Running title: *Bacillus* probiotics in aquaculture

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

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Abstract

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

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

1. Introduction

In the 1870ties, Cohn (1876), Koch (1876) and Tyndall (1877) independently discovered that certain species of bacteria spend at least part of their lives as dormant cellular structures, known as endospores. These bacteria were later classified as *Bacillus* and are now classified in phylum Firmicutes, class Bacilli, and order Bacillales. They are Gram-positive, endosporing, aerobic or facultative anaerobic, characterized by their rod-shaped morphology (between 2.5-10 µm), and are catalase positive. Genus *Bacillus* comprise of approximately 200 bacterial species and are almost ubiquitous in nature; in soil, in compost (*Bacillus composti* and *Bacillus thermophilus*; Yang *et al*. 2013), in extreme environments such as high pH (*Bacillus firmus* OF4; Sturr *et al.* 1994), high temperature (*Bacillus thermophilus*; Yang *et al.* 2013), high salt (*Bacillus halodurans*; Annamalai *et al*. 2013), aquatic environment (Motta *et al*. 2004; Ichimatsu *et al.* 2000) as well as in the gastrointestinal (GI) tract of aquatic animals (Ray *et al*. 2012). They exhibit quite diverse physiological properties such as the ability to produce cellulase, phytase, tannase, chitinase, xylanase, protease and lipase (Ray *et al*. 2012, Ghosh *et al*. 2018) as well as degradation of palm (*Elaeis guineensis*) biodiesel (Lutz *et al*. 2006). Another favourable trait of *Bacillus* is; they produce antimicrobial substances such as peptide and lipopeptide antibiotics and bacteriocins (Stein 2005; Abriouel *et al*. 2011). The sporulation capacity and the production of antimicrobial substances and enzymes confer the capacity of genus *Bacillus* to colonise different habitats and to contribute to nutrition of the host. Moreover, adding selected strains of *Bacillus* as probiotics to control *Vibrio* species in penaeid aquaculture ponds (Moriarty, 1998) and as dietary probiotics (Hong *et al*. 2005) are widely used.

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

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

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

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

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

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

2.1. *Bacillus* in finfish

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

B. sonorensis. Strains of autochthonous enzme-producing *B. sonorensis* with antagonistic feaure were recovered from the DI of mrigal by Dutta & Ghosh (2015), and based on their results, the author suggested that *in vivo* studies merits investigations to clarify their effect on growth performance and health.

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

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

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

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

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

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

2.2. *Bacillus* in shellfish

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

Table 1 here

4. Products by *Bacillus*: exo-enzymes

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

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

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

4.1. Gut associated bacilli and their exo-enzymes

Unlike the ruminants and higher vertebrates, probable contribution of the endosymbionts in the nutritional physiology of fish has been recognized of late (Ray *et al.* 2012). Apart from the endogenous digestive enzymes, exogenous enzymes produced and supplemented by the autochthonous microbiota could be considered as one of the important secondary factors that affects nutrition and feed utilization in fish. In fact, a wide range of enzymes, viz., carbohydrases, phosphatases, esterases, lipases and peptidases produced by gut bacteria might contribute to the digestive processes in fish (Ghosh *et al.* 2018). Extensive studies on Indian major carps (e.g., Ray *et al.* 2012; Mandal & Ghosh 2013; Das & Ghosh 2014; Dutta *et al.* 2015; Dutta and Ghosh, 2015; Banerjee *et al.* 2016; Mukherjee & Ghosh 2016; Mukherjee *et* *al.* 2016; Mukherjee *et al.* 2017) and other teleosts (e.g., Cahil 1990; Ringø *et al*. 1995, 2010, 2016; Llewellyn *et al*. 2014; Al-Hisnawi *et al*. 2014; Hosseinifar *et al*. 2016; Ringø & Song 2016) have indicated the presence of autochthonous gut-associated microorganisms in fish and their beneficial attributes in nutrition. The enzymes of nutritional importance produced by the gut bacteria may be categorized into (1) digestive enzymes, e.g., protease, amylase, lipase etc., and (2) degradation enzymes, e.g., non-starch polysaccharide (NSP) - degrading enzymes, phytase, tannase and chitinase. The review of Ray *et al.* (2012) illustrated contribution of the diverse exo-enzyme producing gut bacteria in the nutrition and well being of the host fish, where gut-associated bacilli were recognized as one of the major groups of bacteria within fish gut. Later, ability of the fish gut-microbiota in enzymatic degradation of plant-derived anti-nutritional factors was displayed by Ghosh *et al.* (2018), in which numerous strains of *Bacillus* were promising. Therefore, the following sub-section will present an extract and update of the research endeavours depicting the occurrence and importance of exo-enzyme producing gut associated bacilli in fish (Table 2).

