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ORIGINAL ARTICLE

Inhibition of *Listeria monocytogenes* growth on vacuum packaged rainbow trout (*Oncorhynchus mykiss*) with carvacrol and eugenol

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Abstract

The aim of this study was to evaluate the effects of carvacrol and eugenol, separately and in combination, on survival of *Listeria monocytogenes*, and sensory and microbiological characteristics in vacuum packaged *Oncorhynchus mykiss* during refrigerated storage (4 ± 1 °C) for 20 days. The control fish fillets were analyzed for microbial (total mesophilic and psychrotrophic bacteria and lactic acid bacteria) and sensory properties. Fish fillets treated with carvacrol, eugenol, and their combination displayed populations of *L. monocytogenes* significantly lower, by 1.35–2.84 log cfu/g, than the control fillets during the whole storage period. No significant differences between groups of fish fillets with different active compound(s) added were noted except at the end of the storage, when the number of *L. monocytogenes* was significantly lower in the fish fillets with eugenol added. Sensory analysis showed that fish fillets with eugenol added were the most acceptable to trained panelists.

Practical applications

Taking into account the increasing need for the production of safe fish and fish products and the fact that carvacrol and eugenol, which exhibited significant antilisterial effect, are generally recognized as safe they can find its practical application in fish industry. Furthermore, as these substances are major constituents of numerous essential oils they can be considered as natural preservatives and used in the organic production as a substitute with synthetic additives which can cause adverse effects.

1 | INTRODUCTION

According to the Food and Agriculture Organization of the United Nations, fish consumption increased from an average of 9.9 kg in the 1960s to around 20 kg in 2015 (FAO, 2016). Fish and fish products are one of the mostly consumed food items, but also are one of the main sources of *Listeria* for humans, and this pathogen can cause severe listeriosis, with symptoms including meningitis, meningoencephalitis, septicemia, and abortion (Wehner et al., 2014).

Listeria monocytogenes is a foodborne pathogen, which is a major public health concern because of its high mortality rate. *L. monocytogenes* has been differentiated into 13 serotypes, where serotype 1/2a has been the most frequently detected in humans (91.8% of human infections in 2013; EFSA/ECDC, 2015). The food industry is registering a steady increase in production, and there is always a need to create a better quality and safer product (Dimitrijević et al., 2015).

Because of the growing popularity of natural and organic foods, there has been a consumer shift away from chemical preservatives in foods, as these compounds exhibited many adverse effects. Thus, the use of natural antimicrobial substances such as essential oils (EOs) or their compounds has gained the interest of the food industry to meet consumers' preferences and replace synthetic additives with more safe natural preservatives. EOs are a mixture of fragrant and very volatile small molecular weight compounds, synthesized in different plant

organs, and constitute a new method for the reduction and elimination of pathogens from food (Bajpai, Baek, & Kang, 2012; Bošković et al., 2013).

As numerous reports have confirmed antioxidant and antimicrobial effects of EOs, they could be good natural preservatives (Amorati et al., 2014; Boskovic et al., 2017; Swamy, Akhtar, & Sinniah, 2016). Most of the EOs and their compounds are generally recognized as safe. Oregano essential oil (OEO), also known as the Mediterranean miracle, is known to possess antibacterial, antiviral, antifungal, antiparasitic, and antioxidant activities. The principal component responsible for the bioactivity of OEO is the small phenolic compound, carvacrol (C; Gaur, Kuhlenschmidt, Kuhlenschmidt, & Andrade, 2018). Eugenol (4-allyl-2-methoxyphenol) is the major compound of the essential oil obtained from cloves (*Eugenia caryophyllus*). Eugenol also has a range of different biological properties confirmed: bactericide, antifungal, larvicidal, antioxidant, and anti-inflammatory, among others (Cansian et al., 2017).

The aim of this study was to evaluate the effectiveness of carvacrol and/or eugenol against *L. monocytogenes*, and their effects on lactic acid bacteria (LAB) and total viable bacteria counts, and on the sensory properties of vacuum packed and stored fresh trout.

