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Prebiotic Evaluation of Copra-Derived Mannooligosaccharides in White-Leg Shrimps

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Abstract

β-Mannooligosaccharides, produced by partial enzymatic hydrolysis of copra pulp residues using recombinant *A. niger* β-mannanase, were evaluated for its potential use as a prebiotic feed supplement for *L. vannamei* culture. Results observed in a 30-day feeding trial showed that dietary supplementation of copra-derived MOS, with doses varying from 4 to 10 g kg^{-1} of dried feed, increased the number of intestinal *Lactobacilli* and *Bifidobacteria* in shrimps fed copra-MOS supplemented diets increased approximately from 150-300%. The number of intestinal presumptive *Vibrio, Coliforms, Clostridia* and *Salmonella* reduced to 39.6-54.1%, 56.9-65.9%, 71.8-86.1% and 100-100%, respectively. In addition, copra-MOS supplement in the feed led to an enhancement of the weight gain, specific growth rate, feed conversion ratio and feed intake of shrimps (P<0.05). After a 7-day challenging test with *Vibrio harveyi* pathogen (~10^6 CFU mL^{-1}) by immersion, the cumulative mortality of the shrimps fed with the diet supplemented 10 g kg^{-1} copra-MOS was 3.5%, that obviously decreased compared to the control shrimps (29.5% mortality). Taking advantage of its intestinal microflora modulation towards increasing probiotic-like bacteria and *Vibriosis* resistance, this cheap oligosaccharide will be valuable in various aquatic animal farming.

Keywords: Copra; Mannooligosaccharides; Prebiotics; *Litopenaeus vannamei*; Growth; Intestine bacteria; Disease resistance

Introduction

Whiteleg shrimp, *Litopenaeus vannamei*, is among high commercial value aquatic species being cultured recently worldwide [1]. However, because of the rapid development, coupled with unusual climate change, the shrimp farming has severely suffered significant economic losses due to viruses and vibriosis diseases [2-4]. Traditional uses of antibiotics for shrimp disease control have been criticized due to the potential development of multi-antibiotic resistance of pathogenic bacteria, reduction in the efficacy of antibiotic treatments, residue accumulation in tissues, and immunosuppression [5-9]. Therefore, using probiotics, prebiotics, and medicinal plants as preventive, environment-friendly and economically efficient alternatives to antibiotic in shrimp farming has been received heightened attention in recent years [10-13].

"Prebiotics are defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth of and/or activating the metabolism of one or a limited number of health-promoting bacteria in the intestinal tract, and thus improve host health" [14]. It have been reported that fructooligosaccharides, inulin, and isomaltooligosaccharides showed the prebiotic characteristic in whiteleg shrimp [15-18].

MOS is basically constituted by linear chains of mannose. Several commercial products of MOS were in trade with various purities and they differ in their molecular structure, depending on the source and processing conditions. The MOS recently used to cultivate shrimps was extracted from yeast cell-wall and it consists of the chain of mannose residues linked together via α-1, 6-glycosidic bond [12,19,20]. Another kind of MOS is β-1, 4-mannooligosaccharides (β-MOS) produced by enzymatic hydrolysis of plant mannans via β-mannanases [21]. This β-MOS is generally purer and shows excellent prebiotic efficacy on humans and terrestrial animals at low doses. However, as for aquatic animals, there is little known about the effectiveness of any β-mannooligosaccharides at present.

Defatted copra meal's carbohydrates is abundant agricultural waste which is discharged by coconut industries. Balasubrtamaniam [22] found that content of copra meal was 23% of mannans and 61% galactomannans. This material is very popular in Vietnam to produce MOS [21].

The aim of this study was to evaluate the effects of copra MOS on growth, survival, intestinal microflora, and bacterial pathogen resistance of whiteleg shrimp.

