

1 **Running title:** *Bacillus* probiotics in aquaculture

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3 **Genus *Bacillus*, promising probiotics in aquaculture: Aquatic animal**  
4 **origin, bioactive components, bioremediation in fish and shellfish**

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28

**29 Abstract**

30 *Bacillus* are Gram-positive spore-forming bacteria that are resistant to aggressive physical and  
31 chemical conditions with various species exhibiting unusual physiological features enabling them  
32 to survive in various environmental conditions including fresh waters, marine sediments, desert  
33 sands, hot springs, Arctic soils and the gastrointestinal (GI) tract of finfish and shellfish. They  
34 are able to rapidly replicate, tolerate a multitude of environmental conditions giving a wide  
35 range of beneficial effects in aquaculture sector. Application of *Bacillus* as probiotics in feed  
36 or as bioremediatory in the aquaculture rearing water is one of the pleasant issues in a  
37 sustainable aquaculture sector. *Bacillus* bacteria can play a desirable role in removal of waste  
38 products from aquaculture environments, maintaining optimum water quality and reducing  
39 stress conditions, that can lead to an optimum immuno-physiological balance, better growth and  
40 survival in the target aquatic animal. The available data show that application of probiotic  
41 *Bacillus* can directly or indirectly enhance the growth and immune status of aquatic organisms.  
42 Probiotic *Bacillus* species can also assist in maintaining a higher density of beneficial bacteria  
43 and a lower load of pathogenic agents in the aquaculture ponds. However, effects of a particular  
44 *Bacillus* species or strain on different finfish/shellfish species, age, growth condition, water  
45 quality and diet types can identify the conditions in which the probiotics could work well. This  
46 review addressed the presence of *Bacillus* in the GI tract of finfish and shellfish, their ability  
47 to produce enzymes and antibacterial compounds, pathogenic bacilli and their efficacy and  
48 potency as probiotics in aquaculture.

49 **Key words:** *Bacillus*, bioremediation, growth, immunity, probiotic

**50 1. Introduction**

51 In the 1870s, [Cohn \(1876\)](#), [Koch \(1876\)](#) and [Tyndall \(1877\)](#) independently discovered that  
52 certain species of bacteria spend at least part of their lives as dormant cellular structures, known  
53 as endospores. These bacteria were later classified as *Bacillus* and are now classified in phylum  
54 Firmicutes, class Bacilli, and order Bacillales. They are Gram-positive, endospore-forming, aerobic or  
55 facultative anaerobic, characterized by their rod-shaped morphology (between 2.5-10  $\mu\text{m}$ ), and  
56 are catalase positive. Genus *Bacillus* comprises of approximately 200 bacterial species and are

57 almost ubiquitous in nature; in soil, in compost (*Bacillus composti* and *Bacillus thermophilus*;  
58 Yang *et al.* 2013), in extreme environments such as high pH (*Bacillus firmus* OF4; Sturr *et al.*  
59 1994), high temperature (*Bacillus thermophilus*; Yang *et al.* 2013), high salt (*Bacillus*  
60 *halodurans*; Annamalai *et al.* 2013), aquatic environment (Motta *et al.* 2004; Ichimatsu *et al.*  
61 2000) as well as in the gastrointestinal (GI) tract of aquatic animals (Ray *et al.* 2012). They  
62 exhibit quite diverse physiological properties such as the ability to produce cellulase, phytase,  
63 tannase, chitinase, xylanase, protease and lipase (Ray *et al.* 2012, Ghosh *et al.* 2018) as well as  
64 degradation of palm (*Elaeis guineensis*) biodiesel (Lutz *et al.* 2006). Another favourable trait  
65 of *Bacillus* is; they produce antimicrobial substances such as peptide and lipopeptide antibiotics  
66 and bacteriocins (Stein 2005; Abriouel *et al.* 2011). The sporulation capacity and the production  
67 of antimicrobial substances and enzymes confer the capacity of genus *Bacillus* to colonise  
68 different habitats and to contribute to nutrition of the host. Moreover, adding selected strains of  
69 *Bacillus* as probiotics to control *Vibrio* species in penaeid aquaculture ponds (Moriarty, 1998)  
70 and as dietary probiotics (Hong *et al.* 2005) are widely used.

71 Optimal GI functionality is essential for sustainable animal production, and effective  
72 functionality of the GI tract and its gut microbiota play an important role in host health (e.g.  
73 Clemente *et al.* 2012; Xiong *et al.* 2017; Ringø *et al.* 2018), and several complex mechanisms  
74 are involved. The microbial community of fish gut are influenced by the anatomy; with or  
75 without stomach, with pyloric caeca (up to 1000) or without, and short and long intestine  
76 (relative intestinal length). In the absence of intestinal microbiota, normal immune development  
77 and function are impaired, protection against infections, gut health as well as contributors to  
78 nutrition (e.g. Rawls *et al.* 2004, 2006; Gómez & Balcázar 2008; Ray *et al.* 2012; Wang *et al.*,  
79 2018; Li *et al.* 2018a). Therefore it is crucial to increase our knowledge on beneficial gut  
80 bacteria, in the context of improved growth performance and health.

81 As alteration of the GI tract microbiota (dysbiosis) may enhance the risk for allergies and other  
82 conditions (Mazmanian *et al.* 2008; Sokol *et al.* 2008), it is of importance to investigate the  
83 intestinal microbiota of endothermic animals as well as aquatic organisms. The presence of  
84 beneficial bacteria such as genus *Bacillus* in the GI tract of aquatic organisms merits  
85 investigations, as autochthonous bacteria, adherent to mucosa, rapidly colonise the digestive  
86 tract at early developmental larval stages (Ringø *et al.* 1996; Hansen & Olafsen 1999; Vadstein  
87 *et al.* 2018).

88 During the last 20 years, numerous studies have been published on *Bacillus* in the intestine of  
89 finfish and shellfish, their potential as probiotics, pathogenicity and their effect on the immune  
90 system (e.g. Abriouel *et al.* 2011; Cha *et al.* 2013; Kim *et al.* 2014; Mukherjee & Ghosh 2016;  
91 Wu *et al.*, 2016; Sreenivasulu *et al.*, 2016; Dash *et al.*, 2018; Bachruddin *et al.* 2018).  
92 Therefore, the current review aimed to present an overview of published data on *Bacillus* in the  
93 GI tract of finfish and shellfish, antagonistic ability, health benefits as probiotics, pathogenicity  
94 and their effect on immunostimulation.

## 95 2. *Bacillus* in the gastrointestinal (GI) tract of finfish and shellfish

96 Optimal GI functionality is of importance for sustainable animal production, and three  
97 components namely diet, mucosa and commensal microbiota are essential for gut health.  
98 Evaluation of the gut microbiota of finfish dates back to late 1920`s and early 1930`s (Reed &  
99 Spence 1929; Gibbons 1933) and since then numerous studies have been published. The GI tract  
100 microbiota in fish is divided into; the GI lumen microbiota (the allochthonous), and those that  
101 adhere to the mucosal surface (the autochthonous microbiota), however, several factors can  
102 modulate the gut microbiota (Ringo *et al.* 2016). The intestinal microbiota contributes to several  
103 essential factors such as pathogen displacement, nutrient- and receptor competition, production  
104 of antimicrobial factors, structural factors (induction of IgA, tightening of tight junction and  
105 immune system development) and several metabolic functions. Of the commensal gut  
106 microbiota of aquatic animals, phylum Firmicutes which include genus *Bacillus* is essential,  
107 and the contribution of Sugita and colleagues (Japanese scientists) and Indian scientists (Ghosh  
108 and colleagues, and Ray and colleagues) can not be underestimated.

109 Since the first studies were published on salmonids by Trust & Sparrow (1974) and Trust (1975),  
110 numerous investigations on the isolations of *Bacillus* in the GI tract of finfish and shellfish  
111 have been carried out. In several *Bacillus* studies showed in Table 1 have, characterized  
112 autochthonous (adherent) *Bacillus* isolated from the GI tract, and this is of importance as they  
113 may contribute to nutrition, inhibit pathogen adherence and colonization, may affect the  
114 immune system and may have potential as probiotics. In Table 1, features of isolated bacilli  
115 are indicated, however, in numerous studies, the intestinal microbiota were only described, and  
116 this is indicated by no information available.

### 117 2.1. *Bacillus* in finfish

118 In numerous studies, counts of presumptive *Bacillus* has been revealed, but without going into  
119 further identification (Table 1). These studies are not discussed in the present review, even  
120 though they investigate dietary effects (e.g. de Paula Silva *et al.* 2011; Green *et al.* 2013; Barreto-  
121 Curriel *et al.* 2018; Nymann *et al.* 2017), effects of antibiotic supplementation (Austin & Al-  
122 Zahrani 1988; Sugita *et al.* 1988a; 1989), production of enzymes (e.g. Sugita *et al.* 1997; Bairagi  
123 *et al.* 2002; Ray *et al.* 2010; Askarian *et al.* 2012), putative probiotics (e.g. Geraylou *et al.* 2014;  
124 Nandi *et al.* 2017), effect of prebiotics (Ringø *et al.* 2006), antimicrobial potential (Sugita *et al.*  
125 1989, 1998), seasonal variations (Hovda *et al.* 2012), effect of stress (Olsen *et al.* 2008) and wild  
126 vs. farmed fish (Strøm & Olafsen 1990; Ramirez & Romero 2017). Readers with special interest  
127 in these studies and other studies only identified *Bacillus* without going into detailed  
128 identification are recommended to have a closer look at the original papers. In the following,  
129 bacteria identified as *Bacillus aerius*, *Bacillus aerophilus*, *Bacillus amyloliquefaciens*, *Bacillus*  
130 *altitudinis*, *Bacillus aryabhattai*, *Bacillus atrophaeus*, *Bacillus cereus*, *Bacillus circulans*,  
131 *Bacillus clausii*, *Bacillus coagulans*, *Bacillus flexus*, *Bacillus licheniformis*, *Bacillus*  
132 *megaterium*, *Bacillus methylotrophicus*, *Bacillus pumilus*, *Bacillus sonorensis*, *Bacillus*  
133 *subtilis*, *Bacillus tequilensis*, *Bacillus thermoamylovorans*, *Bacillus thuringiensis*, *Bacillus*  
134 *stratosphericus* and *Solibacillus silvestris* isolated from the finfish GI tract are discussed.

135 ***B. aerius***. To our knowledge, only one study has isolated this bacterium from the GI tract of  
136 finfish. Dutta *et al.* (2015) reported autochthonous *B. aerius* in the distal intestine (DI) catla  
137 (*Catla catla*) in a study where probiotic characterization of exoenzyme-producing bacteria were  
138 investigated.

139 ***B. aerophilus***. In a study evaluating the autochthonous microbiota of Indian major carp  
140 (*Cirrhinus mrigala*) by cultivation, Mukherjee *et al.* (2016) reported *B. aerophilus*, a bacterium  
141 with probiotic potential based on good growth in intestinal mucus, resistant to diluted bile juice  
142 (2–20%), safe for the target fish, and production of bacteriocin.

143 ***B. amyloliquefaciens***. This bacterium has been isolated in eight finfish studies of eight fish  
144 species, and culture-based methods were used in seven of them (Table 1). The features of most  
145 strains were; probiotic potential, enzyme-production and antagonism, and bacteriocin  
146 production. More recently this bacillar species has been isolated from the intestines of flounder  
147 (Chen *et al.* 2016a), southern flounder (*Paralichthys lethostigma*) (Chen *et al.*, 2016b), rohu

148 (*Labeo rohita*) (Mukherjee *et al.*, 2017) and Inidan major carp (Kavitha *et al.* (2018) using  
149 bculture-based methods, and these studies revealed promising features.

150 ***Bacillus altitudinis*, *Bacillus aryabhatai* and *Bacillus atrophaeus*.** These bacteria, all  
151 autochthonous, have been detected by cultivation in the DI of mrigal (Banerjee *et al.* 2016),  
152 proximal intestine (PI) of walking catfish (*Clarias batrachus*) (Dey *et al.* 2016) and stinging  
153 catfish (*Heteropneustes fossilis*) (Khan & Ghosh 2012). The major feature of these bacilli were  
154 they produce enzymes, and one species, *B. altitudinis*, revealed antibacterial potential.

155 ***B. cereus*.** This bacterium capable by producing amylase, cellulase and protease has been  
156 isolated from the PI and DI of mrigal by Ray *et al.* (2010). In a study evaluated the microbial  
157 diversity in the gut of wild and farmed olive flounder (*Paralichthys olivaceus*), Kim & Kim  
158 (2013) revealed culturable autochthonous *B. cereus* in the DI of wild fish. When investigating  
159 the bacterial community in DI of Atlantic salmon (*Salmo salar*) by cultivation, Askarian *et al.*  
160 (2012) revealed that *B. cereus* was a part of the bacterial community. In a study using culture-  
161 based method by Kavitha *et al.* (2018), *B. cereus* was recovered from intestine of Indian major  
162 carp.

163 ***B. circulans*.** Four studies using cultivation have revealed *B. circulans* in the GI tract of rainbow  
164 trout fed different antibiotics (Austin & Al-Zahrani 1988), and studies evaluating the the  
165 intestinal microbiota of rohu (Ghosh *et al.* 2002), common carp (*Cyprinus carpio*) (Ray *et al.*  
166 2007) and Mossambicus tilapia (Saha *et al.* 2006) (Table 1). In the studies with rohu, common  
167 carp and tilapia, the strains revealed enzyme-producing activities.

168 ***Bacillus clausii*.** Ma *et al.* (2010) recovered this *Bacillus* species from the gut of orange spotted  
169 grouper (*Epinephelus coioides*) demonstrated a remarkable tolerance to mimic GI environment  
170 and antagonistic activities to some potential pathogenic bacteria.

171 ***B. coagulans*.** Ray *et al.* (2010) isolated amylase, cellulase and protease-producing  
172 autochthonous bacteria in the PI and DI of three species of Indian major carps, catla, mrigal and  
173 rohu, by cultivation and one of the strains from PI of catla showed high similarity to *B.*  
174 *coagulans*.

175 ***B. flexus***. To our knowledge, only two studies have revealed this bacterial species in the GI  
176 tract of mrigal (Banerjee *et al.* 2015b) and walking catfish (*Clarias batrachus*) (Dey *et al.*  
177 2016). In the study of mrigal, optimization of strain CMF2 was investigated by various  
178 fermentation parameters, pH, incubation temperature, incubation time, carbon sources,  
179 colloidal chitin etc. to insure optimal chitinase production. In contrast to this study, Dey *et al.*  
180 (2016) investigated the extracellular activities, amylase, protease, cellulase and lipase of several  
181 autochthonous strains isolated from PI and DI, and revealed that strain FG43 showed high  
182 similarity to *B. flexus*.

183 ***B. licheniformis***. *B. licheniformis* has been isolated from several species of cold water and  
184 warm water finfish species with various degree of pathogen inhibition and enzyme production  
185 (Table 1). Ghosh *et al.* (2010) and Mondal *et al.* (2010) isolated this bacilli species from the PI  
186 of rohu and bata (*Labeo bata*) by cultured-based methods. In an investigation by Askarian *et al.*  
187 (2012), *B. licheniformis* was identified in PI and DI of Atlantic salmon by culture method, but  
188 the strain did not display promising exo-enzyme activities or *in vitro* growth inhibition towards  
189 the four pathogens tested. Later, Banerjee *et al.* (2013) revealed that *B. licheniformis* was a  
190 member of the autochthonous enzyme-producing bacteria isolated from PI and DI of two  
191 species of Indian air-breathing fish, murrel (*Channa punctatus*) and stinging catfish using  
192 conventional culture technique. (Kim & Kim (2013) displayed culturable autochthonous exo-  
193 enzyme-producing *B. licheniformis* in the DI of farmed olive flounder. Later on, a strain of *B.*  
194 *licheniformis* was isolated from the mid-intestine (MI) of long whiskers catfish (*Mystus gulio*)  
195 by Das *et al.* (2014), and displayed exo-enzyme activities. Three studies, isolated *B.*  
196 *licheniformis* from the gut of mrigal (Mukherjee *et al.*, 2016), rohu intestine (Mukherjee *et al.*,  
197 2017) and PI of Nile tilapia (*Oreochromis niloticus*) (Ghosh *et al.*, 2017) and revealed pathogen  
198 inhibition- and potential probiotic characteristics.

199 ***B. megaterium***. To our knowledge, several studies have reported *B. megaterium* with some  
200 enzyme producing and antagonistic features in the intestine of fishfish. However, Austin & Al-  
201 Zahrani (1988) in rainbow trout, Green *et al.* (2013) in Atlantic salmon and Kim & Kim (2013)  
202 in the DI of wild olive flounder reported no major features as the authors only focused on gut  
203 microbiota evaluations. In contrast to Saha *et al.* (2006) and (Banerjee *et al.* (2016) who  
204 displayed exo-enzymes producing and exo-producing, and antagonistic *B. megaterium* in the  
205 intestines of Mossambicus tilapia and PI of grass carp (*Ctenopharyngodon idellus*).

