directly measured by ELISA in the skeletal muscle tissue and pursued the co-expression gene network analysis for three diet treatments (n = 9 per treatment): IL-10, IFN-γ, IL-1β, IL-6, IL-18, and TNF-α. Median Fluorescence Intensity (MFI) values from ELISA assays were evaluated through ANOVA for differences due to diet with Tukey's test at a significance level of α = 0.05. We observed that TNF-α was less abundant in canola oil compared to soybean oil and fish oil ($p < 0.05$), as well as IL-10 and IFN-γ ($p < 0.10$) (Table 1).

Identification of co-expressed gene modules

The transcriptome data used in this study were obtained from a previous study[7]. For the three analyzed treatments, a total of 15,378 expressed genes were obtained after quality control and used for further analysis. In this study, we identified clear changes in co-expression between diets, showing how gene expression can be related to cytokines levels.

The modules were chosen due to high correlations (threshold ≥|0.7|). In this way, darkgreen, blue, cyan, green, black, greenyellow, and grey60 were the analyzed modules. To facilitate the comprehension, five correlations

	Treatments				
	Soybean oil	Canola oil	Fish oil		
Cytokines	Mean	Mean	Mean	SEM	*p*-value
IL-10	9.40^{A}	8.62^{B}	9.18^{AB}	0.22	0.08 +
IFN-γ	83.70^{A}	24.48^{B}	55.59^{A}	16.17	0.08 +
IL-1β	11.80	11.02	12.57	0.98	0.59
IL-6	15.90	18.45	17.67	1.74	0.62
IL-18	21.25	108.64	162.36	89.83	0.59
TNF-α	17.33^{A}	12.27^{B}	15.72^{A}	1.26	0.05*

Table 1. Results in MFI with the comparisons among means for every cytokine analyzed in each treatment. Different letters mean that treatments were meaningfully different. *: level of significance of α = 0.05. +: level of significance of α = 0.1. SE: standard error, IL: interleukin, IFN: interferon, TNF: tumoral necrosis factor.

were found, for soybean oil (T1): one in IFN-γ (0.88 and *p*-value 1.6E-09) and another correlation in TNF-α (0.78 and *p*-value 1.7E-06) for the blue module. One negative correlation in IL-18 (−0.78 and *p*-value 3.7E-06) was observed for cyan. One positive correlation was observed in TNF-α (0.94 and *p*-value 1.4E-13) for green module (Fig. 1).

One negative correlation was observed in IL-6 (−0.71 and *p*-value 2.9E-05) for grey60. For canola oil (T2), two correlations were found in two modules: one negative correlation in IL-18 for darkgreen (−0.87 and *p*-value 4.6E-09), and one positive correlation for IL-10 in black (0.74 and *p*-value 1.4E-13). For fish oil (T3), two positive correlations were found in two modules: one correlation in IFN-γ for greenyellow (0.82 and *p*-value 1.3E-07) and another correlation in IL-18 for grey60 (0.96 and *p*-value 3.1E-15) (Fig. 1).

Functional annotation and enrichment: FAANGMine and *BiNGO*

FAANGMine at the University of Missouri completed the modules' functional annotation. To further explore the relationships between genes in clusters correlated with different cytokines, we performed functional enrichment using the Cytoscape plugin *BiNGO*. So, seven modules could be enriched according to their values of Pearson's correlation associated with the cytokine's traits.

In blue module, we identified genes related to biological regulation (False Discovery Rate (FDR) 1.63E-25), signaling (FDR 4.2E-24), regulation of biological process (FDR 9.17E-21), and regulation of cellular process (FDR 9.18E-20) (Fig. 2) (Supplementary Table S1). For the cyan module, genes were enriched for cellular process (FDR 5.37E-22), cellular component biogenesis (FDR 8.98E-17), mitotic cell cycle (FDR 6.25E-14), and cell cycle process (FDR 8.92E-10). Genes in the green module were enriched for cellular macromolecule metabolic process (FDR 1.31E-47), macromolecule metabolic process (FDR 1.61E-43), cellular process (FDR 3.67E-43), and primary metabolic process (FDR 2.79E-32).

