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Original Article

Dietary supplementation with *Ocimum gratissimum* essential oil improves the intestinal bacterial populations of *Piaractus mesopotamicus*

Óleo essencial de *Ocimum gratissimum* melhora a microbiota intestinal do *Piaractus mesopotamicus*

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Abstract

The aim of this study was to evaluate the effects of the dietary supplementation *Ocimum gratissimum* essential oil (EO) on pacu (*Piaractus mesopotamicus*) intestinal microbiota and morphometry. In this study, juveniles of *P. mesopotamicus* were fed with diets supplemented with 0.5 and 1% of *Ocimum gratissimum* EO for 30 days. At the end of this period, the intestinal microbiota of the pacus was relatively quantified through qPCR, and the intestinal morphometry was evaluated using histometric methods. *O. gratissimum* EO feeding supplementation at 1% significantly altered the intestinal microbiota of juvenile pacus, resulting in higher relative quantification of bacteria from the genera *Enterococcus* and *Bacillus* when compared to 0.5% and control groups. *O. gratissimum* EO feeding supplementation presented no effects in intestinal morphometry. Our results suggest that the feeding supplementation of *P. mesopotamicus* with *O. gratissimum* at 1% concentration during 30 days modulates the intestinal bacterial populations but do not cause any changes in intestinal morphometry.

Keywords: clove-basil essential oil, dietary additives, intestinal histomorphometry, intestinal microbioma, neotropical fish.

Resumo

O objetivo deste estudo foi avaliar os efeitos da suplementação alimentar com óleo essencial de *Ocimum gratissimum* (EO) na microbiota intestinal do pacu (*Piaractus mesopotamicus*) e na morfometria intestinal do pacu. Neste estudo, juvenis de *P. mesopotamicus* foram alimentados com dietas suplementadas com 0,5 e 1% de EO de *Ocimum gratissimum* por 30 dias. Ao final deste período, a microbiota intestinal dos pacus foi quantificada relativamente por meio de qPCR, e a morfometria intestinal foi avaliada usando métodos histométricos. A alimentação com suplementação de 1% de EO de *O. gratissimum* alterou significativamente as populações bacterianas intestinais dos juvenis de pacu, resultando em uma maior quantificação relativa de bactérias dos gêneros *Enterococcus* e *Bacillus* quando comparado aos grupos de 0,5% e controle. A suplementação com EO de *O. gratissimum* não causou alterações na morfometria intestinal dos pacus. Nossos resultados sugerem que a alimentação de *P. mesopotamicus* com *O. gratissimum* na concentração de 1% durante 30 dias modula as populações bacterianas intestinais, mas não causa quaisquer alterações na morfometria intestinal.

Palavras-chave: óleo essencial de manjericão-cravo, aditivos dietários, histomorphometria intestinal, microbioma intestinal, peixes Neotropicais.

1. Introduction

Studies have shown the importance of intestinal microbiota modulation for the better productivity of fish (Nayak, 2010; Wang et al., 2018) and have reported the increased safety and beneficial effects of non-antibiotic dietary additives such as prebiotics (Ebrahimi et al., 2012; Munir et al., 2016), probiotics (Giri et al., 2013; Nguyen et al., 2017; Tan et al., 2019) and plant essential oils (EOs) (Zhang et al., 2020; Zheng et al., 2009) on growth improvement and animal health.

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Plant EOs comprise a class of products extracted from plants widely used in the food, hygiene and pharmaceutical industries and have a wide variety of molecules with diverse chemical properties (Freitas Souza et al., 2019). Several studies have reported on the beneficial effects of plant EOs on aquaculture fish by acting as an immunostimulant, such as *Cinnamomum verum* and *Zataria multiflora* (Vaseeharan and Thaya 2014); preventing oxidative stress, such as *Lippia alba* (Saccol et al., 2013); and improving growth parameters, such as *Origanum heracleoticum* (Zheng et al., 2009). Moreover, some plant products, when administered through feeding, have presented bacterial selective effects and modulate the intestinal microbiota and intestinal morphology (Alagawany et al., 2020, Huyben et al., 2021, Zhang et al., 2020).