4.2. Exo-enzyme producing bacilli: digestive enzymes

Endogenous digestive enzymes in fish hydrolyze organic macromolecules (mainly, carbohydrate, protein and lipid) into simpler compounds. In addition, as established for higher vertebrates, supplementation of digestive enzymes (viz., amylase, protease, lipase) from microbial source could be of importance to improve nutrient utilization in fish. Although preliminary studies on microbial amylase activity within fish gut noticed the presence of bacterial amylase, characterization and identification of the specific amylase-producing strains were mostly not carried out (Lesel *et al*. 1986; Das & Tripathi 1991; Bairagi *et al.* 2002). On the other hand, microorganisms with efficient proteolytic activity are widespread in nature because of their rapid growth and *Bacillus* spp. by far the most common among them (Ray *et al.* 2012). To the authors' knowledge, occurrence of proteolytic and amylolytic bacilli (*B. circulans*, *B. cereus* and *B. pumilus*) was first reported in the gut of an Indian major carp (IMC), rohu by Ghosh *et al.* (2002) and correlated it with the feeding habit. However, they didnt quantify enzyme activities. Later, Esakkiraj *et al.* (2009) documented extracellular protease production by *B. cereus* isolated from the gut of flathead grey mullet (*Mugil cephalus*) and indicated that bacterial protease was efficient in utilizing different preparations of tuna-processing wastes, e.g., raw fish meat, defatted fish meat and alkali or acid hydrolysate as nitrogen sources. Mondal *et al.* (2010) detected both protease- and amylase-producing ability of *B. licheniformis* and *B. subtilis* in the gut of bata. Similarly, Ray *et al.* (2010) isolated various strains of bacilli in the gut of three Indian major carps, viz. catla (*B. coagulans*, *B. cereus*); mrigal (*Bacillus* sp., *B. cereus*) and rohu (*Bacillus* sp.), and protein or starch hydrolyzing abilities were demonstrated. Subsequently, presence of amylase- and protease-producing bacilli (*B. thuringiensis*, *B. cereus*, *B. subtilis*, *Bacillus* sp.) in the gut of a marine teleost (Atlantic salmon) were recorded by Askarian *et al.* (2012). Later, *Brevibacillus parabrevis* and *B. licheniformis* isolated from two brackish water fish species, crescent perch (*Terapon jarbua*) and long whiskers catfish (*Mystus gulio)*, respectively, were noticed with considerable amylase and protease activities *in vitro* (Das *et al.* 2014). Since then, diverse *Bacillus* spp. capable of producing amylolytic and proteolytic enzymes were documented from several fish species that included IMCs (Dutta & Ghosh 2015; Dutta *et al.* 2015; Mukherjee & Ghosh 2016; Mukherjee *et al.* 2016; Mukherjee *et al.* 2017); climbing perch (*Anabas testudineus)* (Banerjee *et al.* 2015a); walking catfish (*Clarias batrachus)* (Banerjee *et al.* 2015a; Dey *et al.* 2016) grass carp and rohu (Guo *et al*. 2016; Banerjee *et al*. 2017), striped dwarf catfish (*Mystus vittatus)* (Nandi *et al.* 2017a) and Inidian major carp (Kavitha *et al*., 2018), a brief description of which are presented in Table 2.

