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Molecular effects of mannanase-hydrolyzed coprameal to intestinal immunity in the Pacific white shrimp (*Litopenaeus vannamei*)

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Running title: Shrimp immunity enhanced by mannanase-hydrolyzed copra meal

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33 **Abstract**

34 To mitigate disease outbreak, an alternative approach through enhancing shrimp
35 immunity was explored. Mannan oligosaccharides (MOS) have been previously
36 reported to enhance shrimp immune system. Here, coprameal samples were digested
37 with mannanase to yield MOS, namely, mannanase-hydrolyzed coprameal (MCM) and
38 feasibility of MCM as shrimp immunostimulant in grow-out ponds was determined.
39 Pacific white shrimp (*Litopenaeus vannamei*) were fed with the commercial diet
40 containing 1% MCM as the MCM-supplemented group and compared to the non-MCM
41 control diet. There was no significant difference in survival rates between the MCM-
42 supplemented and the control groups throughout the 4-month-period of the trial ($p >$
43 0.05). Gene expression analysis in shrimp intestines revealed that the transcript levels of
44 antimicrobial peptides (*anti-lipopolysaccharide factor isoform 1* (*alf1*), *penaeidin*
45 *(pen3a)* and *crustin (crus)*) and lysozymes *(lyz)* were not significantly different in the
46 MCM-supplemented group. Meanwhile, *C-type lectin* and *toll-like receptor* transcript
47 levels, whose gene products play roles as pattern recognition proteins, were
48 significantly higher in a group fed with MCM for 2- and 4-month periods than those of
49 the control group ($p < 0.05$). The increased transcript levels of *C-type lectin* and *toll-like*
50 *receptor* provide evidence for potential implementation of MCM as feed supplement to
51 modulate shrimp immune system.

52

53 Keywords: copra meal; mannan oligosaccharides; Pacific white shrimp;
54 immunostimulant

55

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56 **Introduction**

57 Aquaculture production play important role to provide food sources for
58 increasing global population. The global demand for shrimp and prawn production is
59 approximately 70% of crustaceans (FAO 2018). This results in the rapid growth of
60 cultivated shrimp production, in particularly, an expanding of Pacific white shrimp
61 farming throughout many Asian countries. However, the shrimp industry continues to
62 suffer from production losses due to the higher disease outbreak incidents. To mitigate
63 shrimp diseases, much attention has been paid to development of feed additives with
64 potential to enhance growth and immune performance as a mean to prevent disease
65 outbreaks in aquaculture.

66 In mammalians, prebiotics, which are non-digestible carbohydrates, are shown
67 to selectively stimulate growth of beneficial bacteria in host digestive systems, and
68 consequently, providing health benefits to their host animals (Al-Sheraji et al. 2013;
69 Davani-Davari et al. 2019; Manning & Gibson 2004). Recently, prebiotics as
70 immunostimulants have been applied in aquaculture as a promising approach for growth
71 and immune enhancement (Hasan et al. 2019; Luna-González et al. 2012; Sang et al.
72 2010; Staykov et al. 2007). Among those, mannan oligosaccharides (MOS), derived
73 from yeast cell wall (*Saccharomyces cerevisiae*), have been shown to improve growth
74 performance and enhance immune system of many aquatic animals (Dimitroglou et al.
75 2010; Salem et al. 2015; Zhang et al. 2012; Zhang et al. 2020). In crustaceans,
76 applications of MOS as feed additives has been reported to significantly improve the
77 survival rate of crayfish (*Cherax tenuimanus*) (Sang et al. 2010), and enhance growth
78 rates in tropic spiny lobster (*Panulirus ornatus*) (Do Huu & Jones 2014) and black tiger
79 shrimp (*Penaeus monodon)* (Sang et al. 2014; Sang & Fotedar 2010)and freshwater
80 crayfish (*Astacus leptodactylus*) **(**Mazlum et al. **2011).** The dietary MOS from yeast can
81 significantly increase intestinal microvilli length, growth and percent survival rates in
82 the Pacific white shrimp (Gainza & Romero 2020; Zhang et al. 2012)