Ocimum gratissimum (also known as clove-basil) is a widely distributed herb in the tropical region and is commonly present in popular medicine as a treatment for different diseases in humans such as diarrhea, headache and pneumonia (Pessoa et al., 2002). Recently, our group investigated the effects of *O. gratissimum* EO on *Pseudoplatystoma corruscans*, an important species for neotropical aquaculture, and observed its excellent anesthetic performance (da Silva et al., 2020). However, other studies have shown that *O. gratissimum* can present other interesting effects for aquaculture such as antiparasitic and antibiotic effects (Bandeira Júnior et al., 2017; Lima Boijink et al., 2016), stress prevention (Boaventura et al., 2021), blood parameters improvements (Nahak and Kanta Sahu, 2014), faster wound healing (Abdel-Tawwab et al., 2019), higher survival after experimental infection (Brum et al., 2017; Monteiro et al., 2021) and better productive performance (Abdel-Tawwab et al., 2018).

Piaractus mesopotamicus (pacu) is an omnivorous fish that reproduces with a high prolificity (Valladão et al., 2018) and is a great experimental model for studies regarding neotropical fish species due to their close relation to other serrasalmidae species, a family that encloses almost one third of the Brazilian aquaculture production (FAO, 2022). Thus, the present study aimed to investigate if the dietary administration of *O. gratissimum* EO can positively affect the intestinal microbiota and intestinal morphology of *P. mesopotamicus*.

2. Material and Methods

2.1. Ethics

Research on animals was conducted under the approval of the Committee on Animal Use of the Estate University of Mato Grosso do Sul (n° 014/2013).

2.2. Fish and experimental conditions

The experiment was conducted at the city of Aquidauana, Mato Grosso do Sul, Brazil, 20° 28' 30" South and 55° 47' 11" West.

For this study, 144 *P. mesopotamicus* juveniles produced by induced reproduction were acclimated in polyethylene tanks (80 L) with continuous aeration in a flow-through system, with all the tanks receiving water from a dan located nearby the laboratory. Fish were fed a commercial feed (45% of crude protein) until apparent satiation and were reared until reaching a mean weight of 43 g (± 13 g) and mean total length of 13 cm (± 1.2 cm). Then, they were distributed in 12 80-L tanks of the same size and left to acclimate for two weeks under the same conditions.

During the experimental period, one-third of the water in each tank was exchanged daily by syphoning to remove feces. The parameters of water quality were measured daily and maintained at 6.2 ± 2 mg L^{-1} of dissolved oxygen, a pH of 6.8 ± 0.52 and a temperature of 26.3 ± 1.36°C, all of which are considered adequate for *P. mesopotamicus* rearing.

2.3. Extraction and composition of O. gratissimum EO

The EO was obtained using the Clevenger apparatus by hydrodistillation, four 3 hours, of the extract of *O. gratissimum* leaves dried at room temperature. To analyze the EO composition it was used gas chromatography and mass spectrophotometry. Identification was made using the 6th edition of Wiley library, comparison with linear retention indices in the literature (Adams, 2007), interpolation of the retention times of the constituents with those from a series of n-alkanes (C7-C26) injected in the same column and conditions, and according to the equation of Van den Dool and Kratz (Vandendool and Kratz, 1963).

2.4. Experimental design

The tanks were randomly separated in three groups: one control and two treatments, with each group containing four tanks, and each tank containing 12 fish. The treatments consisted of two levels of inclusion of *O. gratissimum* EO: 0.5% and 1.0%, which were added to the commercial feed by aspersion. After the addition of EO, the feeds were left to dry and stored at -8°C until use. The control group was fed a commercial feed (32% of crude protein) without any addition of EO. The fish were fed until apparent satiation twice a day, and the remaining feed was removed after 15 min of administration, when necessary. The experiment was conducted for 30 days.

2.5. DNA extraction and relative quantification of intestinal bacteria through qPCR

After 30 days, two fish from each tank were euthanized using a eugenol solution (50 mg L^{-1}) and a 3-cm portion of the intestine was aseptically collected, measuring after 1 cm from the pyloric cecum. Then, the samples were conditioned in sterile Petri dishes and immersed in phosphate buffer saline (PBS, pH 7.4).

With a surgical blade, the intestine samples were longitudinally opened and placed in 15-mL polystyrene tubes containing 2 mL of PBS. The tubes were vigorously homogenized, the samples were discarded and the remaining solution was centrifuged at 16,000 G for 20 min for the obtention of bacterial pellets. This procedure was repeated to wash the pellets. After the washing, the supernatant was discarded, and the pellets were stored at -80°C for further DNA extraction.

