Intestinal histomorphology of silver catfish fed diets supplemented with *Citrus x Aurantium* essential oil

Histomorfologia intestinal de jundiá alimentado com dietas suplementadas com óleo essencial de *Citrus x Aurantium*

Histomorfología intestinal del bagre plateado alimentado con dietas suplementadas con aceite esencial de *Citrus x Aurantium*

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ABSTRACT  
The aim of this study was to investigate the effects of dietary Citrus x aurantium essential oil (EOCA) on the growth and intestinal histomorphology of silver catfish (Rhamdia quelen) fingerlings. Five diets with different doses of EOCA (0.0, 0.25, 0.5, 1.0, and 2.0 mL/kg diet) were evaluated. At the end of 60 days, the growth performance was positively correlated with dietary EOCA supplementation. The results showed an increase in fold height in the middle intestine (MI) of fish fed the EOCA diet compared to the control group. In the anterior intestine (AI), the enterocyte height increased, while the goblet cell number decreased in all EOCA-supplemented diets. The goblet cell number in the MI and posterior intestine (PI) was higher with EOCA and lowest in the control group. Diets with...
EOCA did not affect the fold height in the PI. Similarly, to the AI, the fold width in PI increased in the 0.5 mL/kg treatment compared to the control group. These results revealed that dietary supplementation with EOCA, primarily at a dose of 0.5 mL EOCA/kg diet, promoted positive effects on the intestinal morphology of *R. quelen*, indicating that the use of EOCA extracted from sweet orange peel, a fruit residue, could be considered in fish diets.

**Keywords:** Histology, Nutrition, Sweet Orange Oil, *Rhamdia quelen*.

**RESUMO**
O objetivo deste estudo foi investigar os efeitos do óleo essencial de *Citrus x aurantium* (EOCA) na dieta sobre o crescimento e histomorfologia intestinal de alevinos de jundiá (*Rhamdia quelen*). Foram avaliadas cinco dietas com diferentes doses de EOCA (0,0, 0,25, 0,5, 1,0 e 2,0 mL/kg de ração). Ao final de 60 dias, o crescimento foi positivamente correlacionado com a suplementação dietética de EOCA. Os resultados mostraram um aumento na altura das pregas no intestino médio (IM) dos peixes alimentados com a dieta contendo EOCA em comparação com o grupo controle. No intestino anterior (IA), a altura dos enterócitos aumentou, enquanto o número de células caliciformes diminuiu em todas as dietas suplementadas com EOCA. O número de células caliciformes no IM e no intestino posterior (IP) foi maior com EOCA e menor no grupo controle. As dietas com EOCA não afetaram a altura das pregas no IP. Da mesma forma que no IA, a largura das pregas no IP aumentou no tratamento com 0,5 mL/kg em comparação com o grupo controle. Esses resultados revelaram que a suplementação dietética com EOCA, principalmente na dose de 0,5 mL de EOCA/kg de ração, promoveu efeitos positivos sobre a morfologia intestinal de *R. quelen*, indicando que o uso de EOCA extraído da casca da laranja doce, um resíduo de frutas, poderia ser considerado em dietas para peixes.

**Palavras-chave:** Histologia, Nutrição, Óleo de Laranja Doce, *Rhamdia quelen*.

**RESUMEN**
El objetivo de este estudio fue investigar los efectos del aceite esencial de *Citrus x aurantium* (EOCA) en el crecimiento y la histomorfología intestinal de los alevines de bagre de plata (*Rhamdia quelen*). Se evaluaron cinco dietas con diferentes dosis de EOCA (0,0, 0,25, 0,5, 1,0 y 2,0 mL/kg). Al final de los 60 días, el rendimiento de crecimiento se correlacionó positivamente con la suplementación dietética con EOCA. Los resultados mostraron un aumento en la altura del alimento en el intestino medio (IM) de los peces alimentados con la dieta EOCA en comparación con el grupo de control. En el intestino anterior (IA), la altura de los enterocitos aumentó, mientras que el número de células de la copa disminuyó en todas las dietas suplementadas con EOCA. El número de células caliciformes en el IM y el intestino posterior (IP) fue mayor con EOCA y menor en el grupo control. Las dietas con EOCA no afectaron la altura del pliegue en el IP, de manera similar a la IA, el ancho del pliegue en el IP aumentó en el tratamiento de 0,5 mL/kg en comparación con el grupo control. Estos resultados revelaron que la suplementación dietética con EOCA, principalmente a una dosis de 0,5 ml de EOCA/kg de dieta, promovió efectos positivos en la morfología intestinal de *R. quelen*, lo que indica que el
uso de EOCA extraído de cáscara de naranja dulce, un residuo de fruta, podría considerarse en las dietas de pescado.

