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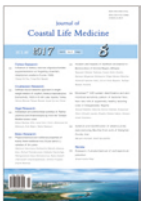

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# Influence of dietary mannan oligosaccharides supplementation on fingerling clownfish, *Amphiprion ocellaris* (Cuvier, 1830)

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ABSTRACT

**Objective:** To test the effects of dietary mannan oligosaccharides (MOS) on clownfish *Amphiprion ocellaris* (*A. ocellaris*).
**Methods:** Six levels (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g/kg diet) of MOS were added to the basal diet (used as control diet) and fed clownfish *A. ocellaris* [total length (24.21 ± 0.16) mm] for 70 days. Growth performance and tissue protein were measured.
**Results:** The result showed that growth rate was enhanced significantly in fish fed 0.10% and 0.15% MOS in diets compared to fish fed other MOS levels or control ($P < 0.05$). Survival rate significantly increased in fish fed diets with 0.20% and 0.25% MOS, compared to control. Fraction rates of protein growth were significantly higher in fish fed diets with 0.15%, 0.20% and 0.25% MOS supplementation than those fish fed other MOS inclusion levels and the control ($P < 0.04$). Dietary MOS concentrations and clownfish growth rates were significantly correlated. Optimal MOS concentration was predicted to be 0.185%, 0.173% and 0.171% at Days 14, 42 and 70, respectively.
**Conclusions:** In conclusion, MOS-supplemented diet benefits for boosting growth, survival and body composition of the clownfish, *A. ocellaris*.

## 1. Introduction

The ornamental clownfish *Amphiprion ocellaris* (Pomacentridae) (*A. ocellaris*) is one of the most popular ornamental marine teleost species. They are artificially bred worldwide to satisfy the demand for the ornamental fish as well as to reduce the catching pressure on the natural ornamental fish stock. In Vietnam, this species was successfully bred and grew in captivity, however, growth and survival rates are still low. Researches have revealed that antibiotics may boost growth and improve feed consumption[1]. In contrast, the use of chemicals and antibiotics carries many risks including toxicity, resistance, residues and possible harm to human health[2]. This has paved the way in finding new replacements for antibiotic[3]. Besides vaccine, probiotics and prebiotics, such as mannan oligosaccharides (MOS) have received high attention.

Prebiotics are non-digestible, but it benefits the host by increase in growth and metabolism of healthy intestinal bacteria of the host[3]. Previous research showed prebiotics can boost growth of many aquaculture species[4-10], boost non-specific immune and pathogen resistance[11] and improve intestinal function and health[4,10,12]. MOS is used commonly in fish and crustacean diet.

There are many studies on effects of MOS on aquatic species, however, to the authors' knowledge, MOS requirement for optimal growth of clownfish is still poorly understood. Inclusion dosage of the prebiotics plays a vital role because inappropriate dose may adversely affect the animals[4,13]. Therefore, the present study aimed to define the optimal range of MOS inclusion in diets on growth performance and protein content of clownfish, *A. ocellaris*.

## 2. Materials and methods

### 2.1. Experimental fish and culture systems

Clownfish *A. ocellaris* were bred and grew at the hatchery at the Institute of Oceanography, Nha Trang, Vietnam. A total of 560 clownfish from the same parents with mean initial total length

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[(24.21 ± 0.16) mm] were randomly stocked in 28 glass tanks (30 cm × 50 cm × 45 cm). Each tank was equipped with a biofilter recirculation system with a circulating rate of ~0.6 L/min. During the experiment, water temperature, pH, ammonia ($NH_4/NH_3$), nitrite ($NO_3$) and salinity, were 28–30 ℃, 8.0–8.3, 0–0.1 mg/L, 0–0.25 mg/L and ~35‰, respectively.

### 2.2. Experimental design

The experiment tested the growth response and mortality of fish fed basal diet added with graded concentrations of MOS for 70 days. A design was used in which the seven dietary treatments were haphazardly allocated to 4 replicate tanks, each with twenty animals in each tank. A total of 28 tanks and 560 fingerling clownfish were stocked.

### 2.3. Experimental diets and feeding

Six levels (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g/kg diet) of MOS were added to a basal diet (content 39.3% crude protein, 8.71% lipid and 19.72 gross energy MJ/kg). Experimental fish were fed 5% of the average biomass in each tank on the first day, and then every day the amount fed was adjusted, based on the amount of uneaten or remaining pellets. Rations were offered to the fish twice daily (1/2 at 08:00 and the remaining 1/2 at 17:00). Status of fish was observed each morning. Uneaten feed and feces were siphoned daily before morning feed. These procedures were followed for 70 days.