On the other hand, in the black module, genes were enriched for cellular respiration (FDR 1.66E-68), generation of precursor metabolites and energy (FDR 9.50E-62), energy derivation by oxidation of organic compounds (FDR 4.29E-61), and electron transport chain (FDR 1.72E-54). Genes in the greenyellow module were enriched for muscle contraction (FDR 2.14E-07), muscle system process (FDR 2.53E-07), regulation of muscle contraction (FDR 1.24E-06), and regulation of muscle system process (FDR 2.63E-06) (Fig. 3) (Supplementary Table S2). Grey60 module was enriched for synapse organization (FDR 1.57E-02), muscle contraction (FDR 2.32E-02), and muscle system process (FDR 2.33E-02). It is important to highlight that the genes identified have important functions related to biological regulation, cellular metabolites, and muscle contraction. By identifying the hub genes, we can gain insight into the mechanisms regulated by different diets. Although the difference in cytokine MFI between the diets was not detected, we observed changes in co-expression that can be explained by other processes, showing the complexity of each one of the treatments (Supplementary Fig. S2 online).

Identification of hub genes in key modules

To obtain the network of genes interacting within these enriched pathways, the results from WGCNA with their relative weights were filtered, including nodes with a weight ≥ 0.15, and 20 neighbor nodes within a distance of two. Where the neighbors match, the degree-in is between one and two nodes inclusive[16,17]. Among 14 modules, six modules were enriched in *BiNGO*, and these six modules presented a correlation between module membership (MM) and gene significance (GS) for each trait higher than 0.20. To focus on the most important relationships within a module, we considered only the top one hub gene by the plugin *CytoHubba* using Maximal Clique Centrality (MCC) in Cytoscape[18]. The hub gene for each module eigengene can be viewed in Table 2 with the FDR for gene ontology (GO) term enriched in *BiNGO*.

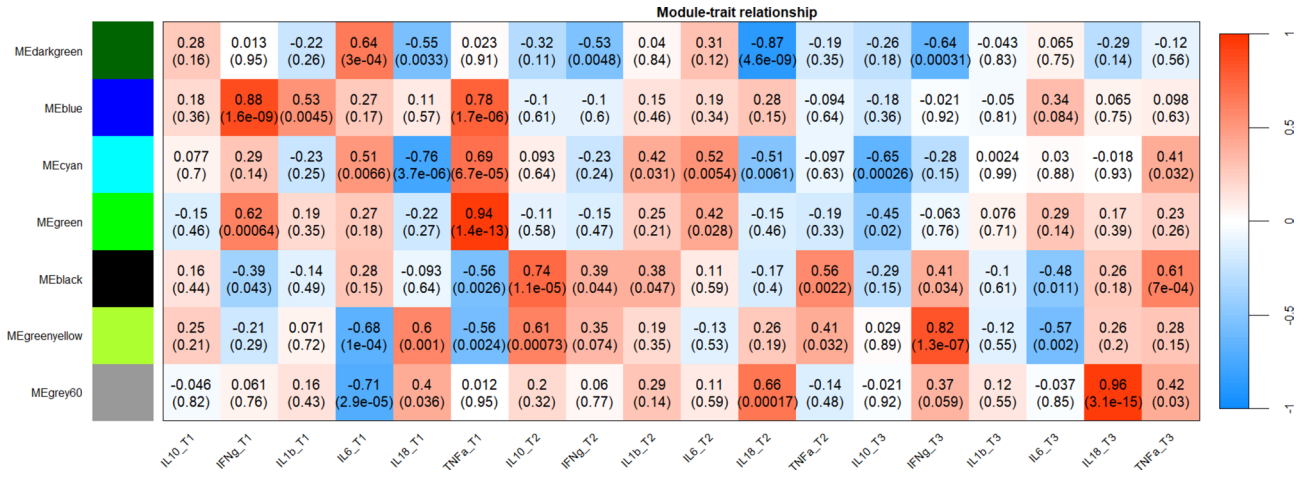

Fig. 1. Heatmap shows in the *y*-axis the module eigengenes associated with the cytokines in each treatment (*x*-axis). Pearson correlation is the top number whereas the *p*-value is the bottom number between brackets. The red colors present a positive correlation and blue colors present a negative correlation. TNFa: tumor necrosis factor-α, IFNg: interferon-γ, IL: interleukin, T1: soybean oil diet, T2: canola oil diet, T3: fish oil diet.

Fig. 2. *BiNGO* enrichment for ME Blue for FDR lower or equal to 0.05. The color scale orange-yellow can be defined by *p*-values (Color-scaled yellow-orange has decreased corrected *p*-values while compared to white ellipses, which have no significative *p*-values).