206 ***B. methylotrophicus***. In a study by [Kim & Kim \(2013\)](#), the authors revealed that *B.*  
207 *methylotrophicus* was a part of microbial diversity in the DI of wild and farmed olive flounder.  
208 *B. methylotrophicus* was also isolated from the EI of channel catfish ([Ran et al. \(2012\)](#)) and from  
209 PI of Indian major carp (*Catla catla*) ([Mukherjee & Ghosh \(2016\)](#)). Recently, this bacilli was  
210 isolated as a part of autochthonous gut microbiota of rohu intestine with a various antagonistic  
211 effect towards pathogenic *Aeromonas* spp. More recently, [Mukherjee et al. \(2017\)](#) and  
212 [Ramirez-Torrez et al. \(2018\)](#) isolated *B. methylotrophicus* with potential probiotic features from  
213 the PI of rohu and EI content of rainbow trout, respectively.

214 ***B. nealsonii***. To our knowledge, only one study has revealed *B. nealsonii* in the intestine of  
215 finfish, rainbow trout, in a study investigated commonly used antibiotics against non-  
216 pathogenic and important fish pathogens ([Yilmaz et al. 2018](#)).

217 ***B. pumilus***. The first study, showing *B. pumilus* in the intestine of fish, rohu, was carried out  
218 by [Ghosh et al. \(2002\)](#). The isolate produced extracellular protease, amylase and cellulase, and  
219 the authors suggested that the bacterial strain might play an important role in rohu fingerlings  
220 nutrition. [Ma et al. \(2010\)](#) recovered *B. pumilus* from the gut of orange spotted grouper. An  
221 allochthonous *B. pumilus* with potential as probiotic was isolated from the EI of channel catfish  
222 by [Ran et al. \(2012\)](#). The autochthonous gut microbiota of brown trout (*Salmo trutta*) was  
223 studied by [Al-Hisnawi et al. \(2015\)](#) and *B. pumilus* was displayed a member of the identified  
224 bacterial community isolated from the fish distal intestine (DI) based on cultivation. In addition,  
225 this bacilli species, autochthonous, with a chitinae- producing character was recovered from the  
226 DI of silver carp (*Hypophthalmichthys molitrix*) by [Banerjee et al. \(2015b\)](#). In their review  
227 devoted to modulation of gut microbiota by dietary manipulations, [Ringø et al. \(2016\)](#) revealed  
228 autochthonous *B. pumilus* in the (DI) of rainbow trout (*Onchorhynchus mykiss*) fed linseed oil.  
229 However, the bacterium was not detected in the DI of fish fed sunflower-, rapeseed- or marine  
230 oil. Strains of enzyme-producing *B. pumilus* showing an antagonist characteristic were isolated  
231 from the PI mrigal and rohu ([Banerjee et al. 2016](#)). Recently, [Ramirez-Torrez et al. \(2018\)](#)  
232 successfully recovered this bacilli species from the EI rainbow trout with a good adhesion  
233 feature.

234 ***B. sonorensis***. Strains of autochthonous enzyme-producing *B. sonorensis* with antagonistic  
235 feature were recovered from the DI of mrigal by [Dutta & Ghosh \(2015\)](#), and based on their



236 results, the author suggested that *in vivo* studies merits investigations to clarify their effect on  
237 growth performance and health.

238 ***B. subtilis***. This species has been revealed in the GI tract of several finfish studies (Table 1).  
239 [Askarian et al. \(2012\)](#) reported it as autochthonous in PI and DI of Atlantic salmon, and similar  
240 findings were later reported by [Green et al. \(2013\)](#). [He et al. \(2013\)](#) investigated the microbial  
241 communities in the intestinal content of black carp (*Mylopharyngodon piceus*), gibel carp  
242 (*Carassius gibelio*) and bluntnose black bream (*Megalobrama amblycephala*) and revealed *B.*  
243 *subtilis*. A *B. subtilis* strain with a remarkable antimicrobial activity against *Edwardsiella*.  
244 *ictaluri* was isolated from the catfish intestine ([Ran et al. 2012](#)). [Banerjee et al. \(2013\)](#)  
245 recovered *B. subtilis* from the PI and DI of Indian air-breathing fish, murrel (*Channa*  
246 *punctatus*) and stinging catfish using base-culture method. Furthermore, based on culture  
247 methods this species has been recovered from DI-content cyprinid species ([Li et al., 2015](#)), the  
248 intestines of southern flounder ([Chen et al., 2016a, 2016b](#)), PI and DI of Nile tilapia ([Talukdar](#)  
249 [et al., 2016](#)) and Inidan major carp ([Kavitha et al. \(2018\)](#)). When discussing the presence of *B.*  
250 *subtilis* it worth to mention isolation of autochthonous *B. subtilis* subsp. *subtilis* from MI of  
251 mrigal ([Das & Ghosh 2013](#)) revealing high phytase activity, which later was used in a solif-  
252 state fermentation study ([Das & Ghosh 2015](#)). A potential probiotic *B. subtilis* subsp. *spizizenii*,  
253 autochthonous, was isolated from DI Indian major carp ([Mukherjee & Ghosh 2016](#)), and the  
254 authors suggested the bacterium to be a bio-control agent, but *in vivo* studies merits  
255 investigations.

256 ***B. tequilensis***. This species is seldomly isolated from finfish, as only one recent study has  
257 revealed its presence in the PI of silver carp ([Banerje et al. 2016](#)). The isolate, HMF6X, revealed  
258 high cellulase and xylanase activities vs. the other isolates isolated from the fish species. In  
259 addition, strain HMF6X revealed antagonistic activity towards *Aeromonas salmonicida*.

260 ***B. thermoamylovorans***. To our knowledge, *B. thermoamylovorans* has only been detected in  
261 one finfish study, by [Nyman et al. \(2017\)](#) investigating the bacterial community in the intestinal  
262 content of Arctic charr (*Salvelinus alpinus*). However, no further evaluation of the extracellular  
263 enzymes-production and the probiotic potential was carried out; topics that merits further  
264 investigations.

265 ***B. thuringiensis***. In a study evaluating the culturable autochthonous gut bacteria in Atlantic  
 266 salmon fed diets with or without chitin, [Askarian et al. \(2012\)](#) revealed that *B. thuringiensis*  
 267 isolated from DI produced enzymes and inhibited *in vitro* growth of four fish pathogens tested.  
 268 Later, [Yilmaz et al. \(2018\)](#) displayed *B. thuringiensis* in the intestine of rainbow trout.

269 ***B. stratosphericus***. Two cultured based studies, revealed cellulase activity by a strain isolated  
 270 from mid intestine (MI) of Piau-com-pinta (*Leporinus friderici*) ([Peixoto et al. 2010](#)). Later  
 271 [Mukherjee et al. \(2016\)](#) demonstrated the probiotic potential of an autochthonous bacteria  
 272 isolated from PI of Indian major carp.

273 ***S. silvestris***. The first study isolating *Bacillus silvestris* was carried out by [Rheims et al. \(1999\)](#)  
 274 isolating the bacterium from forest soil. In 2009, [Krishnamurthi and co-authors](#) re-classified the  
 275 bacterium to *S. silvestris*. To our knowledge, only one study has revealed *S. silvestris* in the GI  
 276 tract of fish; Indian major carp ([Mukherjee et al. 2016](#)).

## 277 **2.2. *Bacillus* in shellfish**

278 The GI tract of shellfish consist of three main segments; foregut (stomach), and mid- and  
 279 hindgut. During the last 20 years, several studies have been published on the gut microbiota of  
 280 shellfish (e.g. [Daniels et al. 2010](#); [Zhu et al. 2016](#); [Li et al. 2018b](#); [Mongkol et al. 2018](#)).  
 281 However, less information is available on *Bacillus* in shellfish intestine (Table 2), compared to  
 282 that reported for fish fish. In shellfish studies, *B. cereus* isolated to tiger shrimp (*Penaeus*  
 283 *monodon*) revealed growth and immune enhancer features ([Chandran et al. 2014](#)), while one  
 284 study by [Vargas-Albores et al. \(2017\)](#) evaluating the gut microbiota of Pacific white shrimp  
 285 (*Litopenaeus vannamei*) by culture-independent method revealed *B. cereus*, *B. thuringiensis*  
 286 and *Bacillus weihenstephanensis* ([Vargas-Albores et al. 2017](#)).

287 **Table 1 here**

## 288 **4. Products by *Bacillus*: exo-enzymes**

289 Genus *Bacillus* is ubiquitous in the environment and, is one of the most commonly used  
 290 probiotics in aquaculture ([Hong et al. 2005](#); [Zokaeifar et al. 2012b](#); [Zokaeifar et al. 2014](#)).  
 291 *Bacillus* spp. are heterogenous, both phenotypically and genotypically ([Slepecky & Hemphill](#)

292 2006). In consequence, they exhibit quite diverse physiological properties, e.g., the ability to  
293 degrade different substrates derived from plant or animal sources that include cellulose, starch,  
294 proteins, hydrocarbons (Lutz *et al.* 2006), and diverse anti-nutritional factors (Ghosh *et al.*  
295 2018). Furthermore, some *Bacillus* species are heterotrophic nitrifiers, denitrifiers, nitrogen-  
296 fixers, iron precipitators, selenium oxidizers, oxidizers and reducers of manganese, facultative  
297 chemolithotrophs, acidophiles, alkalophiles, psychrophiles, thermophiles and others (Priest  
298 1993; Slepecky & Hemphill 2006; Abriouel *et al.* 2011).

299 Being metabolically active, genus *Bacillus* produce a wide arsenal of useful enzymes and  
300 numerous antimicrobial compounds that may include antibacterial, antiviral or antifungal  
301 activity (Prieto *et al.* 2012). The probiotic attributes of the bacilli often lie with their metabolites  
302 that encompass an extensive range of substances with diverse biological functions. Generally,  
303 *Bacillus* spp. is well known as major producers of proteinaceous substances (Zokaeifer *et al.*  
304 2012a), which includes both enzymes and bacteriocins or bacteriocin like inhibitory substances  
305 (BLIS). This section provides an overview on the exo-enzymes and bacteriocins produced by  
306 bacilli and their beneficial attributes, with notes on present status of knowledge and prospects  
307 in aquaculture. Although enzyme-producing ability of bacilli from diverse sources has been  
308 widely known, this section focus on the autochthonous enzyme-producing bacilli recorded from  
309 fish gut. Autochthonous gut-adherent bacteria seemed to be ideal for aquaculture application,  
310 since microorganisms from the fish source might ensure their colonization and enzyme  
311 supplementation within the intestine, and otherwise, would likely to eliminate the risk of  
312 harmful effects by the microorganisms or their metabolites (Ghosh *et al.* 2018).

#### 313 4.1. Gut associated bacilli and their exo-enzymes

314 Unlike the ruminants and higher vertebrates, probable contribution of the endosymbionts in the  
315 nutritional physiology of fish has been recognized of late (Ray *et al.* 2012). Apart from the  
316 endogenous digestive enzymes, exogenous enzymes produced and supplemented by the  
317 autochthonous microbiota could be considered as one of the important secondary factors that  
318 affects nutrition and feed utilization in fish. In fact, a wide range of enzymes, viz.,  
319 carbohydrases, phosphatases, esterases, lipases and peptidases produced by gut bacteria might  
320 contribute to the digestive processes in fish (Ghosh *et al.* 2018). Extensive studies on Indian  
321 major carps (e.g., Ray *et al.* 2012; Mandal & Ghosh 2013; Das & Ghosh 2014; Dutta *et al.*  
322 2015; Dutta and Ghosh, 2015; Banerjee *et al.* 2016; Mukherjee & Ghosh 2016; Mukherjee *et*

323 *al.* 2016; Mukherjee *et al.* 2017) and other teleosts (e.g., Cahil 1990; Ringø *et al.* 1995, 2010,  
324 2016; Llewellyn *et al.* 2014; Al-Hisnawi *et al.* 2014; Hosseinifar *et al.* 2016; Ringø & Song  
325 2016) have indicated the presence of autochthonous gut-associated microorganisms in fish and  
326 their beneficial attributes in nutrition. The enzymes of nutritional importance produced by the  
327 gut bacteria may be categorized into (1) digestive enzymes, e.g., protease, amylase, lipase etc.,  
328 and (2) degradation enzymes, e.g., non-starch polysaccharide (NSP) - degrading enzymes,  
329 phytase, tannase and chitinase. The review of Ray *et al.* (2012) illustrated contribution of the  
330 diverse exo-enzyme producing gut bacteria in the nutrition and well being of the host fish,  
331 where gut-associated bacilli were recognized as one of the major groups of bacteria within fish  
332 gut. Later, ability of the fish gut-microbiota in enzymatic degradation of plant-derived anti-  
333 nutritional factors was displayed by Ghosh *et al.* (2018), in which numerous strains of *Bacillus*  
334 were promising. Therefore, the following sub-section will present an extract and update of the  
335 research endeavours depicting the occurrence and importance of exo-enzyme producing gut  
336 associated bacilli in fish (Table 2).

#### 337 4.2. Exo-enzyme producing bacilli: digestive enzymes

338 Endogenous digestive enzymes in fish hydrolyze organic macromolecules (mainly,  
339 carbohydrate, protein and lipid) into simpler compounds. In addition, as established for higher  
340 vertebrates, supplementation of digestive enzymes (viz., amylase, protease, lipase) from  
341 microbial source could be of importance to improve nutrient utilization in fish. Although  
342 preliminary studies on microbial amylase activity within fish gut noticed the presence of  
343 bacterial amylase, characterization and identification of the specific amylase-producing strains  
344 were mostly not carried out (Lesel *et al.* 1986; Das & Tripathi 1991; Bairagi *et al.* 2002). On  
345 the other hand, microorganisms with efficient proteolytic activity are widespread in nature  
346 because of their rapid growth and *Bacillus* spp. by far the most common among them (Ray *et*  
347 *al.* 2012). To the authors' knowledge, occurrence of proteolytic and amylolytic bacilli (*B.*  
348 *circulans*, *B. cereus* and *B. pumilus*) was first reported in the gut of an Indian major carp (IMC),  
349 rohu by Ghosh *et al.* (2002) and correlated it with the feeding habit. However, they didnt  
350 quantify enzyme activities. Later, Esakkiraj *et al.* (2009) documented extracellular protease  
351 production by *B. cereus* isolated from the gut of flathead grey mullet (*Mugil cephalus*) and  
352 indicated that bacterial protease was efficient in utilizing different preparations of tuna-  
353 processing wastes, e.g., raw fish meat, defatted fish meat and alkali or acid hydrolysate as

354 nitrogen sources. Mondal *et al.* (2010) detected both protease- and amylase-producing ability  
355 of *B. licheniformis* and *B. subtilis* in the gut of bata. Similarly, Ray *et al.* (2010) isolated various  
356 strains of bacilli in the gut of three Indian major carps, viz. catla (*B. coagulans*, *B. cereus*);  
357 mrigal (*Bacillus* sp., *B. cereus*) and rohu (*Bacillus* sp.), and protein or starch hydrolyzing  
358 abilities were demonstrated. Subsequently, presence of amylase- and protease-producing bacilli  
359 (*B. thuringiensis*, *B. cereus*, *B. subtilis*, *Bacillus* sp.) in the gut of a marine teleost (Atlantic  
360 salmon) were recorded by Askarian *et al.* (2012). Later, *Brevibacillus parabrevis* and *B.*  
361 *licheniformis* isolated from two brackish water fish species, crescent perch (*Terapon jarbua*)  
362 and long whiskers catfish (*Mystus gulio*), respectively, were noticed with considerable amylase  
363 and protease activities *in vitro* (Das *et al.* 2014). Since then, diverse *Bacillus* spp. capable of  
364 producing amylolytic and proteolytic enzymes were documented from several fish species that  
365 included IMCs (Dutta & Ghosh 2015; Dutta *et al.* 2015; Mukherjee & Ghosh 2016; Mukherjee  
366 *et al.* 2016; Mukherjee *et al.* 2017); climbing perch (*Anabas testudineus*) (Banerjee *et al.*  
367 2015a); walking catfish (*Clarias batrachus*) (Banerjee *et al.* 2015a; Dey *et al.* 2016) grass carp  
368 and rohu (Guo *et al.* 2016; Banerjee *et al.* 2017), striped dwarf catfish (*Mystus vittatus*) (Nandi  
369 *et al.* 2017a) and Indian major carp (Kavitha *et al.*, 2018), a brief description of which are  
370 presented in Table 2.