2 | MATERIALS AND METHODS

2.1 | Preparation of inoculum

In the present study, *L. monocytogenes* serotype 4b ATCC 19115, serotype 4b NCTC 11994, serotype 4b from smoked herring and serotype 1/2a previously isolated from smoked salmon were used. All isolates were resuscitated in brain–heart infusion (BHI) broth (Oxoid, Hampshire, UK) and incubated at 37 °C for 24 hr, and were then inoculated into fresh BHI and grown in the same conditions. Then, bacterial cultures were mixed in approximately equal proportions to produce the cocktail inoculum of approximately 10^6 log cfu/g.

2.2 | Sample preparation, EO treatment, and packaging

Californian trout (*Oncorhynchus mykiss*) were purchased from a breeding farm, Janj, located in Banja Luka (Bosnia and Herzegovina). The fish were captured with a net and immediately transferred to the laboratory where they were killed and bled by gill cutting. After removal of skin, fish were filleted under hygienic conditions and fillets were divided into four groups.

Fish fillets were inoculated with 10^6 cfu/g of *L. monocytogenes* and then put in brine with salt concentration of 9%. The control group had no active compounds added, whereas in other groups of fillets, 0.5% (% vol/wt) of carvacrol (Carvacrol Natural 99% Sigma–Aldrich, Missouri, USA) or eugenol (Eugenol 99% Sigma–Aldrich), and a combination of these additives at the same levels were added. Then, all fillets were vacuum packed. For vacuum packaging, a Multivac C 500 machine (Multivac Verpackungsmaschinen, Wolfertschwenden, Germany) was used. Trout fillets were packed in a PA/EVOH/PE foil (polyamide/ethylene vinyl alcohol/polyethylene Dynopack, POLIMOON, Kristiansand, Norway), with low permeability to gas. The degree of permeability to O_2 was 3.2 cm^3/m^2/day at 23 °C, to N_2 was 1 cm^3/m^2/day at 23 °C, to CO_2 was 14 cm^3/m^2/day at 23 °C and to water vapor was 15 g/m^2/day at 38 °C.

Packaged trout fillets were stored at low temperature (4 ± 1 °C) and examined on Day 0 and on Days 3, 6, 10, 15, and 20 of storage.

2.3 | Microbiological analyses

For *Listeria* enumeration, 10 g of fish fillet was weighed out aseptically after pack opening, transferred into sterile Stomacher bags and 90 ml of Buffered Peptone Water (BPW; Merck, Germany) was added to each sample, whereas for enumeration of other bacteria, 20 g of fish fillet was weighed out aseptically after pack opening, transferred into sterile Stomacher bags and 180 ml of BPW was added to each sample. The bag contents were homogenized in a Stomacher blender (Stomacher 400 Circulator, Seward, Dominion House, Easting Close, Worthing BN14 8HQ UK) for 2 min. Serial decimal dilutions were prepared and 1 ml or 0.1 ml of appropriately diluted suspension was inoculated directly on the surface of the appropriate media for enumeration of the different bacteria.

Fish fillets were analyzed for *Listeria* spp. at the beginning of the study in order to determine the presence or absence of this pathogen. Inoculated fish fillets were analyzed for *L. monocytogenes*, total viable count (TVC—mesophiles, 30 °C), and LAB count on Day 0 and on Days 3, 6, 10, 15, and 20 of storage.

L. monocytogenes was enumerated on the Agar Listeria acc. to Ottaviani and Agosti (ALOA, Oxoid, Hempshire, UK) and plates were incubated for 24–48 hr at 37 °C according to ISO 11290-1:2017 (2017). TVCs were enumerated on Plate Count Agar (PCA, Merck, Germany) and incubated at 30 °C for 72 hr according to ISO 4833-1:2013 (en; 2003). LAB were enumerated on MRS (Merck) following incubation at 30 °C for 72 hr according to ISO 15214 (1998).

After incubation, plates were examined visually for typical colony types and morphological characteristics associated with each growth medium, the number of colonies was counted, and results were recorded as colony forming units per gram (cfu/g).

2.4 | Sensory evaluation

Six panelists from the Department of Food Hygiene and Technology at the Faculty of Veterinary Medicine, University of Belgrade, evaluated appearance, odor, texture, and total acceptability of fish fillets. Sensory evaluation was performed only on uninoculated samples of fish. Sensory analysis was performed by a quantitative descriptive test (ISO 6658, 2005; ISO 4121, 2003). The evaluation was carried out using a 5-point hedonic scale, where the values 3.5 and higher were considered acceptable.