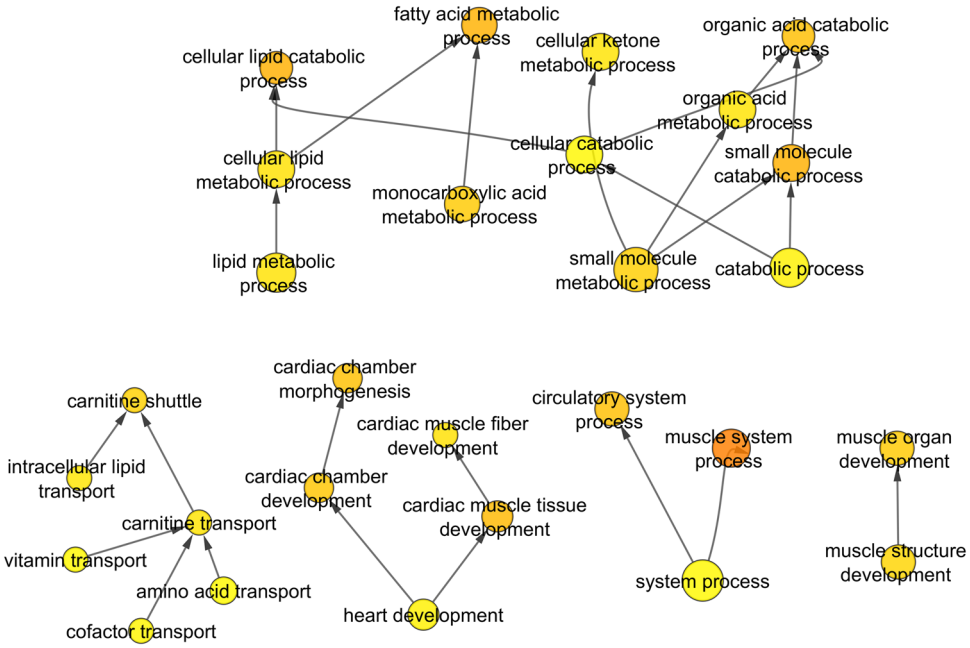

Fig. 3. *BiNGO* enrichment for ME Greenyellow for FDR lower or equal to 0.05. The color scale orange-yellow can be defined by *p*-values (orange has lower corrected *p*-values than yellow).

For the soybean diet, we identified two positive correlations between the blue module eigengene values and both IFN-γ and TNF-α. The LDL receptor-related protein 6 (*LRP6*) is a protein-coding gene linked to protein homodimerization activity and signaling receptor binding in the plasma membrane[19] (Supplementary Fig. S3 online).

Hub gene name	Module Eigengene	Gene description	Cytokines associated	GO Term	FDR
LRP6	Blue	LDL receptor-related protein 6	IFNγ, TNFα	Biological regulation	1.63E-25
TUBB2B	Cyan	Tubulin B 2B Class IIb	IL-18	Cellular process	5.37E-22
HSP90B1	Green	Heat shock protein 90 B family member 1	TNFα	Cellular macromolecule metabolic process	1.31E-47
NDUFA6	Black	NADH:Ubiquinone Oxidoreductase Subunit A6	IL-10	Cellular respiration	1.66E-68
TNNC1	Greenyellow	Troponin C1	IFNγ	Muscle contraction	2.14E-07
MYH8	Grey60	Myosin heavy chain 8	IL-6, IL-18	Muscle contraction	2.32E-02

Table 2. Hub genes with their respective modules, gene description, and GO term enrichment with the lowest FDR.

For the cyan module, the hub gene tubulin β 2 C class IIb *(TUBB2B)* is also a protein-coding gene that includes GTP binding and is a structural constituent of the cytoskeleton[19] (Supplementary Fig. S4 online). In this study, this hub gene was negatively correlated with IL-18 cytokine levels in muscle under the soybean oil diet.

Polyunsaturated fatty acids, mainly EPA, can be protective through inhibition of TNF-α effect of apoptosis and myogenesis[20]. In this way, the heat shock protein 90 β family member 1 (*HSP90B1*) gene encodes a protein that is linked to unfolded protein response and cellular response stimuli, protein is located at the cytosol, endoplasmic reticulum, nucleus, and extracellular[19] (Supplementary Fig. S5 online). Interestingly, the green module eigengenes were positively correlated to TNF-α for the soybean oil diet.

The other hub gene found in the black module was NADH*:* ubiquinone oxidoreductase subunit A6 (*NDUFA6*) which is a protein-coding related to complex I biogenesis and respiratory electron transport in the mitochondrion[19] (Supplementary Fig. S6 online). A positive correlation was found between *NDUFA6* levels in black and IL-10 MFI in muscle in the canola oil diet group. IL-10 is known as an anti-inflammatory cytokine[21].

