Original Article

Dietary *Cirsium arvense* Essential Oil Enhances Immunity and Disease Resistance in Rainbow Trout

Morteza Yousefi[1], Evgeny Vladimirovich Kulikov[1], Olesya Anatolyevna Petrukhina[1], Arfenya Semenovna Karamyan[1], Nadezhda Sachivkina[1], Artyom Brigida[2]

1. Department of Veterinary Medicine, RUDN University, Moscow, Russia.
2. All-Russian Research Institute of Integrated Fish Farming - a branch of the Federal State Budgetary Scientific Institution "Federal Research Center for Animal Husbandry named after Academy Member L. K. Ernst" (L. K. Ernst Federal Research Center for Animal Husbandry), Moscow, Russia.

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ABSTRACT

Background: Fish diseases are among the main challenges in the aquaculture industry. Feed additives are promising agents to control fish diseases by improving their innate immunity.

Objectives: The present study was conducted to assess the effects of dietary supplementation with *Cirsium arvense* essential oil (CAEO) on immunological responses and disease resistance in rainbow trout, *Oncorhynchus mykiss*.

Methods: The fish (mean weight: 31.31±0.15 g) were fed diets containing 0 (Cont), 2 (CAEO2), 4 (CAEO4) and 6 (CAEO6) mL/kg CAEO for 60 days at a daily ration of 5% of biomass (n=3), followed by an experimental infection by *Yersinia ruckeri*.

Results: After 60 days of rearing, dietary CAEO significantly increased serum total protein, total immunoglobulin (Ig), lysozyme, alternative complement (ACH50), respiratory burst activity (measured by nitroblue tetrazolium [NBT] test), skin mucus lysozyme, protease, total Ig, and alkaline phosphatase (ALP). Dietary CAEO significantly decreased post-challenge mortality; Cont, CAEO2, CAEO4, and CAEO6 showed 100%, 58%, 68%, and 48% post-challenge mortalities, respectively. The highest total Ig, NBT, ACH50, mucus protease, and ALP, and post-challenge survival were observed in CAEO6 treatment; whereas, the highest serum total protein, mucus lysozyme, and total Ig were observed in CAEO4 and CAEO6 treatments. Serum lysozyme significantly increased in all CAEO-treated fish.

Conclusion: CAEO can be used as a feed additive for the aquaculture of rainbow trout, as it improves immunity and disease resistance of the fish at a concentration of 6 mL/kg.

Keywords: *Cirsium arvense*, Diet, Aquaculture, Disease, Immunity

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*** Corresponding Author:**
Morteza Yousefi, Associate Professor.
Address: *Department of Veterinary Medicine, RUDN University, Moscow, Russia.*
Phone: *+7 (977) 7563373*
E-mail: *myousefi81@gmail.com*

Introduction

The aquaculture industry has rapidly grown in recent decades and makes a remarkable contribution to supplying high-quality food for humans (Naylor et al., 2021). Rainbow trout, *Oncorhynchus mykiss*, is an aquaculture species characterized by a high growth rate and survivorship (Amiri et al., 2023). The annual global production of rainbow trout was above 952000 metric tons in 2021 (FAO, 2023). However, outbreaks of diseases are among the problems in expanding fish production throughout the world. The genus *Yersinia* is known as a pathogenic bacterium that has a wide range of hosts (Zaidi et al., 2024; Arefpajoohi et al., 2008). One of the widespread diseases in rainbow trout farms is yersiniosis, which is caused by an opportunistic gram-negative bacterium, *Yersinia ruckeri*. This pathogen transmits among a fish population through direct contact and water. The bacterium is present in the fish body for up to 2 months after the outbreak (Tobback et al., 2007).

The annual loss in the aquaculture industry due to diseases has been estimated at around $6 billion in recent decades (Mishra et al., 2018), resulting in research conducted to increase disease resistance in aquaculture. Augmenting nonspecific immunity and antioxidant power is a reliable method for boosting fish health and resistance against opportunistic pathogens (Hoseini et al., 2022; Taheri Mirghaed et al., 2023). This goal can be achieved through the managerial practices (such as maintaining high water quality and reliable stocking density) and using functional feeds.