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

4.3.Exo-enzyme producing bacilli: degradation enzymes

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

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

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

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

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

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

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

4.4. Application of gut associated bacilli in feed-biotechnology

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

Bacterial cellulase supplementation in the formulated diet (as probiotics) or processing of plant feedstuffs with cellulase-producing bacilli have been used in a number of instances (Bairagi *et al*. 2002; Ghosh *et al*. 2004). Ramachandran & Ray (2007) conducted fermentation of black gram seed meal with a *Bacillus* sp. that was effective in reducing crude fibre, tannin and phytic acid. Further, in an attempt to use phytase-producing bacilli in processing of plant ingredient, *B. licheniformis* LF1 and *B. licheniformis* LH1 isolated from rohu were used for fermentation of sesame oilseed meal (Roy *et al*. 2014). Diets incorporated with fermented oilseed meal significantly (P<0.05) improved the apparent digestibility of macro-molecules (protein, lipid) and minerals (phosphorus, calcium, copper, iron and manganese). Another study reporting processing of sesame oil cake by the phytase-producing fish gut bacterium, *B. subtilis* subsp. *subtilis* through SSF resulted in significant (P<0.05) reduction of the anti-nutritients (e.g. phytic acid, tannins, trypsin inhibitor and crude fibre), while levels of free amino acids, fatty acids and different minerals were noticed to be enhanced (Das & Ghosh 2015). Subsequently, fermentation (SSF) of linseed oil-cake with *B. pumilus* LRF1X and *B. tequilensis* HMF6X revealed minor enhancement in the contents of macro-molecules, total free amino acids and fatty acids as compared to the raw substrate (Banerjee & Ghosh 2016). In addition, anti-nutrients (cellulose, hemicelluloses, crude fibre, tannins, phytic acid and trypsin inhibitor) were significantly (P<0.05) reduced and more interestingly, amino acid composition indicated significant (P<0.05) boost in the levels of numerous amino acids (arginine, cystine, histidine, isoleusine, methyonine, phenylalanine, threonine, tryptophan and valine) in the fermented oil-cake, although, lysine and leucine contents were decreased (Banerjee & Ghosh 2016). Therefore, it seems that fermentation by autochthonus exo-enzyme producing bacilli could be effective in improving the feed value of the plant feedstuffs, which would have a great contribution in aquafeed-technology in the upcoming era.

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

The antimicrobial compounds produced by bacteria are usually divided into two major groups; (1) non-ribosomal secondary metabolites, such as peptide or lipopeptide antibiotics and (2) ribosomally synthesized proteins/peptides, such as bacteriocins (Abriouel *et al.* 2011; Lee & Kim, 2011). Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria that often present bactericidal effects against other closely related species (Cotter *et al.* 2005). Bacteriocins are the most abundant and diverse of the bacterially produced antimicrobials (Riley, 2009). These heterogeneous substances portray variable biochemical properties, inhibitory spectra, molecular weights and mechanisms of action (OSullivan *et al.* 2002). Antibiotics have been used in the fish health manager's for the treatment of bacterial diseases in fish since years. However, massive use of broad spectrum antibiotics and antimicrobial drugs increases the selective pressure by developing emergence of bacterial resistance (Verschuere *et al.* 2000). The development of antibiotic resistance among the microorganisms associated with fish diseases has become a global concern during the past years (Kolndadacha *et al.* 2011). Thus, much interest has been paid on the search for novel antibacterial compounds, preferably proteins with prophylactic and/or curative potential, for which the pathogens may not develop resistance (Patil *et al.* 2001). In this regard, bacteriocins from natural sources have been suggested to be an alternative to control the bacterial diseases in aquaculture (Kim *et al.* 2014; Sahoo *et al.* 2016).