The DNA extraction was performed using a KAOVI DNA extraction kit, according to the manufacturer's

protocol. Briefly, 100 mg of the pellet were dissolved in an extraction buffer and 20 µL of lysozyme lysis solution (50 mg mL^{-1}) was added to this solution. The tubes were homogenized and incubated at 37°C during 30 min. After this, 25 µL of proteinase-K (20 mg mL^{-1}) was added, and the solution was homogenized and incubated at 55°C for 30 min. The resultant solution was pipetted in a spin-filter column placed in a 2-mL polystyrene tube, and the column was washed by centrifugation. After this, the column was transferred to a new tube, and the DNA was eluted by the addition of 50 µL deionized water at 65°C. The DNA was quantified by spectrophotometry (NanoDrop), and its integrity was evaluated through electrophoresis in agaroses gel (0.8%). The obtained DNA was then stored at -80 °C for further use.

The quantification of microbiota was performed through the qPCR technique. For this, five pair of primers previously described in the literature were used. The first pair of primers corresponds to the Bacteria domain, and the subsequent pairs correspond to the groups Enterobacteria, Enterococcus, Lactobacillus and Bacillus (Table 1).

The qPCR analysis was performed in the Applied Biosystems Step One sequence detection system (Applied Biosystems USA) using the Platinum SYBR® Green qPCR SuperMix-UDG Kit (Invitrogen Life Technologies USA). The Ct values were obtained using the manufacturer's software.

Data were normalized in relation to total bacteria detection, and the relative quantification was determined using the ΔΔCt method (Schmittgen and Livak, 2008). qPCR data were presented as relative fold change in relation to the control group.

2.6. Intestinal morphometry

Two fish from each tank (n = 8) were euthanized and a sample of intestine was collected as previously described. The intestinal samples were then fixed in formalin (10%) for 24 h and then stored in a 70% ethanol solution. The samples were dehydrated in solutions containing increasing ethanol concentrations, diaphanized in xylol, and embbebed in paraffin. Cross-sections of 5 µm were obtained by semi-serial cuts and stained with hematoxylin and eosin (H&E). Microphotographs were taken using a micro-camera accoupled to an optical microscope (1000x).

All measures were performed using MoticImages Plus 2.0 ML software. The following parameters were analyzed: villi height (considering the distance from the peak of the villi until the muscle layer, in µm), total villi height (villi height + serosa thickness, µm), villi width (transversal cut of villi, in µm), epithelium thickness (µm), muscle thickness (µm), villi area, and number of goblet cells (number of cells per $µm^2$).

3. Statistics

Results were expressed as mean ± standard error. The statistical analyses were performed with the software R V3.0.3. All data were checked for homoscedasticity and normality with Levene's test and Cramer-Von Mises' test, respectively, and were transformed to fit normal distribution using log (x + 1.5). All data were submitted to one-way ANOVA and the resultant means were compared using Tukey's multiple range test ($\alpha<0.05$).

4. Results

In this study, the EO was rich in 1,8-cineole (39.5%), eugenol (14.7%) and β-selinene (12.6%), showing that the chemotype of the *O. gratissimum* used here was a eugenol/1,8-cineole/sesquiterpenes type (Figure 1).

After 30 days of the experiment, relative quantification by qPCR showed that the dietary supplementation with EO interfered in *P. mesopotamicus*' intestinal bacteria profile (Figure 2). Compared to the control group, 1% of the EO supplementation significantly increased the relative quantity of bacteria from the genera *Enterococcus* (P = 0.0478) and *Bacillus* (P = 0.0116). Fish fed with diet supplemented with 1% of EO also presented more intestinal *Lactobacillus* (P = 0.0098) when compared to 0.5% group, but both groups were statistically similar to control. Compared to the control group, fish fed with diet supplemented with 0.5% presented no difference for the Enterobacteriaceae family and from the genera *Enterococcus*, *Bacillus* and

Table 1. Primers used for relative quantification of intestinal bacteria of P. mesopotamicus fed with *O. gratissimum* EO.

