1    **Effects of substrate on shrimp growth, water quality and bacterial community in**

2    **the biofloc system nursing *Penaeus vannamei* postlarvae**

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15    **Abstract**

16    This study aimed to investigate the effects of substrate on water quality, shrimp growth

17    and bacterial community in the biofloc system with a salinity of 5‰. Two treatments,

18    biofloc system with (sB) or without (nB) addition of elastic solid packing filler (nylon)

19    as substrate, were set up. *Penaeus vannamei* postlarvae (PL, ~ stage 15) were stocked at

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20    a density of 4000 PL m$^{-3}$ in each treatment with triplicates for a 28-days culture

21    experiment, taking glucose as carbon source (C:N 15:1). Results showed that the

22    survival rate (96.3±3.6%), FCR (0.76±0.06) and productivity (1.54±0.12 kg m$^{-3}$) in sB

23    treatment were significantly better than those in nB treatment (81.0±7.1%, 0.98±0.08

24    and 1.14±0.09 kg m$^{-3}$, $P<0.05$). All water parameters were in the recommended ranges.

25    Substrate showed significant effect on TAN, TSS, turbidity, biofloc volume, pH and

26    carbonate alkalinity ($P < 0.05$). Actinobacteria (4.0-22.7%), Bacteroidetes

27    (10.4-33.5%), Firmicutes (0.2-11.2%), Planctomycetes (4.0-14.9%) and Proteobacteria

28    (29.4-59.0%) were the most dominant phyla for both treatments. However, the bacterial

29    community in sB treatment showed to be significantly different from that in nB

30    treatment (Jaccard distance 0.94±0.01, $P=0.001$). Substrate showed significant effects

31    on Shannon, Heip, Pielou and Simpson index, as well as relative abundances of

32    Actinobacteria, Bacteroidetes and Planctomycetes ($P < 0.05$). The results suggested

33    that addition of substrate affected the shrimp growth, water quality and bacterial

34    community in the biofloc system nursing *P. vannamei* PL with a 5‰ salinity.

35    **KEYWORDS**

36    *Penaeus vannamei*; Biofloc technology; Low salinity; Substrate; Bacterial community

37    **1  INTRODUCTION**

38    Crustacean is the most valuable group in fish trade in the world, accounting for 23.0%

39    in terms of value with an 8.3% share in culture production, compared to the 65.4% Vs.

40    79.8% of fish, and the 11.6% Vs. 11.9% of mollusks and other aquatic invertebrates

41    (FAO, 2018). Among the cultured crustaceans, *Penaeus vannamei* (Boone, 1931) is the

42    most important species whose production accounted for 53.0% of the total crustaceans

43    production (FAO, 2018).

44    Prenursery of postlarvae (PL) is a very important step during culture of *P.*

45    *vannamei* in many places (Mishra et al., 2008; Rezende et al., 2018). In the

46    conventional prenursery system, PL are intensively stocked at a high density (up to 60

47    PL $L^{-1}$) with small-scale water volume and carefully nursed for 15-60 days (Schveitzer

48    et al., 2017), to save water, land and energy resources, and to deliver large individuals

49    that are resistant to environmental conditions to increase the survival and growth

50    performance during the grow-out phase (Mishra et al., 2008; Rezende et al., 2018).

51    However, in this high-stocking-density system, serious cannibalism behavior,

52    competition for space and degradation of water quality would affect growth and

53    survival of PL (Esparza-Leal et al., 2015). The use of artificial substrate is an effective

54    approach to supply additional surface and refugee for shrimp during prenursery

55    (Tierney et al., 2020). Additionally, biofloc technology (BFT) has also been tried to be

56    used to prenursery of *P. vannamei* PL (Khanjani et al., 2017; Schveitzer et al., 2017), to

57    control the problems usually appeared in the traditional prenursery system, such as

58    biosecurity and toxic ammonia and nitrite (Samocha, 2010), due to the advantages of

59    this technology on nitrogen assimilation *in situ* and pathogen control under minimal or

60    zero water exchange conditions (Avnimelech, 2015; Huang, 2020). Furthermore,

61    several studies have used substrates in biofloc systems to reinforce the controlling of

62    water quality and the growth performance of PL by supplying a natural food through

63    the periphyton growing on the substrate during the prenursery stage (Peiro-Alcantar et

64    al., 2019; Rezende et al., 2018; Tierney et al., 2020).

65        *P. vannamei* is an euryhaline species, and can be cultured at low salinity conditions

66    less than 1.0‰, which is a trend that will continue to grow globally (Roy et al., 2010).

67    Osmoregulation capability of *P. vannamei* develops gradually in the postlarvae stages

68    and can be acclimated to salinities as low as 0.5‰ by stage 12 (Van Wyk et al., 1999),

69    indicating that PL could be nursed under low salinity conditions. As well, biofloc

70    technology has been successfully used for prenursery of *P. vannamei* PL under salinity

71    conditions of 8-16‰ (Esparza-Leal et al., 2016; Luo et al., 2019). However, application

72    of substrate in prenursery of *P. vannamei* PL in the biofloc system under a low-salinity

73    condition has been not documented yet.

74        In this study, it is aimed to estimate the effects of substrate on shrimp growth

75    performance and water quality in the biofloc system nursing *P. vannamei* postlarvae

76    (PL) under a salinity condition as low as about 5‰. Furthermore, the effects of

77    substrate on the bacterial community diversity and composition in this system were

78    also investigated.

79    **2   MATERIAL AND METHODS**

80    **2.1   Ethic statement**

81    The culture experiments were carried out at a local farm of Bifuteng eco-agriculture

82    development Co., Ltd. (BEAD Co., Ltd., Changde, China, Lat. 28°53'57.88" N, Long.

83    111°38'3.08" E), with indoor tanks, under principles in good laboratory animal care,

84    according to the national standard of China (GB/T 35892-2018), 'Laboratory

85    animal-Guideline for ethical review of animal welfare'. The manuscript does not

86    require ethical approval.

87    **2.2   Experimental design**

88    There two treatments with three replicates for each were set up in the present study,

89    biofloc system with (sB) and without (nB) addition of substrate. In the sB treatment,

90    52.4% of the tank internal surface was equally coated with elastic solid packing filler

91    (nylon, with a diameter of 15 cm).