371 Gut bacteria might induce lipolysis either by enzymatic breakdown of triglyceride through  
372 direct bacterial action, or by altering pancreatic lipase activity with bacterial proteases (Ringø  
373 *et al.* 1995). Although reports on specific lipase-producing bacilli from fish gut were scarce,  
374 some of the studies describing amylase, protease or cellulase-producing bacilli within fish gut  
375 also addressed lipolytic activity, if any. Thus, lipase-producing bacilli were detected in the guts  
376 of IMCs (Dutta & Ghosh 2015; Dutta *et al.* 2015; Mukherjee & Ghosh 2016; Mukherjee *et al.*  
377 2016, Mukherjee *et al.* 2017); Atlantic salmon (Askarian *et al.* 2012); brackish water fishes, *T.*  
378 *jarbua* and *M. gulio* (Das *et al.* 2014) catfishes (Dey *et al.* 2016; Nandi *et al.* 2017a) and Nile  
379 tilapia (Ghosh *et al.* 2017).

#### 380 4.3. Exo-enzyme producing bacilli: degradation enzymes

381 Cellulose and hemicelluloses (e.g., xylans) are the major NSPs in plant feedstuffs commonly  
382 encountered by the fish under culture condition, either through natural food (algae,  
383 phytoplankton, detritus, aquatic macrophytes) or formulated diets as there is a thrust to replace  
384 animal sources in fish feed with plant ingredients (rice bran, wheat husks, different oil cakes).

385 The principal endogenous polysaccharide digesting enzymes in fish specifically hydrolyze the  
386  $\alpha$ -glycosidic linkages of starch and yield glucose. However, cellulose remains mostly indigestible  
387 in monogastric animals due to the presence of  $\beta$ -(1 $\rightarrow$ 4) glycosidic linkages and lack of the  
388 endogenous cellulase. Likewise,  $\beta$ -glucanases and  $\beta$ -xylanases capable of digesting other NSPs  
389 are also either rare or not present in fish (Kuźmina 1996). Symbiotic gut microorganisms are  
390 likely to be involved in the fermentative degradation of cellulosic material for the host fish  
391 (Clements 1997). Thus, among the degradation enzymes, emphasis has been given on the ability  
392 of the gut-microbiota to produce cellulase as the major NSP-degrading enzyme (for review, see  
393 Ray *et al.* 2012). Although presence of microbial cellulase within the fish gut was first indicated  
394 in the common carp (Shcherbina & Kazlauskienė 1971), involvement of gut-associated bacilli  
395 in cellulase production was detected much later (Ghosh *et al.* 2002). Protease- and amylase-  
396 producing bacilli (*B. circulans*, *B. cereus* and *B. pumilus*) isolated from rohu were also efficient  
397 in producing cellulase, although none of the enzymes-producing ability was quantified (Ghosh  
398 *et al.* 2002). Importantly, presence of diverse exo-enzyme producing bacilli was correlated it  
399 with the omnivorous feeding aptitude of the concerned carp species. Afterwards, a large number  
400 of cellulose-degrading bacilli have been recorded in the guts of grass carp and tilapia (Saha *et al.*  
401 *et al.* 2006); rohu, catla and mrigal (Ray *et al.* 2010); bata (Mondal *et al.* 2010); pacu (*Piaractus*  
402 *esoptamicus*) and piaucom–piñata (*Leporinus friderici*) (Peixoto *et al.* 2011); walking catfish  
403 (Dey *et al.* 2016) and striped dwarf catfish (Nandi *et al.* 2017a). While considering *in vitro*  
404 cellulase-producing ability of the gut bacilli, Ray *et al.* (2007) noticed that cellulase production  
405 by *B. subtilis* CY5 and *B. circulans* TP3, isolated from the gut of common carp and  
406 Mozambique tilapia, *Oreochromis mossambicus*, respectively was enhanced under optimized  
407 condition through solid-state fermentation (SSF). Further, Peixoto *et al.* (2011) noticed  
408 cellulolytic potential of *B. subtilis* P6 and *Bacillus velesensis* P11, and pH 7.0-9.0 was recorded  
409 for the maximum residual cellulase activity. These observations were instrumental in view of  
410 future utilization of the cellulolytic bacilli in bio-processing of plant feedstuffs *in vitro*.

411 On the contrary, reports on xylanase-producing gut microorganisms in fish are meager (German  
412 & Bittong 2009; Banerjee & Ghosh 2014; Banerjee *et al.* 2016). Banerjee *et al.* (2016) carried  
413 out screening of cellulose and xylan degrading autochthonous gut bacteria from six freshwater  
414 carps. In their study, the strains *Bacillus pumilus* LRF1X, *B. pumilus* CMF1C, *B. tequilensis*  
415 HMF6X, *B. megaterium* CtIF1C and *B. altitudinis* CMH8X revealed both xylan- and cellulose-  
416 degrading ability. Following which, degradation of cellulose and xylan under SSF by *B.*

417 *pumilus* and *B. tequilensis* isolated from the proximal intestines of rohu and silver carp,  
418 respectively, has also been documented (Banerjee & Ghosh 2016). Furthermore, cellulase and  
419 xylanase-producing ability of autochthonous bacilli isolated from the gut of rohu, catla and  
420 mrigal have been reported (Dutta *et al.* 2015; Dutta & Ghosh 2015; Mukherjee *et al.* 2016;  
421 Mukherjee & Ghosh 2016; Mukherjee *et al.* 2017), which are summarized in Table 2.

422 Phytase is another important degradation enzyme, supplementation of which might improve the  
423 availability of phosphorus and other minerals bound to phytic acid by hydrolysis of the phytate  
424 compounds (Oatway *et al.* 2001). Protein rich oil cakes used in aquafeed formulation are the  
425 major source of phytate compounds. Only a few reports have considered exogenous phytase  
426 activity represented by gut bacteria in fish. The first study, indicating phytase-producing bacilli  
427 within fish gut was accomplished by Roy *et al.* (2009), who depicted two phytase-producing  
428 strains of *B. licheniformis* from rohu. Afterward, Askarian *et al.* (2012) demonstrated phytase  
429 activity by autochthonous *B. subtilis*, *B. thuringiensis*, *B. cereus* and *Bacillus* sp. isolated from  
430 the gut of Atlantic salmon fed with or without chitin supplemented diet, although phytase-  
431 producing ability was not quantified. In a comprehensive investigation of phytase-producing  
432 bacteria in freshwater teleosts, Khan & Ghosh (2012) documented *B. subtilis* LB1.4 and *B.*  
433 *atrophaeus* GC1.2 isolated from the digestive tracts of a minor carp, bata and Indian river shad,  
434 *Gudusia chapra*, respectively as efficient phytase-producing strains. Further, phytase-  
435 producing capacity of *B. subtilis* was evaluated *in vitro* in a later study under SSF and phytate-  
436 degrading ability of the strain was established (Khan & Ghosh 2012). In addition, phytase-  
437 producing ability of autochthonous exo-enzyme producing bacilli isolated from the IMCs have  
438 been reported in subsequent studies (Dutta *et al.* 2015; Dutta & Ghosh, 2015; Mukherjee *et al.*  
439 2016; Mukherjee & Ghosh 2016; Mukherjee *et al.* 2017).

440 Tannins are widespread in nature and are considered as the most common among the plant-  
441 derived anti-nutritional factors. Tannin-degrading ability of *Bacillus* and some other genera  
442 have been recorded by Deschamps *et al.* (1980). Presence of tannase-producing microbiota has  
443 been detected in the digestive tract of ruminants feeding on tannin rich forage (Goel *et al.* 2005).  
444 However, information on tannase-producing bacteria from fish gut is scanty as studies carried  
445 out on this topic are inadequate. To the authors' knowledge, only one study illustrated tannase-  
446 producing bacilli in fish gut, where *B. subtilis* KP765736 and *Brevibacillus agri* KP765734  
447 isolated from Nile tilapia represented tannase activity (Talukdar *et al.* 2016). The authors

448 hypothesized that the tannase-producing bacteria established a symbiotic relation with the host  
449 fish and adapted to the neutral or alkaline pH of the fish gut (Talukdar *et al.* 2016).

450 Chitin is considered as the second most abundant biomass in the world after cellulose that forms  
451 a major constituent in many fish food organisms, viz., protozoans, coelenterates, crustaceans,  
452 molluscs, fungi and green algae (Ray *et al.* 2012). Although the first report of bacterial chitin  
453 destruction involved *Bacillus chitinovorius* isolated from an aquatic source (Benecke 1905),  
454 chitinolytic bacilli from fish gut was documented much later. Askarian *et al.* (2012) recorded  
455 chitinase-producing ability of bacilli in the digestive tract of Atlantic salmon fed with (*B.*  
456 *subtilis*) or without (*B. thuringiensis*, *B. cereus* and *Bacillus* sp.) chitin supplemented diets.  
457 Further, Banerjee *et al.* (2015b) detected potent chitinolytic activity of *B. pumilus* KF454036  
458 and *B. flexus* KF454035 isolated from the digestive tracts of silver carp, and mrigal, respectively  
459 and opined that there is possibility of using chitinolytic bacilli from fish gut for chitinase  
460 production or as probiotics to improve feed efficiency in fish.

#### 461 **4.4. Application of gut associated bacilli in feed-biotechnology**

462 Apart from the use of bacilli as probiotic feed supplements, the recent review of Ghosh *et al.*  
463 (2018) proposed to utilize the exo-enzyme producing microorganisms judiciously for bio-  
464 processing of the plant feedstuffs through SSF. Exo-enzyme producing bacilli isolated from  
465 fish gut have been shown to reduce the contents of anti-nutritional factors, viz., fibre, tannin,  
466 phytic acid and trypsin inhibitor in the plant ingredients through SSF (Bairagi *et al.* 2004;  
467 Ramachandran *et al.* 2005; Ramachandran & Ray 2007; Khan & Ghosh 2013). Apart from  
468 elimination of the anti-nutritional factors, increase in nutrient level through microbial synthesis  
469 of essential bio-molecules (viz., amino acids, fatty acids and vitamins) is expected during the  
470 bio-processing through SSF (Banerjee & Ghosh 2016). Thus, SSF has been suggested to  
471 improve digestibility of feedstuffs by augmenting bioavailability of nutrients, reducing  
472 antinutrients and synthesizing bio-molecules (Ghosh *et al.*, 2018). However, in view of  
473 application of the SSF-processed substrate as fish feed ingredient, microbial symbionts  
474 originally isolated from fish gut are preferred in this process so as to avoid likely inclusion of  
475 harmful metabolites in the fermentation-product (Khan & Ghosh 2013).

476 Bacterial cellulase supplementation in the formulated diet (as probiotics) or processing of plant  
477 feedstuffs with cellulase-producing bacilli have been used in a number of instances (Bairagi *et*  
478 *al.* 2002; Ghosh *et al.* 2004). Ramachandran & Ray (2007) conducted fermentation of black  
479 gram seed meal with a *Bacillus* sp. that was effective in reducing crude fibre, tannin and phytic



480 acid. Further, in an attempt to use phytase-producing bacilli in processing of plant ingredient,  
481 *B. licheniformis* LF1 and *B. licheniformis* LH1 isolated from rohu were used for fermentation  
482 of sesame oilseed meal (Roy *et al.* 2014). Diets incorporated with fermented oilseed meal  
483 significantly ( $P<0.05$ ) improved the apparent digestibility of macro-molecules (protein, lipid)  
484 and minerals (phosphorus, calcium, copper, iron and manganese). Another study reporting  
485 processing of sesame oil cake by the phytase-producing fish gut bacterium, *B. subtilis* subsp.  
486 *subtilis* through SSF resulted in significant ( $P<0.05$ ) reduction of the anti-nutrients (e.g. phytic  
487 acid, tannins, trypsin inhibitor and crude fibre), while levels of free amino acids, fatty acids and  
488 different minerals were noticed to be enhanced (Das & Ghosh 2015). Subsequently,  
489 fermentation (SSF) of linseed oil-cake with *B. pumilus* LRF1X and *B. tequilensis* HMF6X  
490 revealed minor enhancement in the contents of macro-molecules, total free amino acids and  
491 fatty acids as compared to the raw substrate (Banerjee & Ghosh 2016). In addition, anti-  
492 nutrients (cellulose, hemicelluloses, crude fibre, tannins, phytic acid and trypsin inhibitor) were  
493 significantly ( $P<0.05$ ) reduced and more interestingly, amino acid composition indicated  
494 significant ( $P<0.05$ ) boost in the levels of numerous amino acids (arginine, cystine, histidine,  
495 isoleucine, methionine, phenylalanine, threonine, tryptophan and valine) in the fermented oil-  
496 cake, although, lysine and leucine contents were decreased (Banerjee & Ghosh 2016).  
497 Therefore, it seems that fermentation by autochthonous exo-enzyme producing bacilli could be  
498 effective in improving the feed value of the plant feedstuffs, which would have a great  
499 contribution in aquafeed-technology in the upcoming era.

##### 500 **5. Bacteriocins produced by *Bacillus* and their antibacterial activity**

501 The antimicrobial compounds produced by bacteria are usually divided into two major groups;  
502 (1) non-ribosomal secondary metabolites, such as peptide or lipopeptide antibiotics and (2)  
503 ribosomally synthesized proteins/peptides, such as bacteriocins (Abriouel *et al.* 2011; Lee &  
504 Kim, 2011). Bacteriocins are ribosomally synthesized antimicrobial peptides produced by  
505 bacteria that often present bactericidal effects against other closely related species (Cotter *et al.*  
506 2005). Bacteriocins are the most abundant and diverse of the bacterially produced  
507 antimicrobials (Riley, 2009). These heterogeneous substances portray variable biochemical  
508 properties, inhibitory spectra, molecular weights and mechanisms of action (OSullivan *et al.*  
509 2002). Antibiotics have been used in the fish health manager's for the treatment of bacterial  
510 diseases in fish since years. However, massive use of broad spectrum antibiotics and  
511 antimicrobial drugs increases the selective pressure by developing emergence of bacterial

512 resistance (Verschuere *et al.* 2000). The development of antibiotic resistance among the  
513 microorganisms associated with fish diseases has become a global concern during the past years  
514 (Kolndadacha *et al.* 2011). Thus, much interest has been paid on the search for novel  
515 antibacterial compounds, preferably proteins with prophylactic and/or curative potential, for  
516 which the pathogens may not develop resistance (Patil *et al.* 2001). In this regard, bacteriocins  
517 from natural sources have been suggested to be an alternative to control the bacterial diseases  
518 in aquaculture (Kim *et al.* 2014; Sahoo *et al.* 2016).

519 Bacteriocins produced by lactic acid bacteria (LAB) are the most widely studied (Nes *et al.*  
520 2007; Ringø *et al.* 2018), while, the *Bacillus* spp. have been less studied in this respect (Abriouel  
521 *et al.* 2011). The genus *Bacillus* includes an assortment of industrially important species and  
522 has a history of safe use in both food and industry (Paik *et al.* 1997). Moreover, investigations  
523 of the antimicrobial potential of genus *Bacillus* is of interest to investigate, as bacilli produce  
524 several bacteriocins or bacteriocin-like inhibitory substances (BLIS) representing different  
525 chemical structures (Von Döhren, 1995; Abriouel *et al.* 2011). The production of bacteriocins  
526 or BLIS has been described for some *Bacillus* species including *B. thuringiensis* (Paik *et al.*  
527 1997), *B. subtilis* (Zheng *et al.* 1999), *B. cereus* (Bizani & Brandelli 2002) and *B.*  
528 *amyloliquefaciens* (An *et al.* 2015). Like the LAB, the genus *Bacillus* also includes  
529 representatives that are generally recognized as safe (GRAS), such as *B. subtilis* and *B.*  
530 *licheniformis* (Smitha & Bhat 2013), and hence can be used in the aquaculture as probiotics for  
531 prevention of some bacterial diseases such motile *Aeromonas* septicemia and vibriosis. *Bacillus*  
532 bacteriocins are increasingly becoming imperative owing to their broader spectra of inhibition  
533 that may include some Gram-negative bacteria and Gram-positive species belonging to genera  
534 of *Aeromonas*, *Edwardsiella*, *Streptococcus*, *Pseudomonas* and *Vibrio* (Dutta & Ghosh 2015;  
535 Chen *et al.* 2016b; Chen *et al.* 2016b; Sumathi *et al.* 2017)

536 The gut microbiota in some finfish species such as Indian carp has been reported to be fairly  
537 dominated by the genus *Bacillus* (Class I of the phylum Firmicutes i.e. the bacilli), that has  
538 become evident through both culture dependent and culture in-dependent methods (Ringø *et al.*  
539 2006; Ghosh *et al.* 2010; Ray *et al.* 2010; Sarkar & Ghosh 2014; Li *et al.* 2015; Das & Ghosh  
540 2015; Mukherjee *et al.* 2016; Yilmaz *et al.* 2018). Further, fish gut associated bacilli are known  
541 to play an important role for prevention of infections in aquaculture by production of  
542 antibacterial substances (e.g. Dimitroglou *et al.* 2011; Mukherjee *et al.* 2017; Nandi *et al.* 2017a;

543 [Nandi et al. 2017b](#); [Ghosh et al. 2017](#)). Although several studies on bacteriocins from *Bacillus*  
544 have exhibited their important aspects of food safety ([Gautam & Sharma 2009](#); [Abriouel et al.](#)  
545 [2011](#); [Nath et al. 2015](#)), very few have addressed the potential application of these antimicrobial  
546 substances to be used against bacterial diseases in aquaculture ([Ran et al. 2012](#); [Kim et al. 2014](#);  
547 [Luo et al. 2014](#); [Guo et al. 2016](#); [Mukherjee et al. 2017](#)). Moreover, studies on fish gut  
548 associated bacilli with regard to likely bacteriocinogenic potential have been rarely addressed  
549 ([Sirtori et al. 2006](#); [Giri et al. 2011](#)). Therefore, it needs extensive study for screening and  
550 characterization of the bacteriocinogenic bacilli from the fish gut and their antibacterial  
551 compounds to obtain more scientific understanding and knowledge to prevent diseases in  
552 aquaculture. This section will present an overview of the diverse classes of bacteriocins  
553 produced by bacilli, and an update on the efficacy of fish gut associated *Bacillus* spp. against  
554 fish pathogens, and their prospective future applications.

