VOL IV

Estudos em Ciências Agrárias e Ambientais

Eduardo Spers
(Organizador)

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APRESENTAÇÃO

É com grande satisfação que apresentamos o volume IV da coletânea **Estudos em Ciências Agrárias e Ambientais**, resultado do esforço colaborativo de pesquisadores de diferentes regiões e instituições, que compartilham aqui reflexões, dados e contribuições relevantes para o avanço do conhecimento técnico-científico em suas áreas de atuação.

Este volume reúne 13 trabalhos organizados em cinco eixos temáticos que refletem a diversidade e complexidade do campo agrário e ambiental contemporâneo: Sistemas de Produção Aquático e Animal; Sustentabilidade Ambiental e Conservação de Recursos Naturais; Sistemas de Produção Vegetal e Agricultura de Precisão e Educação e Inovação no Meio Agrário.

Os temas abordados vão desde o manejo sustentável de recursos naturais, passando por inovações tecnológicas na agricultura e aquicultura, até discussões sobre formação profissional e segurança sanitária nas cadeias produtivas. Essa pluralidade é o reflexo da crescente interdisciplinaridade que caracteriza os estudos agrários e ambientais hoje – exigindo diálogos entre a ciência, a tecnologia, a educação, a economia e a sociedade.

Além da qualidade dos estudos apresentados, destacamos o compromisso dos autores com a pesquisa aplicada, a sustentabilidade e a busca por soluções adaptadas às realidades locais, muitas vezes desafiadoras. A presença de autores da América Latina e Europa também fortalece o caráter internacional da obra, fomentando o intercâmbio de experiências e metodologias.

Agradecemos a todos os autores pela confiança em compartilhar seus trabalhos conosco. Que esta publicação possa inspirar novas pesquisas, colaborações e, acima de tudo, práticas que contribuam com a construção de sistemas agrários e ambientais mais resilientes, justos e inovadores.

Desejamos a todos uma excelente leitura!

Eduardo Eugênio Spers

SUMÁRIO

SISTEMAS DE PRODUÇÃO AQUÁTICO E ANIMAL

CAPÍTULO 1

EFFECTS OF INCLUSION OF PROBIOTIC PEDIOCOCCUS ACIDILACTICI IN DIETS WITH HIGH LEVELS OF SOYBEAN MEAL IN GROWTH AND INTERLEUKINS GENE EXPRESSION OF RAINBOW TROUT (*Oncorhynchus mykiss*)

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ABSTRACT: A 90-days feeding trial was performed to establish the effects of the probiotic *Pediococcus acidilactici* added to diets with high contents of soybean meal in the growth performance, survival rate, proximate composition, and gene expression of the interleukins 1β, 4, 8, 10, and 21 of rainbow trout fingerlings. A basal diet (400 g/kg diet of soybean meal) was added with *P. acidilactici* at 0.2, 2, and 20 g/kg and fed to triplicate groups with initial weight of 2.3 ± 0.1 g (mean ± SE). Growth performance and survival improved with increasing inclusions of the probiotic, while gene expression of the interleukins *il-4* and *il-10* were upregulated as the probiotic increased in the diets. Concentration of *P. acidilactici* adequate for using with high levels

of soybean meal inclusions was of 20 g/kg, as it helps to improve the growth, survival and upregulate the expression of the interleukins IL-4 and IL-10.
KEYWORDS: fingerlings; plant-origin protein; survival; rainbow trout.

1. INTRODUCTION

The production of rainbow trout (*Oncorhynchus mykiss*) relays in aquafeeds that use marine fish derived products as the main sources of protein and lipids (Hardy 2010). The fish meal and oil production will be stable during the next decades, but the increasing demand is surpassing the supply (Gatlin et al., 2007), causing increasing prices of aquafeeds and possibly, their use is no longer viable for aquaculture production (Callet et al., 2017). Plant-based ingredients are the most viable sources to substitute fish ingredients and particularly, soybean meal (SBM) has a high protein content and a well-balanced amino acid profile (Gatlin et al., 2007), is widely available and has low cost (Tacon and Metian 2008). However, inclusion of SBM has been limited due to several negative effects in carnivorous fish, such as retarded growth performance, poor digestibility and in extreme cases, non-infectious enteritis and death (Sealey et al., 2009; Laining et al., 2010; Krogdahl et al., 2010). Thus, higher inclusions of SBM in aquafeeds may require additives which help to improve digestibility proper use of nutrients (Sánchez et al. 2015). Probiotics can help in reducing the negative effects of SBM (Sealey et al., 2009) and their effects in the diets have been poorly investigated: Sealy et al. (2009) reported that fingerlings of rainbow trout tolerate increased levels of SBM by feeding a commercial probiotic including species of yeast and bacteria, while Sánchez et al. (2015) found that inclusion of live yeast (*Saccharomyce*s sp.) improved the growth of juvenile rainbow trout when fed a diet with SBM as the main source of protein.