Materials and Methods

Copra MOS and diet preparation

The prebiotic used in this study, mannooligosaccharides, was produced by partial enzymatic hydrolysis of copra pulp residues obtained from coconut oil processing facilities using a recombinant β-mannanase (from *Aspergillus niger* BK01) [21]. The minimum level of mannooligosaccharides was 90% ww^{-1}. The copra MOS powder was free of antibiotics (tetracycline, chloramphenicol, and chlortetracycline), aflatoxin B1 and bacterial pathogens (*Vibrio cholera, Vibrio parahaemolyticus, Salmonella, E. coli*).

Five diets (control, MOS4, MOS6, MOS8 and MOS10) were formulated with supplementation of various amounts of copra MOS (0, 4, 6, 8, or 10 g) in 1 kg of dried feed. Copra MOS supplied in the powder form was diluted in an adhesive and feed attractant oil (squid liver oil,

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Kaikoh, Japan, 30% omega 3 acid), and then sprayed onto commercial feed pellets (Hipo 1-4 No, CP Vietnam, Vietnam, 42% protein) in all treatments (including control group). Feed, prepared for 10 days, was dried at room temperature for 4 h and then stored at 4°C.

Feeding trials

Juvenile white-leg shrimp (*Litopenaeus vannamei*) was supplied by UNI-President Company (Ninh Thuan, Vietnam) and shipped to the farm of NACMAN (Hai Thanh, Duong Kinh, Haiphong, Vietnam) in a plastic container (250 L) provided with seawater and aeration. The collected shrimp was passed the tests of WSSV, TSV, IHHNV, YHV, and MBV and had no sign of bacterial infections.

Prior to the start of the feeding trials, experimental shrimps were fed with control diet for 30 days to acclimate the experimental diet and condition. After the acclimation period, the experimental design was completely randomized with five treatments. Each diet was randomly assigned to duplicate tanks of shrimp, and each 3 m^3 composite tank (2 m^3 water capacity) was stocked with 200 shrimps per tank (density 100 individual m^{-3}). The mean weight of shrimps used in the experiments was 1.65 ± 0.01 g. Shrimps were fed four times daily at 06:00, 11:00, 16:00, and 21:00 h at about 6-10% body weight, and feeding ration was regulated according to the feed consumption.

Every seven days in the feeding experiment, 10 shrimps per tank were sampled after 3 h feeding to measure the weight of shrimp on a digital balance (Mettler Toledo, AT 200, Greifensee, Switzerland) and to obtain their intestines for the determine of bacterial counts. The cumulative mortality of shrimp during the whole experimental period was recorded.

During the experimental period of feeding experiment (from the start to 30^{th} day), water temperature and water quality parameters were monitored: temperature, 28 ± 2°C, salinity 22 ± 2‰, pH 7.9 ± 0.3, dissolved oxygen, >5 mg L^{-1}. The photoperiod was 12 h light and 12 h dark.

Bacterial quantification

The intestines of ten shrimps collected from each tank were homogenized in sterilized peptone salt buffer (0.1% wv^{-1} casein peptone and 0.85% wv^{-1} sodium chloride) with the ratio of 1:1 (wv^{-1}). Bacterial quantifications for homogenized liquid were made using serial 10 fold dilution in the buffer, followed by plating on the medium agar. Seven types of bacteriological media were used in total plate counts determinations. Among them, tryptone glucose agar (TGA) was used to enumerate the total aerotrophic heterotrophic bacteria which were expressed as total viable bacterial counts (TBC), while thiosulphate citrate bile-salt sucrose (TCBS) agar, Man Rogosa Sharpe (MRS) agar, BDTM Bifidobacterium agar, violet red bile lactose (VRBL) agar and tryptone sulfite cycloserine (TSC) agar, Bismuth Sulphite Agar (BSA) were used to determine selectively the presumptive *Vibrio, Lactobacilli, Bifidobacteria, Coliforms, Clostridium perfringens*, and *Salmonella* counts respectively. After 48 h of incubation at 30°C for total aerotrophic heterotrophic bacteria, *Vibrio* and 37°C for the rest, the number of bacterial colonies was counted and the amount of bacteria was calculated as colony forming unit (CFU). Bacterial counts (BC) were calculated by the formula:

BC (CFU per gram of shrimp intestine)=Number of colonies (CFU)/(Dilution×Weight of shrimp intestine)

Challenge test

A bacterial pathogen, *Vibrio harveyi* associated with whiteleg shrimp's vibriosis disease was obtained from Institute of Biotechnology, Vietnamese Academy of Science and Technology. *V. harveyi* was grown in BOSS broth for 24 h at 30°C. Two hundred whiteleg shrimps (mean weight of 1.65 ± 0.01 g) from each duplicate were immersed in infected seawater (containing final bacteria concentration at 10^6 CFU/ml) for 2 h with a constant aeration in an 8 L plastic container. Then the infected culture was transferred into a 3 m^3-composite tank (2 m^3 water capacity). Shrimps were cultured in conditions described in the feeding trials. The cumulative mortality was calculated for 7 days.

Calculations and statistical analysis

Growth rate was calculated and expressed specific growth rate (SGR) and weight gain (WG) according to the following equation:

$SGR\ (\%\ day^{-1})=100\times(\ln W_e-\ln W_s)/d$, $WG\ (\%)=100\times(W_e-W_s)/W_s$

where W_s and W_e are the weights of the shrimps at the start and end of the growth period, respectively, and d is the number of days, in the growth period.

Feed conversion ratio (FCR)=diet consumed (g)/(W_e-W_s)

Feed intake (FI, g day^{-1})=diet consumed (g)/d/shrimp number

The survival rate in each tank was measured using the following formula:

survival rate (%): SR=100×(n_t/n_o)

where SR is the survival rate; n_t is the number of shrimps at the time t and n_o is the number of shrimp at the commencement.

Cumulative mortality was calculated according to the following equation:

cumulative mortality (%)=100×(n_d/n_o)

where n_d is the number of dead shrimps in the challenge period and no is the number of shrimp at the commencement.

Data were subjected to one-way ANOVA in SPSS 18 (IBM, USA). When appropriate, the Duncan's multiple-range test ($P<0.05$) was applied to evaluate the differences between the means. Data in tables were presented as means ± standard deviation of five tanks.

Results

Bacterial counts in shrimp intestines

Beneficial and harmful bacteria counts were significantly affected by both supplemented-MOS doses ($P<0.05$; Table 1 and Figure 1). The intestinal presumptive *Vibrio* counts (VBC) significantly showed the decreasing tendency with the increasing administration doses of MOS in diets. While the differences in *Lactobacilli, Bifidobacteria, Coliforms* and *Clostridia* counts among shrimp groups showed dissimilar trends to that in VBC. When MOS supplementation was higher than an appropriate level (depending on the group of bacteria in shrimp), the increasing/decreasing trend of these beneficial/harmful bacteria counts was inverted. The maximums of *Lactobacilli* and *Bifidobacteria* counts and the minimums of *Coliforms* and *Clostridia* counts were detected in the shrimp fed with 6 gkg^{-1} MOS-supplemented diet. Interestingly, intestinal *Salmonella* did not present in shrimps fed MOS-supplemented diet at any dose in whole experimental period, while these bacteria appeared in the gut of control shrimps after 1 week of cultivation (data not shown) and then reached cell number up to 80 $CFUg^{-1}$ (at the end of feeding trial).