2.5 | Statistical analysis

In the present experimental design, six randomized samples from each group were analyzed on each examination day. Numbers of microorganisms were transformed into logarithms (log) before statistical analysis. Statistical analysis of the results was conducted using the software GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). The results were expressed as

arithmetic mean ± standard deviation. The effects of different treatments during storage period were appraised by one-factor analysis of variance (ANOVA) with Tukey's multiple comparison test at 95% confidence level (difference considered significant if $p < .05$).

3 | RESULTS

The antimicrobial effects of carvacrol, eugenol, and their combination on fish fillets during storage for 20 days are shown in Table 1.

L. monocytogenes counts ranged between 4.28 and 4.70 log cfu/g at the beginning of the study without significant differences between samples ($p > .05$). The number of *L. monocytogenes* was reduced in all fish fillets during storage by 1.35 (C) to 2.84 (E) log cfu/g. Fish fillets treated with carvacrol, eugenol, or their combination displayed populations of *L. monocytogenes* significantly lower than the control fish fillets ($p < .05$) during the whole storage period. *L. monocytogenes* counts decreased until Day 10, after which, numbers of this pathogen increased in all groups.

The increase was significantly higher ($p < .05$) in the control fish fillets than in fish fillets with added active compounds. No significant differences ($p > .05$) between fish fillets with added active compounds were noted except for the Day 20, when the number of *L. monocytogenes* was significantly lower ($p < .05$) in fish fillets with eugenol compared to fish fillets with both carvacrol and eugenol added. No significant difference ($p > .05$) between the effect of eugenol and carvacrol was noted until the end of the study.

The initial TVC was 3.26 ± 0.16 log cfu/g in the control fish fillets and, significantly lower ($p < .05$) in the fish fillets with EOs added. Numbers of TVC decreased until Day 6 of storage and then increased in all fish fillet groups until the end of the storage period. On Day 20, the mean TVC in control fish fillets was 5.77 log cfu/g and was significantly higher ($p < .05$) than in the other groups of fish fillets. No significant differences ($p > .05$) were observed between fish fillets with added active compounds except on Day 15, when the lowest TVC was recorded in fish fillets with both carvacrol and eugenol added.

At the beginning of the study, LAB were below the limit of detection (<1 log cfu/g) in all fish samples. On Day 6 of storage, the number of LAB ranged between 1.50 ± 0.43 (CO) log cfu/g and 1.87 ± 0.19 (C) log cfu/g. The LAB counts increased until the end of the storage period, but no significant differences were observed between groups ($p > .05$).

Sensory properties (appearance, odor, texture, and overall acceptability) of fish fillets are presented in Table 2.

At the beginning of the study, all sensory property scores for all fish fillets were acceptable, and greater than 4.62. During the first 3 days of storage, no significant differences were observed between compared groups ($p > .05$). From Day 6, sensory scores of the fish fillets decreased in all groups, but greater decreases (less satisfactory scores) were observed in the control fish fillets compared to those with carvacrol and/or eugenol.

On Day 6, the appearance of control fish fillets and those treated with carvacrol was significantly less acceptable ($p < .05$) than the appearance of other fillet groups. However, at the end of the storage period on Day 20, the fish fillets treated with eugenol had the highest appearance scores.

Odor scores did not differ ($p > .05$) between groups during the first 6 days of storage. On Day 10, the odor of control fish fillets and those with carvacrol added was significantly less acceptable compared to that of other fillet groups. Then, until the end of storage, the control fillets received the lowest odor scores, whereas the odor of fillets with carvacrol maintained the same scores as recorded for Day 6; the odor of this fish fillet group was evaluated as the best ($p < .05$) on Day 20 of storage.