83 While MOS used in animal feed additives are mostly derived from yeast, MOS
84 can also be obtained from other natural sources with high mannan sugar contents such
85 as copra meal (Hossain et al. 1996; Kraikaew et al. 2020; Pangsri et al. 2015). Copra
86 meal is a by-product from the coconut milk industry, and it is an alternative source for
87 MOS in a form of β-1, 4 mannooligosaccharides (Ariandi et al. 2015). The potential

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88 applications of MOS from copra meal as immunostimulants have been explored in
89 various animals such as broilers (Prayoonthien et al. 2017; Sundu et al. 2012) and the
90 Pacific white shrimp (Chen et al. 2020; Li et al. 2018; Rungrassamee et al. 2014).
91 Particularly, MOS from copra meal has been reported to improve immune levels and
92 survival rates in the Pacific white shrimp juvenile under a pathogen exposure to *Vibrio*
93 *harveyi* (Rungrassamee et al. 2014), providing a piece of evidence for MOS as a
94 promising shrimp feed additive. Here, we further explored feasibility of MOS through
95 mannanase-hydrolysis of copra meal (MCM) as feed additives in the grow-out pond
96 systems and determined molecular effects on immune gene expression. These findings
97 will lead to further development of MCM (MOS from copra meal) as effective
98 prebiotics for shrimp to maintain their intestinal immunity in the grow-out phase.
99

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100 **Materials and methods**

101 *Preparation of mannanase-hydrolyzed coprameal (MCM)*

102 The copra meals were hydrolyzed using mannanase enzyme to yield mannanase-
103 hydrolyzed coprameal (MCM). Briefly, the defatted copra meals were incubated with
104 0.2-0.6% mannanase solution (15,000unit/g minimum, Shin-Nihon Chemical CO., Ltd.,
105 Aichi, Japan) at 40-70 °C for 18-30 h, and then, dried 2-6 h to obtain MCM product.
106 MCM content analysis was carried out using following methods. Mannobiose was
107 determined by high-performance anion-exchange chromatography coupled with a
108 pulsed amperometric detection (HPAEC-PAD) system, and constituent sugars were
109 analyzed by surfuric hydrolysation. Ashes were determined by a dry-ashing procedure
110 at 55 ± 50°C for 2 h and moisture was determined by oven drying method at 105±1°C
111 for 4 h. Fat was analyzed by a diethyl ether extraction method (AOAC Method 920.39).
112 Protein content was determined with a Kjeldahl method (Lynch & Barbano 1999).

113

114 *Animal facility and feeding trials*

115 A group of 16-day-old Pacific white shrimp postlarvae was cultivated in the grow-out
116 ponds at the animal facility (Terminalia Garden Aquatic development, Taiwan) (Fig.1).
117 Shrimp were reared in brackish water with 0.07-0.15% salt concentrations and divided
118 into two groups: a control feed with commercial feed pellets (a control diet) and a
119 MCM-supplemented group (a test diet) (Table 1). The test diet was formulated to
120 contain 1% of MCM in the commercial feed pellets. Shrimp were fed under these diets
121 and intestine samples (N_{pooled} = 6 with 5 replicates) were aseptically collected at 2- and
122 4- month of the feeding trial period. All tissue samples were stored in RNA*later*® RNA
123 Stabilization Solution (Ambion, USA) to preserve tissue integrity for RNA extraction
124 for gene expression analysis. Water quality in ponds was measured every other day for
125 temperature, pH, and dissolved oxygen and weekly for ammonia–nitrogen, nitrite–
126 nitrogen, and alkalinity levels and maintained at the standard ranges for rearing Pacific
127 white shrimp (Rungrassamee et al. 2013).