In the greenyellow module, the top hub gene was troponin C1 *(TNNC1)* which is a protein-coding gene related to calcium ion binding and actin filament binding located at cytosol and cytoskeletum[19] (Supplementary Fig. S7 online). This hub gene was positively correlated to IFN-γ levels in the fish oil diet. Fish oil consists of saturated fatty acid (SFA), EPA, and DHA, presenting a lower PUFA/SFA ratio because of high levels of SFA as compared to PUFA levels[22].

The other hub gene found in the grey60 module was myosin-heavy chain 8 (*MYH8)* encodes a protein-coding gene associated with ATP hydrolysis activity and actin filament binding located at cytosol and cytoeskeletum[19] (Supplementary Fig. S8 online). In our study, the gene network with *MYH8* as the hub gene was enriched for muscle contraction pathway and was negatively associated with IL-6 in muscle in the soybean oil treatment group as well as positively associated with IL-18 levels in muscle of the fish oil treatment group.

Discussion

The main objectives of this study were to quantify cytokine abundance and to analyze gene co-expression patterns, as well as to evaluate the correlations between co-expressed gene modules and cytokine abundance in the skeletal muscle tissue of pigs fed diets supplemented with 3% of different oil sources. The results identified significant correlations ($r \geq |0.70|$; $p \leq 0.05$) between cytokine abundance and gene modules enriched for specific biological processes.

Differences in cytokine abundance were observed among the dietary treatments. TNF-α levels were significantly lower ($p \leq 0.05$), while IL-10 and IFN-γ showed a trend toward reduction ($p < 0.10$) in the canola oil group. IL-10 is known for its anti-inflammatory properties, particularly through the stimulation of B cells and the inhibition of pro-inflammatory cytokine production by macrophages, including TNF, IL-1, and IL-2[23]. Beyond its immunomodulatory role, IL-10 is also implicated in metabolic processes, as it helps mitigate lipid-induced insulin resistance and contributes to the upregulation of insulin sensitivity[24].

A study conducted in male C57BL/6 mice fed a high-fat diet (HFD; 28% SFA, 30% trans, 28% MUFA, and 14% PUFA) reported elevated IL-10 expression in skeletal muscle, suggesting a beneficial role for this cytokine in maintaining insulin responsiveness[24]. In the present study, diets enriched with soybean and fish oils—both rich in PUFAs—were associated with higher levels of TNF-α, IFN-γ, and IL-10. This indicates that a MUFA-rich diet, such as the canola oil-supplemented diet, resulted in reduced levels of pro-inflammatory cytokines as well as IL-10[22]. Similarly, another study using leptin-deficient (obese and diabetic) rats fed an HFD over an extended period (16 weeks) found evidence of local inflammation, with marked increases in IL-6, IL-1α, and IFN-γ levels in skeletal muscle, alongside elevated IL-10, which was associated with glucose metabolism. The authors proposed that IL-10 may offer protection against insulin resistance in muscle and suggested it as a potential therapeutic target for the treatment of insulin resistance and type 2 diabetes[25].

To better understand the relationship between cytokine levels and gene expression, a co-expression analysis using the WGCNA methodology revealed that the greenyellow module was strongly correlated with IFN-γ levels ($r = 0.82$; $p = 1.3E\text{-}07$) in pigs fed a diet supplemented with 3% fish oil. Functional enrichment analysis of the genes within this module identified pathways related to skeletal muscle contraction and fatty acid metabolism. Although fish oil has been previously associated with anti-inflammatory responses[26], this module includes hub genes involved in mitochondrial β-oxidation, such as *HADHB*, *HADHA, ACADL, CPT2, HADH*, and *CPT1B*—the latter being muscle-specific—as well as genes like *GPAM* (involved in triglyceride synthesis) and *ACADSB*

(involved in acyl-CoA dehydrogenation)[19,27,28]. The reported interaction between IFN-γ and fatty acid oxidation suggests that IFN-γ exposure, particularly in the fish oil group, may enhance mitochondrial β-oxidation[29]. In a previous study involving the same pig population, animals fed fish oil also exhibited significantly higher shear force values (indicating tougher meat) compared to those fed other diets[22]. This increase in shear force may be linked to IFN-γ-associated β-oxidation, potentially impacting muscle structure and meat tenderness.