Palabras clave: Histología, Nutrición, Aceite de Naranja Dulce, Rhamdia quelen.

1 INTRODUCTION

It is well established that proper nutrition is essential for maintaining normal growth and health in all animals, including aquatic species. As such, nutritious diets and appropriate feeding regimes play critical roles in intensive aquaculture (Pohlenz and Gathin, 2014). Nutrition is also crucial for maintaining fish health as it can affect immunocompetence, disease resistance, and stress mediation. Consequently, there is a growing trend towards exploring non-nutritional dietary components to provide various functional attributes (Qi et al. 2009).

Fruits and herbs, along with their by-products, have been considered promising as functional feedstuffs. This is attributed to their broad spectrum of biological activity (Chakraborty and Hancz, 2011; Vaseeharan and Thaya, 2014) and the fact that they leave no residues in the final products, causing no adverse effects on fish, human health, or the environment (Baba et al. 2016; Vicente et al. 2019). Citrus fruits are rich sources of beneficial phytochemicals, such as vitamins A, C, and E, mineral elements, flavonoids, and other compounds. These phytochemicals, consumed through fresh fruits or their derived products, have been suggested to possess a wide variety of biological functions, including antioxidant, anti-inflammatory, anticarcinogenic, and immunostimulant effects, among other functional properties (Acar et al. 2015; Baba et al. 2016). These activities are primarily attributed to components such as terpenes and phenolic compounds (Moufida and Marzouk, 2003; Flamini et al. 2007).

The Citrus genus, including lemon and orange oils, has been the subject of study due to its demonstrated beneficial properties in increasing growth performance, hematological profile, and antioxidant and immunostimulant effects in fish (Acar et al. 2015; Baba et al. 2016; Lopes et al. 2019; Vicente et al. 2019; Acar et al. 2021). Brazil is
the largest producer and exporter of orange juice; according to the Brazilian Department of Agriculture, approximately 50% of worldwide and 80% of Brazilian orange production result in a significant amount of solid and liquid residue, the efficient use of which has recently garnered significant attention (MAPA, 2017).

Gut morphometry is an important tool that can be used to assess the effects of dietary compounds on nutrient absorption, and histopathological studies are essential to reveal positive changes in fish tissues and cells under unfavorable conditions (Bello et al. 2012; Ling et al. 2019). It was previously reported that when included in high doses in the diet of common carp juveniles (*Cyprinus carpio*), the essential oil extracted from *Citrus aurantium* (EOCA) promoted significant histopathological changes in the intestinal tract (Acar et al. 2021). In addition, studies in rats demonstrated a beneficial effect of EOCA on the gastric mucosa (Moraes et al. 2009; Moraes 2013). Given these reports, this study aimed to evaluate the effects of dietary supplementation with EOCA on the intestinal histomorphology of silver catfish, *Rhamdia quelen*. These results will provide relevant information on the advantages of a natural product associated with fish farming and animal welfare.