Bacterial group	Oligonucletide name	Sequency	References
Bacteria domain	DOMBAC-F	5' – CGGYCCAGACTCCTACGGG – 3'	Lee et al. (1996)
	DOMBAC-R	5' – TTACCGCGGCTGCTGGCAC – 3'	
Enterobacteraceae	FamENTER-F	5' – ATTGACGTTACCCGCAGAAGAAGC – 3'	Lunedo et al. (2019)
	FamENTER-R	5' – CTCTACGAGACTCAAGCTTGC – 3'	
Lactobacillus	LACTOBAC-F	5' – CACCGCTACACATGGAG – 3'	Lunedo et al. (2019)
	LACTOBAC-R	5' – AGCAGTAGGGAATCTTCCA – 3'	
Enterococcus	ENTEROC-F	5' – CCCTTATTGTTAGTTGCCATATT – 3'	Lunedo et al. (2019)
	ENTEROC-R	5' –ACTCGTTGTACTTCCCATTGT – 3'	
Bacillus	BACIL-F	5' – GCTGGTTAGAGCGCACGCCTGATA – 3'	Solichová et al. (2019)
	BACIL-R	5' – CATCCACCGTGCGCCCTTTCTAAC – 3'	

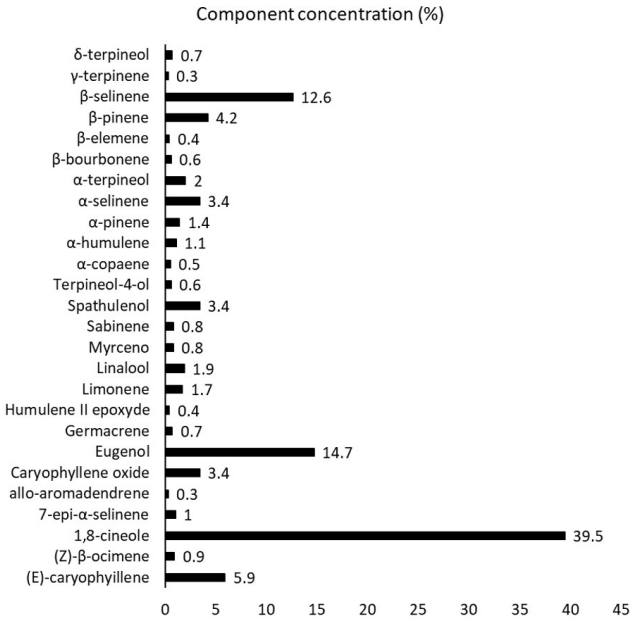

Figure 1. Concentration of each component of *O. gratissimum* determined by gas chromatography.

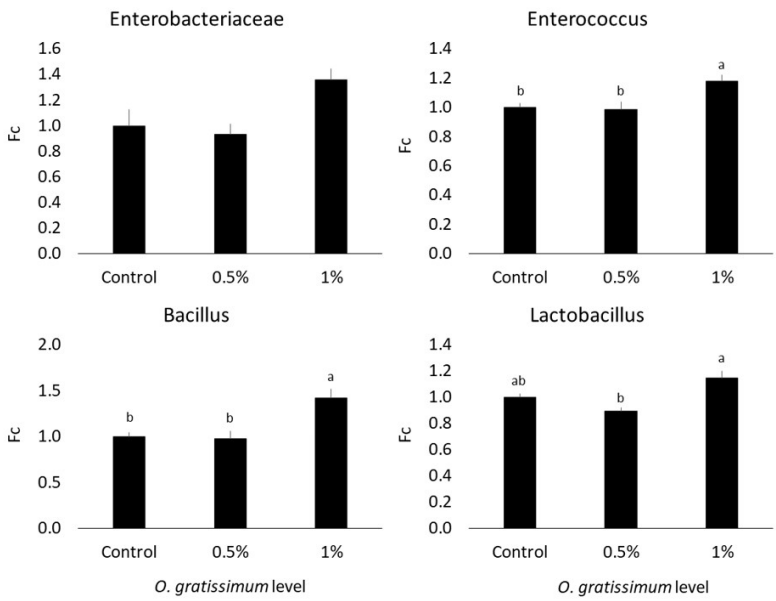

Figure 2. Relative quantification of bacterial groups in *P. mesopotamicus* intestine after O. gratissimum EO feeding. Different letters indicate significant differences according to Tukey's test (α = 5%). Fc: fold change.