92    Before the beginning of the experiment, tanks (width $\times$ length $\times$ depth $= 2 \times 2.5$

93    $\times$ 1.3 m, 6.5 m$^3$ in volume) connected with a Roots blower air pump (CRS-100,

94    Changsheng mechanical and electrical equipment Co., Ltd., Zhangqiu, Shandong,

95    China) were filled 5.0 m$^3$ (1.0 m in depth) culture water with a salinity of about 5.0‰

96    per tank. The water for culture experiment was prepared according to the method of

97    Ray and Lotz (2017), with some modifications. Briefly, artificial sea salt powder

98    (Qianglong corporation, Tianjin, China), and food-grade chemical reagents of KCl,

99    $MgCl_2$, $CaCl_2$ and $NaHCO_3$ were added to tap water, to manage an initial salinity of

100    5.02±0.08‰, with $K^+$, $Mg^{2+}$ and $Ca^{2+}$ concentrations of 301.3±5.9, 925.2±12.1 and

101    306.7±6.9 mg L$^{-1}$, respectively, and a pH value of about 8.0. After that, sterilization

102    with 10.0 mg L$^{-1}$ of chlorinedioxide followed by neutralization with 1.0 mg L$^{-1}$ of

103 ascorbic acid was executed according to the processes of previous studies (Gaona et al.,

104 2017; Lara et al., 2017).

105 At the beginning of the experiment, *P. vannamei* PL was randomly assigned to each

106 tank at a density of 4000 PL per m$^3$. Those PL (~PL15, 2.5±0.4 mg) were kindly

107 supplied from BEAD Co., Ltd. and had been treated with a desalinating and

108 acclimation procedure to adapt the experimental conditions before being assigned (Luo

109 et al., 2019; Van Wyk et al., 1999). Next, during the 28-days experimental period, PL

110 were fed with a commercial formulated shrimp diet (crude protein 40.0%, crude lipid

111 5.0%, crude fibber 5.0%, crude ash 15.0%, moisture 12.0%, Alpha corporation,

112 Jiangmen, Guangdong, China), with a frequency of four times (6:00, 12:00, 18:00,

113 24:00) a day, basing on the feeding table and the estimated total biomass according to

114 methods of Van Wyk et al. (1999). Besides, glucose (food grade, carbohydrate content

115 90.0%, Fufeng biotechnology Co., Ltd., Hohhot, Inner Mongolia Autonomous, China)

116 was added as exogenous carbon source to the experimental tanks, according to an

117 inputted carbon to nitrogen ratio (C:N) of 15:1 (Huang et al., 2021). The inputted C:N

118 was the C:N contained in the inputted materials (feed and carbon source), and

119 determined basing on the carbon contents contained in carbon source and feed (Kumar

120 et al., 2017, Tinh et al. 2021) and the conception that 25% of the feed nitrogen is

121 theoretically converted as shrimp biomass (Piedrahita, 2003). Throughout the whole

122 experimental period, no water exchange was operated, but evaporating loss was

123 complemented with dechlorinated tap water per week.

124    During 14-28 d of the experiment, partial biofloc was removed by using a

125    side-stream settling chamber equipped a submersible pump (JGP-2500L, SENSEN

126    Group Co., Ltd., Zhoushan, China) which ran for 6 h every day at a rate of 2.25 $m^3$ per

127    h to maintain a biofloc volume < 15 mL $L^{-1}$.

128    **2.3   Determination of water parameters**

129    Water temperature, dissolved oxygen and pH were detected each day by using electric

130    analyzers (YSI-ProPlus, Yellow Springs Instruments Inc., OH, USA; pH-100 meter,

131    LICHEN Sci-Tech, Co., Ltd., Shanghai, China). Water samples were collected once a

132    week and passed thought a 0.22-μm pore size microfilter (Xinya purification equipment

133    Co., Ltd, Shanghai, China). Then, the dissolved total nitrogen in water (DTN), total

134    ammonia nitrogen (TAN), nitrite, nitrate, carbonate alkalinity, and total suspended

135    solid (TSS), were determined according to the standard methods (APHA, 1995).

136    Additionally, the whole total nitrogen (WTN) in water body of each treatment was also

137    determined every week with water sample without filtration (APHA, 1995). And the

138    total nitrogen contained in biofloc (BTN) was defined as the total nitrogen content

139    contained in biofloc in per liter water and was determined by the difference between

140    WTN and DTN. With an Imhoff cone, 1-liter water sample was settled for 15 min after

141    which the sediment volume was read as the biofloc volume (settleable solids, BFV)

142    (Avnimelech, 2015), and the supernatant was used to measure the turbidity (APHA,

143    1995).

144    **2.4   Growth performance measurements**

145    Thirty PL were selected randomly and individually weighed to the nearest 0.1 mg with

146    an electric balance (AUX220, Shimadzu, Japan) each week. Survival rate, weekly

147    increment of body weight (wiW), specific growth rate (SGR), feed conversion rate

148    (FCR) and productivity were calculated according the following formulates:

$$\text{Survival rate } (\%) = [(\text{survival shrimp counts})/\text{stocked shrimp counts}] \times 100$$

$$\text{wiW } (\text{g week}^{-1}) = (\text{fbw} - \text{ibw})/\text{culture weeks}$$

$$\text{SGR } (\% \text{ d}^{-1}) = [(\ln \text{fbw} - \ln \text{ibw})/\text{culture days}] \times 100$$

$$\text{FCR} = \text{total mass of feed given}/\text{gained shrimp biomass}$$

$$\text{Productivity } (\text{kg m}^{-3}) = \text{harvested shrimp biomass}/\text{culture water volume}$$

149      Wherein, fbw and ibw represented the final and initial mean body weight of PL,

150    respectively.