As observed in the WGCNA analysis, IFN-γ and TNF-α were positively co-expressed within the blue module in pigs fed the soybean oil diet (r = 0.88; p = 1.6E-09 and r = 0.78; p = 1.7E-06, respectively). Genes within this blue module were significantly enriched for biological regulation processes. Skeletal muscle actively secretes cytokines (or myokines), a process that involves not only muscle fibers but also various resident cell types within the tissue[30,31]. Regarding the regulation of these cytokines, our findings suggest a complex regulatory landscape, indicating that cytokine expression may be modulated both at the level of individual genes and through coordinated gene co-expression networks—patterns that are effectively captured by the sensitivity of the WGCNA approach.

In line with the well-established role of cytokine regulation in metabolic health, our study identified specific gene co-expression modules (blue, green, and grey60) associated with this regulatory process. Notably, genes such as peroxisome proliferator-activated receptor gamma (PPARG) were identified within the blue module, alongside the co-expressed cytokine TNF-α in pigs fed the soybean oil diet. In our previous study, we identified PPARG receptor (downregulated in the soybean oil group), which was enriched in the pathway "Role of IL-6 in Obesity and Type 2 Diabetes in Adipocytes"[7].

All three modules (blue, green, and grey60) showed strong correlations with IFN-γ, TNF-α, and IL-6, particularly evident in the soybean oil diet compared to the canola and fish oil diets. These modules were associated with biologically relevant pathways, including biological regulation, cellular macromolecule metabolic processes, and muscle contraction. These findings provide valuable insights into how dietary oils influence regulatory mechanisms in skeletal muscle. According to the study by Hong et al., diet-induced macrophage infiltration was associated with TNF-α and IL-6 expression, highlighting the interaction between the genes encoding these cytokines[24]. Furthermore, the same authors reported an association between IL-6 and insulin resistance in muscle tissue[24].

The identified hub genes, such as *LRP6, TUBB2B, HSP90B1, TNNC1, NDUFA6*, and *MYH8*, may play key roles in various biological regulatory pathways, cellular processes, macromolecular metabolic processes, cellular respiration, and muscle contraction, and were co-expressed with certain pro- or anti-inflammatory cytokines (Table 2). For instance, *LRP6*, found in the blue module and associated with IFN-γ and TNF-α, is related to the low-density lipoprotein receptor (LDLR) gene family[19]. *TUBB2B*, a beta tubulin isoform correlated with IL-18 in the cyan module, binds GTP and is a major component of cellular microtubules[19]. *HSP90B1*, correlated with TNF-α in the green module, functions in the stabilization and folding of other proteins[19]. *TNNC1*, associated with IFN-γ in the greenyellow module, plays a central regulatory role in skeletal muscle contraction[19]. *NDUFA6*, associated with IL-10 in the black module, acts as an accessory subunit of complex I in the mitochondrial respiratory chain[19]. *MYH8*, associated with IL-6 and IL-18 in the grey60 module, is involved in the generation of mechanical force in motor proteins such as actin[19]. In the present study, several hub genes were found to be associated with pro- and anti-inflammatory cytokines, representing a pioneering effort to establish specific correlations between these hub genes and individual cytokines in skeletal muscle.

The green module showed a positive association with the soybean oil diet and a strong correlation with TNF-α, warranting further investigation. Of particular relevance to this finding is the known inhibitory effect of TNF-α on ligand-dependent transcriptional activity of *PPARG*, suggesting a potential negative interaction between these two factors within this dietary context[32]. Additionally, the identification of *HSP90B1* as a hub gene in this module carries functional significance, as it has been associated with signaling through Toll-like receptors (TLRs 1, 2, 4, 5, 7, and 9)[33]. These observations underscore how specific dietary fatty acids, such as those found in soybean oil, may modulate immune-related pathways through interactions with co-expressed genes. Based on this relationship, it is plausible that TNF-α, *PPARG*, and *HSP90B1*—in connection with fatty acids and their immunological effects—are involved in the regulation of eicosanoid and cytokine synthesis. Moreover, the balance of n-6/n-3 polyunsaturated fatty acids (PUFAs) may also play a critical role in regulating immune responses[34]. Thus, diets rich in PUFAs, particularly from distinct oil sources, may influence not only the modulation of immune response mechanisms but also membrane composition and fluidity via specific receptor-mediated pathways[35].