Functional feeds are those supplemented with beneficial additives, for example, herbal materials, which result in boosting growth, immunity, and disease resistance (Encarnação, 2016). Promotion of the antioxidant power of fish by herbal supplements is due to the presence of active compounds with radical scavenging properties and stimulation of antioxidant enzymes (Mohiseni, 2017). Since some immune responses result in the production of pro-oxidant molecules, a robust antioxidant defense supports a strong immune response and pathogen elimination (Biller & Takahashi 2018).

Also, functional feeds can improve systemic (humoral) and superficial (skin mucosal) nonspecific immune defenses (Adel et al., 2015; Adel et al., 2016; Rashmeei et al., 2020; Taheri Mirghaed et al., 2020; Hoseini et al., 2021). Because surrounding water is the main route of pathogen transmission in aquaculture facilities, skin mucosal immunity is vital for disease prevention in aquaculture practice. If pathogens cross through the surface defense barriers, humoral nonspecific immune components are pivotal for recognition and elimination of them (Hoseini et al., 2023).

Cirsium arvense is an herb abundant in the northern hemisphere, which naturally grows in grasslands (Tiley, 2010). Essential oil of *C. arvense* (CAEO) is rich in α-bisabolol and δ-cadinene (Dehjurian et al., 2017), two sesquiterpenes known for their antioxidant, anti-microbial, anti-cancer, anti-inflammatory, and neuroprotective effects (Pérez-López et al., 2011; Kim et al., 2013; Hui et al., 2015; Eddin et al., 2022). Despite these benefits, CAEO has not been evaluated as a health-promoting agent in aquaculture. The present study, thus, aimed to assess the effects of CAEO on humoral/superficial immune parameters and resistance to *Y. ruckeri* infection in rainbow trout.

Materials and Methods

Preparation of CAEO

C. arvense aerial parts were collected, washed with distilled water, and dried overnight. The herbal materials were then placed in a Clevenger apparatus and heated for 5 h (Hasankhani et al., 2023). The ratio of the herbal materials to water was 1:3 (w:v). After distillation, the essential oil volume was determined and transformed to mass based on its density (0.858 g/mL). The essential oil was then dried on sodium sulfite and kept at 2 °C until further analysis.

Diets and rearing trial

CAEO was added to the fish diets at 0, 2, 4, and 6 mL/kg (corresponding to 0, 1.6, 3.2, and 4.8 g/kg), based on a preliminary study on its in vitro bactericidal activity. The diets were prepared according to Table 1 by mixing the feedstuffs. After adding 0.35 L/kg water to the mixture, the pastes were pelleted using a meat grinder. The pellets were dried at room temperature and kept in the refrigerator until use.

Rainbow trout (~ 31 g individual weight) were purchased from a local farm, transported to the laboratory, and stocked in a tank (2000 L) for 2 weeks to acclimatize to the new conditions. The fish were fed the control diet (Cont) during the acclimation period, then randomly distributed into 12 tanks (300 L capacity), 15 fish per tank. The tanks were filled with dechlorinated (aerated) tap water and continuously aerated. Each of the diets mentioned above was offered to three tanks for 60 days at a daily rate of 5%, divided into three meals. Half of the water in the tanks was replaced with clean water every day (Hoseini et al., 2016). During the rearing period, photoperiod, water temperature, pH, dissolved oxygen, and unionized ammonia were 14:10 (light: dark), 15.3±0.3 ˚C, 7.33±0.3, 6.88±0.2 ppm, and <0.05 ppm, respectively.

Sampling procedure

At the end of the rearing period, the fish were fasted for 24 h (Hoseini et al., 2019) and sampled for assessment of serum and skin mucus immunological parameters. Fifteen fish per treatment were randomly caught and anesthetized by clove extract (150 ppm). The fish were blood-sampled through the caudal vein using 2-mL non-heparinized syringes. After 30 min of clotting at room temperature, the blood samples were centrifuged (3000 rpm; 10 min) and the blood sera were collected in new tubes and kept at -20 ˚C until analysis.

Skin mucus collection was performed according to Ross et al. (2000). Briefly, the anesthetized fish were individually placed in a polyethylene bag containing 10 mL of NaCl solution (50 mM) and gently rubbed for 2 min. After that, the collected skin mucus was poured into a sterile tube, centrifuged at 4 ˚C for 10 min, and the supernatant was kept at -80 ˚C until analysis.