151    **2.5   High-throughput sequencing of 16S rRNA gene and data processing**

152    Along the experimental period, water was sampled every week. Briefly, 50 ml water

153    was collected from each tank of a treatment according to the method of

154    Martínez-Córdova et al. (2018), pooled as one sample and filtered through a 0.22-μm

155    pore size membrane. Then, the membrane was stored at -80 $^{\circ}$C for extraction of

156    bacterial DNA genome with an E.Z.N.A$^{\text{TM}}$ Mag-Bind Soil DNA Kit (OMEGA

157    Bio-Tek, Inc., GA, USA), according to the manufacturers' instructions.

158      The V3-V4 region of 16S rRNA gene of bacterial DNA genome was amplified by

159    using the universal primers, 341F: 5'-CCTACGGGNGGCWGCAG-3' and 805R:

160    5'-GACTACHVGGGTATCTAATCC-3', in a 30-μl mixtures containing microbial

161  DNA (10 ng/μl) 2 μl, forward primer (10 μM) 1 μl, reverse primer (10 μM) 1 μl, and 2X

162  KAPA HiFi Hot Start Ready Mix 15 μl (TaKaRa Bio Inc., Japan), via a procedure with

163  a thermal instrument (Applied Biosystems 9700, USA): 1 cycle of denaturing at 95°C

164  for 3 min, first 5 cycles of denaturing at 95°C for 30 s, annealing at 45°C for 30 s,

165  elongation at 72°C for 30 s, then 20 cycles of denaturing at 95°C for 30 s, annealing at

166  55°C for 30 s, elongation at 72°C for 30 s and a final extension at 72°C for 5 min. The

167  PCR product was purified and recovered with MagicPure Size Selection DNA Beads

168  (TransGen Biotech Co., Ltd., Beijing, China). And then, the purified PCR product was

169  quantified and normalized with a Qubit ssDNA Assay Kit (Life Technologies, USA), to

170  construct the 16S gene library for high-throughput sequencing on the Miseq platform

171  according to the standard protocol (Illumina, USA) by Sangon Biotech (Shanghai) Co.,

172  Ltd. The raw data produced from high-throughput sequencing has been deposited at

173  NCBI with accession number of PRJNA646765.

174     Data processing for the high-throughput sequencing data was carried out under the

175  QIIME 2 (Quantitative Insights Into Microbial Ecology, Version 2019.10) framework

176  (Bolyen et al., 2019). In brief, ambiguous nucleotides, adapter sequences and primers

177  contained in reads, and short reads with length less than 30 bp were removed with the

178  cutadapt plugin (Martin, 2011). After that, bases in the two ends of reads with quality

179  score lower than 25 were trimmed. Thereafter, chimeras were filtered, and pair-ended

180  reads were joined, dereplicated, to obtain high-quality reads which were clustered to

181  operational taxonomic units (OTUs) with an identity of 0.97 by using the Vsearch tool

182  (Rognes et al., 2016). Thereafter, the counts of OTUs were normalized by 16S rRNA

183  gene copy number based on rrnDB database (version 5.6) with the QIIME 2 plugin of

184  q2-gcn-norm.

185  **2.6  Bacterial diversity analysis**

186  Bacterial community diversity was analyzed with Plugin diversity of QIIME 2 basing

187  on the normalized counts of OTUs. Metrics regard to alpha diversity, such as Shannon

188  index, richness indexes of Margalef, dominance indexes of Berger-Parker and Simpson,

189  and evenness indexes of Heip and Pielou, were computed with the diversity plugin of

190  QIIME 2. Besides, beta Jaccard index (distance) were also determined with this plugin,

191  and then significantly tested by using PERMANOVA with 999 permutations

192  (Anderson, 2001). Basing on the OTUs composition of samples, each sample was

193  plotted into a PCA diagram with STAMP (Parks et al., 2014).

194  **2.7  Bacterial composition analysis**

195  OTUs were annotated by referring to the GreenGene database 13.8, collapsed at

196  phylum, class, order, family and genus levels, respectively, with a QIIME 2 Plugin of

197  taxa. At each level, relative frequencies of species were calculated with the normalized

198  counts of OTUs. Differential analysis for phylum proportion between both treatments

199  was conducted with STAMP (Parks et al., 2014) and LEfSe (Segata et al., 2011).

200  **2.8  Statistical analyses**

201    Data was statistically analyzed with the SPSS platform for windows (version 22.0, IBM

202    Co., NY, USA). Student's T Test were executed for comparison of growth parameters

203    between both treatment, as soon as normality distribution and homogeneity of data

204    were proved with Shapiro-Wilk's test and Levene's test, respectively. Or else,

205    non-parametric Mann-Whitney U test was conducted. Percentage data was submitted to

206    arcsine transformation. Furthermore, during each stage, ANOVA with repeated

207    measurements was used to test the significance of main effects and interactions of

208    substrate and time on water parameters, bacterial diversity indices and bacterial

209    composition data under Mauchly's test of Sphericity assuming condition, or else, the $P$

210    value was corrected by using Greenhouse-Geisser test. Differences were considered

211    significant at $P < 0.05$.

212    **3  RESULTS**

213    **3.1  Water quality**

214    The levels of dissolved oxygen and temperature in the present study were above 5.0 mg

215    $L^{-1}$ and 26.0 $^{\circ}$C, respectively (Table 1). The effects of time on both parameters were

216    significant ($P < 0.05$, Table 1). Whereas, substrate showed no significant effect on the

217    levels of dissolved oxygen and temperature ($P > 0.05$, Table 1).

218        The carbonate alkalinity in sB treatment was maintained at a high level of

219    306.5±51.7 mg $L^{-1}$ CaCO$_3$, although it was lower than that in nB treatment (373.7±12.4

220    mg $L^{-1}$ CaCO$_3$, Table 1). The pH values in nB treatment and sB treatment were

221    7.20±0.14 and 7.11±0.03, respectively (Table 1). Both parameters were significantly

222    affected by substrate and time, as well as their interactions ($P < 0.05$, Table 1).

223        The mean concentrations of the three inorganic nitrogen compounds were at low

224    levels in the present study ($< 1.0$ mg $L^{-1}$, Table 1). Those compounds were not

225    significantly affected by substrate, time and their interaction ($P > 0.05$), with

226    exceptions of the substrate main effect and the interaction of substrate and time on TAN

227    ($P < 0.05$, Table 1).

228        No significant substrate effect on WTN (whole total nitrogen in waterbody), BTN

229    (total nitrogen contained in biofloc in per liter water) and DTN (dissolved total nitrogen

230    in waterbody) was found ($P > 0.05$, Table 1). Whereas, the effects of time on those

231    parameters were significant, as well as the interactions of substrate and time on WTN

232    and DTN ($P < 0.05$, Table 1).