In humans, unsaturated fatty acids have been reported to reduce energy intake, triacylglycerol synthesis, and the expression of certain transcription factors, such as peroxisome proliferator-activated receptors (PPARs). Additionally, they increase fatty acid oxidation and high-density lipoprotein cholesterol (HDL-C) levels[36]. PPARs modulate gene expression indirectly, with notable immunomodulatory effects. For example, IFN-γ expression is repressed by PPARG in T cells[37,38]. Furthermore, PPARG expression can be modulated by high-fat diets (HFDs) in both rodents and humans with type 2 diabetes[39,40]. HFDs may induce hyperinsulinemia through enhanced lipogenesis and obesity from gestation into adulthood, downregulate glucose transporters, and contribute to increased insulin resistance[36]. Another cytokine involved in glucose uptake is IL-6, which can improve insulin sensitivity and promote lipid oxidation in skeletal muscle tissue[41].

In our study, we observed strong correlations between IFN-γ in the blue module and IL-6 in the grey60 module, particularly in pigs fed soybean oil compared to those fed canola or fish oils (Fig. 1). These findings reflect potential mechanisms by which dietary PUFAs influence immune and metabolic responses at the gene expression level.

The metabolic role of essential PUFAs—particularly linoleic acid (LA; C18:2 n-6)—helps to explain their pro-inflammatory potential. LA is elongated and desaturated into arachidonic acid (AA; C20:4 n-6), which is the precursor of prostaglandins and leukotrienes produced through the cyclooxygenase and lipoxygenase pathways,

respectively. These eicosanoids act as pro-inflammatory mediators and have been implicated in the pathogenesis of metabolic diseases[21]. Eicosanoids function similarly to hormonal regulators of immune cell activity, inducing inflammatory responses through T cells and lymphocytes[21].

Thus, the interaction between gene expression and cytokine profiles in muscle plays a critical role in identifying inflammation- and metabolism-related pathways in healthy pigs, which serve as a relevant model for human metabolic diseases when fed diets containing different fatty acid sources.

While acknowledging that not all cytokines are directly correlated with specific enriched pathways, our network-based co-expression analysis highlights the utility of systems biology approaches in unraveling complex biological mechanisms across tissues and nutritional contexts. Notably, our study demonstrated how dietary oils modulate immune signaling in skeletal muscle and emphasized the identification of hub genes linked to immune-related metabolic processes. This provides novel insights into the distinct immunomodulatory effects of canola, soybean, and fish oils, and underscores the value of nutrigenomics in exploring gene-diet interactions. Further studies are warranted to investigate direct interactions between cytokines and hub genes revealed in this work.

Methods

Ethical approval

All animal procedures were approved by the Animal Care and Use Committee of Luiz de Queiroz College of Agriculture (ESALQ-USP) (Piracicaba, Brazil, protocol number: 2018.5.1787.11.6 and number CEUA 2018–28) and followed ethical principles in animal research, according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching[42].

Animals and sample collection

Our study included 27 purebred Large White immunocastrated male pigs (*Sus scrofa*), offspring of 32 sows and three sires. These pigs were randomly assigned to one of three dietary treatments (9 animals per treatment): a soybean-corn meal base supplemented with either 3% soybean oil (as reference), 3% canola oil, or 3% fish oil. The sample size was calculated based on the statistical power method according to the experiment by Duan and collaborators[43]. The animals were selected and genotyped for the halothane mutation – *RYR1* gene – and just halothane homozygous negative (NN) pigs were selected[44]. The pigs aged 71 ± 1.8 days were then allocated to the three dietary treatments. The farm was equipped with a hole-dry self-feeder in each pen and a nipple drinker, allowing these animals to have ad libitum access to food and water during the experiment[7,45].

The diets were formulated to have similar digestible energy and metabolizable energy content, according to Almeida and collaborators[22]. We did not use antibiotic growth promoters. According to the manufacturer's recommendations, all the pigs were immunocastrated through 2 mL doses of Vivax® (Pfizer Animal Health, Parkville, Australia) at 105 days and 133 days of age. With 98 days of trial, pigs were slaughtered with a final age of 169 ±7 days and an average final body weight of 133.9 ±9.4 kg. On slaughter, we collected samples of the *Longissimus lumborum* muscle, which were quickly excised, snap-frozen in liquid nitrogen, and stored at −80 °C for posterior analyses of RNA-Sequencing (RNA-Seq), WGCNA, and ELISA. More details can be found in Almeida et al.[22] and Fanalli et al.[7]. All the study is reported following ARRIVE guidelines.