2 MATERIAL AND METHODS

2.1 FISH AND CULTURE CONDITIONS

Four hundred fish (1.96 ± 0.4 g and 6.41 ± 0.3 cm) were placed in a recirculating system with twenty 50 L tanks (20 fish per tank) fed a control diet (see sample collection and analytical methods) and acclimated to laboratory conditions for 10 days. The experimental protocol was approved by the Ethical and Animal Welfare Committee of the UFSM, under registration number 074/2014.
2.2 WATER SAMPLING AND ANALYSES

Water parameters were monitored daily and remained within the desired range throughout the experimental period: temperature 25.4 ± 0.32°C, pH 7.08 ± 0.04, dissolved oxygen levels 6.97 ± 0.3 mg/L, hardness 27 ± 1.5 mg CaCO3/L, alkalinity 51 ± 0.7 mg CaCO3/L, nitrite 0.08 ± 0.03 mg/L, total ammonia 0.75 ± 0.51 mg/L, and un-ionized ammonia 0.039 ± 0.04 mg/L.

2.3 ESSENTIAL OIL EXTRACTION, ANALYSIS, AND IDENTIFICATION OF CONSTITUENTS

The methodology for extracting EOCA from the peel and identifying compounds was described in Lopes et al. (2019). Analysis of the EOCA revealed a predominance of limonene (93.89%), linalool (2.60%), and β-pinene (1.71%). The yield of EOCA was 0.92% v/w.

2.4 DIETS AND EXPERIMENTAL DESIGN

The preparation of the diets was formulated based on the protocols described by Zeppenfeld et al. (2016). All ingredients were finely ground, weighed, and mixed until a homogeneous mixture was achieved. Different doses of EOCA (0.0, 0.25, 0.5, 1.0, or 2.0 mL EOCA per kg of diet) were then added, along with canola oil (Table 1). Subsequently, the mixtures were dried in a forced air circulation oven for 24 hours (40°C). Finally, the pellets were stored in a freezer (at −4°C) until use. Silver catfish were fed the experimental diets until apparent satiation twice daily (9 am and 5 pm) for 60 days. Tanks were cleaned 30 minutes after feeding via siphoning to remove waste.
Table 1. Composition and proximate analysis of the experimental diets

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g kg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean meal</td>
<td>300</td>
</tr>
<tr>
<td>Meat and bone meal</td>
<td>350</td>
</tr>
<tr>
<td>Rice bran</td>
<td>120</td>
</tr>
<tr>
<td>Corn</td>
<td>150</td>
</tr>
<tr>
<td>Canola oil</td>
<td>30</td>
</tr>
<tr>
<td>Salt</td>
<td>10</td>
</tr>
<tr>
<td>Vitamin and mineral premix*</td>
<td>30</td>
</tr>
<tr>
<td>Phosphate dicalcium</td>
<td>10</td>
</tr>
</tbody>
</table>

Analysed proximate composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter</td>
<td>954.6</td>
</tr>
<tr>
<td>Crude protein</td>
<td>388.3</td>
</tr>
<tr>
<td>Ether extract</td>
<td>105.4</td>
</tr>
<tr>
<td>Mineral matter</td>
<td>142.9</td>
</tr>
</tbody>
</table>

* Vitamin and mineral mixture (minimum levels per kilogram of product) – folic acid: 250 mg, pantothenic acid: 5.000 mg, antioxidant: 0.60 g, biotin: 125 mg, cobalt: 25 mg, copper: 2.000 mg, iron: 820 mg, iodide 100 mg, manganese: 3.750 mg, niacin: 5.000 mg, selenium: 75 mg, vitamin A: 1.000, 000 UI, vitamin B1: 1.250 mg, vitamin B12: 3.750 mcg, vitamin B2: 2.500 mg, vitamin B6: 2.485 mg, vitamin C: 28.000 mg, vitamin D3: 500.000 UI, vitamin E: 20.000 UI, vitamin K: 500 mg, zinc: 17.500 mg.

Source: Authors.