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

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

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

5.1. Classes of bacteriocins produced by *Bacillus*

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

To date, most of the classification efforts are made with the bacteriocins from LAB. The classification scheme of LAB bacteriocins was primarily developed by Klaenhammer (1993), who grouped bacteriocins into four distinct classes with sub-classes: Class I or lantibiotics (post-translationally modified, thermostable peptides, containing lanthionine or derivatives, <5 kDa); Class II (unmodified small heat-stable peptides, <10 kDa); Class III (unmodified large heat-labile peptides, >30 kDa) and Class IV (large complex proteins, containing carbohydrates or lipid moieties). This grouping has formed the basis of all subsequent classification schemes for bacteriocins produced by the Gram-positive bacteria. Readers with special interest are referred to the reviews of Mokoena (2017) and Ringø *et al.* (2018) where updated classification schemes of bacteriocins produced by the LAB have been presented. Genus *Bacillus* may be considered as the second most important group for the production of bacteriocins and BLIS after the LAB. It was evident that the bacteriocins produced by *Bacillus* spp. display broad range of molecular mass; e.g. the smallest bacteriocin-like compound (800 Da) is produced by *B. licheniformis* (Teixeira *et al.* 2009), whereas *B. thuringiensis* produced the largest bacteriocin (950 kDa) such as thuricin (Cherif *et al.* 2001). Many *Bacillus* bacteriocins belong to the lantibiotics, post-translationally modified peptides commonly dispersed among different bacterial groups. Lantibiotics are the best-described antimicrobial peptides with regard to biosynthesis mechanisms, genetic determinants and peptide structure. Members of the genus *Bacillus* also produce many other unmodified bacteriocins within Class II of LAB bacteriocins (Klaenhammer 1993; Drider *et al.* 2006; Nes *et al.* 2007), which includes the pediocin-like bacteriocins (Class IIa) and the two-peptide bacteriocins (Class IIb), while others show completely novel peptide sequences (Abriouel *et al.* 2011). Some of the well described bacteriocins produced by *Bacillus* spp. are, subtilin by *B. subtilis* (Banerjee & Hansen 1988), cerein by *B. cereus* (Oscariz *et al.* 1999), bacillocin 490 by *B. licheniformis* (Martirani *et al.* 2002), haloduracin by *B. halodurans* (Lawton *et al.* 2007), thuricin by *B. thuringiensis* (Gray *et al.* 2006), subtilosin by *B. amyloliquifaciens* (Sutyak *et al.* 2008), and megacin by *B. megaterium* (Kiss *et al.*, 2008), and they are mostly active against Gram-positive organisms *such as Listeria monocytogenes,Gardnerella vaginalis, Streptococcus agalactiae*, *Staphylococcus aureus* and *Leuconostoc mesenteroides* In view of the increasing number of bacteriocins described within different bacterial groups such as lactic acid bacteria, it is very difficult to ascertain a combined classification scheme of bacteriocins (Nes *et al.* 2007). Therefore, the *Bacillus* bacteriocin classification system may be adopted independently even though some compounds produced by both *Bacillus* and LAB have very similar characteristics. Consequently, a simple classification scheme for the bacteriocins / BLIS produced by the *Bacillus* spp. was proposed by Abriouel *et al.* (2011) that holds three classes of bacteriocins (Table 3): Class I (antimicrobial peptides that undergo post-translational modifications); Class II (small nonmodified and linear peptides, heat and pH stable, 0.77–10 kDa) and Class III (large proteins with phospholipase activity, >30 kDa).

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; Lalloo *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 speceis (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 from aquatic sources and identification of bacteriocins produced by aquatic animals/environmets are scarce (Table 4).