Lactobacillus. However, in relation to the group fed with diet supplemented with 1%, the 0.5% group presented significantly lower quantities of *Enterococcus*, *Bacillus* and *Lactococcus* bacteria.

Regarding intestinal morphometry (Table 2), fish fed with diet supplemented with 0.5% EO showed lower villi thickness (P = 0.0097), muscle layer thickness (P = 0.0019) and villi area (P = 0.0038) when compared to fish fed 1% of EO. However, the only significant difference presented by 0.5% EO group when compared to control was the decrease of muscle layer thickness. Fish fed with diet supplemented with 1% of EO showed no differences when compared to the control regarding total villi height, villi height and muscle layer thickness (Figure 3).

No natural deaths were observed during the whole the experimental period.

Table 2. Intestinal histological morphometry of P. mesopotamicus fed with O. gratissimum EO for 30 days.

Parameter	Control	0.5%	1%	*P*-value
Total Villi Height (µm)	250 ± 27	128 ± 30	230 ± 18	0.1410
Villi Height (µm)	238 ± 26	120 ± 28	219 ±18	0.1230
Villi Thickness (µm)	73 ± 8 ab	59 ± 9 b	84 ± 4 a	0.0097
Muscle Layer Thickness (µm)	35 ± 2 a	21 ± 5 b	35 ± 3 a	0.0019
Villi Area (µm²)	16554 ± 2620 ab	5463 ± 1732 b	19629 ± 1540 a	0.0038
Goblet cells (cells)	2.6 ± 0.9	3.0 ± 0.5	0.6 ± 0.7	0.2020

Different letters indicate significant differences according to Tukey's test (α = 5%).

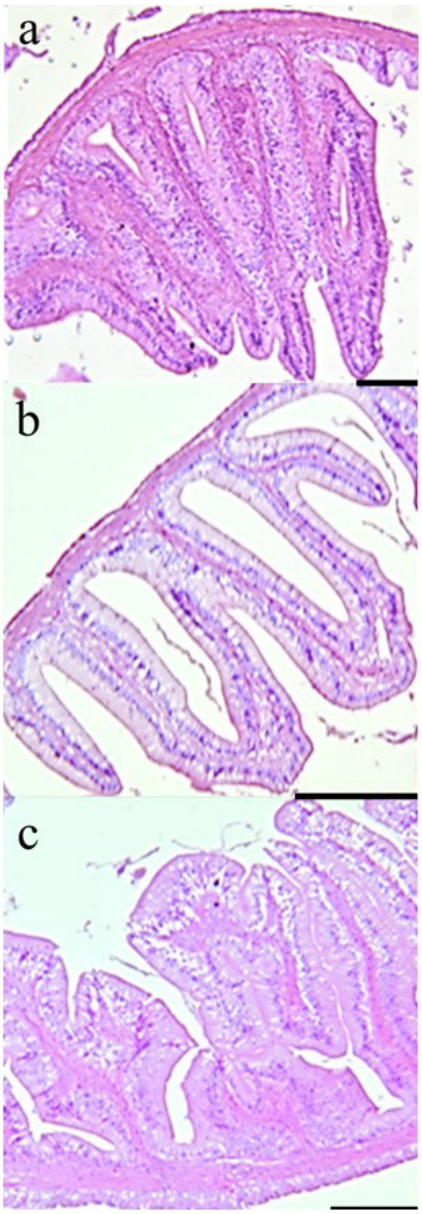

Figure 3. Representative microphotographs of *P. mesopotamicus* fed with control diet (A), 0.5% (B) and 1% (C) of *O. gratissimum* EO.

5. Discussion

Several studies have reported the effects of *O. gratissimum* on teleost species (Abdel-Tawwab et al., 2018, 2019; Boaventura et al., 2021; Brum et al., 2017; Monteiro et al., 2021; Nahak and Kanta Sahu, 2014), but this is the first that evaluated the effects of *O. gratissimum* EO on *P. mesopotamicus*, an important neotropical fish species.