### 2.4. Sampling and data collection

All the fish were weighed and total length was measured at Days 0, 14, 42 and 70. At the end of the experiment, one fish from each cage (4 animals in each treatment) was randomly sampled and sacrificed for measurement of muscle composition. Fish were starved for 24 h prior to measuring or sampling.

### 2.5. Chemical analysis of culture fish

A pooled sample of 10 fish before the feeding trial was randomly collected and then stored at 20 ℃ for initial body protein analysis. Muscles of six fish in each treatment were sampled for protein analysis at the end (Day 70). Crude protein was measured following the Kjeldahl standard method, with the procedure described by Do Huu and Jones[4].

### 2.6. Data calculation

Growth rate [ultiaverage weekly length gain (LG, mm/week)], daily growth coefficient (DGC, %/day) and survival were computed by following equations[5]: LG (mm/week) = $(L_t - L_o)$/week; DGC (%/day) = $100 \times (L_t^{1/3}-L_o^{1/3})$/days; Survival rate = $100 \times (N_t/N_o)$, where $L_t$ and $L_o$ are total lengths of clownfish at the end (Day 70) and initial length (mm) respectively, $N_o$ is the initial number of fish and $N_t$ is the number of fish at the end of the experiment. Fraction rate of protein growth was calculated as $K_s$ (%/day) = $100 \times \{[\ln(P_t) - \ln(P_o)]\}$/days[14], where $P_t$ and $P_o$ are protein content of clownfish at time t (Day 70) and beginning, respectively.

### 2.7. Prediction of MOS concentration for highest growth response of clownfish

The response of fish to dietary MOS concentrations was estimated from growth data. Each growth data set at each time point with respect to concentrations of dietary MOS was analyzed by response models as described by Do Huu *et al.*[15].

Dose-response analysis was performed using eight nutritional models including 1 compartmental model, two broken lines (linear ascending and quadratic ascending), 1 saturation kinetics, 2 logistic models (3 and 4 parameters) and the two exponential and sigmoidal models. In each case, the levels of MOS were independent variable and growth was the dependent variable. The response parameter that gave the most significant effect was the mean length of clownfish and this was selected as the output response for each of the models. Absolute weight and LG, DGC were also considered but rejected. The model selected for further analysis of results was based on the low sum of squared residuals and high values of $R^2$. In present study, the compartmental model $y = a \times e^{-bx}(1 - e^{-c(x-d)})$ was the most appropriate and was used for analysis, where $y$ is the growth response of clownfish, $a$ is theoretical maximum, $b$ is intercept, $c$ is nutrient rate constant, and $d$ is kinetic order of the response when $x = 0$[16].

### 2.8. Statistical analysis

Data are presented as mean ± SEM. To compare length and growth rate of fish among diet treatments, ANOVA tests and least significant difference were used. Differences were significant when $P < 0.05$. Non-parametric Kruskal-Wallis test was used to compare survival rate. The relationships between MOS levels supplemented in the diets and growth data were tested by Pearson correlation ($r$). All statistical analysis were performed in SPSS 18 (IBM, Chicago, IL).

## 3. Results

### 3.1. Growth performance of clownfish fed different diets

The length of fish at the start of the experiment was uniform among the treatments [(24.21 ± 0.16) mm]. There was no significant difference in average length of fish between treatments at commencement, or at Days 14 and 42 (ANOVA, $P \geqslant 0.621$). However, significant differences of fish length among diets were detected at the end of the trial (Day 70).

At the end of the experiment (Day 70), fish fed with MOS-supplemented diets out-performed those fish fed on the basal diet. Mean length ranged from 28.69 mm to 30.53 mm. The greatest increase in length was in the group of fish fed 0.15% MOS [(30.53 ± 0.80) mm], followed by fish fed diet 0.10% MOS [(30.16 ± 0.09) mm] with no significant difference ($P = 0.320$). The lengths of fish in those two diet treatments were significantly higher than those fish fed the control diet and other MOS-supplemented diets ($P \leqslant 0.001$). The lowest length increment was in the group of fish fed the control diet [(28.69 ± 0.50) mm], while the mean lengths were 29.20 mm, 29.44 mm, 29.21 mm and 29.16 mm in the groups of fish fed diets with 0.05%, 0.20%, 0.25 and 0.30% MOS, respectively. There was no significant difference in the mean lengths of those groups ($P \geqslant$

0.18) (Figure 1).