2.5 GROWTH PERFORMANCE

The fish were weighed and measured at 0, 30, and 60 days of the experiment before each biometry. Final body weight (FW) and specific growth rate (SGR) were calculated using the appropriate formulas where necessary. The description of the growth performance of silver catfish fed different doses of EOCA is detailed in Lopes et al. (2019).
2.6 SAMPLE COLLECTION AND HISTOLOGICAL ANALYTICAL METHODS

At the end of the feeding trial, the fish were fasted for 24 hours before sampling and anesthetized for 3 minutes with eugenol at 40 mg L\(^{-1}\), followed by euthanasia via spinal cord sectioning. The intestines of five fish per treatment were collected and fixed in Bouin’s solution for 24 h (Layton et al., 2019). Afterward, rings of tissue were sampled from the anterior, middle, and posterior regions and washed in 70% ethanol. The samples were then dehydrated in a graded ethanol solution and embedded in historesin (Historesin Embedding Kit, Leica Microsystems®) following standard histological procedures. Embedded tissues were transversely sectioned following the intestinal lumen. Semi-serial cuts, with a thickness of 3 µm, were obtained using a semi-automatic microtome (Leica RM 2265®). One histological glass slide was prepared from each tissue, with 5 cuts. The histological slides were prepared according to the protocols of Harris hematoxylin–eosin, following the methodology described by Prophet et al. (1992), and later observed and documented with a photographic camera (CC50HD, Leica®) attached to a microscope (Leica® DM 750) and measured in ImageJ (Figure 1). To assess morphological changes, mucosal folds were measured according to the generally described method (Escaffre et al. 2007; Ferreira et al. 2016):

- hF: height of fold from the base to apex (5 folds per intestine region, totalling 15 measurements per animal);
- WF: width of the fold, measured at both the base and the apex (5 folds measured per intestinal region, totalling 30 measurements per animal);
- HE: height of enterocytes, measured on both sides of the fold (5 folds measured per intestinal region, totalling 30 measurements per animal);
- GC: total number of goblet cells per fold (5 folds counted per intestinal region, totalling 15 measurements per animal).
Figure 1 - Schematic representation of the morphometric parameters of the intestine in *Rhamdia quelen*. (A) hF: height of fold; wF: width of fold; (B) hE: height of enterocyte; GC: goblet cells. **Staining:** Toluidine blue. **Scale bars:** A: 100 µm; B: 50 µm.

2.7 STATISTICAL ANALYSIS

Final body weight and SGR were subjected to regression analyses. The means found for all replicates were considered, and the best model was chosen based on the p-value. The intestinal histomorphology data were subjected to regression (orthogonal contrast), but as they did not follow a specific mathematical pattern, potential differences between treatments were tested using one-way analysis of variance (ANOVA) followed by the Duncan test. The results are expressed as the mean ± standard error (SEM). Analyses were performed using Statistica Software 7.0 (StatSoft, Tulsa, OK, USA) and GraphPad Prism 7.0 (GraphPad Software, Inc., CA, USA), with p < 0.05 considered significant.
3 RESULTS

3.1 GROWTH PERFORMANCE AND SURVIVAL

Fish survival was not affected significantly by the experimental diets. After 60 days the FW and SGR increased proportionally with dietary EOCA supplementation (Figure 2). Additional details of the growth performance of silver catfish fed dietary EOCA supplementation are detailed in Lopes et al. (2019).

Figure 2 - Growth performance of silver catfish *Rhamdia quelen* fed diets supplemented with different doses of *Citrus x aurantium* essential oil (EOCA). (A) FW: final weight, $Y = 1.4133x + 15.463$, $R^2 = 0.9592$ ($p = 0.040$). (B) SGR: specific growth rate, $Y = 0.1378x + 3.4363$, $R^2 = 0.9384$ ($p = 0.027$), where $y =$ final weight (g) and SGR (%/day), respectively, and $x =$ dose of EOCA (mL per kg diet).

3.2 HISTOLOGICAL PARAMETERS

3.2.1 Anterior Intestine

The height of folds in the anterior intestine was not affected in fish fed the EOCA diet. Fish fed a diet containing 0.5 mL EOCA kg$^{-1}$ exhibited the widest folds. Silver catfish fed all treatments supplemented with EOCA showed a higher height of enterocytes
and lower numbers of goblet cells in the anterior intestine compared to control fish (Figure 3).