#### 555 **5.1. Classes of bacteriocins produced by *Bacillus***

556 Genus *Bacillus* are known to produce a wide variety of  
557 antimicrobial substances that include peptide or lipopeptide antibiotics, bacteriocins and  
558 bacteriocin-like inhibitors ([Stein 2005](#), [Sumi et al. 2015](#)). Antimicrobial peptides produced by  
559 bacteria through ribosomal synthesis are generally referred to as bacteriocins, which are a  
560 heterologous group of proteinaceous antimicrobial substances and known to produce by  
561 bacteria from every major group ([Riley & Wertz 2002a,b](#)). Many other antimicrobial substances  
562 that are not well characterized or the peptide nature of the compound has not been confirmed  
563 and they are referred to as bacteriocin-like inhibitory substances (BLIS) ([Abriouel et al. 2011](#)).  
564 The *Bacillus* group of bacteria often produce lipopeptide antibiotics by non-ribosomal synthesis  
565 (e.g. iturins). This section will present an overview on the classification of bacteriocins or BLIS  
566 produced by the bacilli excluding the non-ribosomally synthesized peptides.

567 To date, most of the classification efforts are made with the bacteriocins from LAB. The  
568 classification scheme of LAB bacteriocins was primarily developed by [Klaenhammer \(1993\)](#),  
569 who grouped bacteriocins into four distinct classes with sub-classes: Class I or lantibiotics  
570 (post-translationally modified, thermostable peptides, containing lanthionine or derivatives, <5  
571 kDa); Class II (unmodified small heat-stable peptides, <10 kDa); Class III (unmodified large  
572 heat-labile peptides, >30 kDa) and Class IV (large complex proteins, containing carbohydrates  
573 or lipid moieties). This grouping has formed the basis of all subsequent classification schemes

574 for bacteriocins produced by the Gram-positive bacteria. Readers with special interest are  
575 referred to the reviews of [Mokoena \(2017\)](#) and [Ringø et al. \(2018\)](#) where updated classification  
576 schemes of bacteriocins produced by the LAB have been presented. Genus *Bacillus* may be  
577 considered as the second most important group for the production of bacteriocins and BLIS  
578 after the LAB. It was evident that the bacteriocins produced by *Bacillus* spp. display broad  
579 range of molecular mass; e.g. the smallest bacteriocin-like compound (800 Da) is produced by  
580 *B. licheniformis* ([Teixeira et al. 2009](#)), whereas *B. thuringiensis* produced the largest  
581 bacteriocin (950 kDa) such as thuricin ([Cherif et al. 2001](#)). Many *Bacillus* bacteriocins belong  
582 to the lantibiotics, post-translationally modified peptides commonly dispersed among different  
583 bacterial groups. Lantibiotics are the best-described antimicrobial peptides with regard to  
584 biosynthesis mechanisms, genetic determinants and peptide structure. Members of the genus  
585 *Bacillus* also produce many other unmodified bacteriocins within Class II of LAB bacteriocins  
586 ([Klaenhammer 1993](#); [Drider et al. 2006](#); [Nes et al. 2007](#)), which includes the pediocin-like  
587 bacteriocins (Class IIa) and the two-peptide bacteriocins (Class IIb), while others show  
588 completely novel peptide sequences ([Abriouel et al. 2011](#)). Some of the well described  
589 bacteriocins produced by *Bacillus* spp. are, subtilin by *B. subtilis* ([Banerjee & Hansen 1988](#)),  
590 cerein by *B. cereus* ([Oscariz et al. 1999](#)), bacillocin 490 by *B. licheniformis* ([Martirani et al.](#)  
591 [2002](#)), haloduracin by *B. halodurans* ([Lawton et al. 2007](#)), thuricin by *B. thuringiensis* ([Gray](#)  
592 [et al. 2006](#)), subtilosin by *B. amyloliquifaciens* ([Sutyak et al. 2008](#)), and megacin by *B.*  
593 *megaterium* ([Kiss et al., 2008](#)), and they are mostly active against Gram-positive organisms  
594 such as *Listeria monocytogenes*, *Gardnerella vaginalis*, *Streptococcus agalactiae*,  
595 *Staphylococcus aureus* and *Leuconostoc mesenteroides*. In view of the increasing number of  
596 bacteriocins described within different bacterial groups such as lactic acid bacteria, it is very  
597 difficult to ascertain a combined classification scheme of bacteriocins ([Nes et al. 2007](#)).  
598 Therefore, the *Bacillus* bacteriocin classification system may be adopted independently even  
599 though some compounds produced by both *Bacillus* and LAB have very similar characteristics.  
600 Consequently, a simple classification scheme for the bacteriocins / BLIS produced by the  
601 *Bacillus* spp. was proposed by [Abriouel et al. \(2011\)](#) that holds three classes of bacteriocins  
602 ([Table 3](#)): Class I (antimicrobial peptides that undergo post-translational modifications); Class  
603 II (small nonmodified and linear peptides, heat and pH stable, 0.77–10 kDa) and Class III (large  
604 proteins with phospholipase activity, >30 kDa).

605

## 5.2. Pathogen inhibition by bacilli and their bacteriocinogenic activity

Bacterial antagonism is a regular incident in nature that might play a vital role in maintaining the balance between potentially beneficial and pathogenic microorganisms associated with fish (Balcázar *et al.* 2006; Pandiyan *et al.* 2013). Growth inhibition of pathogenic bacteria by the beneficial bacteria could be due to the individual or combined production of antibacterial metabolites (e.g., bacteriocins, siderophores, lysozymes, proteases), competition for essential nutrients, alteration of pH by organic acid production and competitive exclusion (Verschuere *et al.* 2000; De Vrese & Schrezenmeir 2008; Laloo *et al.* 2010; Mukherjee & Ghosh 2016). Among these, antimicrobial peptides or bacteriocins have received major attention as an alternative bio-control agent limiting colonization of pathogenic bacteria in GI fish (Ghanbari *et al.* 2013). Consequently, *Bacillus* spp. isolated from different environments presented a huge variety of species producing bacteriocins or BLIS, which displayed antimicrobial activity against other bacteria including fish pathogens (Abriouel *et al.* 2011; Sahoo *et al.* 2016). In general, the majority of aquatic origin bacteriocins with antagonistic activities against bacterial pathogens have been reported from the marine fish with a lesser extent from freshwater fish species (Sahoo *et al.* 2016). Moreover, bacteriocinogenic bacilli directly recovered from fish and/or characterizations of the bacteriocins are sparse (Sirtori 2006; Bhaskar *et al.* 2007; An *et al.* 2015). Compare to allochthonous microbiota, the use of autochthonous microbiota and their metabolites might be preferred to induce disease resistance e.g. inhibiting the pathogens and avoid their likely harmful effects on the host fish and the normal microbiota (Ringø *et al.* 2018). Numerous studies have revealed that genus *Bacillus* is a part of the autochthonous bacterial community in fish (Ray *et al.* 2012; Ghosh *et al.* 2018). In some aquaculture species such as Indian carp, the justification of using bacilli or bacteriocinogenic bacilli of autochthonous nature might be associated with the bacterial strains, target pathogens and the hosts, all essentially share the same ecological niche (Mukherjee *et al.* 2016; 2017). In view of application of bacteriocinogenic bacilli, research endeavours towards disease resistance or pathogen inhibition in aquaculture may be grouped into three categories: (1) bacteriocin production has not been confirmed, but assumed to be the reason behind pathogen inhibition, (2) proteinaceous nature of the BLIS has been confirmed with partial characterization, and (3) specific bacteriocins produced by bacilli has been characterized and identified. The most reports are associated with the first two categories. In contrast, studies on bacteriocinogenic bacilli

637 from aquatic sources and identification of bacteriocins produced by aquatic  
638 animals/environments are scarce (Table 4).

639 During the last two decades, information on antagonism by fish gut associated bacilli against  
640 diverse pathogenic bacteria has become available, and this section presents an overview on the  
641 pathogen inhibitory bacilli isolated from different fish species (Table 5). Antimicrobial  
642 substances produced by bacilli isolated from GI tracts of Japanese coastal fish (Sugita *et al.*  
643 1998) and an Indian major carp (IMC), rohu (Giri *et al.* 2011) have been reported. Antibacterial  
644 compound produced by *B. licheniformis* P40 isolated from an Amazon basin fish *Leporinus* sp.  
645 was bactericidal and bacteriolytic to *Listeria monocytogenes* (Cladera-Olivera *et al.* 2004). In  
646 addition, *B. subtilis* SG4 (Ghosh *et al.* 2007), *B. aerius* CCH1A and *B. sonorensis* CCH1Ph  
647 (Dutta *et al.* 2015) isolated from mrigal, *C. mrigala*; *B. methylotrophicus* isolated from channel  
648 catfish, *Ictalurus punctatus* (Ran *et al.* 2012) and catla, *Catla catla*, (Mukherjee & Ghosh  
649 2016); and *B. cereus* and *B. circulans* obtained from the GI tract of some other fish species  
650 (Laloo *et al.* 2010; Geraylou *et al.* 2014) were established as antagonistic against different  
651 strains of *Aeromonas hydrophila* pathogenic to fish. Strains of *B. methylotrophicus* isolated  
652 from soil or channel catfish intestine inhibited fish pathogens causing enteric septicaemia (*E.*  
653 *ictaluri*) and motile aeromonad septicaemia (*A. hydrophila*) (Ran *et al.* 2012). Another strain,  
654 *B. subtilis* BHI344, isolated from the GI tract of channel catfish was shown to depressed growth  
655 of pathogenic *A. hydrophila*, *A. sobria*, and *A. caviae*, *in vitro* (Luo *et al.* 2014); while strain  
656 *B. sonorensis* CM2H3L isolated from the gut of mrigal inhibited *in vitro* growth of *A.*  
657 *salmonicida* (Dutta & Ghosh 2015). Mukherjee *et al.* (2016) revealed that *B. stratosphericus*  
658 KM277362, *B. aerophilus* KM277363, *B. licheniformis* KM277364 and *S. silvestris*  
659 KM277365 isolated from the GI tract of mrigal inhibited *in vitro* growth of *A. hydrophila*, *A.*  
660 *salmonicida*, *P. fluorescens* and *P. putida*. Inhibition of pathogenic aeromonads was also  
661 revealed by *B. methylotrophicus* (NR116240), *B. amyloliquefaciens* (NR117946) and *B.*  
662 *licheniformis* (NR118996) isolated from rohu (Mukherjee *et al.* 2017). However,  
663 characterization and identification of the antibacterial compounds produced by these gut  
664 bacteria was not evaluated in the above mentioned studies, and inhibition was assumed to be  
665 due to bacteriocins or BLIS. Some of these observations made an attempt for partial purification  
666 and characterization of the inhibitory substances, and the proteinaceous nature of the BLIS were  
667 confirmed (Giri *et al.* 2011; Mukherjee *et al.* 2017). Among three bacilli species; *B. subtilis*, *B.*  
668 *cereus* and *B. amyloliquefaciens* recovered from the gut of IMC, only *B. amyloliquefaciens*

669 demonstrated antagonistic activity against three fish pathogens *A.*  
670 *hydrophila*, *Acinetobacter* sp. and *Acinetobacter tandoii* (Kavitha *et al.*, 2018). *B. subtilis*  
671 (ATCC 6633) inhibited *in vitro* growth of *A. hydrophila* and *P. fluorescens* (Aly *et al.* 2008),  
672 while *B. subtilis* strains isolated from grass carp intestine exhibited inhibitory activities against  
673 fish pathogenic bacteria, including *A. hydrophila*, *A. punctata*, *E. ictaluri*, *A. punctata f.*  
674 *intestinali*, *Vibrio flurialis*, and *Str. agalactiae*, but the inhibitory effect varied with highest  
675 effect against *A. hydrophila* and *A. punctata* (Guo *et al.*, 2016). In a study by Banerjee *et al.*  
676 (2017), *B. subtilis* LR1 isolated from the GI tract of rohu exhibited a bacteriocin (~50 kDa)  
677 with inhibitory activity against four fish pathogens; *B. mycoides*, *A. salmonicida*, *P. fluorescens*  
678 and *A. hydrophila*. *B. subtilis* and *B. amyloliquefaciens* recovered from GI tract of marine fish  
679 (*Paralichthys lethostigma*) and cultured pond of sea cucumber exhibited antagonistic activity  
680 against *V. anguillarum*, *V. harveyi*, *V. vulnificus*, *Streptococcus* sp. and *Staphylococcus aureus*  
681 (Chen *et al.*, 2016a). Among the eight *Bacillus* strains isolated from the intestine of the fish  
682 species; *Scophthalmus maximus*, *Paralichthys olivacues*, *Epinephelus coioides* and  
683 *Clupanodon punctatus*, revealed *B. amyloliquefaciens* M001 more antagonistic activity against  
684 multiple aquatic bacterial pathogens including *V. anguillarum*, *V. campbellii*, *V. vulnificus*, *V.*  
685 *parahamolyticus*, *Streptococcus* sp. and *Edwardsiella tarda* (Chen *et al.* 2016b). It is worth to  
686 mention that very few of the well-characterized bacteriocins produced by fish gut associated  
687 *Bacillus* spp., e.g., *Bacillus* sp. P45 isolated from *Piaractus mesopotamicus*, Amazon basin  
688 fish (Sirtori *et al.*, 2006) and *B. amyloliquefaciens* isolated from the marine fish *Epinephelus*  
689 *areolatus* (An *et al.*, 2015) have been investigated. However, it has become evident that several  
690 reports suggested that fish gut associated bacilli displayed inhibitory activity against the major  
691 fish pathogens, which could be due to bacteriocins. However, purification and characterization  
692 of the specific bio-active compounds and mechanism behind the pathogen inhibition are still  
693 less understood in most of the cases.

694 The use of purified bacteriocins in aquaculture is a controversial issue, as the main concern  
695 would be application of these compounds to the farmed fish that are aquatic (Rather *et al.*,  
696 2017). Instead, application of the bacteriocinogenic strains as probiotics have been suggested  
697 (Gatesoupe 2008; Karthikeyan & Santhosh, 2009; Issazadeh *et al.* 2012). In reality, this could  
698 be a more rational and economically feasible approach than application of purified bacteriocins  
699 considering the fact that the ability of the bacteriocinogenic bacilli to sporulate would enable  
700 them to establish within the Gi tract of the hosts and in the aquatic environments (Rather *et al.*

701 2017). Another benefit by using live *Bacillus spp.* is that they are not generally involved in the  
702 processes of horizontal gene transfer with Gram-negative bacteria (e.g. *Vibrio* and *Aeromonas*  
703 spp.), and thus are unlikely to acquire genes of antibiotic resistance or virulence from these  
704 species (Moriarty, 1999). The production of antimicrobial substances and sporulation capacity  
705 confer *Bacillus* strains with a double advantage in terms of their survival in different habitats.  
706 Furthermore, the proteinaceous nature of the bacteriocins or BLIS implies putative degradation  
707 of the anti-microbial substance within the GI tract of fish, suggesting their use as prophylactic  
708 or therapeutic feed supplements (Ringø *et al.* 2018). However, subsequent studies on  
709 purification, characterization and identification of the bacteriocins from bacilli are necessary to  
710 realize effectiveness of the vast array of compounds produced by diverse species and also to  
711 develop a sustainable strategy to explore the potential bacteriocinogenic bacilli for disease  
712 resistance and pathogen prevention in aquaculture.