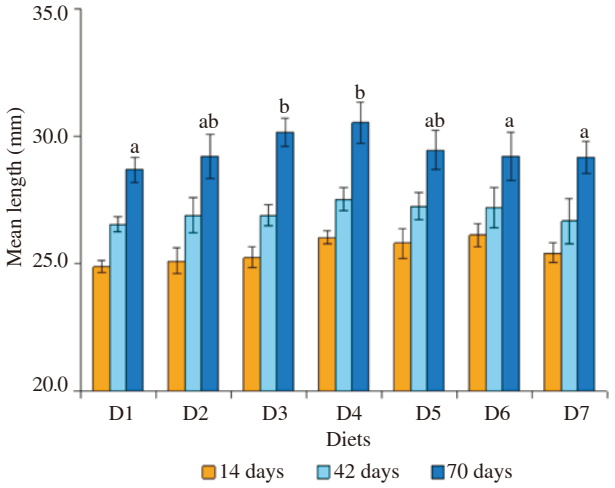


**Figure 1.** Average DGC (%/day) (± SEM) of clownfish fed different concentrations of MOS.
D1: Control; D2: 0.05% MOS; D3: 0.10% MOS; D4: 0.15% MOS; D5: 0.20% MOS; D6: 0.25% MOS; D7: 0.30% MOS. Different letters indicate significant differences between treatments.

At Day 70, DGC was highest in the fish fed 0.15% MOS (DGC = 0.34%/day), followed by the group of fish fed 0.10% MOS in the diet (DGC = 0.32%/day), but there was insignificant difference ($P = 0.24$). The DGCs of those two groups were significantly higher than fish fed the control diet and those groups of fish fed other concentrations of MOS ($P \leqslant 0.03$). There was no significant difference ($P \geqslant 1.27$) in DGCs of fish fed 0.05%, 0.20%–0.30% of MOS inclusions or the control diet.

### 3.2. Survival rate

At the end of the experiment (Day 70), survival rates of fish ranged from 90.00% to 98.33% (Figure 2). The highest survival rates were for fish fed diets 0.20% and 0.25% MOS which were both 98.33%. The survival rates of those two groups of fish were significantly higher than those fish fed the control and 0.3% MOS inclusion diets ($P \leqslant 0.04$). The lowest survival rates were for fish fed control and 0.3% MOS, which were 90.00% and 90.95%, respectively, and with no significant difference ($P = 0.81$). Survival rate of fish fed 0.05% and 0.25% MOS both was 96.67%.

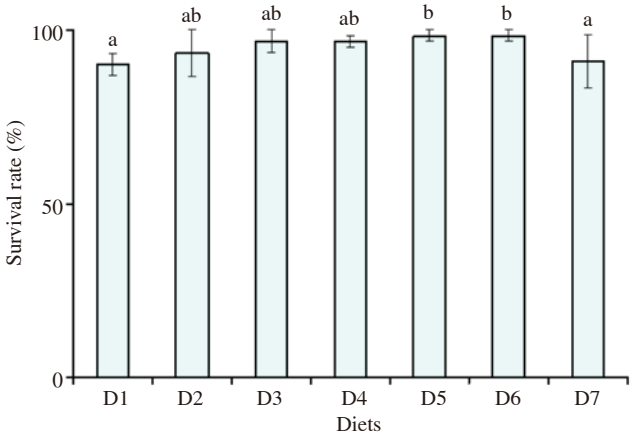


**Figure 2.** Survival rate of clownfish fed different levels of MOS (0%–0.3%).
D1: Control; D2: 0.05% MOS; D3: 0.10% MOS; D4: 0.15% MOS; D5: 0.20% MOS; D6: 0.25% MOS; D7: 0.30% MOS. Different letters indicate significant differences between treatments.

### 3.3. Protein content and $K_s$ (%/day)

At Day 0, protein content in muscle of clownfish was uniform among the treatments (12.33% ± 0.01%) without significant difference (ANOVA, $P \geqslant 0.371$). At Day 70, our results revealed that protein content in the muscle of clownfish ranged from 13.15% in the group fed the control diet to 16.25% in the group fed diet 0.2% MOS. There was no significant difference in protein content of fish fed the different diets ($P \geqslant 0.05$).

$K_s$ of fish fed diets 0.15%, 0.20% and 0.25% MOS were highest, and there was no significant difference between them ($P = 0.645$). The $K_s$ of those three groups of fish was significantly higher than those fish fed the control diet, diets 0.05% and 0.10% MOS ($P \leqslant 0.038$). The lowest $K_s$ were in groups of fish fed control diet, diets supplemented with 0.05% and 0.10% MOS, with no significant difference between them ($P = 0.231$) (Figure 3).