The determination of EO composition after the extraction process is of significant importance in order to determine the main active compounds presented by the EO, the chemotype of the plant cultivar used for its manufacturing and its potential uses. In this study, composition analysis showed higher concentrations of 1,8-cineole, eugenol and β-selinene when compared with the other compounds, placing the *O. gratissimum* specimens in the eugenol/1,8-cineole/sesquiterpenes chemotype. Here, eugenol was the most abundant substance found in the *O. gratissimum* EO, which corroborates the findings of Bandeira Júnior et al. (2017) and Monteiro et al. (2021). Commonly found in clove EO, eugenol is well known for its anesthetic action (Barbas et al., 2021; Tarkhani et al., 2017a, b), but some studies also examined its antiparasitic (Lima Boijink et al., 2015) and antibacterial effects (Sutili et al., 2014) 1,8-cineole, also known as eucalyptol, has also been reported on as an anesthetic for fish. However, when administered through feeding, 1,8-cineole was reported on as a way to prevent oxidative stress in *Oncorhynchus mykiss* (Taheri Mirghaed et al., 2018) and *Cyprinus carpio* (Taheri Mirghaed et al., 2019). β-selinene is commonly reported in *O. gratissimum* composition (Bandeira Júnior et al., 2017; Monteiro et al., 2021); however, no studies investigated its action in fish using the purified form.

Evaluation of the intestinal microbiota is considered an important tool for assessing the host's physiological, immunological and nutritional status. In this study, 1% *O. gratissimum* EO dietary supplementation for 30 days affected the intestinal microbiota of *P. mesopotamicus* by increasing bacteria of the genus *Enterococcus*, *Bacillus* and *Lactobacillus*. Lactic acid bacteria such as *Enterococcus* sp. and *Lactobacillus* sp. as well as bacteria from the genus *Bacillus* have an important role in the maintenance of homeostasis in mucous environments by producing antimicrobial substances that act against pathogens or competing for cell-surface and mucin-binding sites (Kuebutornye et al., 2020, Liu et al., 2013). Additionally, these bacteria can

contribute to improved fish nutrition and growth through the production of enzymes and the production of essential amino acids and vitamins, such as vitamin B, which is vital for the energy metabolism of fish (Nayak, 2021).

According to the consulted literature, no studies have evaluated the effects of *O. gratissimum* EO on fish intestinal bacterial profile. While feeding *Sciaenops ocellatus* with the EO of a close relative to *O. gratissimum*, *O. Americanum*, Sutili et al., (2016) observed higher concentrations of linalool (33.54%) and lower concentrations of 1,8-Cineole (12.89%) when compared to our EO, and according to DGGE analysis of the microbial DNA, no changes in the fish intestinal bacterial community were noted after the experimental period. These findings suggest that the high concentration of 1,8-cineole observed in the present study may be the major cause of the changes in the intestinal bacterial profile. EOs of other plants have also been described as modulators of intestinal bacterial profile, such as *Cymbopogon citratus* and *Pelargonium graveolens* (Al-Sagheer et al., 2018) and *Origanum* sp. (Zhang et al., 2020).

In this study, *O. gratissimum* EO affected the intestinal morphology differently depending on the concentration. The most prominent effects were observed in fish fed 1% of EO, as they presented a higher villi thickness and area than the other groups. This may be attributed to the increase in lactic acid bacteria observed in this group when compared to 0.5% group. Lactic acid bacteria are known to produce short-chain fatty acids, which can improve the proliferation of enterocytes and increase the villi extension and nutrient absorption area (Abdel-Latif et al., 2020).

Despite those differences between treatments regarding intestinal morphology, none of them presented any significant difference when compared with the control group. According to the consulted literature, it is relatively common to observe no differences in the intestinal morphology after the supplementation of diets with plant derivatives such (Hernández et al., 2015; Valladão et al., 2017) which may come from interspecific characteristics, supplementation dose and period of feeding. However, morphometrical improvements due to dietary supplementation with *O. gratissimum* leaf extract have been reported in *Clarias gariepinus* (Abdel-Tawwab et al., 2018), and in other fish species using plant EOs either obtained in a laboratory or using commercial blends (Huyben et al., 2021, Zeng et al., 2015).

6. Conclusions

In conclusion, in this study, it was observed that the feeding of *P. mesopotamicus* with diet supplemented with *O. gratissimum* EO at a concentration of 1% for 30 days result in beneficial modulation of intestinal microbiota and an increase in intestinal nutrient absorption area. More studies should be conducted to determine the optimal period of EO feeding.

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