713 **Tables 2 to 5 here**

#### 714 **6. *Bacillus* as bioremediation of water quality**

715 The aquatic ecosystems including aquaculture sector is the first ecosystem that greatly affected  
716 by different sources of pollutions, and bioremediation by probiotic bacteria is a process in which  
717 beneficial bacteria are used to clean up contaminated water and soil. Generally a successful  
718 bioremediation of rearing water by probiotics can occur via several ways including (i)  
719 optimizing nitrification rates to maintain a low ammonia concentration, (ii) optimizing  
720 denitrification rates to remove the excess nitrogen from the ponds as nitrogen gas, (iii)  
721 maximizing sulphide oxidation to decrease the accumulated hydrogen sulphide, (iv)  
722 maximizing carbon mineralization to carbon dioxide to minimize the sludge accumulation, (v)  
723 maximizing primary pond productivity to enhance the production of the growing target aquatic  
724 animal as well as the secondary crops and (vi) maintaining a diverse and stable pond community  
725 to avoid from the dominance of the undesirable species in the ponds. Probiotics like *Bacillus*  
726 can modulate the microbial communities in water and sediment leading to a reduction and/or  
727 elimination of some pathogenic agents as well as improving the growth and survival of the  
728 target animal. However, the efficacy of probiotics is associated with a good understanding the  
729 nature of bacterial competition between species or strains.



730 Among probiotics bacteria, are *Bacillus* sp. especially associated with improved water quality.  
731 The rationale is that Gram-positive bacteria are efficient in converting organic matter into CO<sub>2</sub>  
732 compared to Gram-negative bacteria (Kumar *et al.*, 2016). The buildup of dissolved and  
733 particulate organic matter is very common phenomenon observed during shrimp production  
734 cycles, however high levels of Gram-positive bacteria (e.g., probiotic *Bacillus*) can be used to  
735 minimize the levels of organic carbon in the system. Bacterial species belongs to the *Bacillus*  
736 genera are also known to be a potential bio-remediators for organic wastes (Thomas & Ward,  
737 1992). These probiotic bacteria regulate the population of beneficial microbiota in aquatic  
738 environment and control the pathogenic microorganism and subsequently enhances the  
739 decomposition of undesirable organic substances in the water and sediments (Chávez-Crooker  
740 & Obrique-Contreras, 2010).

741 When certain *Bacillus* strains are added to the water in a sufficient density, they may cause a  
742 significant impact, by competing with the bacterial microbiota naturally exist for the available  
743 organic matter e.g. uneaten feed and aquatic animal feces. Such biologically interaction activity  
744 is a reflecting of not only the strain capacity for the enzyme production, but also some  
745 environmental conditions such as water temperature, dissolved oxygen, pH, organic and  
746 inorganic substances. For instance, Yu *et al.* (2012) demonstrated that among nine strains of  
747 ammonia nitrogen degradation bacteria of wastewater origin, *B. amyloliquefaciens* revealed  
748 highest degrading activity than the other strains at 35°C, pH 7.0 and rotation 200 r/min  
749 (aeration). At high temperature (>35°C) and pH (>7) was degradation activity gradually  
750 reduced. In an early study, Focht & Verstraete (1977) suggested that temperature is critical by  
751 influencing growth, absorption and utilization of substances via changing the enzyme  
752 activities. In addition, environmental alkaline/acidic condition influence the degradation of  
753 ammonia nitrogen by some *Bacillus* bacteria. The degradation rate also declined with an  
754 increase in carbon source (glucose) level, probably due to glucose overloaded that inhibited  
755 the degrading level of ammonia nitrogen, indicating that ammonia nitrogen consumed in  
756 medium is utilized to proliferate the new bacterial cells. Further, the optimal shaking speed (as  
757 source of oxygen) to reach a maximum ammonia nitrogen degradation was obtained at 200  
758 r/min and decreased gradually thereafter indicating that some *Bacillus* species can perform a  
759 better activity for removal of ammonia from the water column under aeration condition than  
760 anaerobic one.

761 Studies demonstrated the efficacy of some commercial and indigenous *Bacillus* as probiotics  
762 and suggested that bacilli improved the water quality parameters in cultured ponds or  
763 recirculation systems. Members with this trait, including *B. subtilis*, *B. licheniformis*, *B. cereus*,  
764 *B. coagulans* are suggested as suitable for bioremediation of organic detritus (Divya *et al.*,  
765 2015), although they do not seem to be present in sufficient population level in the water  
766 column or sediment. *B. subtilis* and *B. licheniformis* are two suitable candidates for  
767 bioremediation of aquaculture rearing water (Singh, 2002), and some details of efficacy of  
768 *Bacillus* probiotics in aquaculture rearing water is presented in Table 6.

#### 769 **6.1. *Bacillus* probiotic efficacy on rearing water of fish**

770 Chen & Chen (2001) revealed that a combination application of *B. subtilis* and *B. megaterium*  
771 (supplemented twice a week) into rearing recirculating-water systems of red-parrot fish (male  
772 midas cichlid *Cichlasoma citrinellum* × female redhead cichlid (*C. synspilum*) not only  
773 significantly maintained total ammonia nitrogen (TAN), chemical oxygen demands (COD) and  
774 water transparency values to the lower levels than the control, but also reduced the high  
775 mortality of fish reared at high-density cultured environment. The low values of TAN and  
776 COD and the higher water transparency in the water by *Bacillus*-treatment could be attributed  
777 to the presence of *Bacillus* spp. that were counted as the dominant bacterial species in the water  
778 of the treated group. In a study by Laloo *et al.* (2007), a combined supplementation of *B.*  
779 *subtilis*, *B. mycoides* and *B. licheniformis* originally recovered from mud sediment and  
780 common carp intestinal tract at 7-day intervals was able to significantly reduce ammonia,  
781 nitrite, nitrate and phosphate ions in the rearing water of common carp held in recirculation  
782 tanks at 20°C. *B. subtilis* secretes two enzymes of nitrate and nitrite reductases that under  
783 nitrogen limitation condition, they reduce nitrate to ammonia (via nitrite), as *B. subtilis* is able  
784 to use nitrate or nitrite as sole source of nitrogen and also as the electron acceptors for anaerobic  
785 respiration (Laloo *et al.*, 2007). Heterotrophic nitrification by *Bacillus* has also been reported  
786 by Mevel & Prieur (2000). Such biological functions by *B. subtilis* and *B. cereus* grown under  
787 aerobic, facultative aerobic and anaerobic conditions make them able to change nitrogen  
788 metabolism that facilitate both nitrification and denitrification processes. Naderi-Samani *et al.*  
789 (2016) demonstrated that inclusion of *Bacillus* sp. in the rearing tank water of common carp  
790 significantly decreased TAN compared to control, but nitrate increased. When a combination  
791 of *B. pumilus* and *Lb. delbrueckii* probiotics applied to the rearing water of common carp in a

792 biofloc culture system with molasses as carbon source, no change was displayed in  
793 temperature, pH and dissolved oxygen compared vs. the control, whereas a sudden decline in  
794 TAN was observed in the water of the probiotic treated groups after the 7th week and onwards  
795 following 20% water exchange (Dash *et al.*, 2018). In addition, an increase in total suspended  
796 solids (TSS) and total dissolved solid (TDS), and a fluctuation in nitrate level was observed.  
797 Use of *B. pumilus* as a known nitrogen removal bacterium along with periphytic algae available  
798 in such biofloc system could efficiently reduce total ammonia and nitrite in an aquaculture  
799 system as demonstrated by Banerjee *et al.* (2010) who used *B. pumilus* in rearing water of *P.*  
800 *monodon*. Furthermore, no significant change was seen in pH, salinity, dissolved oxygen and  
801 temperature values. In a previous study, Ghosh *et al.* (2008) evaluated the supplementation  
802 effect of an indigenous isolate of *B. subtilis* strain recovered from the intestine of mrigal into  
803 the rearing water of four ornamental fish species; *Poecilia reticulata*, *P. sphenops*, *Xiphophorus*  
804 *helleri* and *X. maculatus* at four different concentrations every 15 days interval significantly  
805 reduced the levels of dissolved organic matter, TAN as well as total counts of coliforms and  
806 motile *Aeromonas* compared to control ones suggesting a well inhibitory activity by *B. subtilis*  
807 towards some Gram-negative bacteria. More recently, Zhang *et al.* (2013) demonstrated that  
808 supplementation of a indigenous *B. subtilis* strain isolated from grass carp to a fish pond for  
809 7 days significantly reduced ammonia, nitrite and total nitrogen levels in water over an extended  
810 period compared with the control group, but nitrate did not show significant difference. Also,  
811 the microbial diversity increased in the probiotic treated groups, and could be due to secretion  
812 of some antimicrobial substances such as coagulin, amicoumacin and subtilisin by *B. subtilis*,  
813 findings that Cutting (2011) suggested to suppress the growth of competing microbes and  
814 enteric pathogens. The interesting finding that the probiotic treated group was dominated by  
815 *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* may be interest as *Proteobacteria*, has  
816 previously been shown to play a role in nitrogen removal (Labbe' *et al.* 2007; Shapleigh, 2011).  
817 Prophylactic efficacy of *B. subtilis* in rearing water of *Tilapia nilotica* previously infected with  
818 *Flavobacterium columnare* improved some water quality parameters (TAN, dissolved oxygen  
819 and pH) and survival of fish (Mohamed *et al.*, 2011) suggesting the production of an  
820 antagonistic capacity by *B. subtilis* to columnaris disease. Despite an adequate data reported on  
821 the positive effects of bacilli probiotics as bioremediation of water quality of aquaculture  
822 species, scarce data are available on their negative effects as water quality bioremediators.  
823 Gupta *et al* (2016) reported that an eight-week application of *Paenibacillus polymyxa* ( $10^3$ - $10^5$

824 cfu/ml) as probiotic in common carp rearing water demonstrated no significant effect on some  
825 water quality parameters including pH, total ammonia, nitrite and dissolved oxygen.

## 826 **6.2. *Bacillus* probiotic efficacy in rearing water of shrimp**

827 [Dalmin et al \(2001\)](#) in a study demonstrated that application of an indigenous *Bacillus* spp in  
828 rearing water of *P. monodon* culture was able to maintain the optimum transparency and low  
829 organic carbon of the pond waters combined with a decrease in *Vibrio* count in the water  
830 column. [Matias et al \(2002\)](#) compared the efficacy of two commercial probiotic products on  
831 the water quality parameters of commercial *P. monodon* grow-out ponds, and relatively a lower  
832 concentration in TAN, nitrate, COD and biological oxygen demand was seen in the early culture  
833 phase in ponds treated with mixture of *Bacillus* sp and *Saccharomyces* sp than treated ponds with  
834 mixture of *Bacillus* sp., *Nitrosomonas* sp. and *Nitrobacter* sp. Additionally, no significant  
835 change was recorded in values of salinity, ammonia, hydrogen sulphide, while dissolved  
836 oxygen and transparency values were slightly increased, while pH value was almost constant.  
837 Use of commercial probiotics (a mixture of *Bacillus* sp., *Saccharomyces cerevisiae*,  
838 *Nitrosomonas* sp. and *Nitrobacter* sp in the rearing water of *L. vannamei* ponds improved  
839 density of beneficial bacteria, dissolved oxygen and water transparency, but reduced  
840 concentrations of total inorganic nitrogen, phosphate and COD ([Wang et al., 2005](#)). Also, no  
841 significant variation was seen in pH value of probiotic treated group, while pH level in control  
842 one was significantly increased after a remarkable decrease during first 40 days of the trail. The  
843 average counts of *Bacillus* sp., ammonifying bacteria, and protein mineralizing bacteria were  
844 also significantly higher in probiotic treated ponds compared to control ones. Further,  
845 application of the probiotics significantly improved dissolved oxygen, while COD was  
846 decreased. A higher counts of protein mineralizing bacteria and ammonifying bacteria in the  
847 probiotic treated ponds indicating an efficient mineralization and relation with a decrease in  
848 values of COD, dissolved inorganic nitrogen and phosphorus. Also, a more stable in pH value  
849 (7.8-8.6) was seen in the water of probiotic treated ponds than control ones (7.23-9.26). Such a  
850 lower fluctuation in the pH might be due to a lower source of carbon dioxide and total alkalinity  
851 in the water column providing a better environmental condition for the shrimp growth.  
852 Additionally, higher values of dissolved oxygen and transparency in water column of probiotic  
853 treated ponds could be due to a higher density of phytoplankton in the treated ponds as was  
854 confirmed by the workers. A reduction in COD was also seen by [Porubcan \(1991\)](#) who used a

855 *Bacillus* sp. probiotic into pond aerators of shrimp with an increase in final production. Use of  
856 a mixture of some species of commercial *Bacillus* strains including *B. subtilis* and *B.*  
857 *licheniformis* (Sanolife MIC) in hatcheries of *P. monodon* and *L. vannamei* were able to  
858 improve the water quality and reduced the density of *Vibrio* bacteria in water column (Decamp  
859 *et al.*, 2008). However, the exact water quality parameters were not described in their works. In  
860 a study by Rahiman *et al* (2010) a lower value in ammonia, nitrite and pH was seen in the  
861 *Bacillus* sp treated water of *M. rosenbergii* culture during 60 days cultivation period, while no  
862 change was observed in dissolved oxygen value compared to control groups. *In vitro*  
863 bioremediation assessment of three indigenous *Bacillus* species; *B. pumilus*, *B. licheniformis*  
864 and *B. subtilis* isolated from marine water and soil sample showed that these bacilli bacteria  
865 were able to reduce TAN with an optimum growth occurred at occurred at 30 °C, pH 7.5, and  
866 1.5% NaCl (Devaraja *et al.*, 2013). Also, under *in vivo* condition, *B. pumilus* reduced TAN  
867 concentration in the rearing water of *P. monodon*. de Paiva Maia *et al.* (2013) appraised the  
868 efficacy of commercial *Bacillus* probiotics on the bacterial population and phytoplankton  
869 concentration in intensive *L. vannamei* culture with recirculation system. The results indicated  
870 that probiotics bacteria improve total heterotrophic bacteria count in the sediment and caused  
871 marked change in the percentage value of Pyrrophyta concentration, environmental quality of  
872 water and sediment in ponds with recirculation system. The change in heterophilic bacteria was  
873 probably due to addition of molasses as a source of carbohydrate that is important for the  
874 survival and growth of heterogenic bacteria in water column. It is also worth to say that under  
875 such a closed recirculation system; an aeration is important to provide sufficient dissolved  
876 oxygen in the production system to prevent the limitation of the *Bacillus* probiotic efficiency.  
877 Application of an indigenous probiotic *B. subtilis* once a week started 2 days after rearing  
878 beginning on the water quality and bacterial community of *L. vannamei* culture enhanced the  
879 water quality values by a decreasing in pH, nitrite, water transparency and soluble reactive  
880 phosphorus and an increasing in COD and *Chlorophyl* a density (Wu *et al.*, 2016). The probiotic  
881 also affected the bacterial community of culturing water at different culture stages with a more  
882 impact was seen on the early and middle phases of shrimp culture than in the late phase,  
883 probably due attribution of a better initial colonization and multiplication of probiotic by  
884 peptone addition in the water column, similar to Lara-Anguiano *et al.* (2013) who demonstrated  
885 that use of molasses as a fertilizer increased *Bacillus* spp. density in water column. However,  
886 probiotic could not directly maintain this effect in the late culture stage probably because of a

887 high organic load in the water and an increasing of indigenous microorganism observed at this  
888 stage. It seems application of encapsulated probiotics in water column prefers the beneficial  
889 bacteria in shrimp and culture water and enhanced water quality for the levels of pH, ammonia  
890 and nitrite of culture water. This was supported by [Nimrat et al. \(2012\)](#) who revealed lower  
891 level in pH, ammonia and nitrite in the treated shrimp compared to the controls, when two  
892 mixtures of *Bacillus* species (first mixture-*B. thuringiensis*, *B. megaterium*, *B. polymyxa*, *B.*  
893 *licheniformis*, *B. subtilis* and second mixture-*B. subtilis*, *B. polymyxa*, *B. megaterium*, *B.*  
894 *circulans*, *B. pumilus*) in the form of microencapsulated probiotic using *Artemia* in the rearing  
895 water of *L. vannamei* were used. Also, higher densities of total heterotrophic bacteria and  
896 *Bacillus* count were obtained in shrimp and water of the treated groups than in controls.  
897 [Zokaeifar et al. \(2014\)](#) reported that application of *Bacillus* mixture in culture water of *L.*  
898 *vannamei* resulted in significant improvement in water quality parameters, i.e., reduction in  
899 ammonia, nitrite and nitrate ions and confers beneficial effect on growth performance, digestive  
900 enzyme activity, immune response and disease resistance against *V. harveyi*. Furthermore,  
901 assessment of some water quality parameters including salinity, carbonates, bicarbonates, total  
902 alkalinity, Mg hardness, Ca hardness, total hardness, ammonia and pH in rearing water of *L.*  
903 *vannamei* treated with *B. pumilus* resulted in a slight increase in pH, total alkalinity and  
904 hardness, but with a fluctuation in other parameters ([Sreenivasulu et al., 2016](#)).