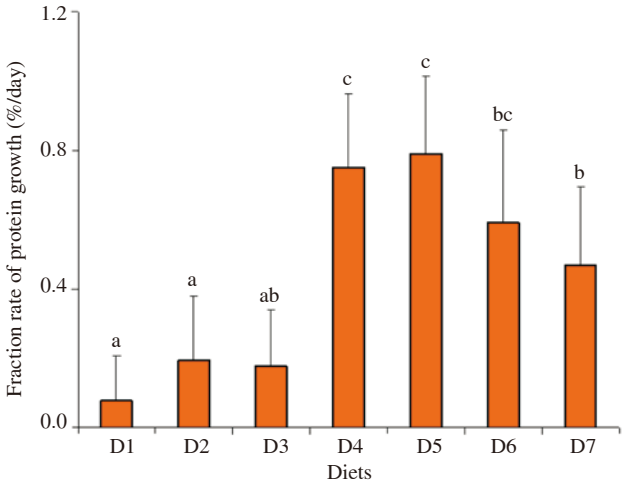


**Figure 3.** Fraction rate of protein growth.
D1: Control; D2: 0.05% MOS; D3: 0.10% MOS; D4: 0.15% MOS; D5: 0.20% MOS; D6: 0.25% MOS; D7: 0.30% MOS. Different letters indicate significant differences between treatments.

### 3.4. Relationship between MOS levels and growth of fish

Pearson correlation analysis showed that dietary MOS concentrations were significant positive correlations to growth of clownfish ($P \leqslant 0.017$). Among growth performance data, MOS levels showed higher correlations with the mean total length of fish ($P = 0.001$) (Table 1).

**Table 1**
Pearson correlation ($r$) matrix amongst dietary MOS inclusion levels (%) and growth rate of clownfish at Days 14, 42 and 70.

| Time | | L (mm) | W (g) | LG (mm/week) | DGC (%/day) |
|---|---|---|---|---|---|
| Day 14 | $r$ | 0.311** | 0.179* | 0.213** | 0.201** |
| | Significance | 0.000 | 0.013 | 0.003 | 0.005 |
| Day 42 | $r$ | 0.379** | 0.104* | 0.189* | 0.172* |
| | Significance | 0.000 | 0.017 | 0.012 | 0.015 |
| Day 70 | $r$ | 0.288** | 0.271** | 0.389** | 0.179* |
| | Significance | 0.000 | 0.000 | 0.000 | 0.011 |

*: Correlation is significant at the 0.05 level (2-tailed); **: Correlation is significant at the 0.01 level (2-tailed). L: Length of fish; W: Weight of fish.

### 3.5. Predicting optimal level of MOS requirement for maximal growth of clownfish

When dose response was analyzed by a compartmental model[17], growth at Day 70 and MOS-suplemnted levels positively correlated ($R^2$ = 61.19%). The predicted optimal levels of MOS supplementation in the diet reduced as the weight of fish increased. The predicted optimal MOS requirement for maximal growth of clownfish was 0.185%, 0.173% and 0.171% at Days 14, 42 and 70, respectively (Figure 4, Table 2).

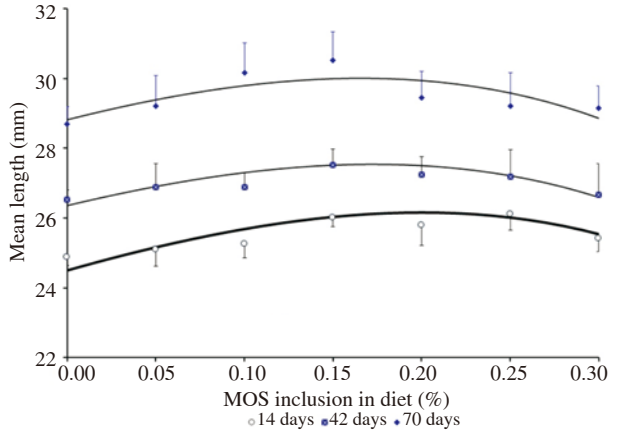


**Figure 4.** Estimation of optimal MOS concentration in the clownfish diet for maximal growth (mean total length) of clownfish, *A. ocellaris* using a compartmental model.