Analysis of serum and mucus immunological parameters

Total immunoglobulin (Ig) concentrations of the serum and mucus samples were determined according to Siwicki and Anderson (1993). After determination of total protein concentrations, the samples were mixed with polyethylene glycol (12%) for 2 h at room temperature, then centrifuged. Total protein concentrations were determined again and subtracted from the previous total protein of each sample to calculate total Ig concentrations. Soluble protein concentration in the mucus samples was determined according to Bradford (1976); whereas, total protein concentrations in the serum samples were determined using a commercial kit (Zist Chem Co., Tehran, Iran).

Lysozyme activities in serum and mucus samples were determined based on the lysis of *Micrococcus luteus* at 450 nm (Ellis, 1990). Briefly, 25 µL of the samples were mixed with 1 mL of the bacterial suspension (in phosphate buffer pH 6.2), and a decrease in optical density was recorded for 5 min. Serum activity of the alternative complement pathway (ACH50) was determined according to hemolysis of sheep erythrocytes, using veronal buffer (containing ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, magnesium, and gelatin). ACH50 activity was calculated as the dilution factor of the samples producing 50% hemolysis (Yano, 1992). Mucus protease activity was determined through the hydrolysis of AZOcasein according to Iversen and Jørgensen (1995). In contrast, mucus alkaline phosphatase (ALP) activity was measured by a commercial kit (Parsazmun Co., Tehran, Iran) and a spectrophotometer (UNICO UV-2100, USA).

Blood respiratory burst (assessed by NBT) activity was measured according to Stasiak and Baumann (1996) at 620 nm, using nitroblue tetrazolium as substrate. The activity of ALP was measured using commercial kits (Parsazmun Co., Tehran, Iran) and based on the protocol provided by the company.

Bacterial challenge

Pathogenic bacteria *Y. ruckeri* (KC291153) were isolated from moribund fish and identified by morphological and biochemical characteristics. The bacterium was cultured on tryptone soya agar medium, and a NaCl solution (0.85%) was used to prepare a bacterial suspension. Then, 10 fish per replication (30 fish per treatment) were anesthetized, and 0.1 mL of bacterial suspension at a concentration of 107 CFU/mL was intraperitoneally injected into each fish (Oroji et al., 2021). The pathogenic concentration of bacteria was determined using a pretest and LD_{50} calculation. Then, the cumulative mortality of fish was recorded within 14 days after challenge.

Statistical analysis

This study was conducted as a completely randomized design, and the sub-samples from each tank were averaged for statistical analysis. The data were subjected to one-way ANOVA, after confirming normality (Shapiro-Wilk test) and homoscedasticity (Levene's test). Pair comparisons among the treatments were conducted using the Tukey test. $P<0.05$ was considered a significant difference. Statistical analyses were performed using SPSS software, version 22.

Results

Dietary CAEO supplementation significantly increased serum lysozyme activity and total protein levels, compared to Cont treatment. There was no significant difference in serum lysozyme activity among CAEO2, CAEO4, and CAEO6 treatments. The highest serum

Table 1. Composition of the control diet

Ingredients (g/kg)		Cont	CAEO2	CAEO4	CAEO6
Corn meal		100	98.4	96.8	95.2
Wheat meal		160	160	160	160
Soybean meal		150	150	150	150
Soybean oil		65	65	65	65
Fishmeal[1]		172	172	172	172
Poultry byproduct[2]		340	340	340	340
Vitamin premix[3]		5	5	5	5
Mineral premix[3]		5	5	5	5
Methionine		1	1	1	1
Lysine		2	2	2	2
CAEO		0	1.6	3.2	4.8
Proximate composition	Moisture	91	90.8	90.2	91.1
	Crude protein	411	410	408	409
	Crude fat	159	158	161	160
	Crude ash	79.2	78.9	78.4	79.3

[1]Crude protein 64%; crude fat 10%, [2]Crude protein 54%; crude fat 22%, [3]Bought from Amineh Gostar Co. (Tehran, Iran).

total protein level was found in CAEO4 and CAEO6 treatments. There were no significant differences in serum ACH50, total Ig, and NBT between Cont and CAEO2 treatments; both were significantly lower than those of CAEO4 and CAEO6 treatments. The highest ACH50, total Ig, and NBT were related to CAEO6 treatment (Table 2).