Protein extraction and quantification

The chosen cytokines were based on a previous study conducted by the group according to Fanalli et al.[7,46,47]. The tissue homogenate preparation was made using 100 µg of the *Longissimus lumborum* muscle with the addition of 990 µL of RIPA 1% buffer (RIPA Lysis Buffer, 10X, Merck) and 10 µL of protease inhibitor 10% (Protease Inhibitor Cocktail Set I 100X, Merck). Next, we homogenize the tissue by using the turrax (Ultra-turrax T10 basic, Ika) for 30 s. Then, the samples were centrifuged 10,000 g for ten minutes at 4 °C. Following the centrifugation, the samples were aliquoted and stored at −80 °C until immunoassay. After the protein extraction, the samples were quantified with PierceTM BCA Protein Assay Kit (ThermoFisher Scientific) and measured by 520 nm in the spectrophotometer. The concentration in ng/mL was calculated using the following Eq. (1).

$$\frac{\left(\left(\frac{Finalabsorbance-0.0542}{0.0011}\right)*50\right)}{1000} \quad (1)$$

Multiplex sandwich ELISA

According to the manufacturer's instructions, the immunoassay was realized with the multiplex MILLIPLEX® Map Kit (Merck KGaA, Darmstadt, Germany). Briefly, fluorescent magnetic beads coated with capture antibodies were sonicated, vortexed, and diluted. Standards were prepared via serial dilution, and quality controls were reconstituted. Following a plate wash, standards, quality controls, and samples were added to designated wells along with the diluted beads. The plate was sealed and incubated overnight at 2–8 °C to allow for analyte binding. After washing, biotinylated detection antibodies were added, followed by streptavidin–phycoerythrin. Following a final wash, drive fluid was added to resuspend the beads. Then, the samples were read in the equipment Luminex® 200™, HTS, FLEXMAP 3D® software (Merck KGaA, Darmstadt, Germany). The results considered were Median Fluorescence Intensity (MFI).

RNA-Seq, quality control, alignment, and normalization

All the sequencing analyses were realized at the Genomic Center in the Animal Biotechnology Laboratory at ESALQ/USP, Piracicaba, Sao Paulo, Brazil. Total RNA was extracted from the skeletal muscle using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Quantification, purity, and

integrity of total RNA were evaluated by the Nanodrop 1,000 and Bioanalyzer. For all samples, the RNA integrity number (RIN) was between 7.8 and 10.0. To prepare the library, 2 µg of total RNA was used according to the TruSeq RNA Sample Preparation Kit v2 Guide protocol (Illumina, San Diego, CA). To calculate the average library size, we used the Agilent Bioanalyzer 2,100 (Agilent, Santa Clara, CA, USA). The quantification of the libraries was made using quantitative PCR with the KAPA Library Quantification Kit (KAPA Biosystems, Foster City, CA, USA). The quantified samples were diluted, labeled by a barcode, and pooled to be run in different lanes using the TruSeq DNA CD Index Plate (Illumina, San Diego, CA, USA). All the samples were sequenced across five lanes on the flow cell using the TruSeq PE Cluster Kit v4- cBot-HS kit (Illumina, San Diego, CA, USA). They were clustered and sequenced using HiSeq 2,500 equipment (Illumina, San Diego, CA, USA) with a TruSeq SBS Kit v4-HS (200 cycles) according to the manufacturer's instructions. More details can be viewed in a previous publication by our group[7].

The quality of the raw RNA-Seq reads was checked by FastQC version 0.11.8 [http://www.bioinformatics.bbrc.ac.uk/projects/fastqc/]. Thus, we removed the sequencing adaptors, index, barcodes, and low-complexity reads using the TrimGalore version 0.6.5 [http://www.bioinformatics.babraham.ac.uk/projects/trim_galore]. Phred score higher than 33 and reads length bigger than 70 nucleotides were kept to the following steps[7].

The alignment was made with the pig reference genome Sus scrofa 11.1, available at Ensembl [http://www.ensembl.org/Sus_scrofa/Info/Index]. The read counts of mRNAs for all annotated genes were calculated using Bowtie2 version 2.4.3[48].

Gene expression levels were normalized using transcripts per million (TPM) calculated by RNA-Seq by Expectation–Maximization (RSEM) version 1.3.1. This method estimates the number of fragments originating from each gene in each replicated library, effectively handling reads that map ambiguously between isoforms and genes, thus reducing variations in total read counts across samples[49]. Fifty percent of rarely or lower expressed genes were removed from further analysis.