During the last two decades, information on antagonism by fish gut associated bacilli against diverse pathogenic bacteria has become available, and this section presents an overview on the pathogen inhibitory bacilli isolated from different fish species (Table 5). Antimicrobial substances produced by bacilli isolated from GI tracts of Japanese costal fish (Sugita *et al.* 1998) and an Indian major carp (IMC), rohu (Giri *et al.* 2011) have been reported. Antibacterial compound produced by *B. licheniformis* P40 isolated from an Amazon basin fish *Leporinus* sp. was bactericidal and bacteriolytic to *Listeria monocytogenes* (Cladera-Olivera *et al.* 2004). In addition, *B. subtilis* SG4 (Ghosh *et al.* 2007), *B. aerius* CCH1A and *B. sonorensis* CCH1Ph (Dutta *et al.* 2015) isolated from mrigal, *C. mrigala*; *B. methylotrophicus* isolated from channel catfish, *Ictalurus punctatus* (Ran *et al.* 2012) and catla, *Catla catla*, (Mukherjee & Ghosh 2016); and *B. cereus* and *B. circulans* obtained from the GI tract of some other fish species (Lalloo *et al.* 2010; Geraylou *et al.* 2014) were established as antagonistic against different strains of *Aeromonas hydrophila* pathogenic to fish. Strains of *B. methylotrophicus* isolated from soil or channel catfish intestine inhibited fish pathogens causing enteric septicaemia (*E. ictaluri*) and motile aeromonad septicaemia (*A. hydrophila*) (Ran *et al.* 2012). Another strain, *B. subtilis* BHI344, isolated from the GI tract of channel catfish was shown to depressed growth of pathogenic *A. hydrophila*, *A. sobria*, and *A. caviae*, *in vitro* (Luo *et al.* 2014); while strain *B. sonorensis* CM2H3L isolated from the gut of mrigal inhibited *in vitro* growth of *A. salmonicida* (Dutta & Ghosh 2015). Mukherjee *et al.* (2016) revealed that *B. stratosphericus* KM277362, *B. aerophilus* KM277363, *B. licheniformis* KM277364 and *S. silvestris* KM277365 isolated from the GI tract of mrigal inhibited *in vitro* growth of *A. hydrophila*, *A. salmonicida*, *P. fluorescens* and *P. putida*. Inhibition of pathogenic aeromonads was aslo revealed by *B. methylotrophicus* (NR116240), *B. amyloliquefaciens* (NR117946) and *B. licheniformis* (NR118996) isolated from rohu (Mukherjee *et al.* 2017). However, characterization and identification of the antibacterial compounds produced by these gut bacteria was not evaluated in the above mention studies, and inhibition was assumed to be due to bacteriocins or BLIS. Some of these observations made an attempt for partial purification and characterization of the inhibitory substances, and the proteinaceous nature of the BLIS were confirmed (Giri *et al.* 2011; Mukherjee *et al.* 2017). Among three bacilli species; *B. subtilis*, *B. cereus* and *B. amyloliquefaciens* recovered from the gut of IMC, only *B. amyloliquefaciens* demonstrated antagonistic activity against three fish pathogens *A. hydrophila*, *Acinetobacter* sp. and *Acinetobacter tandoii* (Kavitha *et al*., 2018). *B. subtilis* (ATCC 6633) inhibited *in vitro* growth of *A. hydrophila* and *P. fluorescens* (Aly *et al*. 2008), while *B. subtilis* strains isolated from grass carp intestine exhibited inhibitory activities against fish pathogenic bacteria, including *A. hydrophila*, *A. punctata, E. ictaluri*, *A. punctata f. intestinali*, *Vibrio flurialis*, and *Str. agalactiae*, but the inhibitory effect varied with highest effect against *A. hydrophila* and *A. punctata* (Guo *et al*., 2016). In a study by Banerjee *et al.* (2017), *B. subtilis* LR1 isolated from the GI tract of rohu exhibited a bacteriocin (~50 kDa) with inhibitory activity against four fish pathogens; *B. mycoides*, *A. salmonicida*, *P. fluorescens* and *A. hydrophila*. *B. subtilis* and *B. amyloliquefaciens* recovered from GI tract of marine fish (*Paralichthys lethostigma*) and cultured pond of sea cucumber exhibited antagonistic activity against *V. anguillarum, V. harveyi, V. vulnificus, Streptococcus* sp. and *Staphylococcus aureus* (Chen *et al*., 2016a). Among the eight *Bacillus* strains isolated from the intestine of the fish species; *Scophthalmus maximus, Paralichthys olivacues, Epinephelus coioides* and *Clupanodon punctatus,* revealed *B. amyloliquefaciens* M001 more antagonistic activity against multiple aquatic bacterial pathogens including *V. anguillarum, V. campbellii, V. vulnificus, V. parahamolyticus, Streptococcus* sp. and *Edwardsiella tarda* (Chen *et al*. 2016b). It is worth to mention that very few of the well-characterized bacteriocins produced by fish gut associated *Bacillus* spp., e.g., *Bacillus* sp. P45 isolated from *Piaractus mesopotamicus*, Amazon basin fish (Sirtori *et al.*, 2006) and *B. amyloliquefaciens* isolated from the marine fish *Epinephelus areolatus* (An *et al.*, 2015) have been investigated. However, it has become evident that several reports suggested that fish gut associated bacilli displayed inhibitory activity against the major fish pathogens, which could be due to bacteriocins. However, purification and characterization of the specific bio-active compounds and mechanism behind the pathogen inhibition are still less understood in most of the cases.

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

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

Tables 2 to 5 here

6. *Bacillus* as bioremediation of water quality

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

Among probiotics bacteria, are *Bacillus* sp. especially associated with improved water quality. The rationale is that Gram-positive bacteria are efficient in converting organic matter into CO_2 compared to Gram-negative bacteria (Kumar *et al*., 2016). The buildup of dissolved and particulate organic matter is very common phenomenon observed during shrimp production cycles, however high levels of Gram-positive bacteria (e.g., probiotic *Bacillus*) can be used to minimize the levels of organic carbon in the system. Bacterial species belongs to the *Bacillus* genera are also known to be a potential bio-remediators for organic wastes (Thomas & Ward, 1992). These probiotic bacteria regulate the population of beneficial microbiota in aquatic environment and control the pathogenic microorganism and subsequently enhances the decomposition of undesirable organic substances in the water and sediments (Chávez-Crooker & Obreque-Contreras, 2010).