Dietary CAEO supplementation significantly increased mucus lysozyme activity and total Ig levels, compared to Cont treatment. The highest mucus lysozyme and total Ig levels were found in CAEO4 and CAEO6 treatments. There were no significant differences in mucus protease activity between Cont and CAEO2 treatments; both were significantly lower than those of CAEO4 and CAEO6 treatments. The highest mucus protease was found in CAEO6 treatment. Mucus ALP activity increased significantly in CAEO6 treatment, compared to the other treatments. There was no significant difference in mucus ALP activity among Cont, CAEO2, and CARO4 treatments (Table 2).

Fish mortality after *Y. ruckeri* infection significantly decreased in CAEO treatments, compared to Cont treatment. Cont, CAEO2, CAEO4, and CAEO6 showed 100, 58, 68, and 48% post-challenge mortalities, respectively, which significantly differed from each other (Figure 1).

Discussion

Boosting the innate immunity system of the fish is an eco-friendly way to manage disease in aquaculture. It is a prophylactic practice that prevents antibiotic use/abuse in the industry. Herbal feed additives are great agents to achieve this goal as they are generally safe and have benefits other than immune-boosting, such as improving fish growth and antioxidant capacity. This research is the first one assessing the benefits of dietary CAEO on managing yersiniosis in rainbow trout.

Humoral immune components participate in the systemic immune responses, whereas mucosal immunity prevents pathogens from crossing the epithelial surfaces in fish (Yousefi et al., 2024). Improvements in these immune parameters may help fish resist opportunistic pathogens. Complement proteins recognize foreign cells and lyse them. Furthermore, the complement system contributes to the adaptive immune system through antigen presentation

Table 2. Immunological parameters in serum and mucus of rainbow trout fed diets supplemented with 0 (Cont), 2 (CAEO2), 4 (CAEO4), and 6 (CAEO6) mL/kg *C. arvense* essential oil (CAEO)

Sample Type	Immunological Assays	Cont	CAEO2	CAEO4	CAEO6
Serum	Total protein (g/dL)	2.2±0.2[c]	2.9±0.3[b]	3.9±0.4[a]	4.6±0.4[a]
	Lysozyme (U/mL)	52.5±3.5[b]	63.4±5.4[a]	68.3±6.7[a]	73.5±7.1[a]
	ACH50 (U/mL)	21.2±2.7[c]	25.4±3.1[c]	36.5±1.5[b]	42.8±1.4[a]
	Total Ig (mg/mL)	1.4±0.2[c]	1.8±0.4[c]	2.6±0.2[b]	3.8±0.3[a]
	Nitroblue tetrazolium (optical density)	0.34±0.04[c]	0.42±0.05[c]	0.64±0.02[b]	0.85±0.05[a]
Mucus	Lysozyme (U/mL)	33.2±3.5[c]	41.3±4.2[b]	55.6±5.6[a]	68.5±8.2[a]
	Total Ig (mg/dL)	1.7±0.2[c]	2.9±0.5[b]	4.2±0.4[a]	4.4±0.5[a]
	Protease (U/mL)	8.2±2.2[b]	9.5±2.1[b]	12.3±1.5[ab]	14.5±1.6[a]
	ALP (U/mL)	3.4±0.5[b]	3.8±0.2[b]	3.7±0.4[b]	4.5±0.3[a]

Significant differences among the treatments are shown by different superscripted letters (P<0.01; Tukey).

and inflammation (Bavia et al., 2022). Respiratory burst activity of blood leukocytes leads to the production of a huge amount of superoxide anion for killing pathogens (Hampton et al., 2020). Lysozyme is found in blood and skin mucus and acts as a bactericidal agent, effective against gram-positive bacteria (Song et al., 2021). Immunoglubins, found in circulation and epithelial surfaces, are compartments of the adaptive immune system, but basal levels of Ig can be used as an indicator of the immune system's strength (Ebrahimzadeh Mousavi et al., 2024). Skin mucus protease participates in the surface immune function by destabilizing and continuously sloughing off the mucus surface that leads to inhibition of pathogen localization (Hoseini et al., 2023). Skin mucus ALP also participates in the surface barrier immunity by suppressing inflammation associated with pathogen invasions (Hoseini et al., 2023). The present study showed that dietary CAEO can improve various immune indicators in the fish blood, serum, and skin mucus. Although there is no study regarding the benefits of dietary CAEO on the humoral and mucosal immunity of fish, essential oils have been shown to boost immune strength. For example, dietary supplementation with *Santalum album* essential oil significantly increased serum lysozyme, complement, and blood phagocytosis in Nile tilapia, *Oreochromis niloticus* (Mansour et al., 2024). Rainbow trout treated with dietary *Dracocephalum kotschyi* essential oil showed boosted lysozyme, complement, total Ig, protease, and ALP in serum and/or skin mucus (Hafsan et al., 2022).