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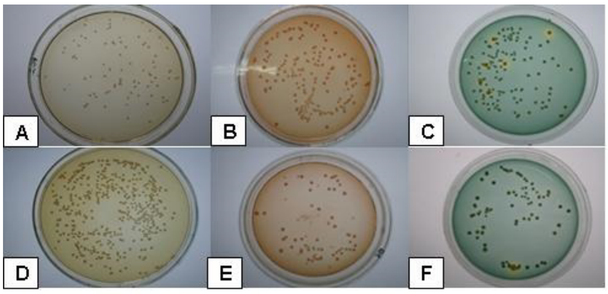
Bacteria	*Lactobacilli*	*Bifidobacteria*	*Vibrios*	*Coliforms*	*Clostridia*	*Salmonela*
Control	9.40 ± 0.14^{a}	4.30 ± 0.28^{a}	359.5 ± 3.54^{e}	36.25 ± 4.60^{b}	6.25 ± 0.07^{d}	0.8 ± 0.01
MOS4	13.85 ± 0.21^{b}	6.45 ± 0.35^{b}	217.0 ± 2.83^{d}	15.40 ± 1.70^{a}	1.76 ± 0.08^{c}	0 ± 0.00
MOS6	27.70 ± 0.42^{e}	15.65 ± 0.49^{e}	202.0 ± 2.83^{c}	12.35 ± 0.92^{a}	0.87 ± 0.05^{a}	0 ± 0.00
MOS8	23.40 ± 0.28^{d}	12.58 ± 0.46^{d}	183.0 ± 4.24^{b}	13.90 ± 1.84^{a}	1.35 ± 0.07^{b}	0 ± 0.00
MOS10	19.00 ± 0.28^{c}	8.35 ± 0.35^{c}	165.0 ± 2.83^{a}	15.60 ± 6.15^{a}	1.58 ± 0.03^{c}	0 ± 0.00
P (df, F)	<0.05 (4, 1009.13)	<0.05 (4, 197.26)	<0.05 (4, 807.44)	<0.05 (4, 25.70)	<0.05 (4, 798.63)	<0.05 (4, 5.32)

(*) Data (mean ± SD) in the same column not sharing a common superscript was significantly different (Duncan's multiple-range test, P<0.05).

Table 1: The bacterial counts in the intestine of *Litopenaeus vannamei* fed with copra MOS-supplemented diets (10^4 $CFUg^{-1}$) (*).



A, B, C: colonies of Lactobacillus, Coliforms, Vibrio in control shrimp intestine; D, E, F: colonies of Lactobacillus, Coliforms, Vibrio in shrimp intestine fed with 8 gkg^{-1} copra-MOS-supplemented diets for 4 weeks.

Figure 1: Colonies of intestine bacteria.

Parameters	WG (%)	SGR (%/d)	FCR	FI (g/d)	SR (%)
Control	135.43 ± 0.82^{a}	2.99 ± 0.01^{a}	1.46 ± 0.01^{d}	3.48 ± 0.04^{a}	93.5 ± 0.71
MOS4	238.11 ± 0.76^{b}	4.06 ± 0.01^{b}	1.17 ± 0.01^{c}	4.55 ± 0.00^{d}	96.2 ± 0.28
MOS6	239.04 ± 3.79^{b}	4.07 ± 0.04^{b}	1.11 ± 0.01^{b}	4.35 ± 0.03^{c}	96.4 ± 0.42
MOS8	240.87 ± 3.80^{b}	4.09 ± 0.04^{b}	1.08 ± 0.02^{a}	4.25 ± 0.05^{b}	99.2 ± 0.28
MOS10	248.49 ± 3.44^{c}	4.16 ± 0.03^{c}	1.03 ± 0.01^{a}	4.20 ± 0.04^{b}	98.8 ± 0.07

(*) Data (mean ± SD) in the same column not sharing a common superscript was significantly different (Duncan's multiple-range test, P<0.05)

Table 2: Growth, feed utilization and survival of whiteleg shrimp fed with copra MOS-supplemented diets for 30 days (*).

Growth performance and fees utilization

Whiteleg shrimps fed the diets supplemented with copra MOS demonstrated better growth performance and feed utilization during 30-day feeding trial (Table 2 and Figure 2). Shrimps fed all levels of experimental MOS had significantly higher (P<0.05) weight gain, specific growth rate, feed conversion ratio and feed intake values than shrimps fed the basal diet did. However there were no differences among growth performance parameters (WG, SGR) of shrimps fed 0.4, 0.6 and 0.8% of MOS. While feed utilization values (FCR, FI) did not significantly (P>0.05) differ between the diets supplemented with 0.8 and 1.0% of copra MOS, although shrimps fed higher MOS level had better numerical values. Survivals of shrimps during the 30-day trial were high (93-99%) in all treatments.