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

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

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

Chen & Chen (2001) revealed that a combination application of *B. subtilis* and *B. megaterium* (supplemented twice a week) into rearing recirculating-water systems of red-parrot fish (male midas cichlid *Cichlasoma citrinellum* × female redhead cichlid (*C. synspilum*) not only significantly maintained total ammonia nitrogen (TAN), chemical oxygen demands (COD) and water transparency values to the lower levels than the control, but also reduced the high mortality of fish reared at high-density cultured environment. The low values of TAN and COD and the higher water transparency in the water by *Bacillus*-treatment could be attributed to the presence of *Bacillus* spp. that were counted as the dominant bacterial species in the water of the treated group. In a study by Lalloo *et al*. (2007), a combined supplementation of *B. subtilis, B. mycoides* and *B. licheniformisa* originally recovered from mud sediment and common carp intestinal tract at 7-day intervals was able to significantly reduce ammonia, nitrite, nitrate and phosphate ions in the rearing water of common carp held in recirculation tanks at 20ºC. *B. subtilis* secretes two enzymes of nitrate and nitrite reductases that under nitrogen limitation condition, they reduce nitrate to ammonia (via nitrite), as *B. subtilis* is able to use nitrate or nitrite as sole source of nitrogen and also as the electron acceptors for anaerobic respiration (Lalloo *et al*., 2007). Heterotrophic nitrification by *Bacillus* has also been reported by Mevel & Prieur (2000). Such biological functions by *B. subtilis* and *B. cereus* grown under aerobic, facultative aerobic and anaerobic conditions make them able to change nitrogen metabolism that facilitate both nitrification and denitrification processes. Naderi-Samani *et al.* (2016) demonstrated that inclusion of *Bacillus* sp. in the rearing tank water of common carp significantly decreased TAN compared to control, but nitrate increased. When a combination of *B. pumilus* and *Lb. delbrueckii* probiotics applied to the rearing water of common carp in a biofloc culture system with molasses as carbon source, no change was displayed in temperature, pH and dissolved oxygen compared *vs*. the control, whereas a sudden decline in TAN was observed in the water of the probiotic treated groups after the 7th week and onwards following 20% water exchange (Dash *et al*., 2018). In addition, an increase in total suspended solids (TSS) and total dissolved solid (TDS), and a fluctuation in nitrate level was observed. Use of *B. pumilus* as a known nitrogen removal bacterium along with periphytic algae available in such biofloc system could efficiently reduce total ammonia and nitrite in an aquaculture system as demonstrated by Banerjee *et al*. (2010) who used *B. pumilus* in rearing water of *P. monodon.* Furthermore, no significant change was seen in pH, salinity, dissolved oxygen and temperature values. In a previous study, Ghosh *et al*. (2008) evaluated the supplementation effect of an indigenous isolate of *B. subtilis* strain recovered from the intestine of mrigal into the rearing water of four ornamental fish species; *Poecilia reticulata, P. sphenops, Xiphophorus helleri* and *X. maculatus* at four different concentrations every 15 days interval significantly reduced the levels of dissolved organic matter, TAN as well as total counts of coliforms and motile *Aeromonas* compared to control ones suggesting a well inhibitory activity by *B. subtilis* towards some Gram-negative bacteria. More recently, Zhang *et al.* (2013) demonstrated that supplementation of a indigenous *B. subtilis* strain isolated from grass carp to a fish pond for 7 days significantly reduced ammonia, nitrite and total nitrogen levels in water over an extended period compared with the control group, but nitrate did not show significant difference. Also, the microbial diversity increased in the probiotic treated groups, and could be due to secretion of some antimicrobial substances such as coagulin, amicoumacin and subtilisin by *B. subtilis*, findings that Cutting (2011) suggested to suppress the growth of competing microbes and enteric pathogens. The interesting finding that the probiotic treated group was dominated by *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* may be interest as *Proteobacteria,* has previously been shown to play a role in nitrogen removal (Labbe´ *et al*. 2007; Shapleigh, 2011). Prophylactic efficacy of *B. subtilis* in rearing water of *Tilapia nilotica* previously infected with *Flavobacterium columnare* improved some water quality parameters (TAN, dissolved oxygen and pH) and survival of fish (Mohamed *et al*., 2011) suggesting the production of an antagonistic capacity by *B. subtilis* to columnaris disease. Despite an adequate data reported on the positive effects of bacilli probiotics as b ioremediation of water quality of aqucualture species, scarece data are available on their negative effects as water quality bioremediators. Gupta et al (2016) reported that an eight-week application of *Paenibacillus polymyxa* (10^3-10^5