905 In a consequence, biological methods to treat water in aquaculture systems is regarded as the  
906 most promising treatment technology and is being widely used to minimize toxic nitrogenous  
907 compounds in aquaculture systems, and application of some bacteria such as *Bacillus* have been  
908 recognized as beneficial in converting hazardous organic wastes into environmentally safe  
909 compounds via ammonification and nitrification processes ([Roa et al. 1997](#); [Mevel & Prieur,](#)  
910 [2000](#)). Improving of the rearing water quality parameters by *Bacillus* probiotics will enhance  
911 the health status of aquatic animals via providing an optimum environmental condition that can  
912 result in an approach in an optimum physiological condition as well. Also, *Bacillus* probiotics  
913 suppress the density of potential pathogenic microorganisms via bacterial competition causing  
914 a reduction stress culture condition and improving the animal immune-physiological balance.  
915 Under such optimum animal physiological and environmental conditions, the cultured animal  
916 can reflect a better growth performance and survival. However, it is worth to say that *Bacillus*  
917 species are not a highly ammonia cleaner, and no single *Bacillus* strain been so far reported to  
918 remove above 90% of ammonia ([Meng et al. 2009](#); [Xie et al. 2013](#)). Thus, to simultaneously

919 remove the ammonia and nitrite from aquaculture rearing waters, a combination of a high  
 920 cleaner ammonia bacteria plus a *Bacillus* species as nitrifying probiotic could be recommended  
 921 as bioremediation purposes in the rearing water of cultured aquatic animals as [Jawahar](#)  
 922 [Abraham et al. \(2004\)](#) showed that a mixture of *Nitrosomonas* sp. and *Bacillus* sp. was the most  
 923 efficient in removing 96% TAN in microcosm experiments. Although, most of researchers who  
 924 used bacilli probiotics as a supplementary diet did not measure the water quality parameters,  
 925 it seems oral application may improve the water quality conditions as a 21-day use of *B.*  
 926 *licheniformis* and *B. flexus* in diet of *L. vannamei* illustrated a significant reduction in total  
 927 ammonia nitrogen and chemical oxygen demand of the shrimp rearing water ([Cai et al., 2019](#)).

928 **Table 6 here**

## 929 **7. *Bacillus* as probiotics**

### 930 **7.1. *Bacillus* as probiotics in fish**

931 Over the past decade there were increasing attempts to evaluate different types of bacteria  
 932 as probiotic in aquaculture (e.g. [Ringø et al., 2018](#); [Hoseinifar et al., 2018](#)). Among  
 933 promising bacterial strains, *Bacillus* sp. showed beneficial effects on growth performance  
 934 and disease resistance of different fish species ([Dawood et al., 2017](#); [Fečkaninová et al.,](#)  
 935 [2017](#); [Dawood & Koshio, 2016](#); [Li et al., 2018](#); [Hoseinifar et al., 2016](#)). Regarding the  
 936 studies on *Bacillus* sp. it seems that the main focus was on *B. subtilis* ([Zhang et al., 2014](#)).  
 937 The present section summarized and discuss the effects of different species of *Bacillus* on  
 938 growth performance, immune parameters and resistance against diseases.

#### 939 **7.1.1. *Bacillus* probiotics as growth promoter**

941 It is now well accepted that the costs of diet composes a large portion of farmers costs; reported  
 942 to be around 50-60% ([Amiri et al., 2017](#)). Therefore, several researchers are working on  
 943 different approaches to decrease the costs, especially by using different types of growth  
 944 promoters ([Ng & Koh, 2016](#); [Hoseinifar et al., 2017b](#)). Growth promotion by dietary  
 945 administration of probiotics has been reported in different fish species ([Hai, 2015](#)), and the  
 946 mode of actions proposed for growth enhancement is producing exogenous enzymes by  
 947 probiotics as well as improving the intestinal physiology ([Hoseinifar et al., 2017a](#)). Regarding  
 948 different *Bacillus* species there were contradictory reports ([Table 7](#)). Even, in case of identical

949 probiotic and fish species, different authors reported different results. This can be attributed to  
950 difference in intestinal microbiota, life stages and culture condition of target aquatic organisms.  
951 To the best of our knowledge, the first study using *B. subtilis* as fish growth promoters was  
952 done by [Kumar et al. \(2006\)](#), by feeding rohu different levels ( $0.5, 1.0$  and  $1.5 \times 10^7$  CFU g<sup>-1</sup>)  
953 of *B. subtilis* for 15 days and observed significant increase in weight gain. Later, [Bagheri et al.](#)  
954 (2008) supplemented rainbow trout fry diet with varying levels ( $4.8 \times 10^8, 1.2 \times 10^9, 2.01 \times 10^9,$   
955  $3.8 \times 10^9, 6.1 \times 10^9$  CFU g<sup>-1</sup>) of commercial *B. subtilis*. After a short administration duration  
956 period (13 days), probiotic fed fish showed noticeable increase in weight gain, specific growth  
957 rate (SGR) and feed conversion ratio (FCR). The best growth promotion was obtained when  
958 fish fed with  $3.8 \times 10^9$  CFU g<sup>-1</sup> probiotic. Moreover, in a 28-days feeding trial, [Liu et al. \(2012\)](#)  
959 investigated the effects of oral administration of *B. subtilis* E20 ( $10^4, 10^6$  and  $10^8$  CFU g<sup>-1</sup>) on  
960 growth performance parameters of orange-spotted grouper, and revealed that dietary probiotic  
961 significantly increased feeding efficiency and weight gain. The authors suggested that this  
962 improvement can be attributed to provision of nutrient and exogenous enzymes e.g. protease  
963 and lipase by *B. subtilis*. It has been reported that *Bacillus* sp. can supply essential nutrients,  
964 such as amino acid, and vitamins K and B<sub>12</sub> for host organism which can have beneficial effects  
965 on growth performance ([Sun et al., 2012](#); [Liu et al., 2012](#)). [Wu et al. \(2012\)](#) used another strain  
966 (*B. subtilis* Ch9) in grass carp diet. Fish were fed with different levels ( $1.0 \times 10^9, 3.0 \times 10^9$  and  
967  $5.0 \times 10^9$  CFU kg<sup>-1</sup>) of probiotic for 56 days, and at the end of feeding trial, probiotic fed fish  
968 had significantly higher weight gain, SGR and FCR. The best results achieved when fish fed  
969 with  $3.0 \times 10^9$  CFU kg<sup>-1</sup> of *B. subtilis*. Besides, the authors noticed significant increase of  
970 digestive enzymes activity and improved intestinal morphology in probiotic fed fish. Similarly,  
971 dietary administration of *B. Subtilis* ( $1 \times 10^{10}$  CFU g<sup>-1</sup>) significantly increased final weight, FCR  
972 and protein efficiency ratio of olive flounder compared to those fish fed basal diet ([Cha et al.,](#)  
973 [2013](#)). Furthermore, supplementation of Nile tilapia diet with  $5 \times 10^6$  CFU g<sup>-1</sup> level of *B. subtilis*



974 significantly improved growth performance parameters (Telli *et al.*, 2014). The survival rate and  
975 weight gain of Nile tilapia (65.5 g) were significantly increased when fish were fed with *B.*  
976 *subtilis* at  $1 \times 10^7$  /g for two months (Aly *et al.* 2008). Recently, Liu *et al.*, (2017) revealed in  
977 a 8-week experiment that dietary administration of *B. subtilis* HAINUP40 ( $10^8$  cfu/g<sup>-1</sup>)  
978 effectively enhances growth performance, intestinal probiotic recovery, digestive enzyme  
979 activities in Nile tilapia ( $95 \pm 8$  g). Dietary administration of *B. subtilis* ( $0.42 - 1.35 \times 10^7$  cfu  
980 g<sup>-1</sup>) in juvenile large yellow croaker (*Larimichthys crocea*) (7.82 g) in floating sea cages for 10  
981 weeks provided a better growth performance at the higher dosage of probiotic than lower  
982 dosage, suggesting a significant effect by probiotic dosage optimization (Ai *et al.*, 2011) as  
983 similar results were revealed when Nile tilapia was orally fed high dosage of *B.*  
984 *amyloliquefaciens* for two months (Reda *et al.*, 2015). Reda *et al.* (2015) revealed that high  
985 dosage of the bacilli resulted in an increase of intestinal villi heights, higher numbers of goblet  
986 cells and intraepithelial lymphocytes in fish intestine vs. control and/or low supplemented  
987 levels. Further, higher levels of protein and lipid contents were measured in fish carcass fed  
988 higher level of the bacilli. However, Gobi *et al.* (2016) in a study with catfish (*Pangasius*  
989 *hypophthalmus*) revealed that application of *B. licheniformis* Dahb1 ( $10^5$  cfu mL<sup>-1</sup>) was more  
990 effective on growth than higher dosage indicating of host and probiotic specific species.  
991 Further, juvenile Asian sea bass (*Lates calcalifier*) fed diets supplemented with different dosages  
992 of the combination of *B. licheniformis* and *B. subtilis* showed significantly a better growth at  
993  $\times 10^6$  CFU g<sup>-1</sup> than higher or lower levels of the probiotics after being fed for 2 months (Adorian  
994 *et al.*, 2018). Also, a better performance in term of body composition, total protein and digestive  
995 enzymes (protease, lipase, and amylase) were seen in this dosage than others suggesting the  
996 dosage optimization could play a significant role on the fish growth status.

997

998 In addition to *B. subtilis*, some attempts have been also carried out to demonstrate the possible  
999 effects of other *Bacillus* species as probiotic on the growth of fish. [Bandyopadhyay and](#)  
1000 [Mohapatra \(2009\)](#) studied the effects of *B. circulans* PB7 isolated from the intestine of *Catla*  
1001 *catla* as feed additive in *C. catla* fingerlings diet. Fish were fed on various levels of probiotic  
1002 including  $2 \times 10^4$ ,  $2 \times 10^5$ ,  $2 \times 10^6$  CFU  $100 \text{ g}^{-1}$  for 60 days. At the end of feeding trial, the authors  
1003 observed positive effects on growth performance parameters. The best results were obtained  
1004 when fish were fed on  $2 \times 10^5$  CFU  $\text{g}^{-1}$ . Also, in a 30 days study, [Sun et al. \(2010\)](#) administered  
1005 a single dose of two species of Bacilli in orange spotted grouper diet. The authors supplied  
1006 probiotic strain from the gut of orange spotted grouper. However, unlike previous studies with  
1007 Bacilli probiotics, these probiotics were unable to affect growth performance parameters.  
1008 Similarly, the effects of *B. amyloliquifaciens* was evaluated in in a 90-days feeding trial with  
1009 Nile tilapia ([Silva et al., 2015](#)). Fish were fed  $1 \times 10^6$  CFU  $\text{g}^{-1}$ ,  $5 \times 10^6$  CFU  $\text{g}^{-1}$ , and  $1 \times 10^7$  CFU  
1010  $\text{g}^{-1}$  levels of probiotic. The result revealed that this probiotic had no significant effect on Nile  
1011 tilapia. A six-week dietary administration of *B. amyloliquifaciens* in turbot demonstrated a  
1012 marginal enhancement in the fish growth performance, but digestive enzyme activities i.e.  
1013 protease, amylase in hepatopancreas, protease activity in intestine and lipase activity in stomach  
1014 of treated fish significantly showed an increase compared to the control fish ([Chen et al. 2016b](#)).  
1015 When silver carp larvae were fed with *B. latrospores* and *B. licheniformis* via rotifers  
1016 (*Brachionus plicatilis*) as live probiotic vehicle, a better growth performance was seen in treated  
1017 fish than control one ([Sahandi et al., 2012](#)). However, the water quality including water  
1018 temperature used is not stated. A 62-day oral application of *B. velezensis* previously isolated  
1019 from rearing water of salmon improved the growth and antioxidant capability of juvenile  
1020 Atlantic salmon reared in a recirculating aquaculture ([Wang et al., 2019](#)). They reported that a  
1021 combined administration of *B. velezensis* and *R. mucilaginosus* demonstrated a better effect on  
1022 growth performance, feed utilization, immune response, and antioxidant capability and  
1023 mortality, suggesting a more beneficial effect of multi-species probiotic than single one. Such  
1024 beneficial effect was also found by [Giri et al., 2014](#)) when *B. subtilis* in combination with *P.*  
1025 *aeruginosa* + *L. plantarum* were orally used in *Labeo rohita* with a better result in improvement  
1026 of weight gain, SGR and FCR than fish received *B. subtilis* alone. However, in a study by [Doan](#)  
1027 [et al. \(2018\)](#) no significant difference was seen on the growth of tilapia fed either *B. velezensis*  
1028 in single form or in a combination with *L. plantarum* for 30 days.

1029 Beside the oral administration of *Bacillus*, a few studies attempted to assess the effect of bath  
1030 application of *Bacillus* on fish growth. For instance, in a study with Nile tilapia, [Zhou et al.](#)  
1031 [\(2010\)](#) used *B. coagulans* as water probiotic. The culture water was treated with a single dose  
1032 ( $10^7$  CFU ml<sup>-1</sup>) of probiont for 40 days, the authors stated that fish in probiotic treated group  
1033 had significantly higher final weight and SGR compared to the control one. Also, 56-days  
1034 application of *B. subtilis* ( $10^7$  CFU ml<sup>-1</sup> with 2 days interval) as water additive caused a  
1035 remarkable increase in final weight and specific growth weight in the probiotic treated fish  
1036 [\(Zhou et al., 2010\)](#). However, the mode of action of water probiotic on fish growth performance  
1037 warranted further research works.

#### 1038 **7.1.2. *Bacillus* probiotics as enhancer of immune status and disease resistance**

1039 Among different probiotic strains, there are some reports on beneficial effects of *Bacillus* sp. on  
1040 the immune functions of fish. The first study on using of *B. subtilis* as immunostimulant was  
1041 performed by [\(Newaj-Fyzul et al., 2007\)](#). The authors isolated *B. subtilis* AB1 from the  
1042 digestive tract of rainbow trout and supplemented rainbow trout diet with a single dose ( $10^7$  g  
1043 kg<sup>-1</sup>) for 14 days. Interestingly, probiotic fed fish showed significant increase of immune  
1044 parameters including respiratory burst, serum and gut lysozyme, peroxidase, phagocytic killing,  
1045 total and a1-antiprotease activities. Furthermore, probiotic fed fish demonstrated higher  
1046 resistance against *Aeromonas* sp. Also, [Liu et al. \(2012\)](#) studied protective effects of *B. subtilis*  
1047 E20 isolated from fermented boiled soybeans, and revealed a significant increase in some  
1048 immune parameters including lysozyme, phagocytosis, respiratory burst and complement  
1049 activities. When treated fish were challenged with *Streptococcus* sp an increase resistance was  
1050 seen indicating a positive correlation between immune responses and survival. Also, the most  
1051 beneficial effects was noticed in fish fed with higher levels of the probiotic. Similarly, dietary  
1052 *B. subtilis* protected olive flounder against *Streptococcus iniae* [\(Cha et al., 2013\)](#). This was  
1053 attributed to positive effects of *B. subtilis* on immune system, as the authors observed  
1054 significantly higher responses of respiratory burst, lysozyme, superoxide dismutase and  
1055 myeloperoxidase activities in probiotic fed fish. In two separate studies with Nile tilapia, [Zhou](#)  
1056 [et al. \(2010\)](#) and [Telli et al. \(2014\)](#) studied possible effects of *B. subtilis* on some immune  
1057 parameters. However, the studies were different both in terms of administration dose ( $10^7$  CFU  
1058 ml<sup>-1</sup> or  $5 \times 10^6$  CFU g<sup>-1</sup>) and route (oral or bath). Interestingly, both studies showed that this  
1059 probiont significantly increased the fish immune responses including lysozyme, phagocytosis,

1060 catalase and myeloperoxidase activities. Nile tilapia fed *B. subtilis* demonstrated an  
1061 enhancement in some immune parameters including nitroblue tetrazolium assay, neutrophil  
1062 adherence and lysozyme activity compared with the untreated control group (Aly et al. 2008).  
1063 When yellow croaker was fed with *B. subtilis*, a significant enhancement was seen in fish  
1064 immune responses and survival after challenging with *V. harveyi*. It was interesting that the  
1065 addition of fructooligosaccharide as a feed source of probiotic in fish diet did not significantly  
1066 affect immune responses, disease resistance and growth performance of treated fish (Ai et al.,  
1067 2011). *B. subtilis* LR1 isolated from the intestine of rohu could effectively reduce bacterial  
1068 pathogenicity in Indian major carp (Banerjee et al., 2017). In a study by Liu et al. (2017) oral  
1069 use of *B. subtilis* HAINUP40 enhanced some immune responses and disease resistance of Nile  
1070 tilapia challenged with *Str. agalactiae* (Table 7).