128

129 *Total RNA extraction and cDNA synthesis*

130 Intestine tissues were homogenized in TRI REAGENT® (Molecular Research
131 Center, USA) for RNA extraction according to supplier's instruction. RNA pellets were

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132 washed twice with 500 µL of 75% ice-cold ethanol, air-dried for 5 min, and dissolved in
133 50 µL diethylpyrocarbonate (DEPC)-treated water. To remove potential genomic DNA
134 contamination, all RNA samples were treated with DNaseI enzyme (RQ1 RNase-free
135 DNaseI, Promega, USA) for 60 min at 37 °C. The DNaseI treated-RNA samples were
136 purified via phenol:chloroform extraction and precipitated with 1/10 volume of 3M
137 sodium acetate and 1 volume of isopropanol as previously done (Rungrassamee et al.
138 2010). All treated-RNA samples were confirmed to be free from genomic DNA via
139 PCR reaction. RNA concentration was quantified with a Nanodrop ND-8000
140 spectrophotometer (NanoDrop, UK). An ImProm-II™ Reverse Transcriptase System kit
141 (Promega, USA) was used to synthesize the first strand cDNA using 1.5 µg of the
142 DNA-free RNA sample as a template and each reaction was carried out according to the
143 supplier's protocol. The concentration of cDNA was measured by a Nanodrop ND-8000
144 spectrophotometer.
145
146 *Realtime PCR analysis of immune related genes transcript levels in response to MCM*
147 *supplement diet*
148 To determine MCM effects on expression levels of host immune genes, cDNA
149 from each shrimp group after 2- and 4-month of the feeding trial periods were used as
150 templates in realtime PCR analysis using a CFX96™ realtime system (Bio-rad
151 Laboratories, USA). The transcript levels of immune-related genes chosen were anti-
152 lipopolysaccharide factor1 (*alf1*), crustin (*crus*), c-type lectin (c-*lec*), lysozyme (*lyz*),
153 penaedin3 (*pen3a*) and Toll1 (*Toll1*) and the elongation factor-1 alpha (*EF1a*) was used
154 as an internal control. Each realtime PCR reaction contained IQ™ SYBR Green
155 Supermix (Bio-Rad, USA), gene-specific primer pair (1.25 µM, Table 2) and cDNA
156 (100 ng). The realtime PCR cycling parameters used were 95 °C for 3 min, 40 cycles of
157 at 95 °C for 30 sec, 57 °C for 20 sec and 72 °C for 30 sec. The fluorescent signal
158 intensities were recorded at the end of each cycle. Melting curve analysis was
159 performed from 55°C to 95 °C with continuous fluorescence measurement at every 0.5
160 °C increment. Relative abundance of the target immune genes in intestines of the
161 Pacific white shrimp was determined by the ΔΔct method (Livak & Schmittgen 2001).
162 The relative fold change for each target gene was compared between the MCM-
163 supplement to the control groups at the same time point.