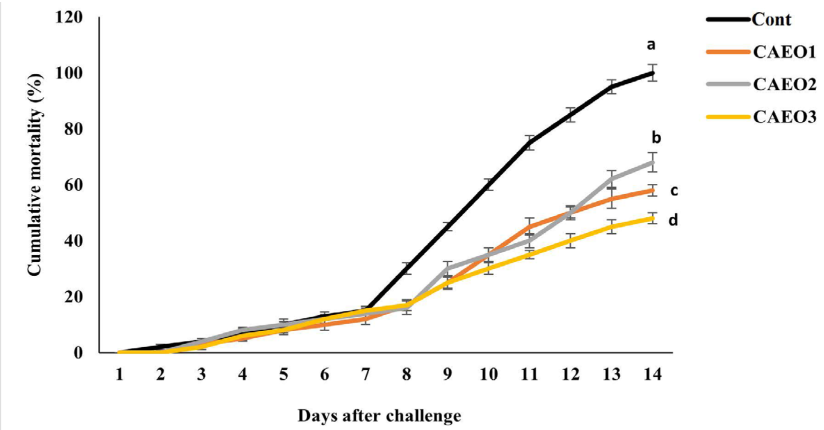

Figure 1. Cumulative mortality of rainbow trout fed diets supplemented with 0 (Cont), 2 (CAEO2), 4 (CAEO4), and 6 (CAEO6) mL/kg CAEO and challenged by *Y. ruckeri*

Note: Significant differences among the treatments are shown by different letters (P<0.01; Tukey).

Fish fed the diets supplemented with CAEO showed higher resistance against *Y. ruckeri* infection in the present study, which may be due to the enhanced immune response. The augmentation in the systemic and mucosal immune system before the bacterial challenge put the fish in a suitable position to combat the injected pathogens. Such a boosted innate immunity may have beneficially reduced the bacterial load and localization, thereby mitigating post-challenge mortality (Hoseini et al., 2022). On the other hand, it has been shown that a weak antioxidant capacity suppresses immune responses to pathogens, as some immune responses are oxidative (e.g. superoxide anion production) and need proper antioxidant capacity to prevent host cell damage (Biller & Takahashi, 2018). CAEO possesses antioxidant characteristics (Hossain et al., 2016); thus, it is speculated that the higher disease resistance in the present study can be partially attributed to improvements in the antioxidant capacity in the fish. Similar to the present results, dietary S. album essential oil increased disease resistance of Nile tilapia, along with boosting serum lysozyme, complement, blood phagocytosis, and hepatic antioxidant capacity (Mansour et al., 2024). Dietary administration of *D. kotschyi* and oregano essential oils also led to similar results in rainbow trout (Hafsan et al., 2022) and common carp, *Cyprinus carpio* (Zhang et al., 2020), respectively.

Overall, this study for the first time showed that dietary CAEO potentiate to boost systemic/mucosal immunity in rainbow trout. Such improvements may be responsible for the higher disease resistance of the fish after *Y. ruckeri* infection. According to the present study, 6 mL CAEO per kg trout diet is adequate to excrete the benefits mentioned above.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of Peoples' Friendship University of Russia, Moscow, Russia (Protocol No.: 9a, 2024).

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Authors' contributions

Conceptualization, supervision, data curation, funding acquisition, formal analysis, and review & editing: Morteza Yousefi; Project administration, methodology, and investigation: Morteza Yousefi, Evgeny Vladimirovich Kulikov, and Olesya Anatolyevna Petrukhina; Writing the original draft: All authors.

Conflict of interest

The authors declared no conflict of interest

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