233        The biofloc volume (BFV) levels in nB treatment and sB treatment were similar,

234    with a value of 8.8±2.3 and 8.3±2.3 mL $L^{-1}$, respectively (Table 1). However, the total

235    suspended solids (TSS) and turbidity in sB treatment (491.3±150.2 mg $L^{-1}$, and

236    299.5±92.0 nephelometric turbidity units, NTU) were higher than those in nB treatment

237    (148.5±31.3 mg $L^{-1}$ and 111.9±56.3 NTU, Table 1). Substrate showed significant main

238    effects on those three parameters ($P < 0.05$, Table 1). The effects of time on BFV and

239    turbidity were also significantly, as well as the interaction of substrate and time on BFV

240    ($P > 0.05$, Table 1).

241    **3.2   Growth performance**

242 During the 28-days culture experiment, the average body weights of shrimp in sB

243 treatment were higher than those of nB treatment (Fig. 1). At the end, although the final

244 body weight in sB treatment (0.40±0.03 g) was not significantly different from that of B

245 treatment (0.36±0.04 g, $P$ = 0.596, Table 2), the survival rate (96.3±3.6%) and

246 productivity (1.54±0.12 kg m$^{-3}$) of the former treatment were significantly higher than

247 those of the latter (81.0±7.1% and 1.14±0.09 kg m$^{-3}$, $P$ < 0.05, Table 2). The feed

248 conversion rate (FCR) in sB treatment (0.76±0.06) was significantly lower than that in

249 nB treatment (0.98±0.08, $P$ = 0.044, Table 2). There no significant difference was

250 observed between both treatments for the weekly increment of body weight (wiW) and

251 specific growth rate (SGR) of shrimp during the culture experiment ($P$ > 0.05, Table 2).

252 **3.3 Bacterial diversity**

253 The Shannon index for bacterial community in sB treatment (6.77±0.18) was

254 higher than that in nB treatment (6.14±0.07, Table 3). Substrate, time and their

255 interaction showed significant effects on this index ($P$ < 0.05, Table 3). The Shannon

256 index in sB treatment obtained the highest value at 7 d and then slightly decreased, but

257 that in nB treatment peaked at 21 d (Fig. 2 a).

258 Higher OTU counts (6238.3±353.8) and Margalef index (387.7±28.0) was

259 observed in sB treatment, when compared to that in nB treatment (5713.5±368.2 and

260 343.9±22.0, Table 3). Both indexes significantly affected by time ($P$ < 0.05), but not by

261 substrate ($P$ > 0.05, Table 3). Those two indexes showed similar changing trends with

262 the Shannon index in each treatment (Fig. 2 b and c).

263    The evenness indexes, Heip index and Pielou index, in sB treatment (0.018±0.001

264    and 0.538±0.003) and nB treatment (0.013±0.001 and 0.493±0.01, Table 3) peaked at

265    14 d and 21 d, respectively (Fig. 2 d and e). The main effects and interactions of

266    substrate and time on both indexes were significant ($P < 0.05$), with exception of the

267    interaction on Heip index ($P = 0.544$, Table 3).

268    The Simpson index in sB treatment (0.955±0.003) was higher than that in nB

269    treatment (0.927±0.010, Table 3). This index in sB treatment displayed a peak value at

270    14 d, but that in nB treatment showed a decreasing trend during experiment (Fig. 2 f).

271    Substrate and time significantly affected this index ($P < 0.05$, Table 3). Whereas, the

272    Berger-Parker index in sB treatment (0.152±0.014) was lower than that in nB treatment

273    (0.193±0.020, Table 3). Substrate was not significantly affected this index ($P > 0.05$,

274    Table 3). The change trend of Berger-Parker index in the present study was contrary to

275    that of the Simpson index in each treatment (Fig. 2 g).

276    The bacterial community distances within nB treatment and sB treatment were

277    0.76±0.36 and 0.77±0.37, respectively (Table 4). PCA analysis also showed that water

278    samples collected from the same treatment were closer than those from the other

279    treatment, basing on the OTUs composition (Fig. 3). In addition, the bacterial beta

280    diversities between both treatments were significantly different with a Jaccard distance

281    of 0.94±0.01 ($P = 0.001$, PERMANOVA, Table 4).

282    **3.4   Bacterial composition profile**

283      Thirty phyla were assigned for the OTUs in the present study. The most dominant phyla

284      in both treatments were Actinobacteria (4.0-22.7%), Bacteroidetes (10.4-33.5%),

285      Firmicutes (0.2-11.2%), Planctomycetes (4.0-14.9%) and Proteobacteria (29.4-59.0%)

286      (Fig. 4 a). Besides, Chloroflexi and Verrucomicrobia were one of the dominant phyla in

287      nB treatment (0.1-11.4%) and sB treatment (0.3-16.2%), respectively (Fig. 4a). Among

288      those seven phyla mentioned above, the proportions of Chloroflexi, Planctomycetes

289      and Verrucomicrobia were significantly different between both treatments from a point

290      of view of the whole experimental period ($P < 0.05$, Fig. 4 b). The main effects and

291      interactions of substrate and time on proportions of those seven phyla were significant

292      ($P < 0.05$), except the substrate effects on Firmicutes and Proteobacteria, and the

293      interaction of substrate and time on Planctomycetes ($P > 0.05$, Table 5).

294      The bacterial composition profiles on class, order, family and genus levels were

295      showed in Fig. 1S-Fig. 4S, respectively.

296      The LEfSe analysis showed that an unassigned genus and the phylum

297      Verrucomicrobia were the most significant biomarkers for nB and sB, respectively (Fig.

298      5).

299      **4   DISCUSSION**

300      **4.1   Effects of substrate on water quality**

301      All the water parameters determined in the present study were within the acceptable

302      range for culturing *P. vannamei* (Furtado et al., 2014; Hargreaves, 2013; Schveitzer et

303      al., 2013a; Van Wyk et al., 1999; Xu et al., 2012; Zhang et al., 2017). Addition of

304    substrate showed significantly effect on TSS in the present study ($P < 0.05$), with

305    higher level in sB treatment than that in nB treatment. Consistently, Schveitzer et al.