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164
165 *Statistical analysis*
166 Statistical analysis was conducted using SPSS of Windows version 15.0 to
167 perform Student's t test analysis for significant differences in shrimp weights or gene
168 expression analysis (Landau & Everitt 2004).
169
170 **Results and discussion**
171 *MCM component analysis*
172 In this work, MOS supplement was derived in a form of mannanase-hydrolyzed
173 coprameal (MCM). MCM yield was ~95% of the copra meal. The content analysis of
174 MCM revealed high composition of carbohydrates (55.0%), followed by crude proteins
175 (22.9%), fats (9.3%), moisture (7.0%) and ashes (5.8%) (Table 3). The free sugar
176 contents in MCM were mainly mannobiose sugars (10.3%) and others were glucose,
177 mannose, fructose, sucrose and mannotriose. Among those sugars, mannobiose has been
178 reported for their important role as an immune modulator (Patel & Goyal 2012; Tiwari
179 et al. 2020). For instance, mannobiose is able to activate innate immune response in
180 chicken under *in vivo* and *in vitro* studies (Agunos et al. 2007; Duarte et al. 2014; Ibuki
181 et al. 2010; Ibuki et al. 2011). This suggests that MCM supplementation in this study
182 could potentially be used to enhance disease protection in animals.
183
184 *Expression analysis of the immune related in response to MCM supplementary diet*
185 Since shrimp digestive system, especially its intestine can be prone to pathogen
186 invasion (Aguirre Guzman et al. 2010; Soonthornchai et al. 2015), it is important to
187 determine approaches to modulate intestinal immune level. Hence, we selected genes
188 encoding the antimicrobial peptides (*alf1*, *pen3a* and *crus)*, lysozyme (*lyz*), C-type
189 lectin *(c-lectin)*, and Toll-like receptor protein (*Toll1)* as our genes of interest due to
190 their important roles in digestive tract of the shrimp as part of host defense mechanisms
191 against pathogens (Tassanakajon et al. 2013). To evaluate molecular effects of MCM
192 supplement on host immune system, the relative expression levels of the
193 aforementioned transcripts were compared in shrimp intestines at 2-month and 4-month
194 after feeding with the MCM-supplement diet to those fed with the non-MCM as a
195 control diet (Fig. 2). Our result showed that the MCM as dietary supplement did not

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196 affect the transcript levels of *alf1, pen3a* and *crus*. Additionally, the group fed with
197 MCM showed a significant decreased in transcript level of *lyz* at the 2-month of the
198 MCM-feeding period ($p < 0.05$), however the transcript level of *lyz* was not
199 significantly different to the non-MCM diet at 4-month-period. This suggests that MCM
200 did not have a direct effect on modulating antimicrobial peptides including lysozyme. In
201 contrast, the *c-lectin* and *Toll1* expression levels were significantly higher than the
202 control diet by ~2-fold when given MCM supplement diet at 2- and 4-month periods (p
203 < 0.05). This suggests that MCM supplement specifically increases expression levels of
204 *c-lectin* and *Toll1*, whose gene products play role as pattern recognition proteins. The
205 encoded C-type lectin is a member of lectin family proteins that has been reported to
206 bind to carbohydrate in a calcium-dependent manner (Weis et al. 1998). It plays
207 important role in pathogen recognition by detecting conserved pathogen cell wall
208 components of Gram-negative bacteria and further induces immune response to
209 invaders (Bi et al. 2020; Medzhitov & Janeway 2002; Wang & Wang 2013). Lectins
210 show strong bacterial-agglutination and opsonic activity, which facilitate phagocytosis
211 (Luo et al. 2006). In the Chinese white shrimp, C-type lectin has been demonstrated *in*
212 *vivo* to mediate in *V. anguillarum* clearance (Wang et al. 2009). Thus, the induction of
213 *C-lectin* could provide a local immune response and protection in Pacific white shrimp
214 intestine against bacterial pathogen invasion. Similarly, *Toll1*, encodes for Toll-like
215 receptor protein, was also significantly increased at 2- and 4-month of the MCM
216 supplemented group in comparison to the control group. Toll-like receptor proteins have
217 been reported in mammals to play roles in viral pathogen detection and mediate
218 responses to those viruses(Takeuchi & Akira 2009). Interestingly, the function of *Toll1*
219 in the Pacific white shrimp has not yet been linked to viral diseases protection in shrimp
220 (Labreuche et al. 2009), but it has been shown to involve in responses to bacterial
221 pathogen (Liu et al. 2016). This suggests that MCM is a promising candidate to increase
222 disease resistance to bacterial pathogens in Pacific white shrimp farming. Further
223 investigation on optimal dosages and formulation for the MCM supplement should be
224 conducted for future application.