On the other hand, the probiotic *Pediococcus acidilactici* has been reported to improve morphology of intestinal microvilli, enterocyte endocytic activity, reduce intestinal alterations caused by bacterial infection (Merrifield et al., 2010; Merrifield et al., 2011; Harper et al., 2011, Al-Hisnawi et al., 2019). In zebrafish (*Danio rerio*), use of *P. acidilac*tici improved the growth performance, immune responses, and reproductive performance (Arani et al., 2019; Arani et al., 2021). Torrecillas et al. (2018) reported that use of *P. acidilactici* and mannooligosaccharides in juvenile European sea bass (*Dicentrachus labrax*) improved the growth and disease resistance when fed diets with low inclusion of fish meal and oil. Thus, P*. acidilactici* seems to have a positive effect on intestine health and may be included in feeds with high content of plant-origin proteins and the objective of the present research was to determine the effects of different concentrations of *P. acidilactici* added to diets with high contents of SBM in the growth performance, survival rate, proximate composition, and gene expression of the interleukins 1β, 4, 8, 10, and 21 of fingerlings of rainbow trout.

2. MATERIALS AND METHODS

2.1. EXPERIMENTAL DIETS

The formulation of the basal diet was done according with Cruz et al. (2011), with soybean meal (providing 75% of the protein portion) and fish meal (providing 25% of the protein portion) as protein sources (Table 1). *Pediococcus acidilactici* (Bactocell®, Lallemand, Canada, 1 x10^{10} CFU/g) was added to the basal diet with at 0.2 (*Pa*0.2), 2 (*Pa*2) and 20 (*Pa*20) g/kg of diet, by spraying the bacteria previously diluted in distilled water, following the manufacturer´s instructions. The basal diet without addition of bacteria was used as control.

Table 1. Basal composition of the experimental diets fed to rainbow trout fingerlings. The proximate composition was of (% dry weight basis ± SD); crude protein, 42.1 ± 1.0; crude lipids. 11.2 ± 0.7, and ash, 9.8 ± 0.3.

Ingredients	g/kg
Fish meal[1]	200
Soybean meal[2]	400
Cod liver oil[3]	50
Soybean lecithin[3]	50
Dextrin[3]	100
Mixture of vitamin and minerals[4]	40
Wheat gluten[5]	50
α-celullose[5]	110

[1]Vimifos S.A. de C.V., Mexico

[2]Pisa S.A. de C.V., Mexico

[3]Drotasa S.A. de C.V., Mexico

[4]Sigma Aldrich Co., MO, USA

[5]DSM Nutrition Products of Mexico, Mexico

2.2. EXPERIMENTAL ORGANISMS

Organisms of 60-days after hatching were obtained from the commercial farm “El Xote” (municipality of Jilotzingo, State of Mexico, Mexico) and transported to Laboratorio de Producción Acuícola ubicated at the municipality of Tlalnepantla, Estado de Mexico. Organisms were kept in 1000-L tanks with continuous filtration, aeration and fed with a commercial feed (Growth-fish trucha, Malta Texco de Mexico S.A. de C.V., Mexico) for three weeks until the start of the feeding trial.

2.3. FEEDING TRIAL

The feeding trial was performed in a recirculating system with 12 polypropylene tanks of 100-L each. Thirteen fish with an initial weight of 2.3 ± 0.1 g (mean ± SE) were randomly allocated in each tank. Each diet was fed to triplicate tanks at 7% of the total body weight and daily ration was divided into two equal feedings at 09:00 and 16:30 hours. Organisms were weighed every 10 days, and the ration size was adjusted accordingly. During the trial, water conditions were (mean ± SE) as follows: dissolved oxygen, 5.0 ± 0.1 mg/L; ammonia, 0.01 ± 0.001 mg/L; pH, 8.1 ± 0.2 and temperature of 19 ± 2 °C. All tanks were maintained under natural cycle of 12 h light, 12 h darkness. The trial was conducted for a period of 90 days and at the end, the organisms were starved for 24 h and weighed to obtain the growth performance. Then, 10 fish of each treatment were euthanized with an overdose of clove oil. Samples of liver and muscle were obtained for protein and lipid contents, while distal intestine samples were taken for the gene expression analysis.