306    (2013b) also observed significant effect of substrate on TSS with higher levels in the

307    biofloc systems with substrate. Whereas, in another previous study, the TSS levels in

308    the biofloc systems with substrate were lower than that in the system without substrate

309    (Rezende et al., 2018). It is proposed here that substrates with different shape have

310    different effects on TSS. For example, nylon or polyester fiber net with a small mesh

311    size (1-2 mm) has the ability to filter the water and capture biofloc, thereby reducing the

312    amount of solids in the rearing water, leading to the lower TSS value (Rezende et al.,

313    2018). On the contrary, the substrate with a larger mesh size, such as 2.5 cm (Tierney et

314    al., 2020), would filter both water and biofloc. Similarly, in the present study, elastic

315    solid packing filler (nylon) was used as substrate which is a long open space composed

316    with nylon yarns radially fixed to a central rope. Thus, the flocs would be not retained

317    within this open space of substrate, especially under the rough aeration condition of the

318    biofloc system, leading to an increasing of TSS level in water body.

319       In the present study, it was found that the turbidity levels in both treatments were

320    increased continuously. This might be contributed to the removal operation for biofloc

321    during 14-28 d of the experiment in the present study. The turbidity in the present study

322    was determined on water sample without big-size biofloc which settled by using an

323    Imhoff cone for 15 min, indicating that this parameter was correlated to the content of

324    small-size biofloc. Previous studies have showed that the bacterial groups are different

325    between small- and big-size bioflocs (Chen et al., 2019; Huang et al., 2020). Therefore,

326    the removal operation in this study might make settleable solids (big-size biofloc) as

327    well as bacterial groups attached be removed from the water column, which would

328    improve the growth of bacteria adhering on small-size biofloc retained in the water

329    body, by reducing the competition from bacteria relating with big-size biofloc, and in

330    turn, prompting formation of small-size suspended biofloc and increasing the turbidity.

331        Substrate showed significant effect on turbidity in the present study ($P = 0.002$). It

332    was thought that the addition of artificial substrates may influence the water circulation

333    in the tanks, leading to smaller turbulence in the water, and in turn to facilitating

334    sedimentation or particle aggregation and formation of large flocs, which reduces the

335    suspended solids and thus the turbidity in the water column (Ferreira et al., 2016;

336    Fleckenstein et al., 2020). However, in the present study, the turbidity level in sB

337    treatment was found to be higher than that in nB treatment. It was speculated that

338    substrate might play a positive role in the process of turbidity increasing caused by the

339    removal operation for big-size biofloc discussed above.

340        Substrate also showed significant effect on carbonate alkalinity in the current study

341    ($P < 0.001$). And the carbonate alkalinity in sB treatment was lower than that in nB

342    treatment in the current study, indicating that more alkalinity was consumed in biofloc

343    system with substrate. This might be contributed to the different bacterial communities

344    which led to different bacterial activities consuming $HCO_3^-$ (Ebeling et al., 2006).

345    Corroboratively, more diverse bacterial community in sB treatment (Shannon index

346    6.77±0.18) than that in nB treatment (Shannon index 6.14±0.07), and different bacterial

347    communities between both treatments (Jaccard distance 0.94±0.01, $P < 0.001$) were

348    observed in the present study. Nevertheless, the inferring function profile of bacterial

349    community should be investigated to better understand the difference on $HCO_3^-$

350    consumption between both treatments next.

351    The results of ANOVA with repeated measurements showed that addition of

352    substrate showed significantly effects on TAN, biofloc volume (BFV), and pH in the

353    present study ($P < 0.05$). Meanwhile, the interactions of substrate and time on most

354    water parameters, such as whole total nitrogen in waterbody (WTN), total dissolved

355    nitrogen (DTN), TAN, BFV, carbonate alkalinity and pH, were significant in the

356    present study, indicating that the changing styles of these parameters along with time

357    elapsing were different between the biofloc systems with and without addition of

358    substrate. Rezende et al. (2018) also observed that some water parameters were

359    significantly affected by the interactions of the rearing day and treatment (substrate).

360    **4.2   Effects of substrate on shrimp growth**

361    The body weight of shrimp in treatment with substrate would be significantly prompt,

362    when compared to that without substrate (Olier et al., 2020; Schveitzer et al., 2013b),

363    via forming periphytons on substrate as supplementary natural food which could be fed

364    by shrimp for 24 h a day (Anand et al., 2019; Asaduzzaman et al., 2008; Kumar et al.,

365    2017). In the present study, the final body weight, weekly increment of body weight

366    (wiW) and specific growth rate (SGR) of shrimp in sB treatment were elevated by

367    10.0-11.1%, compared to those in nB treatment, which were similar with the results

368    (8.4-11.0%) observed by Tierney et al. (2020). Previous studies showed that the raw

369     material (Mani et al., 2021; Peiro-Alcantar et al., 2019; Rezende et al., 2018) and shape

370     (Fleckenstein et al., 2020) of substrates could affect its elevating efficiency on growth

371     performance of shrimp. For example, periphyton nutritional values were shown to be

372     dependent on substrate type (Tinh et al., 2021). And natural or vegetable substrates was

373     found to be more beneficial to growth of shrimp than artificial substrates (Mani et al.,

374     2021). It is also thought that the substrate composed of individual sheets with a large

375     opening or mesh size and a low overall surface area would show less improving effect

376     on shrimp production metrics. For instance, Ferreira et al. (2016) found that the

377     addition of substrate with an amount equivalent 400% of the lateral area of the tanks

378     significantly elevated the body weight of shrimp, but that with 200% did not. Similarly,

379     Fleckenstein et al. (2020) and Tierney et al. (2020) also observed that addition of

380     substrate only increased the total in-tank surface area by 13.4% and 21%, respectively,

381     did not lead to significant effect on any shrimp production metrics. In this study, the

382     substrate used was a long open space composed with nylon yarns radially fixed to a

383     central rope, covering only 52.4% of the tank internal surface, which might be

384     contributed to the non-significant benefit for the final body weight, wiW and SGR of

385     shrimp in sB treatment, compared to those in nB treatment ($P > 0.05$).