There were no changes in the texture of the fish fillets during the first 3 days of storage ($p > .05$). Fish fillets with carvacrol achieved significantly lower ($p < .05$) texture scores on Day 6. On Day 15, there was no statistically significant difference between fish fillets treated with carvacrol, eugenol, or their combination. The texture scores decreased during storage in all groups. However, at the end of the

TABLE 1 Effect of carvacrol, eugenol, and their combination on *Listeria monocytogenes*, TVC, and LAB in fish meat during storage at 4 °C for 20 days

		Day					
	Group	0	3	6	10	15	20
L. monocytogenes	CO	4.48 ± 0.31^{A}	2.06 ± 0.41^{A}	2.39 ± 0.27^{A}	2.49 ± 0.24^{A}	2.87 ± 0.37^{A}	$3.13 \pm 0.25^{A,C}$
	C	4.70 ± 0.49^{A}	1.05 ± 0.07^{B}	1.26 ± 0.57^{B}	1.00 ± 0.22^{B}	2.01 ± 0.08^{B}	$1.95 \pm 0.41^{B,C}$
	E	4.69 ± 0.32^{A}	1.01 ± 0.18^{B}	1.36 ± 0.35^{B}	0.74 ± 0.44^{B}	1.76 ± 0.28^{B}	1.85 ± 0.20^{B}
	C + E	4.28 ± 0.76^{A}	1.07 ± 0.26^{B}	0.94 ± 0.34^{B}	1.17 ± 0.36^{B}	2.04 ± 0.39^{B}	2.93 ± 0.34^{C}
TVC	CO	3.26 ± 0.16^{A}	1.88 ± 0.45^{A}	2.67 ± 0.20^{A}	3.26 ± 0.18^{A}	4.63 ± 0.23^{A}	5.77 ± 0.36^{A}
	C	2.64 ± 0.31^{B}	1.56 ± 0.40^{A}	1.48 ± 0.25^{B}	2.08 ± 0.56^{B}	2.71 ± 0.18^{B}	3.38 ± 0.36^{B}
	E	$2.80 \pm 0.60^{A,B}$	1.62 ± 0.13^{A}	1.41 ± 0.18^{B}	2.37 ± 0.16^{B}	3.67 ± 0.31^{C}	3.85 ± 0.56^{B}
	C + E	2.57 ± 0.23^{B}	1.62 ± 0.49^{A}	1.40 ± 0.45^{B}	2.45 ± 0.28^{B}	2.68 ± 0.78^{D}	3.71 ± 0.66^{B}
LAB	CO	nd	nd	1.50 ± 0.43	2.42 ± 0.35	2.10 ± 0.28	2.52 ± 0.29
	C	nd	nd	1.87 ± 0.19	2.23 ± 0.33	2.00 ± 0.46	2.13 ± 0.22
	E	nd	nd	1.81 ± 0.11	2.08 ± 0.33	1.75 ± 0.26	2.31 ± 0.38
	C + E	nd	nd	1.69 ± 0.45	2.07 ± 0.65	2.04 ± 0.17	2.21 ± 0.28

Note. Different letters (A–C) indicate statistically significant differences between columns ($p < .05$). Abbreviations: nd = not determined; TVC = total viable count; LAB = lactic acid bacteria.

TABLE 2 Sensory properties of vacuum packaged fish, stored at 4 ± 1 °C (mean ± *SD*)