2.4. PROXIMATE COMPOSITION

Protein contents of muscle and liver were determined by acid digestion and further distillation according with the AOAC (1990) methods, while lipids were extracted with chloroform and methanol and determined following the technique reported by Blight and Dyer (1959).

2.5. QUANTITATIVE RT-PCR ANALYSIS

Total RNA from the intestine tissue samples was extracted according with Blaufuss et al. (2020), using TRIzol reagent (Invitrogen, Carlsbad, USA) and quantified with an RNA BR assay kit (Invitrogen, Carlsbad, USA) in a Qubit 4 (Invitrogen, Carlsbad, USA) fluorometer. Samples of RNA were diluted to contain the same quantity nuclease-free water and then, treated with DNase (DNase I, Invitrogen, Carlsbad, USA) and reverse transcribed into cDNA (High-capacity cDNA reverse transcription kit, ThermoFisher Scientific, Waltham, USA) according with the manufacturer instructions. The resulting samples with cDNA were stored at -80 °C until further analysis. The primers of interleukins 4 (*il-4*), 8 (*il-8*), 10 (*il-10*), and 21 (*il-21*) were designed to be intron-free, while those of interleukin 1ß (*il-1ß*) were reported by Vélez-Calabria et al. (2021) (Table 2).

Each sample was amplified by duplicates in 0.1 ml microtubes by using a MyGo mini-RT-PCR system (IT-IS International. Stokesley, England) and a SensiFAST SYBER no-ROX kit (Meridian Bioscience, Memphis, USA) with melt curves to assess product specificity. The genes were normalized against ß-actin expression and quantified using the ΔΔCt methodology reported by Livak and Schmittgen (2001).

Table 2. Primer sequences of rainbow trout genes used for quantitative polymerase chain reaction.

Gene	Accession	Primers
il-1ß	AJ223954	*f.* ACA TTG CCA ACC T CA TCA TCG *r.* TTG AGC AGG TCC TTG TCC TTG
il-4	AB574337	*f.* ACC ACC ACA AAG TGC AAG GAG TTC T *r.* CAC CTG GTC TTG GCT CTT CAC AAC
il-8	AJ279069	*f.* CTC GCA ACT GGA CTGACA AA *r.* TGG CGT ACA TCC TGA TGC TC
il-10	AB118099	*f.* GGA TTC TAC ACC ACT TGA AGA GCC C *r.* GTC GTT GTT GTT CTG TGT TCT GTT GT
il-21	FM883701	*f.* CAA CAG TGT GAT GTC GAA CGC TC *r.* CCT TGG CAG ACT GTT TTC TCT CTC C
ß-actin	AJ438158.1	*f.* TGG GGC AGT ATG GCT TGT ATG *r.* CT. TGG CAC CCT AAT CAC CTC T

2.6. STATISTICAL ANALYSIS

Values of growth performance, protein and lipids contents of muscle and liver, and gene expression were tested for normality and homoscedasticity with the Kolmogorov-Smirnov test and Levene´s test, respectively. Then, one-way ANOVA was performed using Prism software (v. 10.2.2, GraphPad Software, San Diego, CA, USA). Significant differences were evaluated with the post hoc with a Fisher´s LSD test, with a significant level of 5% ($P < 0.05$) for each set of comparisons.

3. RESULTS

Growth performance and survival rates of juvenile rainbow trout fed diets additions with soybean and the inclusion of different concentrations of *P. acidilactici* are show in Table 3. After 90 days, weight gain (WG) was significantly lower in organisms fed with diets *Pa*0.2 and *Pa*2 when compared with the juveniles fed the *Pa*20. Concentration. A similar trend was observed for the values of specific growth rate (SGR). Regarding feed intake (FI) and feed conversion efficiency (FCE) were found no significant differences were observed, but higher values were observed in the fish fed Control diet. Survival rate was higher showed direct relationship with *P. acidilactici* concentration, but no significant differences were observed among the treatments.