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

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

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

Bacillus sp. probiotic into pond aerators of shrimp with an increase in final production. Use of a mixture of some species of commercial *Bacillus* strains including *B. subtilis* and *B. licheniformis* (Sanolife MIC) in hatcheries of *P. monodion* and *L. vannamei* were able to improve the water quality and reduced the density of *Vibrio* bacteria in water column (Decamp *et al*., 2008). However, the exact water quality parameters were not described in their works. In a study by Rahiman *et al* (2010) a lower value in ammonia, nitrite and pH was seen in the *Bacillus* sp treated water of *M. rosenbergii* culture during 60 days cultivation period, while no change was observed in dissolved oxygen value compared to control groups. *In vitro* bioremediation assessment of three indigenous *Bacillus* species; *B. pumilus*, *B. licheniformis* and *B. subtilis* isolated from marine water and soil sample showed that these bacilli bacteria were able to reduce TAN with an optimum growth occurred at occurred at 30 °C, pH 7.5, and 1.5% NaCl (Devaraja *et al*., 2013**)**. Also, under *in vivo* condition, *B. pumilus* reduced TAN concentration in the rearing water of *P. monodon*. de Paiva Maia *et al*. (2013) appraised the efficacy of commercial *Bacillus* probiotics on the bacterial population and phytoplankton concentration in intensive *L. vannamei* culture with recirculation system. The results indicated that probiotics bacteria improve total heterotrophic bacteria count in the sediment and caused marked change in the percentage value of Pyrrophyta concentration, environmental quality of water and sediment in ponds with recirculation system. The change in heterophilic bacteria was probably due to addition of molasses as a source of carbohydrate that is important for the survival and growth of heterogenic bacteria in water column. It is also worth to say that under such a closed recirculation system; an aeration is important to provide sufficient dissolved oxygen in the production system to prevent the limitation of the *Bacillus* probiotic efficiency. Application of an indigenous probiotic *B. subtilis* once a week started 2 days after rearing beginning on the water quality and bacterial community of *L. vannamei* culture enhanced the water quality values by a decreasing in pH, nitrite, water transparency and soluble reactive phosphorus and an increasing in COD and *Chlorophyl* a density (Wu *et al*., 2016). The probiotic also affected the bacterial community of culturing water at different culture stages with a more impact was seen on the early and middle phases of shrimp culture than in the late phase, probably due attribution of a better initial colonization and multiplication of probiotic by peptone addition in the water column, similar to Lara-Anguiano *et al*. (2013) who demonstrated that use of molasses as a fertilizer increased *Bacillus* spp. density in water column. However, probiotic could not directly maintain this effect in the late culture stage probably because of a high organic load in the water and an increasing of indigenous microorganism observed at this stage. It seems application of encapsulated probiotics in water column prefers the beneficial bacteria in shrimp and culture water and enhanced water quality for the levels of pH, ammonia and nitrite of culture water. This was supported by Nimrat *et al.* (2012) who revealed lower level in pH, ammonia and nitrite in the treated shrimp compared to the controls, when two mixtures of *Bacillus* species (first mixture-*B. thuringiensis, B. megaterium, B. polymyxa, B. licheniformis, B. subtilis* and second mixture-*B. subtilis, B. polymyxa, B. megaterium, B. circulans, B. pumilus)* in the form of microencapsulated probiotic using *Artemia* in the rearing water of *L. vannamei* were used. Also, higher densities of total heterotrophic bacteria and *Bacillus* count were obtained in shrimp and water of the treated groups than in controls.