386       Nevertheless, in the present study, the survival rate and productivity in sB

387     treatment significantly increased when compared with those in nB treatment ($P < 0.05$).

388     Substrate could act as a refuge to add space and reduce the cannibalism behavior,

389     leading to more survival. And this increased space by addition of substrate reduced the

390     relative stocking density, which is beneficial for shrimp growth. An inverse

391     relationship between stocking density and growth performance of shrimp was found in

392     previous studies (Esparza-Leal et al., 2015; Esparza-Leal et al., 2020 ; Liu et al., 2017;

393     Panigrahi et al., 2019; Peixoto et al., 2018). Moreover, the feed conversion rate (FCR)

394     in sB treatment significantly decreased, compared with that in nB treatment ($P < 0.05$).

395     Bioflocs which mostly grew on tank wall or substrate as periphyton could be grazed by

396     shrimp as natural supplementary food (Tinh et al., 2021), reducing input of artificial

397     feeds, and consequently decreasing the FCR (Avnimelech, 2015). Undoubtedly,

398     substrate addition facilitates this feeding behavior of shrimp by supplying a niche for

399     preying on biofloc (Anand et al., 2019; Asaduzzaman et al., 2008).

400     **4.3   Effects of substrate on diversity of bacterial diversity**

401     Generally, highly diverse bacterial community including 18-40 phyla were observed in

402     waterbody of biofloc systems raising *P. vannamei* (Baliga et al., 2021; Ferreira et al.,

403     2021; Huerta-Rabago et al., 2019; Vargas-Albores et al., 2019), with a Shannon index

404     as high as 5.31-7.23 (Addo et al., 2021; Chen et al., 2019; Gao et al., 2019; Huang et al.,

405     2021; Jiang et al., 2020; Kim et al., 2021; Mariana et al., 2018). Similarly, in the

406     present study, 30 phyla were observed in both treatments with a Shannon index of

407     6.14-6.77. In the biofloc system, the environment rich in organic matter could form

408     several trophic levels which facilitate the growth of a number of bacterial phyla

409     (Fleckenstein et al., 2020), resulting in a high bacterial diversity.

410     Between both treatments in the present study, the beta diversity Jaccard index was

411     significantly different, indicating different bacterial communities in both treatments.

412   Corroboratively, from a point of view of the whole experimental period, higher

413   Shannon index, OTU counts, Margalef index, Heip index, Pielou index and Simpson

414   index, but lower Berger-Parker index of bacterial community in sB treatment were

415   observed when compared to those in nB treatment, indicating higher bacterial diversity,

416   richness and evenness, but lower dominance of predominant species (Magurran, 2004),

417   in the biofloc system with addition of substrate (sB treatment).

418       The results of ANOVA with repeated measurements revealed that substrate showed

419   significant effects on some alpha diversity indexes, such as Shannon index, Heip index,

420   Pielou index and Simpson index ($P < 0.05$). Meanwhile, the interactions of substrate

421   and time on Shannon index and Pielou index were also significant ($P < 0.05$), indicating

422   that the changing styles of those indexes over time were different between treatments in

423   the present study. Consistently, the bacterial community in sB treatment obtained the

424   most diverse situation at 7 d with the highest Shannon index, but in nB treatment, the

425   Shannon index increased gradually and peaked at 21 d, indicating that substrate

426   addition accelerated the formation of high-diverse bacterial community in the biofloc

427   system.

428       All alpha diversity indexes were significantly affected by time ($P < 0.05$),

429   indicating a time-dependent succession pattern of bacterial diversity in biofloc systems

430   regardless of addition of substrate, which was also observed in other studies (Jiang et al.,

431   2020; Kim et al., 2021; Wang et al., 2020; Xu et al., 2019).

432   **4.4   Effects of substrate on bacterial composition**

433    In general, Proteobacteria and Bacteroidetes were found to be the most dominant phyla

434    in marine biofloc systems rearing *P. vannamei* (Ferreira et al., 2021; Huerta-Rabago et

435    al., 2019;  Jiang et al., 2020; Mariana et al., 2018; Martínez-Córdova et al., 2018;

436    Vargas-Albores et al., 2019), as well as in biofloc systems with low salinities (Addo et

437    al., 2021; Huang et al., 2021). Similarly, in the present study, those two phyla together

438    accounted for 41.33-82.44% and 55.56-74.69% in nB treatment and sB treatment,

439    respectively. Proteobacteria have a diverse phenotypic and phylogenetic lineage and

440    widely dispersed in the environment rich in organic compounds (Kersters et al., 2006;

441    Kirchman, 2002; Rud et al., 2017), such as biofloc systems. Bacteroidetes serve as a

442    degrader of polymeric organic matter and are found in a range of habitats (Thomas et al.,

443    2011; Woebken et al., 2007). Additionally, Actinobacteria, Chloroflexi, Firmicutes,

444    Planctomycetes and Verrucomicrobia were important phyla in both treatments of the

445    present study, which were also commonly detected in biofloc systems stocking

446    white-leg shrimp (Deng et al., 2018; Ferreira et al., 2021; Huang et al., 2021;

447    Huerta-Rabago et al., 2019; Jiang et al., 2020; Luis-Villaseñor et al., 2016;

448    Martínez-Córdova et al., 2018; Martins et al., 2020; Schveitzer et al., 2020).

449    Substrate showed significantly main effects on the proportions of most of the seven

450    predominant phyla mentioned above in this current study. It is considered that substrate

451    could supply attaching material for species which grow adhesively, and tend to adsorb

452    the bacteria with particle-attaching or biopolymer-degrading capability (Huang et al.,

453    2020). For example, the filamentous Chloroflexi grow on other microorganisms and

454    substrate (Kragelund et al., 2007; Miura et al., 2007); and Bacteroidetes prefer for

455    polymers and are frequently found attaching to surfaces, colonizing on particles (Deng

456    et al., 2019; Fernández-Gómez et al., 2013; Kirchman, 2002; Morgan-Sagastume et al.,

457    2008). Although Planctomycetes grow as single free-swimming cells, they also appear

458    as single cells within flocs (Morgan-Sagastume et al., 2008), and are usually found to

459    adhere to the surfaces of invertebrates (Fuerst et al., 1997), detrital aggregates (Crump

460    et al., 1999; Delong et al., 1993), and flocs (Huang et al., 2020). Actinobacteria are

461    chemoorganotrophic bacteria (saprophytes) with branched filaments (Madigan et al.,

462    2015) and have been described as small free-living cells (Ghai et al., 2013), leading to a

463    higher proportion in free-living fraction than that in particle-attached fraction

464    (Bachmann et al., 2018; Huang et al., 2020; Mestre et al., 2017). Therefore, the free- or

465    attaching-living characteristic of those phyla might contribute to the variations of their

466    abundance between both treatments in the present study.