Figure 3- Morphometry of the anterior intestine from the silver catfish *Rhamdia quelen* fed diets containing different concentrations of essential oil *Citrus x aurantium*. (a) Height of fold (µm); (b) Width of fold (µm); (c) Height of enterocyte (µm); (d) Number of goblet cells (per fold). Values are reported as mean ± SEM (n = 5). Same letters indicate no significant difference between treatments (one-way ANOVA and Duncan’s test, P < 0.05).

Source: Authors.

3.2.2 Middle Intestine

In the middle intestine, the highest fold height was observed in all treatments with EOCA diet. Dietary supplementation with EOCA did not affect the width of folds or enterocyte height in the middle intestine. The number of goblet cells in this section was higher in fish fed all treatments with EOCA diet compared to control fish (Figure 4).
3.2.3 Posterior Intestine

Regarding the posterior intestine, all dietary concentrations of EOCA did not significantly affect the height of the folds. The widest width of folds and highest enterocyte height were observed in fish fed the diet containing 0.5 mL EOCA kg\(^{-1}\). The number of goblet cells was higher in fish fed diets containing 0.25 and 1.0 mL EOCA kg\(^{-1}\) (Figure 5).
Figure 5 - Morphometry of the posterior intestine from the silver catfish *Rhamdia quelen* fed diets containing different concentrations of essential oil *Citrus x aurantium*. (a) Height of fold (µm); (b) Width of fold (µm); (c) Height of enterocyte (µm); (d) Number of goblet cells (per fold). Values are reported as mean ± SEM (n = 5). Same letters indicate no significant difference between treatments (one-way ANOVA and Duncan's test, P < 0.05)

**POSTERIOR INTESTINE**

![Graphs showing morphometry](image)

Source: Authors.

4 DISCUSSION

Besides assessing the impact of diet on growth and feed utilization efficiency in fish, it is also vital to understand the mechanisms responsible for the observed performance. These mechanisms include, among others, nutrient digestibility, absorption in the digestive system, and retention in the body (Aanyu et al. 2014). The efficiency at which ingested diets are digested largely determines growth performance (Cook et al. 2000).

The analysis of the EOCA showed a predominance of limonene followed by linalool and β-pinene, consistent with previous reports on orange essential oils (Boussaada and Chemli, 2007; Omodamiro and Umekwe, 2013; Zarrad et al. 2015; Lopes et al. 2019; Silva et al. 2023). Limonene is associated with several beneficial aspects in the diet. Moraes et al. (2009) demonstrated that limonene increased gastric mucus production in mice, indicating a gastroprotective effect. In Nile tilapia (*Oreochromis niloticus*), Aanyu et al. (2018) showed the up-regulation of several genes involved in
growth, intestinal digestion, and absorption associated with limonene supplementation. In rainbow trout, *Oncorhynchus mykiss*, it was observed that the limonene of orange peel essential oil acted as a protector against *Yersinia ruckeri*, suggesting its potential use in production and reducing resistance to antibiotics in teleosts and humans (Gültepe, 2020).

Only a few studies have evaluated the relationship between promoter additives, primarily essential oils (EOs), and intestinal histology in fish. The evaluation of the histological structure of digestive organs in fish fed new promoter additives provides valuable information about digestive capacity and potential animal health (Caballero et al. 2003; Marković et al. 2012). The response of EO will depend on concentration and species of fish tested. In this sense, Sheikhzadeh et al. (2017) demonstrated that the highest growth in fish can partly be attributable to maintaining the function and structure of the intestine, leading to an increased digestive capacity of the gut. For example, the inclusion of up to 2.0 mL *Citrus latifolia* essential oil (EOCL) kg⁻¹ diet did not promote growth; however, fish fed 1.0 and 2.0 mL EOCL kg⁻¹ diet presented higher survival, and all EOCL groups had increased intestinal fold height and length compared to the control group (Lopes et al. 2020). Our results indicated that dietary supplementation with EOCA has a direct impact on fish growth. This could be attributed to the effects of the various bioactive compounds of *Citrus* EOs (Zou et al. 2016), their interactions, or the interdependent effects of their active components (Acar et al. 2015; Lopes et al. 2020). However, we observed that even low doses of EOCA dietary supplementation can have positive effects on *R. quelen* intestinal morphology, indicating that different concentrations of *Citrus* EOs could be used for different objectives.