1071 Apart from *B. subtilis*, the immune responses of fish treated with other bacilli have been also  
1072 demonstrated by some researchers. Sun et al. (2010) fed orange spotted grouper with previously  
1073 isolated *B. clausii* and *B. pumilus* from this fish gut at a single dose ( $10^8$  CFU g<sup>-1</sup>) for 60 days.  
1074 The results revealed that both *Bacillus* bacteria were able to significantly increase phagocytosis,  
1075 and complement C3, but no significant effect on lysozyme and superoxide dismutase activities.  
1076 Such results suggest a beneficial effect of endogenous *Bacilli* on fish immune responses.  
1077 However, more details works are requested to judge such results. For example, a correlation  
1078 of efficacy of the probiotic on immune status with the survival of target animal after being  
1079 challenged with a virulent pathogen is essential to approve the efficacy of the probiotic. The  
1080 immunomodulatory and disease controlling effects of *B. amyloliquefaciens* have been studied  
1081 in two separate studies on *C. catla* (Das et al., 2013) and Nile tilapia (Selim & Reda, 2015). In  
1082 both trials the probiotics increased immune parameters including serum and mucus lysozyme,  
1083 serum and mucus myeloperoxidase, respiratory burst, nitric oxide, serum bactericidal activity  
1084 and some immune genes expression. Interestingly, *B. amyloliquefaciens* increased mucosal  
1085 immunity of catla as well as resistance against *E. tarda* (Das et al., 2013). Further, the possible  
1086 effects of two *Bacillus* species (*B. coagulans* MTCC 9872, and *B. licheniformis* MTCC 6824)

1087 on non-specific immunity of common carp and fish protection against *A. hydrophila* infection  
1088 was evaluated by Gupta *et al.* (2014). After 80-days application of a single dose ( $10^9$  CFU  $g^{-1}$ ),  
1089 both probiotics significantly increased lysozyme, myeloperoxidase and respiratory burst  
1090 activities. This increase in immune parameters also resulted in noticeably protection against *A.*  
1091 *hydrophila*. A 14-days oral application of *B. amyloliquefaciens* G1 ( $3 \times 10^7$  and  $3 \times 10^9$  cfu/g feed) in  
1092 eel (*Anguilla anguilla*) resulted in cumulative mortalities of 69.24% and 30.76% lower than in the high  
1093 and low probiotic-cell-density groups, respectively compared to control groups, when fish were  
1094 challenged with *A. hydrophila* at  $1 \times 10^9$  cfu/cells (Lu *et al.*, 2011). Use of *B. amyloliquefaciens* in diet  
1095 of tilapia increased the erythrocyte and leukocyte counts, hemoglobin content and hematocrit, but higher  
1096 level of bacilli provided a better effect on serum total protein and globulin levels (Reda *et al.*, 2015).  
1097 When turbot was orally subjected to *B. amyloliquefaciens*, significantly a higher activity of sera  
1098 superoxide dismutase and total protein content as well as a higher relative per cent of survival (62.7%)  
1099 were obtained after challenging fish with *V. anguillarum* (Chen *et al.*, 2016b). Again, oral administration  
1100 of trout with 1:1 mixture of *B. subtilis* and *B. licheniformis* spores (BioPlus2B) could improve the  
1101 survival and immune responses (antibody titer, lymphocyte and total protein) of fish against *Yersinia*  
1102 *ruckeri* challenge (Raida *et al.*, 2003), suggesting that *Bacillus* and *Yersinia* may share antigens with  
1103 some similarity to expand certain B- or T-lymphocyte clones induced by the *Bacillus* that could confer  
1104 some protection against yersiniosis. Asian sea bass fed combined bacilli probiotics (*B.*  
1105 *licheniformis* and *B. subtilis*) demonstrated an enhancement in immunocompetent cell  
1106 populations (leukocytes) (Adorian *et al.*, 2018).

1107 Use of a lower dosage ( $10^5$  cfu/ml) of *B. licheniformis* provided a better efficacy on catfish  
1108 immune parameters and antioxidant than higher dosage ( $10^7$  cfu/ml) (Table 7) (Gobi *et al.*,  
1109 2016). Again, a better protective effect was seen when fish were challenged with *V.*  
1110 *parahaemolyticus*. Such a promising result via bath administration of bacilli raised various  
1111 issues including effectiveness of different administration methods, dosage of bacilli and  
1112 duration of its application as well as the species of target fish. Innate immunological variables

1113 including lysozyme activity, respiratory burst assay, myeloperoxidase content, catalase and  
 1114 superoxidase dismutase activities were improved when common carp were treated by *P.*  
 1115 *polymyxa* as water additive probiotic for 8 weeks at 29.5°C (Table 7) (Gupta *et al.*, 2016). Such  
 1116 enhancement in immune variables was confirmed by an increase resistance in the treated fish  
 1117 challenged with *A. hydrophia*. Atlantic salmon fed either with single *B. velezensis* or in  
 1118 combination with *R. mucilaginosa* demonstrated an enhancement in immune responses (Table  
 1119 7) and disease resistance after fish being challenged with *A. salmonicida* (Wang *et al.*, 2018).  
 1120 Further, application of *B. velezensis* either in a single form or in combination with *L. plantarum*  
 1121 significantly enhanced various innate immune responses and resistance against *S. agalactiae*  
 1122 infection in Nile tilapia compared to control fish (Doan *et al.*, 2018). Giri *et al.* (2014) revealed  
 1123 that application of *B. subtilis* as single probiotic or in combination with *P. aeruginosa* + *L.*  
 1124 *plantarum* enhanced some immune responses and diseases resistance against *A. hydrophial* in  
 1125 of rohu compared to control one.

1126 Recently, the specific strains probiotics in specific host has been an attractive subject for the  
 1127 researchers, but minimum data is available related to aquaculture species. In a study by  
 1128 Galagarza *et al.* (2018) some strains of *B. subtilis* stimulated immune responses both locally  
 1129 and systemically in tilapia. More recently, it has been shown that *Bacillus* spores can be used  
 1130 as a perfect oral vaccine delivery system for their forceful specialty, gene operability, safety  
 1131 and adjuvant property. Jiang *et al.* (2019) demonstrated that higher survival and specific Ig M  
 1132 and IgZ as well as an up-regulation of some immune-related genes in grass carp orally  
 1133 vaccinated with recombinant *B. subtilis* spores carrying grass carp reovirus VP4 protein after  
 1134 the immunized fish being challenged with the virus.

1135

**Table 7 here**

## 1136 7.2. *Bacillus* as probiotics in shellfish culture

1137 Among the several probiotic candidates in shrimp aquaculture, *Bacillus* has brought  
 1138 encouraging results (Zokaeifar *et al.*, 2012b). *Bacillus* have been used to improve the growth  
 1139 performance, enhance immune response and disease management (e.g. Shen *et al.*, 2010;  
 1140 Keysami *et al.*, 2012). In addition, it is also well documented that *Bacillus* species are able to  
 1141 secrete a wide range of extracellular substances and antimicrobial peptides, which improves the  
 1142 water quality, feed digestion and absorption, boost shrimp health and immunity, promotes  
 1143 growth and reproduction and survival against pathogenic microorganism (Zokaeifar *et al.*,

1144 2012b; NavinChandran *et al.*, 2014; Chai *et al.*, 2016; Laranja *et al.*, 2017). Detail of effects of  
1145 *Bacillus* as probiotics on growth performance, immune responses and disease resistances in  
1146 shrimp culture are presented in Table 8.

#### 1147 7.2.1. *Bacillus* probiotics as growth promoter

1148 In shrimp aquaculture, manipulation of microbiota using probiotics have been reported as a  
1149 worthy practice to control or inhibits the pathogenic bacteria, improves digestive enzyme  
1150 activity and growth performance and enhance immune response of host against pathogenic  
1151 infection or physical stress (Balcázar *et al.*, 2006; Pérez *et al.*, 2010). One of the expected  
1152 advantage of *Bacillus* as probiotics is a direct growth-promoting effect by induction of digestive  
1153 enzymes, including protease and amylase, which consequently stimulates the natural digestive  
1154 enzyme activity of the host (Liu *et al.*, 2009; Wang, 2007). In shrimps, it has been reported that  
1155 *Bacillus* sp. have contributes to host nutrition, especially by supplying fatty acids and vitamins  
1156 and improves growth and survival in *P. monodon* postlarvae without water exchange (Devaraja  
1157 *et al.*, 2013; NavinChandran *et al.*, 2014; Kumar *et al.*, 2016). Zokaeifar *et al.* (2012b, 2014)  
1158 demonstrated that *Bacillus* probiotic enhances the digestive enzyme activity, growth  
1159 performance in shrimps. The results further revealed that adminsitartion of *B. subtilis* to *L.*  
1160 *vannamei* increased the activity of protease and amylase digestive enzyme and subsequently  
1161 improves the growth of shrimp juveniles i.e., final weight and weight gain(Zokaeifar *et al.*,  
1162 2012b; Zokaeifar *et al.*, 2014). Nimrat *et al.* (2012) and Sadat Hoseini Madani *et al.* (2018)  
1163 assessed the effect of commercial *Bacillus* probiotics application in growth performance,  
1164 bacterial number, feed efficiency, body composition during rearing of *L. vannamei*. The study  
1165 pointed out that *L. vannamei* with *Bacillus* probiotics added to their experimental tanks had  
1166 significantly higher effect on their length gain %, weight gain %, average daily gain, SGR %  
1167 and FCR than with the control. The probiotic bacteria also improves feed utilization of *L.*  
1168 *vannamei* postlarvae. Bachruddin *et al.* (2018) reported that probiotics treatment which includes  
1169 *Bacillus* into the culture water white shrimp, *L. vannamei* significantly improves weight gain,  
1170 total length, FCR of shrimp species. Kongnum & Hongpattarakere (2012) and Chai *et al.* (2016)  
1171 investigated the effects of *Bacillus* probiotic bacteria isolated from intestine of a healthy, wild  
1172 shrimps on the growth rate in *L. vannamei*. The results of these studies show that *Bacillus*  
1173 probiotics reduced shrimp culture risks because of stressful conditons or facotors and improved  
1174 the growth performance, i.e., total weight, relative growth rate %, FCR, digestion and nutrient

1175 absorption of shrimp. In a study by [Olmos et al. \(2011\)](#) best growth performance in term of  
 1176 weight gain and food-conversion ratio was observed in juvenile *L. vannamei* orally fed with *B.*  
 1177 *subtilis* compare to both control and the fish fed with *B. megaterium* suggesting probiotic  
 1178 species selection is an important factor. Dietary application of *B. licheniformis* either LS-1 or  
 1179 *B. flexus* LD-1, or in a combined form demonstrated an enhancement in the weight gain and  
 1180 SGR of *L. vannamei* and improve water quality suggesting of dual beneficial effects of *Bacillus*,  
 1181 both to the host and to the rearing water ([Cai et al., 2019](#)).

1182 Apart from beneficial role of probiotics bacteria in shrimp growth, there are also few reports  
 1183 which suggests that application of poly- $\beta$ -hydroxybutyrate (PHB), a bacterial storage  
 1184 compound deposited intracellularly in amorphous state in inclusion in the cytoplasm  
 1185 accumulated as a cellular energy and carbon reserve by large variety of bacterial species, have  
 1186 been reported as a biocontrol agent for crustacean culture and [found](#) promising to control  
 1187 vibriosis ([Jiang et al., 2008](#); [Rebah et al., 2009](#); [Borah et al., 2002](#); [Defoirdt et al., 2007](#); [Wang](#)  
 1188 [et al., 2012](#); [Sui et al., 2012](#)). *Bacillus* are commonly found in the intestine of shrimp species  
 1189 like *B. megaterium* and *B. pasteurii* have been shown to accumulate PHB in the range 11 % up  
 1190 to as high as 79 % on cell dry weight under optimized conditions ([Singh et al., 2009](#); [Kaynar](#)  
 1191 [& Beyatli, 2009](#)). [Laranja et al. \(2014\)](#) assessed the curative action of PHB accumulating  
 1192 *Bacillus* species on growth and abiotic stress. The study revealed that PHB accumulating  
 1193 *Bacillus* bacteria isolated from Philippine shrimp culture ponds, improves the growth  
 1194 performance, i.e., average body weight, average body length *P. monodon* postlarvae and  
 1195 ammonia stress ([Laranja et al., 2014](#)).

### 1196 **7.2.2. *Bacillus* probiotics as an enhancer of immune status and disease resistance**

1197 Products which can enhance host immunity and disease resistance of shrimps have probiotics  
 1198 gathered much interest during the last decade ([Tseng et al., 2009](#); [Kumar et al., 2016](#)). Among  
 1199 probiotic bacteria, *Bacillus* species have been demonstrated as harmless bacteria that promotes  
 1200 the health of the host animal by stimulating the innate immune response and improve resistance  
 1201 towards pathogenic microbial infection ([Rengpipat et al., 2000](#); [Sánchez-Ortiz et al., 2016](#);  
 1202 [Laranja et al., 2017](#)).

1203 Earlier studies with *Bacillus* suggested that the probiotic bacteria were able to enhance provide  
 1204 resistance of shrimp species (e.g. *P. monodon*) when challenged with *Vibrio harveyi* ([Rengpipat](#)



1205 *et al.*, 1998a; Rengpipat *et al.*, 1998b). However, the mode of action was only understood after  
1206 few authors started describing the underlying mechanism including immune response, whether  
1207 invertebrates including shrimps are able to mount an immune response with some of the  
1208 attributes of the vertebrate's immune system. Rengpipat *et al.* (2000) observed that *Bacillus*  
1209 probiotics (e.g. *Bacillus* S11) stimulates the immune response by activating phenoloxidase,  
1210 phagocytosis and antimicrobial activity in hemolymph of *P. monodon* and improves survival  
1211 and disease resistance against *V. harveyi* (Rengpipat *et al.*, 2000). It has been also shown that  
1212 feeding *Bacillus* probiotics can increase the survival of shrimp species against bacterial and  
1213 viral pathogens through immune modification, e.g. *B. subtilis* E20, isolated from fermented  
1214 soyabean have been reported to increase resistance to white shrimp (*L. vannamei*) against *V.*  
1215 *alginolyticus* through increase in phenoloxidase, and phagocytic activity, whereas, feeding  
1216 *Bacillus* OJ, to white leg shrimp enhance the immune response (phenoloxidase, phagocytosis,  
1217 etc.) and provide protection against white spot syndrome virus (WSSV) (Tseng *et al.*, 2009; Li  
1218 *et al.*, 2009). Feeding of *L. vannamei* with *Bacillus* probiotics as a supplemented diet decreased  
1219 the total viable counts of bacteria and the *Vibrio* count in the shrimp intestine (Li *et al.* 2009).  
1220 Bath administration of *B. fusiformis* ( $10^5$  cfu mL<sup>-1</sup>) added either as daily or as interval day  
1221 increased survival in both treatments in the larvi-culture system of *L. vannamei* (Guo *et al.*,  
1222 2009).  
1223 *B. cereus* enhanced various immunological variables including phenoloxidase, lysozyme,  
1224 respiratory burst, bactericidal activity in *P. monodon* (Chandran *et al.*, 2014). Such  
1225 enhancement of immune status was confirmed by higher a survival seen in the treated shrimp  
1226 after being challenged with *V. harveyi*.

1227 Moreover, *B. subtilis* E20, isolated from natto (fermented soyabean human health food), has  
1228 also been reported to improve some immune responses variables such as lysozyme and  
1229 prophenoloxidase I and II, survival and stress tolerance including water temperature, salinity  
1230 and nitrite-N in white shrimp (Liu *et al.*, 2010).

1231 A higher survival level together with a better stress tolerance to ammonia oxygen deficiency was  
1232 seen in *L. vannamei* orally fed with *B. subtilis* than control group (Olmos *et al.* 2011) that could be  
1233 due to ammonia conversion-transformation by the *B. subtilis* probiotic, less content-production of  
1234 ammonia by the functional feed and ammonia less oxygen consumption by the functional feed  
1235 which can result in extensive physiological and biochemical functions.