	Day					
Group	**0**	**3**	**6**	**10**	**15**	**20**
Appearance						
CO	4.75 ± 0.22	4.77 ± 0.27	4.83 ± 0.19^{A}	4.25 ± 0.24	3.75 ± 0.23	2.45^{A} ± 0.38
C	4.78 ± 0.25	4.77 ± 0.17	3.73 ± 0.22^{B}	3.77 ± 0.22	3.23 ± 0.17	2.32^{A} ± 0.19
E	4.73 ± 0.20	4.80 ± 0.19	4.80 ± 0.18^{A}	3.57 ± 0.42	3.23 ± 0.22	3.78^{B} ± 0.17
C + E	4.75 ± 0.19	4.75 ± 0.19	3.75 ± 0.19^{B}	3.78 ± 0.18	3.17 ± 0.17	2.73^{A} ± 0.22
Odor						
CO	4.93 ± 0.22	4.72 ± 0.21	4.17 ± 0.21	3.73 ± 0.22^{A}	2.42 ± 0.42^{A}	1.63 ± 0.21^{A}
C	4.73 ± 0.22	4.82 ± 0.19	4.20 ± 0.21	3.72 ± 0.22^{A}	3.70 ± 0.24^{B}	3.83 ± 0.19^{B}
E	4.80 ± 0.20	4.85 ± 0.19	4.35 ± 0.19	4.22 ± 0.19^{B}	3.72 ± 0.23^{B}	3.25 ± 0.20^{C}
C + E	4.78 ± 0.23	4.78 ± 0.19	4.18 ± 0.25	4.27 ± 0.20^{B}	3.45 ± 0.42^{B}	2.77 ± 0.21^{D}
Texture						
CO	4.73 ± 0.19	4.78 ± 0.21	4.72 ± 0.23^{A}	3.75 ± 0.21^{A}	2.83 ± 0.21^{A}	2.23 ± 0.22
C	4.68 ± 0.21	4.73 ± 0.19	4.23 ± 0.23^{B}	3.83 ± 0.20^{A}	3.32 ± 0.17^{B}	2.37 ± 0.38
E	4.78 ± 0.17	4.73 ± 0.21	4.70 ± 0.21^{A}	3.78 ± 0.19^{A}	3.30 ± 0.20^{B}	2.50 ± 0.45
C + E	4.68 ± 0.25	4.75 ± 0.23	4.75 ± 0.19^{A}	4.28 ± 0.21^{B}	3.28 ± 0.21^{B}	2.67 ± 0.19
Overall acceptability						
CO	4.65 ± 0.15	4.85 ± 0.19	4.25 ± 0.19	3.83 ± 0.20^{A}	2.58 ± 0.38^{A}	1.70 ± 0.21^{A}
C	4.73 ± 0.20	4.75 ± 0.19	4.17 ± 0.20	4.13 ± 0.20^{AB}	3.58 ± 0.38^{B}	2.25 ± 0.23^{B}
E	4.78 ± 0.19	4.67 ± 0.21	4.32 ± 0.19	4.18 ± 0.19^{B}	3.37 ± 0.34^{B}	2.75 ± 0.19^{B}
C + E	4.62 ± 0.19	4.77 ± 0.17	4.33 ± 0.20	4.22 ± 0.19^{B}	3.72 ± 0.21^{B}	2.27 ± 0.21^{B}

Note. Different letters (A–D) in the column indicate statistically significant differences ($p < .05$).

storage, no significant differences between textures of the examined groups of fish fillets were observed ($p > .05$).

Until Day 10 of storage, there were no differences in overall acceptability between groups of fish fillets. From Day 10, the control fish fillet group achieved significantly lower scores than fish fillets with active substances added ($p < .05$).

4 | DISCUSSION

Most studies agree that, generally, EOs are slightly more active against gram-positive than gram-negative bacteria (Boskovic et al., 2015; Burt, 2004), which can explain effects of the active compounds used in the present study. However, some studies indicate that the gram-positive *L. monocytogenes* is more resistant to EOs than other bacteria and resistance could be comparable to that of some gram-negative bacteria (Gómez-Estaca, De Lacey, López-Caballero, Gómez-Guillén, & Montero, 2010; Kim, Marshall, Cornell, Preston III, & Wei, 1995; Oussalah, Caillet, Saucier, & Lacroix, 2007). As EOs are complex mixtures, their antibacterial activity is based on several targets in the cell and cannot be attributed to a single compound (Bajpai et al., 2012; Burt, 2004).

Carvacrol, one of the major components of oregano, is able to change the permeability of bacterial membranes, thanks to a phenolic hydroxyl group able to form hydrogen bonds with active sites of target enzymes (Picone et al., 2013). In addition, these natural antimicrobials can promote the leakage of contents out of the cell, and depletion of the microbial cell proton motive force and of the ATP pool, which eventually lead to leaking of intracellular constituents, coagulation of cell contents, lysis, and cell death (Nazzaro, Fratianni, De Martino, Coppola, & De Feo, 2013). In the present study, carvacrol significantly decreased the number of *L. monocytogenes*, confirming results obtained from previous studies. Many studies recorded that among different active compounds of EOs, carvacrol had the strongest antibacterial effect. However, results from the current study showed that eugenol exhibited an even greater antilisterial effect.

Eugenol, the active compound extracted from the dried flower buds of clove (a major component, approximately 85%), can destroy the cell wall of bacteria. Permeability of the membrane cell is increased, causing intracellular ingredients to leak from the cell, especially electrolytes including K^+, Ca^{2+}, and Na^+, which are necessary for the maintenance of the energy status, regulation of metabolism, solute transport, and so forth. This can result in changes of cell membrane structure, detrimentally affect cell metabolism and lead to cell death (Cox et al., 2001).