Table 3. Growth performance and survival of rainbow trout fingerlings fed diets with high content of soybean meal and the addition of several concentrations of Pediococcus acidilactici for a period of 90 days. Data are the means of triplicate groups ± SE. Means with different letters in the same column differ significantly (P < 0.05).

	FW (g)	WG (%)	SGR (%/day)	FI	FCE	Survival
Control	4.7 ± 0.7	96 ± 23ab	0.7 ± 0.1ab	0.27 ± 0.05	0.5 ± 0.1	59 ± 16
*Pa*0.2	3.8 ± 0.3	72 ± 12b	0.5 ± 0.07b	0.20 ± 0.02	0.4 ± 0.05	64 ± 5
*Pa*2	3.8 ± 0.2	70 ± 3b	0.5 ± 0.02b	0.18 ± 0.01	0.4 ± 0.04	77 ± 4
*Pa*20	4.8 ± 0.3	110 ± 10a	0.8 ± 0.05a	0.22 ± 0.05	0.5 ± 0.04	74 ± 6

Table 4 shows protein and lipid contents in muscle and liver. Increasing protein values were observed in muscle as the probiotic concentration increased in diets, but no significant differences were observed. Regarding protein contents in liver, no significant differences were observed among the treatments. Lipid contents in muscle were significantly higher value than observed in organisms fed diet *Pa*20 with respect those fed the *Pa*0.2 and *Pa*2. Lipids in liver values did not show significant differences among treatments, but higher contents were observed in organisms fed diets *Pa*0.2 and *Pa*2.

Table 4. Protein and lipid contents of muscle and liver of rainbow trout fingerlings fed diets with high content of soybean meal and the addition of several concentrations of *Pediococcus acidilactici* for a period of 90 days. Data are the means of triplicate groups ± SE. Means with different letters in the same column differ significantly (P < 0.05).

	Muscle		Liver	
	Protein	Lipid	Protein	Lipid
Control	12.8 ± 2.4	5.9 ± 1.9ab	16.6 ± 1.7	5.9 ± 1.9
*Pa*0.2	12.5 ± 2.1	3.9 ± 0.2ab	19.6 ± 3.4	9.2 ± 0.9
*Pa*2	14.3 ± 1.8	3.9 ± 0.2ab	14.5 ± 2.0	8.3 ± 1.3
*Pa*20	15.9 ± 0.2	7.9 ± 0.3a	15.2 ± 2.3	4.5 ± 2.1

Figure 1 shows the relative gene expression of proinflammatory interleukins (*il-1β*, *il-8* and *il*-21) in the intestine of rainbow trout juveniles. Expressions of *il-1β* (Figure 1A) were down regulated in the organisms fed diets Control, *Pa*0.2 and *Pa*2, while treatment *Pa*20 was up regulated, but no significant differences were observed. Significant higher values were found in the expression of the *il-8* when juveniles were fed with *Pa*0.2 and compared with those fed with Control and *Pa*20 (Figure 1B). Finally, the expression of *il-21* (Figure 1C) was down regulated in groups feeding the Control, *Pa*0.2 and *Pa*2 diets, but no significant differences were observed when compared with the group fed *Pa*20 diet. The expression of anti-inflammatory interleukins is shown in Figure 2, and increased values of expression of *il-4* were observed as the concentrations of the probiotic also increased, however no significant differences were observed (Figure 2a). Regardless of the different probiotic concentrations, the expression of *il-10* was significantly up regulated when compared with the fish fed the Control diet (Figure 2B).

Figure 1. Gene expression (as ΔΔCt values) of proinflammatory interleukins (A) 1β (*il-1β*), (B) 8 (*il-8*), and (C) 21 (il-21) from the distal intestine of rainbow trout fingerlings fed diets with high content of soybean meal and the addition of several concentrations of *Pediococcus acidilactici* for a period of 90 days. Each bar represents the means of triplicate groups ± SE. Bars with different letters differ significantly ($P < 0.05$).