225 Here, we report that the feed supplement containing mannanase-hydrolyzed
226 coprameal (MCM) was able to induce transcript levels of pathogen recognition proteins
227 in the Pacific white shrimp. The significant increase was observable on Month 2 of the

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228 feeding trial and the level remained similar on Month 4. Although future shrimp
229 experiments fed with a range of MCM concentration might be necessary to fully
230 elucidate beneficial effects of MCM. Our results provide a promising evidence for
231 further implementation of MCM as feed supplements or immunostimulants to enhance
232 shrimp health. Additionally, a comparison of shrimp survival rates under pathogen
233 challenge between the MCM supplement shrimp group to non-MCM supplement
234 control will strengthen the potential application of MCM as shrimp immunostimulant.
235
236 **Conflict of interest**
237 Yasuhiko Yoshida, Motohiro Maebuchi and Masahisa Ibuki are employees of Fuji Oil
238 Co., Ltd. or Japan Nutrition Co., Ltd., whom kindly supplied mannanase-hydrolysis of
239 copra meal (MCM) used in this study. They had no roles in experimental design and
240 data analysis.
241
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246
247

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430 **Table 1**. Composition of feed diets used in this study.
431

Raw material	% Feed composition	
	Control diet	MCM supplement diet
Wheat flour	35	35
Fish meal	20	20
Squid meal	10	10
Water	30	29
Vitamin and yeast mix	5	5
MCM	0	1

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435 **Table 2.** Primer sequences used in realtime PCR analysis
436

Gene	GenBank Accession Number	Primer	Sequence (5'-3')
Anti-lipopolysaccharide factor1 (*alf1*)	EW713395	alf1_F alf1_R	AGGACCTCATCCCTTCGCTAGT GGACACCACATCCTGCCATTGA
Crustin (*crus*)	AF430074	crus_F crus_R	CCACAACCTGTTCCAACGACTACAA AAACCTGCGATCCGAAGAATGAGG
C-type lectin (c-*lec*)	JF834160	lec_F lec_R	CGCTGATGGCTCGGATGAGA AGGCTGAGTTCGGTGGCAATAG
Lysozyme (*lyz*)	AF425673	lyz_F lyz_R	GGAGTTCGAGTCGTCCTTCAACA GTAGTCGCTTCCGCACCAGTA
Penaedin3 (*pen3a*)	Y14926	pen3a_F pen3a_R	CGTGGTCTGCCTGGTCTTCTT CAAAGGTCTCACGAAGGGTGGT
Toll1 (*Toll1*)	DQ923424	Toll1_F Toll1_R	CGAGAGCGAGTTGGACGAGAAG CCTGTGGGTGTGGCATGATGTA
Elongation factor-1 alpha (*EF1a*)	GU136229	EF1a_F EF1a_R	CGTCGCTTCCGACTCGAAGAA TGGCAATCAAGCACAGGTGAGTAG

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440 **Table 3**. Content analysis of mannanase-hydrolyzed coprameal (MCM).
441

	Component in MCM (%)	
General composition	Crude protein	22.9
	Crude fat	9.3
	Ash	5.8
	Moisture	7.0
	Carbohydrate	55.0
Free sugar contents	Glucose	1.5
	Mannose	2.6
	Fructose	1.8
	Sucrose	4.6
	Mannobiose	10.3
	Mannotriose	2.1

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445 **Figure Legends**

446

447 **Figure 1.** Experimental overview for evaluating effects of mannanase-hydrolysis of
448 copra meal (MCM) as immunostimulant in Pacific white shrimp, *Litopenaeus vannamei*
449 in a grow-out pond. The Pacific white shrimp postlarvae were divided into two groups:
450 a control feed with commercial feed pellets and a 1% MCM-supplemented group. The
451 intestine samples ($N_{pooled} = 6$ with 5 replicates) were collected at 2- and 4- month of the
452 feeding trial period for gene expression analysis.

453

454 **Figure 2.** Relative gene expression analysis of immune-related genes in intestines of
455 Pacific white shrimp after 2-month and 4-month feeding period with the mannanase-
456 hydrolyzed coprameal-supplemented diet group (MCM). The fold changes of transcript
457 of antimicrobial peptides (*alf1*, *pen3a* and *crus)*, lysozyme (*lyz*), C-type lectin *(c-lectin)*,
458 and Toll-like receptor protein (*Toll1)* were determined by real-time PCR in relative to
459 the control diet. An error bar represents standard deviation, which was calculated from
460 five replicates for each sample. Asterisk indicates significant difference in fold-changes
461 between groups fed with MCM and non-MCM containing diets ($p < 0.05$).