Previous studies reported the effect of plant EOs and extracts against some pathogens, including *L. monocytogenes*, on food model systems (Boziaris, Proestos, Kapsokefalou, & Komaitis, 2011; Solomakos, Govaris, Koidis, & Botsoglou, 2008). In the study of Miyague, Macedo, Meca, Holley, and Luciano, (2015), carvacrol showed the strongest activity against *L. monocytogenes*. In contrast, some researchers indicated that carvacrol had no antibacterial effect against *L. monocytogenes* in steak, likely because of the presence of certain food components (Veldhuizen, Creutzberg, Burt, & Haagsman, 2007).

As many EOs also show synergistic antimicrobial activity when used in combination, this property could reduce the amount of EO needed and its potential impacts on sensory quality. However, in the present study, a synergistic effect of the two EOs was not observed. In fact, both carvacrol and eugenol separately exhibited better

antibacterial effects than when they were used in combination. Even so, the combination of these active compounds significantly reduced populations of *L. monocytogenes* compared to the control.

At the start of the study period, LAB were not a significant part of the natural microbiota of the fish used. However, during cold storage of fish under vacuum packaged conditions, LAB usually become the predominant bacterial type (Gram & Dalgaard, 2002). That was the case in the present study, where on Day 20, the population of LAB was ~2.5 log cfu/g, indicating substantial growth in the untreated fish. Because LAB can be spoilage bacteria, it is important to control their growth. In comparison with untreated control fish, the final LAB count (Day 20) in fish with added carvacrol was lower by approximately 0.4 log cfu/g, but differences were not significant.

Generally, bacterial counts of fresh trout have been reported to be in the range of 3–4 log cfu/g. This initial load of bacteria could be because of contamination during handling, harvesting, and processing (Nowzari, Shábanpour, & Ojagh, 2013). Initial TVCs in the fish used in the present study were relatively low, indicating good hygiene of the material. During storage, the mean TVC in control fish fillets reached ~5.8 log cfu/g, whereas the increase of this bacterial group was significantly lower in fish with added active compounds, because of the their antibacterial activity. There are differing data in the literature about the limit of TVC that indicates fish spoilage (Pacquit et al., 2007). Even so, some authors indicate that the upper limit for fish spoilage is 10^7 cfu/g (Koutsoumanis, 2001; Olafsdottir et al., 2004). In the present study, this TVC count was not reached in any fish.

The use of EOs in foods is limited, despite their antimicrobial properties, primarily because of their effect on the organoleptic properties of food. Some studies showed that EOs positively influenced the acceptability of food. Changes in sensory food characteristics depend on the chemical composition of the EOs examined, the properties of the food and the preferences of panelists (Boskovic et al., 2017; Burt, 2004). In view of their organoleptic properties, EOs could most readily be incorporated in manufactured foods that are traditionally associated with herbs (savory dishes, such as meat and fish dishes, cheese, vegetable dishes, soups, and sauces; Burt, 2004).

The sensory properties of all fish fillets with added active compounds derived from EOs were acceptable to our panelists. During storage, sensory properties of the fish changed as a result of bacterial growth and oxidation processes and scores decreased, but greater decreases (less satisfactory scores) were observed in the control fish compared in fish with carvacrol and eugenol added. This can be explained because of the lower number of bacteria in these fish fillets. Carvacrol on cod fillets produced a "distinctive but pleasant" flavor, which decreased gradually during storage at 2 °C (Mejlholm & Dalgaard, 2002). Kim et al. (1995) reported that, on fish, carvacrol is said to produce a "warmly pungent" aroma. However, in the present study, eugenol tended to be more acceptable than carvacrol to panelists, even though significant differences were not observed.

5 | CONCLUSION

The present study showed that eugenol, carvacrol, and their combination exhibited strong antimicrobial activity against *L. monocytogenes*. However, eugenol caused the greatest reduction of this pathogen and trout fillets with added eugenol were sensorially evaluated as the best by trained panelists. Our results suggest that the eugenol, carvacrol, or their combination could be used for controlling bacterial growth and survival in vacuum packaged *O. mykiss*.

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