Mortality of the shrimps challenged with *Vibrio harveyi*

Cumulative mortality of shrimps shows the risk of this animal population dying from the disease during an experimental period. In the 7-day challenge test with *Vibrio harveyi* pathogen, mortality of shrimps fed 1% copra MOS supplemented diet (experimental group) decreased compared to shrimps fed basal diet (control group). The cumulative mortalities of experimental group and control group were 3.5 ± 2.1% and 29.5 ± 4.2%, respectively (Table 3). The numbers of Vibrio in the shits of experimental and control groups were 200 ± 46 ($CFUg^{-1}$) and 250,000 ± 94,328 (10^3 $CFUg^{-1}$), respectively.

Discussion

Results from the present study clearly indicate that the dietary supplementation of copra MOS exerted positive effect on growth performance and feed utilization of whiteleg shrimps, with shrimps fed 10 gkg^{-1} MOS-supplemented diet exhibiting the highest weigh gain and specific growth rate (P<0.05) in all experimental groups. The improved growth observed in this study was consistent with that reported for shrimps fed diet supplemented other prebiotics including MOS extracted from cell wall of yeast [18]. However, the copra mannooligosaccharides' growth-promoter effect distinguishes from previous studied prebiotics (fructooligosacchrides, isomaltooligosaccharides) [15,16], because in this study, the copra MOS led to an enhancement of weight gain and specific growth rate as well as survival obviously in the conditions in which shrimps have excellent growth and survival rate. While both Li et al. [16] and Luna-González et al. [17] found no significant increase in weight and survival of *L. vannamei* fed with fructans (scFOS, inulin) dose varying from 0.025 to 1.00% of diet in the similar high growth conditions. Li et al. [15] found dietary isomaltooligosaccharides (IMO) alone had no obvious effect on survival of shrimp as IMO dose increased from 0 to 0.2% [16]. In addition, copra MOS supplementation up to 1% ww^{-1} did not show any inhibition in growth, survival and feed efficiency of shrimps.

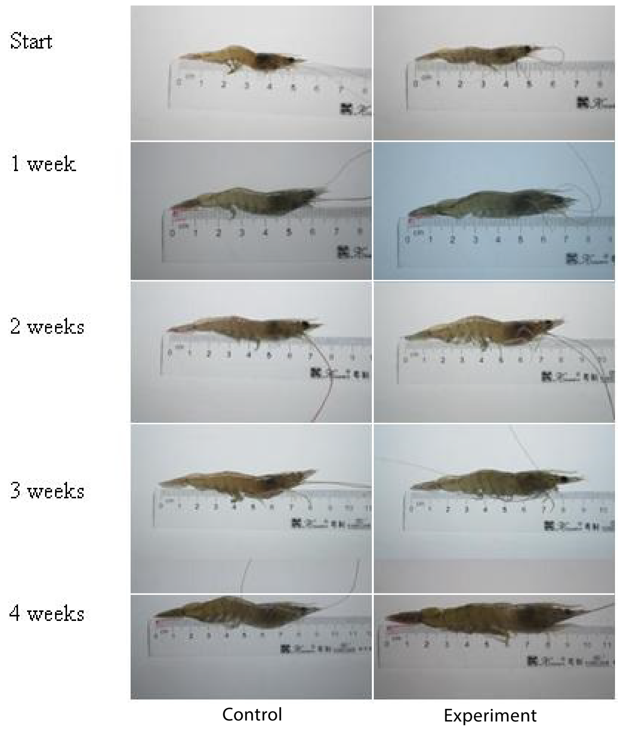


Figure 2: Effect of copra MOS-supplementation to growth of white leg shrimps.