It is noteworthy that the observed alterations in the intestinal morphology of *R. quelen* have been associated with beneficial effects in several studies in other animals, resulting in an increased absorption surface area in the intestinal mucosa (Ferreira et al. 2016; Goulart et al. 2018; Hamidian et al. 2018; Laudadio et al. 2011). Supplementation with EOCA also promoted improvements in the heights of intestinal villi in the middle intestine of *R. quelen*. These villi are responsible for water-soluble nutrient absorption (Sundell and Rønnestad, 2011). The observed increase in the height of folds is highly desirable, as shorter villi and deeper crypts are associated with increased susceptibility to
diseases caused by intestinal pathogens (Ferreira et al. 2012). The higher the height of the intestinal villi, the better the digestion and nutrient absorption, which is reflected in positive effects on animal performance.

Similar to our results, Lopes et al. (2020) observed that dietary supplementation with EOCL increased the fold height and length in the intestine of tambaqui, *Colossoma macropomum*, which probably increased the area available for nutrient and ion absorption. But in counterpart to our findings, ACAR et al. (2021) observed that common carp fed high doses of EOCA (10 and 15 mL kg diet$^{-1}$) showed cellular infiltration and hyperemia in the lamina propria and submucosa in the intestine. Apparently these histopathological changes are toxic or allergic reactions to the high doses of EOCA. Branco et al. (2011) reported that the improved histological structure (number and height of folds) is an indication of enhanced nutrient and electrolyte absorption capacity. This increase may be greater due to the higher turnover rate caused by stimuli resulting from the action of active principles of plants and their essential oils.

A dose-response relationship of EOCA in the diet in relation to growth was observed, but this pattern was not observed in intestinal histomorphology. However, this difference in relation to the dietary addition of an EO and physiological responses is common (Saccol et al. 2013; Zeppenfeld et al. 2016; Lopes et al. 2020).

An improvement in the width of folds was observed in the anterior and posterior intestine, mainly in fish fed the diet containing 0.5 mL EOCA kg$^{-1}$. Besides the increased absorption area, wider folds can lead to slower chyme transit, which allows better digestion and increased absorption (Cao and Wang, 2009; Kapoor et al. 1975). Furthermore, the higher enterocyte height in the anterior and posterior intestinal segments may be indicative of an increase in cytoplasmic content due to absorption (Escaffre et al. 2007). The increase in surface area promoted by EOCA is highly desirable because it can enhance the digestive and absorptive processes, leading to improved performance of the animals, as observed in this study.

The number of goblet cells per fold presented different responses to the EOCA treatment, with a decrease in the anterior and an increase in the middle and posterior intestine. As observed by Tugnoli et al. (2020), the decrease in goblet cell number can be
related to a high priority in stimulating enterocyte proliferation, since this region is the main site of nutrient absorption (Sundell and Ronnestad, 2011). This hypothesis is supported by the increase in enterocyte height observed in the anterior intestine in all EOCA treatments. On the other hand, the middle intestine presented a higher number of goblet cells and no difference in enterocyte height in all EOCA treatments. It is well known that mucins aid in lubrication, nutrient absorption, and provide important cofactors for food breakdown (Carrassón et al. 2006; Grau et al. 1992). Thus, the increase in goblet cell population in the middle and posterior regions can represent beneficial effects in nutrient absorption.

5 CONCLUSION

The present study showed that dietary supplementation with EOCA (primarily at 0.5 mL EOCA kg diet⁻¹) promoted positive effects on the intestinal morphology of *R. quelen* and indicates that the use of essential oil extracted from sweet orange peel, a fruit residue, could be considered in fish diets.
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