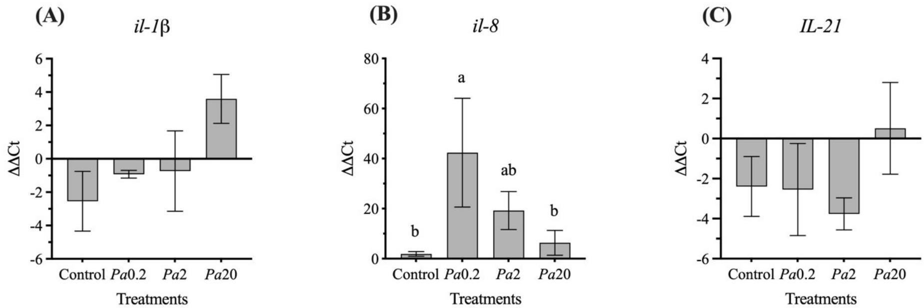
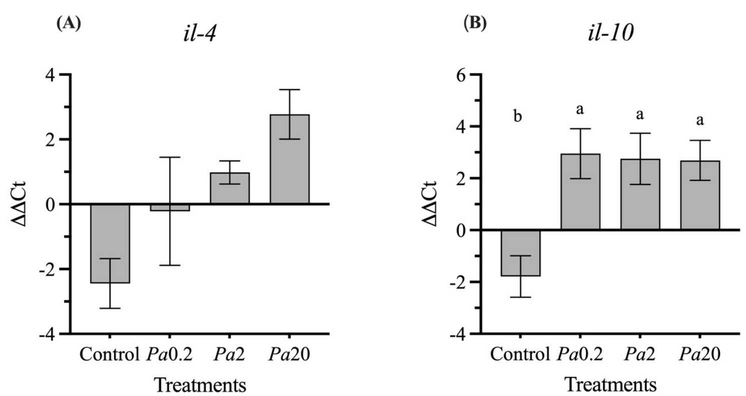


Figure 2. Gene expression (as ΔΔCt values) of anti-inflammatory interleukins (A) 4 (*il-4*) and (B) 10 (*il-10*) from the distal intestine of rainbow trout fingerlings fed diets with high content of soybean meal and the addition of several concentrations of *Pediococcus acidilactici* for a period of 90 days. Each bar represents the means of triplicate groups ± SE. Bars with different letters differ significantly ($P < 0.05$).



4. DISCUSSION

Due to its high protein content, amino acid profile, availability, and relative low cost, the SBM is an alternative to fish meal as protein source in aqua feeds. However, its inclusion in high quantities is still limited as it has several negative effects in carnivorous fish, such as retarded growth performance, poor digestibility and in extreme cases, non-infectious enteritis and death (Laining et al., 2010; Krogdahl et al., 2010). The effects of inclusion of different concentrations of the probiotic *P. acidilactici* in diets with high levels of soybean meal for rainbow trout fingerlings are presented for the first time.

Use of soybean meal as main source of protein in diets for carnivorous fish affects growth performance (Gatlin et al., 2007), due to the presence of several compounds known as antinutrients (Laining et al., 2010). Addition of enzymes (Cruz et al., 2011) or probiotics (Sánchez et al., 2015) showed to improve the growth performance. Fingerlings fed diet *Pa*20 showed higher growth performance, so the inclusion of *P. acidilactici* has a positive effect on growth, which was previously reported by Merrifield et al. (2011) who fed rainbow trout with a commercial diet and Torrecillas et al. (2018) fed European sea bass with a plant-protein base diet. The positive effect of this probiotic in growth, seems to be related to the concentrations in fed, as previously reported by Arani et al. (2018) in zebrafish.

Inclusion of *P. acidilac*ti did not have any effect on proximate composition of muscle and liver, with the exception for lipid contents in muscle of fingerlings fed diet *Pa*20 that were significantly higher than the other treatments. However, such values are similar to lipid contents previously reported in muscle for rainbow trout fed diets with inclusion of different soybean products (Enriquez et al., 2015; Aguillón et al., 2017; Segura et al., 2021).

Use of soybean meal has been reported to cause upregulation of immune-related genes and resulting in inflammation and enteritis in salmonids (Krogdahl et al., 2010; Tsai et al. ,2023). The expression of five interleukins was assessed, three proinflammatory (the *il-1*β, *il-8*, and *il-21*) and two anti-inflammatories (*il-4* and *il-10*). Interleukins are a group of cytokines which are involved in the regulation of several immune responses (Secombes et al., 2011; Sakai et al., 2021).