Experiments	Cumulative mortality (%)	*Vibrio* in shrimp shit	
		Initial cell number (CFU g^{-1})	Final cell number (CFU g^{-1})
Control	29.5 ± 4.2	30,000	250,000,000 ± 94,328,000
Copra MOS, 1%	3.5 ± 2.1	30,000	200 ± 46

(*)Data expressed in means ± standard deviation, means followed by the same letters are not significantly different according to Duncan's multiple-range test, P<0.05

Table 3: Effect of copra-derived MOS on the survival rate (%) of shrimp (*Litopenaeus vannamei*) challenged with *Vibrio harveyi* 7 days after infection (*).

Showing quantitative changes in major groups of intestinal bacteria in whiteleg shrimp due to copra MOS supplementation, our results can contribute in the knowlegde of β-MOS in general, its prebiotic activity and application potential on aquatic animals *in vivo*. Copra MOS supplementation in range of concentration from 4 to 10 gkg^{-1} in diet showed an impressive capacity of improving beneficially intestinal bacteria population in the *Litopenaeus vannamei*. The intestinal *Lactobacilli* and *Bifidobacteria* counts were higher approximately from 1.5 to 3.0 times compared to those in the control shrimps. While fructans including FOS and inulin showed the low *Lactobacilligenic* effect in whiteleg shrimp [23]. Yeast-derived MOS supplementation only increased slightly gut *Bifidobacteria* in carp [19] and cobia larvae [24]. Judged from these data, MOS produced during the process of the controlled enzymatic hydrolysis of copra by endomannanase might be a good Bifidus factor in aquatic animals. However, it is important to note that copra-MOS supplementations at higher concentrations of 8 gkg^{-1} improved the lactic acid bacteria lower than 6 gkg^{-1} copra-MOS supplementation did. Maybe excessive proliferation of a or some lactic acid bacteria species selectively stimulated by copra-MOS might negatively impact the counts of Bifidobacteria and other lactic bacteria in shrimp guts that the mechanism needs to be investigated.

In this study, copra-MOS supplementations also effectively decreased the counts of potentially harmful intestinal bacteria. Comparing to control shrimps, the VBC, Coliforms, *Clostridia* and *Salmonella* counts in shrimps fed with copra-MOS-supplemented diets were lower with the percentage of 39.6-54.1%, 56.9-65.9%, 71.8-86.1% and 100-100%, respectively. The results of VBC reduction in this study agreed with those in some prebiotic tests on aquatic animals [18,23]. However, in the work of Zhou et al. [23] there was only 1.23% reduction of presumptive intestinal Vibrio count in Pacific white shrimp fed with 1.6 g ScFOS. Zhang et al. [18] also found slight reduction of VBC in sea cucumbers with the FOS-supplemented concentrations. Because of the lower impact of *Clostridia* and *Salmonella* on shrimp-disease breakout, data on effects of MOS and prebiotics on these harmful bacteria in shrimps so far has not received much attention. The intestinal *Clostridia* reduction means the limitation to decline the animal's digestive enzymes [25]. Thus copra MOS could be able to enhance the digestion of shrimps. *Salmonella* was a potential pathogen in human and invertebrates, so *Salmonella* reduction capacity of dietary copra MOS *in vivo* is worth to ensure safety of seafood. Results in this study showed that copra MOS supplementation in diet could be a feasible way to reduce bacterial infections in *Litopenaeus vannamei*.

Some studies have reported prebiotic oligosaccharides effectively enhanced the animal's immune system and improved the resistance to pathogenic bacteria disease [15,16,26]. Dietary copra MOS was also found to reduce strongly cumulative mortality of whiteleg shrimp in the present study. The increase in resistance against *Vibrio harveyi* in shrimp fed copra MOS-supplemented diet can be possibly explained on the basis of stimulation of probiotic-like bacteria.

In conclusions, this is the first determination on the impact of mannooligosaccharides derived from copra on whiteleg shrimp's health. Supplementation of 4 to 10 gkg^{-1} copra MOS in diet can significantly improve growth, feed conversion, modulating intestinal microflora and enhance the resistance against *Vibrios*, *E. coli* and *Salmonella* of whiteleg shrimp. This kind of prebiotic will gain a great deal of interest for aquaculture applications in large scale due to an increasing demand towards food safety and sustainable development.

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