1236 [Dong et al. \(2014\)](#) evaluated the role of *Bacillus* as protective agent and immunomodulator in  
1237 *Marsupenaeus japonicus* juveniles against temperature stress. The study revealed that *Bacillus*  
1238 improved growth, minimize damage caused by free radicals generated from insufficient oxygen  
1239 metabolism due to high temperature and to enhance immune response in *M. japonicus* during  
1240 the high temperature farming period. In another study, administration of *Bacillus* (e.g., *Bacillus*  
1241 *subtilis*, *B. licheniformis*) in *L. vannamei* culture water, have been shown to enhance immune  
1242 response, i.e., prophenoloxidase (ProPO), peroxinectin (PE), lipopolysaccharide and  $\beta$ -1,3-  
1243 glucan binding protein (LGBP), lysozyme and serine protein ([Zokaeifar et al., 2012b](#); [Zokaeifar](#)  
1244 [et al., 2014](#); [Sadat Hoseini Madani et al., 2018](#)). Moreover, the probiotic bacteria also enhances  
1245 disease resistance in white shrimp juveniles against pathogenic *V. harveyi* ([Zokaeifar et al.,](#)  
1246 [2012b](#); [Zokaeifar et al., 2014](#))

1247 Alternatively, some studies revealed that *Bacillus* probiotics bacteria, isolated from same  
1248 environmental condition or system where they will be applied, can boost the cellular and  
1249 humoral component of innate immunity in shrimp species. *B. cereus* enhanced various  
1250 immunological variables including phenoloxidase, lysozyme, respiratory burst, bactericidal  
1251 activity in *P. monodon* ([Chandran et al., 2014](#)). Such enhancement of immune status was  
1252 confirmed by higher a survival seen in the treated shrimp after being challenged with *V. harveyi*.  
1253 ([Chandran et al., 2014](#)). Later in 2016, [Chai et al](#) shows that *Bacillus* probiotic PC465 strain,  
1254 isolated from gut of *Fenneropenaeus chinensis*, enhances immunity including ProPO,  
1255 peroxinectin, penaeidin, thioredoxin, lectins, haemocyanin and crustin and provide protection  
1256 against white spot syndrome virus infection in *L. vannamei* ([Chai et al., 2016](#)). Another  
1257 interesting observation was noted by some authors that *Bacillus* species (like *B. thuringiensis*)  
1258 have shown to carry poly-beta-hydroxybutyrate (PHB), a well-known bacterial storage  
1259 compound and a polymer of the short chain fatty acids  $\beta$ -hydroxybutyrate, that exert its  
1260 beneficial effect by stimulating both the specific and non-specific immune mechanism in  
1261 aquatic animals ([Suguna et al., 2014](#); [Defoirdt et al., 2007](#)). Further, few authors have  
1262 highlighted the beneficial role of PHB-accumulating *Bacillus* on immunity and survival of  
1263 shrimp species against pathogenic microorganism ([Laranja et al., 2014](#)). [Laranja et al. \(2017\)](#)  
1264 reported that *Bacillus* strain with PHB can able to stimulates innate immune response in *P.*  
1265 *monodon* postlarvae. The results explained that, *P. monodon* postlarvae fed with PHB containing  
1266 *Bacillus* sp. JL47 enriched *Artemia* larvae, increase the relative expression of innate immune  
1267 genes, i.e., ProPO, transglutaminase (Tgase) and heat shock protein 70 (Hsp70) after *V.*

1268 *campbellii* challenge. More recently, innate immune enzyme activities, digestive enzyme  
1269 activities, stress tolerance and disease resistance to *V. harveyi* have been reported in *L.*  
1270 *vannamei* after shrimp being fed *B. licheniformis* and *B. flexus* either in single or in a combined  
1271 form for 21 days at 28°C (Cai *et al.*, 2019).

1272  
1273

**Table 8 here**

#### 1274 **8. Pathogenic *Bacillus***

1275 Few reports describing pathogenesis of *Bacillus* affecting fish and shellfish. Furthermore, no  
1276 report has been published on the experimental pathogenicity of these *Bacillus* spore formers in  
1277 aquatic animals so far. Goodwin *et al.* (1994) reported *B. mycoides* as the causative agent of a  
1278 superficial epizootic disease in commercial channel catfish in Alabama, USA. The affected fish  
1279 had pale areas or ulcers on the dorsal area and focal necrosis of epaxial muscle with chains of  
1280 Gram-positive bacilli identified as *B. mycoides*. When healthy catfish were subjected to the  
1281 isolated bacterium either intramuscularly or subcutaneously at  $1.6 \times 10^4$  cfu/fish, development  
1282 of lesions resembled those in natural epizootic were seen. A possible explanation for the  
1283 congestion and lack of bleeding seen in the affected fish could be due to toxin products by these  
1284 bacilli bacteria e.g. *B. cereus* that are able to cause a disseminated intravascular coagulation.

1285 Wang *et al.* (2000) reported a new bacterial white spot syndrome caused by *B. subtilis* in  
1286 cultured tiger shrimp in Malaysian shrimp farming. The affected shrimp showed white spots  
1287 similar to symptoms caused by white spot viral disease (WSVD), but the affected shrimp were  
1288 active and grew normally with no significant morbidity and mortality. The appeared white spots  
1289 were lichen-like with the puncture centers unlike the melanized dots in the white spots caused  
1290 by WSVD. Microscopy evaluations revealed degeneration and discoloration of the cuticle of  
1291 the epicuticle and underlying cuticular layers were detected. It was suggested that such disease  
1292 may be associated with the regular use of *B. subtilis* probiotic in shrimp ponds. *B. subtilis* can  
1293 excrete enzymes of protease, amylase, glucanase and lipase (Shady, 1997) suggesting that the  
1294 bacterium ability to lyse the shrimp cuticle composition i.e. chitin, calcium carbonate and lipid  
1295 (Branson, 1993).

1296 In an experimental study by Sineva *et al.* (2009) expression of *B. cereus* hemolysin II in *B.*  
1297 *subtilis* rendered the bacterium being pathogenic for the crustacean *Daphnia magna* when was  
1298 challenged with the expressed *B. subtilis* at  $0^4$ - $10^6$  cfu L<sup>-1</sup> at water temperature  $20 \pm 5^\circ\text{C}$ . The

1299 lethal concentrations 50% (LC<sub>50</sub>) on the fifth day of the experiment for the expressed *B. subtilis*  
1300 and *B. cereus* were  $5.4 \times 10^5$  and  $4.5 \times 10^5$  cfu mL<sup>-1</sup>, respectively.

1301 More recently, a new bacterial white patch disease caused by *B. cereus* has been reported from  
1302 different *L. vannamei* aquaculture farms industry in India in 2015 (Velmurugan *et al.*, 2015).  
1303 The disease caused a continuous morbidity and mortality, and the affected shrimps showed  
1304 white opaque patches in the carapace, necrosis, whitish blue coloration, loss of appetite and  
1305 pale white muscles. A mortality up to 70% was observed within 3-5 days at the acute stage of  
1306 the disease outbreak. The isolated *B. cereus* strains represented high virulence factors including  
1307 hemolytic and lipase activities, and mortality up 100% occurred in *L. vannamei* and *Artemia*  
1308 *franciscana* after challenging them with the bacterium intraperitoneally at 10<sup>6</sup> cfu/shrimp or via  
1309 bath at 10<sup>8</sup> cfu/ml in both shrimp and *Artemia* at unknown water temperature. *B. cereus* is  
1310 capable of proliferating in a wide range of environments including soils, clays, sediment, dust,  
1311 mineral water, processed foods, and is able to secrete protease, amylase, glucanase and lipase  
1312 (Hendriksen *et al.*, 2006), thus it may be able to invade the suppressed aquatic animals under  
1313 an adverse environmental condition in which is suitable for expressing of the virulence genes  
1314 of the bacterium (Andreeva *et al.*, 2007).

### 1315 9. Safety of *Bacillus*

1316 The possible use of *Bacillus* as probiotics in aquatic animal feed or supplementation to the  
1317 rearing water entering the human food chain should be considered as an important public health  
1318 issue. However, there is no data available related to the risk of human food contamination with  
1319 *Bacillus* used in aquatic animals. Transfer of an antibiotic resistance via the presence of  
1320 transmissible antibiotic resistance genes in some probiotic bacteria is one of the critical risk  
1321 issues. Also, infections from the probiotics and existing of enterotoxins and emetic toxins in  
1322 probiotic bacteria is another important risk requires serious attention in aquaculture industry.  
1323 However, Almost all published data relating to *Bacillus* probiotics in aquaculture are  
1324 associated with their efficacy rather than the safety. It is also worth to say that safety evaluation  
1325 and data on a specific *Bacillus* strain probiotic must not be considered common to similar  
1326 probiotic strain because the safety and risk assessment of each probiotic should be concerned  
1327 based on a case-by-case basis. The severity of a negative effect by a particular probiotic is  
1328 associated with the level of susceptibility of immune-physiological conditions of the target

1329 aquatic animal e.g. early hatched fish larvae. Therefore, it is possible that a *Bacillus* probiotic  
1330 strain is considered as a safe under particular conditions but it is unsafe in other conditions.  
1331 Like antibiotics, it is feasible to say that there is no specific probiotic to be regarded as 100%  
1332 safe. Also, the existence of unwanted/contaminated bacteria or their toxic substances in the  
1333 form of a probiotic can be also considered as another important safety and quality issue.  
1334 Sometimes such contaminants may be a more significant issue than the specific quality of the  
1335 probiotics. At the present time probiotics such as *Bacillus* species used in aquatic animal feed  
1336 or in their water cultures are generally considered as safe. However, some of the bacterial  
1337 probiotic species potentiate some risks of transmission of antibiotic resistance to some  
1338 pathogenic bacteria or production of some their enterotoxins (Anadón *et al.*, 2006).

#### 1339 **9.1. Risks related to the use of *Bacillus* as probiotics**

1340 Despite, *Bacillus* used as probiotics in water or as feed supplement to aquatic animals are  
1341 relatively safe, it is worth to say that protection of human, animals and the environment from  
1342 the potentially unsafe probiotic bacteria is an essential issue. Generally, the risks associated  
1343 with the use of *Bacillus* as probiotics in aquaculture sector can be summarized as suggested by  
1344 Marteau (2001), FAO/WHO (2002), Doron & Snyderman (2015); (a) transfer of antibiotic  
1345 resistance from the probiotics to other pathogenic bacteria, (b) GI or systemic infection of the  
1346 target animal fed with the probiotic, (c) GI or systemic infection of the consumers of the animal  
1347 products produced by animals fed with the probiotics, (d) GI or systemic infection of the  
1348 handlers of animal or aquatic animal feed, (e) release of infectious bacteria or their toxic  
1349 substances to the environment from the animal production system, (f) sensitization of the  
1350 external tissues such as skin, eye and mucus membrane in the handlers of the probiotics, (g)  
1351 toxic effects in the host due to the production of toxins by the bacteria contaminated in the  
1352 probiotics and (h) hypersensitivity of the immune system reactions in susceptible hosts.

#### 1353 **9.2. Risk assessment**

1354 Assessment of *Bacillus* probiotics in aquatic animal diets or in their rearing water against the  
1355 potential risks is a significant issue The *Bacillus* used as probiotics need to be recognized to  
1356 strain level with any infection in humans and aquatic animals. Also, such probiotic must not  
1357 carrier the transferable antibiotic resistance genes. *Bacillus* able to produce toxins or cause  
1358 hypersensitivity reactions in the target host are not suitable for probiotics. Since 2007 the  
1359 European Food Safety Authority (EFSA) has been using a concept [Qualified Presumption of

1360 Safety, QPS), European approach for the assessment of the safety of probiotics] as a generic  
1361 risk assessment tool to assess the safety of a microorganism such as *Bacillus* (EFSA, 2007).  
1362 *Bacillus* as spore-forming bacteria are becoming popular as the promising probiotics for use in  
1363 aquatic animal feed or in their rearing water because of their tolerance to fluctuations in the  
1364 water temperatures that make them easier to handle during manufacture, storage and  
1365 transportation of feed. A number of 13 *Bacillus* species including *B. subtilis*, *B.*  
1366 *amyloliquefaciens*, *B. licheniformis*, *B. coagulans* and *B. megaterium* have been recognized by  
1367 EFSA (2013) to be used as probiotics for animal feed including fish and shellfish. The safety  
1368 of these *Bacillus* species was detected based on the absence of enterotoxins and emetic toxins.  
1369 However, it is important to note that some *Bacillus* bacteria such as *B. anthracis*, *B. cereus*, *B.*  
1370 *thuringiensis* are serious pathogens in humans and animals (e.g. Damgaard *et al.*, 1997;  
1371 Hernandez *et al.*, 1998; Little & Ivins, 1999; Kotiranta *et al.*, 2000.; Raymond *et al.*, 2010).  
1372 Despite a good information available about the pathogenesis of *B. anthracis* and *B. cereus*, no  
1373 evidence of pathogenic effects for other endospore-forming bacteria is available. The emetic  
1374 toxin (cereulide), enterotoxins haemolysin, non-haemolytic enterotoxin and cytotoxin products  
1375 by *B. cereus* are well known toxins that can affect humans (e.g. Granum & Lund, 1997; Schoeni  
1376 & Lee Wong, 2005). In a study by From *et al.* (2005) from 333 strains of different *Bacillus*  
1377 species, eight strains of *B. subtilis*, *B. mojavensis*, *B. pumilus* and *B. fusiformis* were able to  
1378 produce emetic toxins and cytotoxins. Also, some disorders such as cattle mastitis (Parkinson  
1379 *et al.*, 1999) and cattle abortion (Agerholm *et al.*, 1997) have been reported by *B. cereus* and *B.*  
1380 *licheniformis*, but no data available regarding aquatic animals. There are also some antibiotic  
1381 resistance reports in *Bacillus subtilis* showing this bacterium carriers conjugative transposons  
1382 e.g. Tn5397, which is able to transfer resistance to tetracycline encoded by the tet(M) gene  
1383 (Mullany *et al.*, 1990; Roberts *et al.*, 1999) and tet(L) gene (Phelan *et al.*, 2011) as well as  
1384 macrolide-lincosamide-streptogramin B resistance determinants on the plasmid (Monod *et al.*,  
1385 1986).

## 1386 10. Conclusions

1387 Several reports exist in finfish and shellfish regarding the presence of *Bacillus* in the GI tract.  
1388 However, when investigating *Bacillus* in the GI tract, one major concern popped up; several  
1389 studies have focus to characterize the GI lumen communities (the allochthonous microbiota)  
1390 and the gut microbiota from faecal samples, while fewer studies have focus on bacteria that  
1391 adhere to the mucosal surface (the autochthonous microbiota) which may be important in

1392 specialized physiological functions. We therefore recommend in future studies, more focus on  
1393 the autochthonous gut microbiota.

1394 Most previous studies evaluating *Bacillus* in the GI tract of finfish and shellfish were based on  
1395 culture-based approaches, but this approach may be question. Although there is a discussion  
1396 among scientists about the value and need of using culture-based techniques vs. culture-  
1397 independent approaches, it is apparent that viable cells are valuable to culture collections, in  
1398 vaccine production, as well as their use in probiotics and synbiotic studies. During the last  
1399 decades, the denaturing gradient gel electrophoresis (DGGE) method has been commonly used,  
1400 but the method only detect 1-2% of the microbial diversity. Therefore, we recommend; next-  
1401 generation sequencing when evaluating the gut microbiome, including the presence of *Bacillus*  
1402 species in the GI tract of finfish and shellfish species.

1403 Use of *Bacillus* bacteria as probiotics in feed or as biodegrading bacteria in the ponds rearing  
1404 water and soil is now a sustainable motion to reduce the environmental impact induced by  
1405 aquaculture industry. There are at least ten species of *Bacillus* used as the main components of  
1406 commercial probiotic (bioremediation) products for improvement of water quality of aquatic  
1407 animals. Bioremediation by some *Bacillus* strains in finfish and shellfish pond waters have  
1408 revealed not only an increase in the bioremediation efficacy, but also improved survival of the  
1409 cultured animals. These probiotic *Bacillus* species are a well tool by maintaining a higher  
1410 density of beneficial bacteria and a lower load of pathogenic agent in the ponds. As probiotics,  
1411 *Bacillus* play a significant role in maintaining optimum water quality parameters particularly  
1412 toxic gases including ammonia, nitrite, nitrate, hydrogen sulphide and carbon dioxide  
1413 throughout the growing period with a significant reduction of stress level. Elimination of  
1414 stressors, thus lead to an optimum immuno-physiological balance in the target animal with a  
1415 better growth performance and survival rate as the final consequence of the aquaculture activity.  
1416 Therefore, the application of *Bacillus* as probiotics in aquaculture is growing rapidly,  
1417 particularly in the regions where intensive aquaculture systems have been developed. *Bacillus*  
1418 probiotics as components of biocontrol products either in feed or in water column and sediment  
1419 are often used as a mixture of different species to provide a range of beneficial effects on  
1420 aquaculture systems. *Bacillus* as the ubiquitous bacteria in sediments are also naturally ingested  
1421 by aquatic animals. Also, study about the effects of a particular *Bacillus* species or strains on  
1422 different fish and shellfish species, age, growth condition, water quality condition and diet types

1423 can identify the condition in which the probiotics could work well. *Bacillus* spp. are not  
 1424 generally involved in horizontal gene transfer processes with Gram-negative bacteria e.g.  
 1425 *Vibrio* and *Aeromonas*. Thus, obtaining antibiotic resistance genes from these *Bacillus*  
 1426 probiotics is doubtful. *Bacillus* probiotics are able to rapidly replicate, tolerate a multitude of  
 1427 environmental conditions giving a wide range of beneficial effects in aquaculture sector. Also,  
 1428 the sporulation process by *Bacillus* probiotics enables them for the simple process and  
 1429 formulation as well shelf-stable probiotic spore products. However, some *Bacillus* species used  
 1430 as probiotics e.g. *B. subtilis* produce cytotoxic and emetic toxins. Therefore, detailed safety  
 1431 studies are recommended for these bacterial strains before to be used as the safe probiotics.

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1435

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