Statistical analysis and WGCNA

Data were analyzed as randomized complete block design using MIXED procedure of SAS (SAS Institute Inc. Cary, NC). The dependent variable (Y) was treated by each cytokine (IL-10, IFN-γ, IL-1β, IL-6, IL-18, and TNF-α); diet, sire, and block as fixed effects to adjust cytokine abundance (removing the confounding by these effects), and ε is the residual vector. Tukey's test was performed with a significance level of $\alpha \leq 0.05$.

Adjusting phenotypes was done using a simple linear model in R, with a vector of means of phenotypic values of sire as a fixed effect. Thus, the adjusted phenotype was obtained by Eq. (2).

$$\widehat{Y}* = \mu + y - X\widehat{\beta} + \varepsilon \tag{2}$$

W here $\widehat{Y}^*$ is the adjusted/corrected phenotype, µ the general mean vector for the phenotype; y is the phenotype vector; X is the incidence matrix for fixed effects; $\hat{\beta}$ is the fixed effects vector (sire); ε is the residual vector[50].

We employed Weighted Gene Co-expression Network Analysis (WGCNA)[51], a system biology approach implemented in the WGCNA package with R software, to identify clusters of genes (modules) exhibiting similar expression patterns in porcine skeletal muscle. This approach allows for the correlation of gene modules with relevant biological traits, such as cytokine profiles[52]. Normalized TPM data from skeletal muscle samples were used to construct a signed network based on Pearson correlation between genes. A soft threshold power of 14 ($R^2 = 0.76$, Supplementary Fig. S1 **a**) was applied to transform the correlation matrix into a weighted adjacency matrix, representing the connection strength between genes. The topological overlap matrix (TOM) was then calculated based on dissimilarity measures (1 – TOM) to capture the interconnectedness of genes within the network.

Modules were identified using hierarchical clustering with a minimum module size of 30 genes. Genes not assigned to any module were grouped into the grey module. Highly correlated modules were further merged based on the dissimilarity of their eigengenes (first principal component of each module), which represent the overall expression profile of the module[51,52]. A dissimilarity threshold of 0.25 (equivalent to a correlation of 0.75) was used for module merging (Supplementary Fig. S1 **c**). Module-trait relationships were assessed by correlating module eigengenes (MEs) with cytokine profiles (IFN-γ, IL-1β, IL-18, IL-10, IL-6, and TNF-α) measured in the same skeletal muscle samples. Modules exhibiting a strong correlation (Pearson's correlation ≥|0.7|) with at least one cytokine were selected for further analysis (Fig. 1).

To identify potential biological functions associated with selected modules, we focused on genes exhibiting a strong MM and GS relationship. Specifically, genes with their values of filiation to the GS over MM higher than 0.2[53]. These genes, characterized by high intramodular connectivity, are likely to represent key drivers (hub genes) of the biological processes within their respective modules[52,54].

Filtering nodes, functional annotation, enrichment analysis, and hub gene identification

After filtering, nodes, that presented a weight ≥ 0.15[17], were considered for functional annotation and enrichment in all the modules. Nodes with at least 20 neighbors within distance 2 and a degree-in that is between 1 and 2 inclusive were used to show the *BiNGO* enrichment analysis. The darkgreen module could not be enriched because only one node was found above 0.15 in the filtering step, despite having a negative correlation with IL-18, there were not enough nodes identifying hub genes after filtering by weight ≥ 0.15.

The functional annotation was realized using the online software FAANGMine v.1.3 from the University of Missouri [https://faangmine.rnet.missouri.edu/]. The gene ontology and pathway enrichment analyses were realized in Cytoscape v.3.10.0 plugin *BiNGO*[55], a hypergeometric test to assess overrepresentation in a FDR of 0.05 for the GO term in Biological Process. Hub genes were found in the Cytoscape plugin *cytoHubba* using MCC[18].

Data availability

The dataset supporting this study is available in the European Nucleotide Archive (ENA) repository (EMBL-EBI), under accession PRJEB52629. https://www.ebi.ac.uk/ena/browser/view/PRJEB52629

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Author contributions

F.N.C., C.K.T., J.E.K., and A.S.M.C. contributed to the conception and design. F.N.C., C.K.T., and A.S.M.C. performed formal and data analysis. F.N.C. performed statistical analysis. F.N.C. and A.S.M.C. prepared the original draft. F.N.C., C.K.T., J.D.G., and A.S.M.C. wrote the obtained manuscript. B.P.M.S., J.D.G., M.C.D., C.S.O., L.E.N., J.E.K., and A.S.M.C. F.N.C. and A.S.M.C. critically reviewed and provided feedback on the original manuscript. A.S.M.C. obtained financial support. All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to A.S.M.C.