467        Although Firmicutes are microorganisms to grow as single free-swimming cells

468    making them more likely to remain in suspension (Morgan-Sagastume et al., 2008).

469    And Proteobacteria survive as free-living to live suspended in the water column,

470    although some species might attach to particles, surfaces, or cells (Fernández-Gómez et

471    al., 2013; Morgan-Sagastume et al., 2008; Rud et al., 2017; Schveitzer et al., 2020),

472    substrate did not show significant effect on those two phyla in this present study. And

473    the reason should be investigated next.

474        The dominant phyla observed in the present study have variable functions. For

475    instance, Proteobacteria play important roles in the process of nutrient cycling and the

476    mineralization of organic compounds (Kersters et al., 2006; Kirchman, 2002; Rud et al.,

477   2017); Bacteroidetes produce extracellular enzymes with degradative capabilities to

478   serve as degraders of polymeric organic matter (Kirchman, 2002; Thomas et al., 2011;

479   Woebken et al., 2007); Chloroflexi reductively dehalogenate organic chlorinated

480   compounds to obtain energy (Gupta, 2013); Actinobacteria are important in the nutrient

481   and biomaterial recycling (Goodfellow and Williams, 1983), responsible for degrading

482   nucleic acids and proteins (Madigan et al., 2015), and for the production of many

483   hydrolytic enzymes (chitinase, chitinolytic, fatty acid, cellulolytic, nitrile hydrolyzing,

484   amylolytic, proteolytic, and lipolytic) (Das et al., 2008); Planctomycetes have function

485   to degrade the biopolymers of adsorbate (Woebken et al., 2007). Therefore, the

486   different bacterial community profiles between both treatments in the current study

487   might contribute to different activities of nitrogen and carbon metabolism in their water

488   body. However, the inferring functions of bacterial community should be induced next

489   to directly estimate the effects of addition of substrate on the bacterial activities.

490       The interactions of substrate and time on proportions of most of those phyla (except

491   Planctomycetes) also significant in the present study, indicating different development

492   styles of bacterial composition profile between both treatments. Furthermore, all the

493   seven dominant phyla in the present study were significantly affected by time ($P <$

494   0.05), indicating a time-dependent dynamics of bacterial composition in biofloc

495   systems, similar with that observed in other studies (Baliga et al., 2021; Gao et al., 2019;

496   Jiang et al., 2020; Martínez-Córdova et al., 2018; Xu et al., 2019).

497   **5   CONCLUSION**

498      In the present study, addition of elastic solid packing filler (nylon, with a diameter

499      of 15 cm) as substrate that coated 52.4% of the tank internal surface in the biofloc

500      system nursing *P. vannamei* PL with a salinity of 5‰ showed positive effects on

501      shrimp growth performance. Furthermore, substrate also showed significant main

502      effects or interactions with time on most water parameters as well as bacterial diversity

503      and composition.

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**Table 1** Water parameters in the biofloc systems with (sB) and without (nB) addition of elastic solid packing filler (nylon) as substrate, during the 28-days experiment culturing *Penaeus vannamei* at a salinity of 5.0‰

| parameters | Treatments | | *P*-value | | |
| --- | --- | --- | --- | --- | --- |
| | nB | sB | Substrate | Time | Interaction |
| TSS (mg $L^{-1}$) | 148.5±31.3 | 491.3±150.2 | 0.009 | 0.116 | 0.594 |
| WTN (mg $L^{-1}$) | 76.8±11.2 | 76.3±9.6 | 0.057 | <0.001 | 0.013 |
| DTN (mg $L^{-1}$) | 13.0±2.6 | 14.7±3.6 | 0.890 | <0.001 | <0.001 |
| BTN (mg $L^{-1}$) | 63.8±9.3 | 61.6±12.5 | 0.052 | <0.001 | 0.085 |
| TAN (mg $L^{-1}$) | 0.55±0.25 | 0.43±0.06 | 0.010 | 0.227 | 0.002 |
| Nitrite (mg $L^{-1}$) | 0.37±0.28 | 0.10±0.03 | 0.352 | 0.348 | 0.342 |
| Nitrate (mg $L^{-1}$) | 0.51±0.14 | 0.56±0.08 | 0.924 | 0.274 | 0.252 |
| Biofloc volume (mL $L^{-1}$) | 8.8±2.3 | 8.3±2.3 | 0.044 | <0.001 | <0.001 |
| Turbidity (NTU) | 111.9±56.3 | 299.5±92.0 | 0.002 | 0.005 | 0.059 |
| CAK (mg $L^{-1}$ $CaCO_3$) | 373.7±12.4 | 306.5±51.7 | <0.001 | <0.001 | <0.001 |
| Temperature ($^o$C) | 26.9±0.7 | 27.1±0.6 | 0.656 | <0.001 | 0.012 |
| pH | 7.20±0.14 | 7.11±0.03 | 0.001 | 0.001 | <0.001 |
| Dissolved oxygen (mg $L^{-1}$) | 5.85±0.32 | 5.63±0.59 | 0.215 | <0.001 | 0.764 |

Note: BTN, the total nitrogen contained in biofloc; CAK, carbonate alkalinity; DTN, the dissolved total nitrogen in water; NTU, nephelometric turbidity units; TAN, total ammonia nitrogen; TSS, total suspended solids; WTN, the whole total nitrogen in water.