**Table 2**
Nutritional requirement of MOS based on mean length over time for clownfish, *A. ocellaris* fed diet supplemented with MOS.

| Time | Maximal MOS requirement | Goodness of fit ($R^2$) | *a* | *b* | *c* | *d* | SSE |
|---|---|---|---|---|---|---|---|
| 14 days | 0.185 | 65.10 | 139.225 | -1.900 | -0.280 | 0.691 | 1.225 |
| 42 days | 0.173 | 81.20 | 54.814 | -1.482 | -0.943 | 0.695 | 0.279 |
| 70 days | 0.171 | 61.19 | 61.144 | -1.481 | -0.923 | 0.690 | 0.952 |

It is determined by compartmental model $y = a \times e^{-bx}(1 - e^{-c(x-d)})$. *y* is mean length (mm), *x* is concentrations of MOS in diets (%), *b* is the intercept, *a* is the theoretical maximum, *c* is the nutrient rate constant, and *d* is the kinetic order of the response when $x = 0$. SSE: Sum of squared errors.

## 4. Discussion

This study emphasized the benefit of dietary MOS to growth and survival of clownfish, *A. ocellaris*. Growth and survival of fingerling fish were improved when fed diets with 0.10%–0.15% MOS inclusion. This was in agreement with other studies that reported the important role of MOS on boosting growth and survival of aquatic species[4,10,18-20]. In contrast, dietary MOS supplementation did not improve growth in sturgeon, *Acipenser oxyrinchus desotoi*[21] or in common carp, *Cyprinus carpio*[22]. The benefit of MOS inclusion may be species-specific.

There is no significant difference in the protein content of clownfish fed different concentration of MOS. Our results are similar to Do Huu and Jones[4] findings that protein content in lobster fed control and MOS-supplemented diets did not differ significantly, and similar body composition did not change in Asian seabass (*Lates calcarifer*) fed MOS[23]. However, this contradicts to the study that showed the protein content in sea bass (*Dicentrarchus labrax*) increased with the increased levels of dietary MOS[18]. Although our study showed protein content of clownfish did not differ between the treatments, $K_s$ of fish fed diets containing 0.15%, 0.20% and 0.25% MOS were highest. $K_s$ values of those fish were significantly different in comparison to fish fed control and fish fed 0.05% and 0.10% MOS-supplemented diet. Therefore, $K_s$ might be a better indicator to evaluate protein utilization in clownfish with respect to dietary MOS supplementation. Prebiotic inclusion in the diet significantly improved protein utilization in striped catfish, *Pangasianodon hypophthalmus*[19]. Similar inclusion of MOS in the diet enhances utilization of essential fatty acid in gilthead sea bream, *Sparus aurata*[10]. Similarly, the feed conversion ratio and protein efficiency ratio were significantly higher in *Macrobrachium rosenbergii* fed Agrimos[24].

Determination of optimal nutrient requirement plays a vital role in aquaculture. Providing a nutrient at levels which are higher or lower than required may result in adverse effects[4,13]. Most studies on benefits of MOS have focused on one or two 'recommended' inclusion concentration, rather than examining the inclusion rates directly. There are some studies which investigate a range level of MOS in the diet such as 0%–0.3% in carp (*Cyprinus carpio*)[25] and 0%–0.8% MOS in the diet of shrimp *Litopenaeus vannamei*[26], however, they did not determine an optimal level of MOS in the diet. The only prediction of MOS requirement for maximal growth was done on lobster, *Panulirus homarus*[4].

There were eight models of nutrient requirement used in this study in which the model that has the highest goodness of fit ($R^2$) was selected to estimate the optimal level of MOS required for growth. According to Ryan[27], the goodness of fit ($R^2$) is the main element to evaluate the corelations between the model and the effect. In the present study, the use of numerous nutritional models is very similar to previous research on crustaceans[4,13]. Similar to the finding in this study, both the research on crustaceans also found a negative effect on growth when nutrient supplements were out of the optimal dose. The optimal MOS inclusion diminishes as size increases for *Panulirus homarus* lobster[4]. As size influences the dietary MOS requirement on other species, further testing on different sizes of clownfish should be conducted experimentally.

The feed trial reported here has confirmed that the inclusion of MOS in diets promotes high growth, survival rates of clownfish, *A. ocellaris*. The optimal level of dietary MOS for maximal growth of this species was also determined, indicating that MOS at 1.0 to 1.5 g/kg is recommended. Further research is needed to determine MOS concentration to optimize growth of clownfish exposed to various environmental conditions and stressors. It is also recommended that the effects of dietary MOS on different life stages should be examined.

## Conflict of interest statement

We declare that we have no conflict of interest.

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