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Fig 1

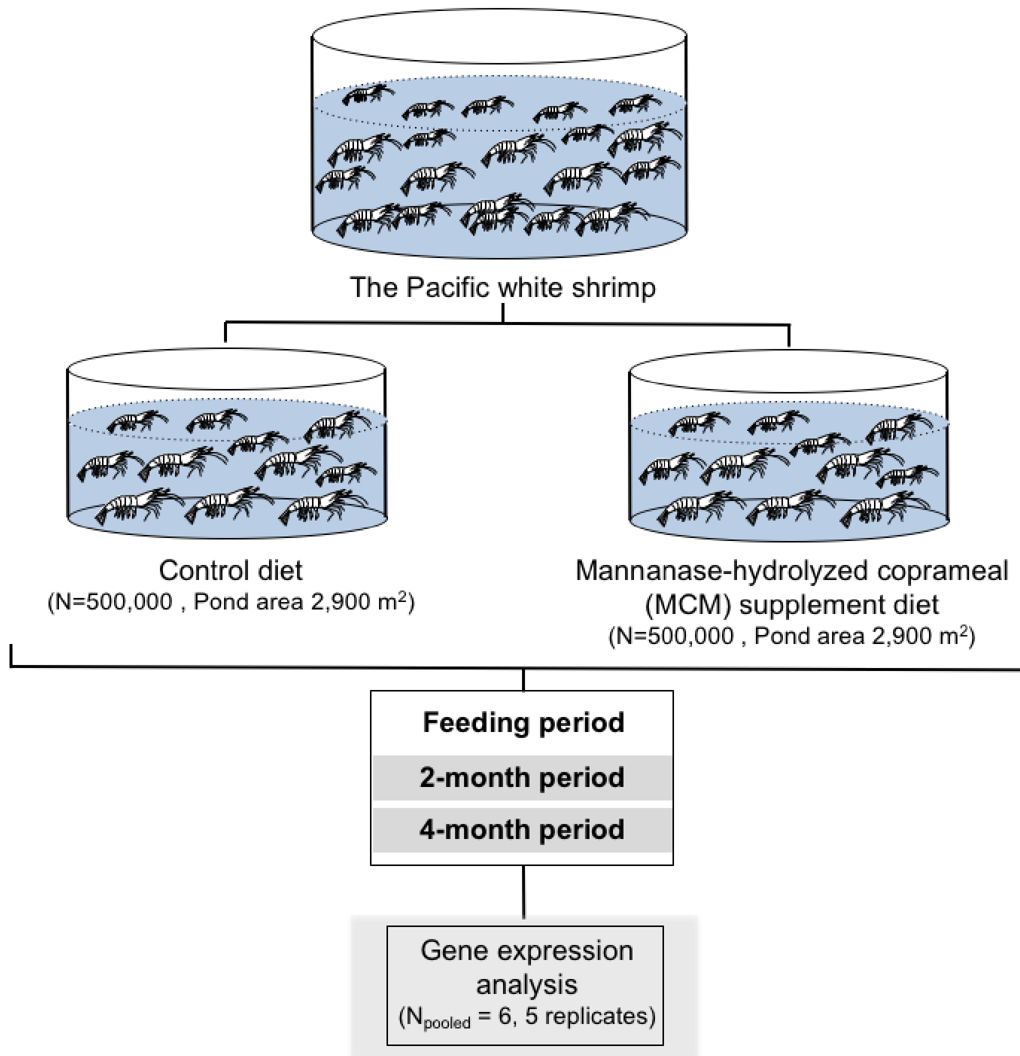

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Fig. 2