**Table 2** Growth performance of *Penaeus vannamei* postlarvae cultured in the biofloc systems with (sB) and without (nB) addition of elastic solid packing filler (nylon) as substrate, during the 28-days experiment at a salinity of 5.0‰

| parameters | B | sB | *P*-value |
|---|---|---|---|
| Final mean body weight (g) | $0.36 \pm 0.04$ | $0.40 \pm 0.03$ | 0.596 |
| Survival rate (%) | $81.0 \pm 7.1$ | $96.3 \pm 3.6$ | 0.011 |
| FCR | $0.98 \pm 0.08$ | $0.76 \pm 0.06$ | 0.044 |
| wiW (g week$^{-1}$) | $0.090 \pm 0.014$ | $0.099 \pm 0.008$ | 0.652 |
| SGR (% d$^{-1}$) | $17.7 \pm 0.5$ | $18.1 \pm 0.4$ | 0.581 |
| Productivity (kg m$^{-3}$) | $1.14 \pm 0.09$ | $1.54 \pm 0.12$ | 0.029 |

Note: FCR, feed conversion rate; SGR, specific growth rate; wiW, weekly increment of body weight.

**Table 3** Bacterial alpha diversity indexes in the biofloc systems with (sB) and without (nB) addition of elastic solid packing filler (nylon) as substrate, during the 28-days experiment culturing *Penaeus vannamei* at a salinity of 5.0‰

| Indexes | Treatments | | *P*-value | | |
|---|---|---|---|---|---|
| | B | sB | Substrate | Time | Interaction |
| Shannon | 6.14±0.18 | 6.77±0.07 | 0.002 | <0.001 | <0.001 |
| OTU counts | 5713.5±368.2 | 6238.3±353.8 | 0.427 | 0.024 | 0.572 |
| Margalef | 343.9±22.0 | 387.7±28.0 | 0.367 | 0.007 | 0.471 |
| Heip | 0.013±0.001 | 0.018±0.001 | 0.026 | 0.012 | 0.544 |
| Pielou | 0.493±0.012 | 0.538±0.003 | 0.024 | <0.001 | 0.016 |
| B-P | 0.193±0.020 | 0.152±0.014 | 0.098 | 0.002 | 0.676 |
| Simpson | 0.927±0.010 | 0.955±0.003 | 0.043 | <0.001 | 0.127 |

OTU, operational taxonomic unit; B-P, Berger-Parker index.

**Table 4** Bacterial community distance between the biofloc systems with (sB) and without (nB) addition of elastic solid packing filler (nylon) as substrate, during the 28-days experiment culturing *Penaeus vannamei* at a salinity of 5.0‰

| Group 1 | Group 2 | Jaccard distance | Permutations | pseudo-*F* | *P*-value |
|---------|---------|------------------|--------------|------------|-----------|
| B | B | $0.761 \pm 0.361$ | - | - | - |
| sB | sB | $0.768 \pm 0.365$ | - | - | - |
| B | sB | $0.941 \pm 0.007$ | 999 | 3.96 | 0.001 |

**Table 5** Proportions (%) of dominant phyla in the biofloc systems with (sB) and without (nB) addition of elastic solid packing filler (nylon) as substrate, during the 28-days experiment culturing *Penaeus vannamei* at a salinity of 5.0‰

| Phylum | Treatments | | *P*-value | | |
| --- | --- | --- | --- | --- | --- |
| | nB | sB | Substrate | Time | Interaction |
| Actinobacteria | 13.67±3.34 | 10.00±4.26 | <0.001 | <0.001 | <0.001 |
| Bacteroidetes | 23.18±4.01 | 16.87±5.57 | <0.001 | <0.001 | <0.001 |
| Chloroflexi | 3.79±2.57 | 0.63±0.22 | <0.001 | 0.001 | 0.002 |
| Firmicutes | 4.53±2.09 | 5.92±1.79 | 0.087 | 0.001 | 0.010 |
| Planctomycetes | 8.57±2.10 | 12.49±1.00 | 0.006 | 0.035 | 0.486 |
| Proteobacteria | 43.82±6.10 | 45.90±2.46 | 0.405 | <0.001 | 0.038 |
| Verrucomicrobia | 0.63±0.18 | 6.06±3.42 | <0.001 | <0.001 | <0.001 |

**Fig. 1** Mean body weight of *Penaeus vannamei* postlarvae cultured in the biofloc systems with (sB) and without (nB) addition of elastic solid packing filler (nylon) as substrate, with time elapsing during the 28-days experiment at a salinity of 5.0‰. Error bar indicates ± standard deviation (SD).

**Fig. 2** Alpha diversity indexes of bacterial communities in the biofloc systems with (sB) and without (nB) addition of elastic solid packing filler (nylon) as substrate, with time elapsing during the 28-days experiment culturing *Penaeus vannamei* at a salinity of 5.0‰.

**Fig. 3** PCA diagram plotting samples basing on their OTU composition profiles. Samples were collected from the biofloc systems with (sB) and without (nB) addition of elastic solid packing filler (nylon) as substrate at time points of 7, 14, 21 and 28 d during experiment culturing *Penaeus vannamei* at a salinity of 5.0‰.

**Fig. 4** Heatmap displaying bacterial composition profiles at phylum level (a) and differential phyla (b) in the biofloc systems with (sB) and without (nB) addition of elastic solid packing filler (nylon) as substrate, with time elapsing during the 28-days experiment culturing *Penaeus vannamei* at a salinity of 5.0‰.

**Fig. 5** Cladogram (a) and Linear discriminant analysis (LDA) scores of LEfSe (linear discriminant analysis effect size) analysis showing biomarkers in the biofloc systems with (sB) and without (nB) addition of elastic solid packing filler (nylon) as substrate, with time elapsing during the 28-days experiment culturing *Penaeus vannamei* at a salinity of 5.0‰.

a

- nB
- sB

a: f_Ellin6075
b: o_RB41
c: c_Chloracidobacteria
d: p_Acidobacteria
e: g_Arthrobacter
f: g_Unassigned_1
g: g_Leadbetterella
h: g_Fluviicola
i: f_Saprospiraceae
j: p_GN02
k: g_Lutibacterium
l: g_Unassigned_2
m: f_Bacteriovoracaceae
n: g_Marinobacter
o: g_HTCC
p: f_HTCC2188
q: g_Unassigned_2
r: f_Verrucomicrobiaceae
s: o_Verrucomicrobiales
t: c_Verrucomicrobiae
u: p_Verrucomicrobia

b