The IL-1β and IL-8 play an important role in the inflammatory response (Seibel et al., 2022) and both enzymes are complementary by stimulating the T cells, increasing the phagocytic activity, antibody production (IL-1β) and inducing the cell migration (IL-8). The expression of *il-1*β induces the upregulation of *il-8* (Secombes et al., 2001) and usually, their expression shows similar patterns (Vélez-Calabria et al., 2021). However, during this experiment the expression of *il-8* decreased as the *P. acidilactici* inclusion increased. In fish, the upregulation of the *il-1*β has been reported during bacterial infection (Sakai et al., 2021), use of dietary prebiotic and probiotic (Sealey et al., 2009; Standen et al., 2013; Al-Hisnawi et al., 2018; Jaramillo-Torres et al., 2019) and when fed soybean meal as main protein source (Torrecillas et al., 2018). Upregulation of *il-1*β in fingerlings fed diet *Pa*-20 seems to be related to the concentration of *P. acidilactici*, rather than inclusion of soybean meal as protein source. Previous reports indicate that increased immune responses were observed when *P. acidilactici* was added in 4 x 10^6 CFU/g diet for zebrafish (Arani et al.,

2018) and 2.1 x 10^6 CFU/g diet for rainbow trout (Al-Hisnawi et al., 2019), inclusions that are similar to the concentration on diet *Pa*-20. Regarding the expression of *il-21,* it shows a similar trend to *il-1*β with upregulation of the group *Pa*20 and downregulation of Control, *Pa*0.2 and *Pa*2. IL-21 is a novel cytokine that in preliminary studies with fish shows to be involved in inflammatory response to bacterial (Li et al., 2022) and viral (Yu et al., 2020) infections. It seems that upregulation of *il-21* is associated with the inclusion of *P. acidilactici* at the level of 20 g/kg, as observed previously for zebrafish (Arani et al., 2018) and rainbow trout (Al-Hisnawi et al., 2019).

Regarding the anti-inflammatory interleukins, *il-4* and *il-10* expressions were upregulated with the inclusion of *P. acidilactici*, regardless of the concentration in diets. IL-4 is involved in the responses that increase mucus production, tissue repair, regulating the inflammatory process and inducing the upregulation of *il-10* (Zhang et al., 2023). On the other hand, IL-10 has been reported to possess a more anti-inflammatory role than IL-4, as it can suppress various immune response (Bottiglione et al., 2020). Additionally, it has been reported that IL-10 downregulates the *il-1*β in Atlantic salmon and rainbow trout (Sequeida et al., 2020). The observed expressions of *il-4* and *il-10* indicate that the inclusion of *P. acidilactici* helps in reducing the inflammation caused by soybean meal.

5. CONCLUSION

Our results show that after 90-days of feeding, *P. acidilactici* helps to reduce the intestinal inflammation caused by the inclusion of 400 g/kg of soybean meal in rainbow trout fingerlings. Considering the growth performance and the expression of the different interleukins (particularly the IL-4 and IL-10), the concentration of 20 g/kg seems to be adequate for using with high levels of soybean meal. Though, it is necessary to feed the probiotic for longer periods to see how the probiotic maintains the anti-inflammatory effect throughout longer periods of time and analyze other genes which may help us to understand the mechanism of *P. acidilacitic* in the intestine of rainbow trout.

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SOBRE O ORGANIZADOR

EDUARDO EUGENIO SPERS realizou pós-doutorado na Wageningen University (WUR), Holanda, e especialização no IGIA, França. Possui doutorado em Administração pela Universidade de São Paulo (USP). Foi Professor do Programa de Mestrado e Doutorado em Administração e do Mestrado Profissional em Comportamento do Consumidor da ESPM. Líder do tema Teoria, Epistemologia e Métodos de Pesquisa em Marketing na Associação Nacional de Pós-Graduação e Pesquisa em Administração (ANPAD). Participou de diversos projetos de consultoria e pesquisa coordenados pelo PENSA e Markestrat. É Professor Titular no Departamento de Economia, Administração e Sociologia, docente do Mestrado em Administração e Coordenador do Grupo de Extensão MarkEsalq no campus da USP/Esalq. Proferiu palestras em diversos eventos acadêmicos e profissionais, com diversos artigos publicados em periódicos nacionais e internacionais, livros e capítulos de livros sobre agronegócios, com foco no marketing e no comportamento do produtor rural e do consumidor de alimentos.

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