Zokaeifar *et al.* (2014) reported that application of *Bacillus* mixture in culture water of *L. vannamei* resulted in significant improvement in water quality parameters, i.e., reduction in ammonia, nitrite and nitrate ions and confers beneficial effect on growth performance, digestive enzyme activity, immune response and disease resistance against *V. harveyi*. Furthermore, assessment of some water quality parameters including salinity, carbonates, bicarbonates, total alkalinity, Mg hardness, Ca hardness, total hard ness, ammonia and pH in rearing water of *L. vannamei* treated with *B. pumilus* resulted in a slight increase in pH, total alkalinity and hardness, but with a fluctuation in other parameters (Sreenivasulu *et al*., 2016).

In a consequence, biological methods to treat water in aquaculture systems is regarded as the most promising treatment technology and is being widely used to minimize toxic nitrogenous compounds in aquaculture systems, and application of some bacteria such as *Bacillus* have been recognized as beneficial in converting hazardous organic wastes into environmentally safe compounds via ammonification and nitrification processes (Roa *et al*. 1997; Mevel & Prieur, 2000). Improving of the rearing water quality parameters by *Bacillus* probiotics will enhance the health status of aquatic animals via providing an optimum environmental condition that can result in an approach in an optimum physiological condition as well. Also, *Bacillus* probiotics suppress the density of potential pathogenic microorganisms via bacterial competition causing a reduction stress culture condition and improving the animal immune-physiological balance. Under such optimum animal physiological and environmental conditions, the cultured animal can reflect a better growth performance and survival. However, it is worth to say that *Bacillus* species are not a highly ammonia cleaner, and no single *Bacillus* strain been so far reported to remove above 90% of ammonia (Meng *et al*. 2009; Xie *et al*. 2013). Thus, to simultaneously remove the ammonia and nitrite from aquaculture rearing waters, a combination of a high cleaner ammonia bacteria plus a *Bacillus* species as nitrifying probiotic could be recommended as bioremediation purposes in the rearing water of cultured aquatic animals as Jawahar Abraham *et al*. (2004) showed that a mixture of *Nitrosomonas* sp. and *Bacillus* sp. was the most efficient in removing 96% TAN in microcosm experiments. Alghough, most of reserchers who used bacilli probiotics as a supplementary diet did not measaure the water quality parameters, it seems oral application may improve the water quality condtions as a 21-day use of *B. licheniformis* and *B. flexus* in diet of *L. vannamei* illustrated a significant reduction in total ammonia nitrogen and chemical oxygen demand of the shrimp rearing water (Cai *et al*., 2019).

Table 6 here

7. *Bacillus* as probiotics

7.1. *Bacillus* as probiotics in fish

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

7.1.1. *Bacillus* probiotics as growth promoter

It is now well accepted that the costs of diet comproses a large portion of farmers costs; reported to be around 50-60% (Amiri *et al.*, 2017). Therefore, several researchers are working on different approaches to decrease the costs, especially by using different types of growth poromoters (Ng & Koh, 2016; Hoseinifar *et al.*, 2017b). Growth promotion by dietary adminitsration of probiotics has been reported in different fish species (Hai, 2015), and the mode of actions proposed for growth enhancement is producing exogenous enzymes by probitoics as well as improving the intetsinal physiology (Hoseinifar *et al.*, 2017a). Regarding different *Bacillus* species there were contradictory reports (Table 7). Even, in case of identical probitoic and fish species, different authors reported different results. This can be attributed to difference in intestinal microbiota, life stages and culture condition of target aquatic organisms. To the best of our knowledge, the first study using *B. subtilis* as fish growth promoters was done by Kumar *et al.* (2006), by feedind rohu different levels (0.5, 1.0 and 1.5 ×10^7 CFU g^{-1}) of *B. subtilis* for 15 days and observed significant increase in weight gain. Later, Bagheri *et al.* (2008) supplemented rainbow trout fry diet with varying levels (4.8×10^8, 1.2×10^9, 2.01×10^9, 3.8×10^9, 6.1×10^9 CFU g^{-1}) of commercial *B. subtilis*. After a short administration duration period (13 days), probiotic fed fish showed noticeable increase in weight gain, specific growth rate (SGR) and feed conversion ratio (FCR). The best growth promotion was obtained when fish fed with 3.8×10^9 CFU g^{-1} probiotic. Moreover, in a 28-days feeding trial, Liu *et al.* (2012) investigated the effects of oral administration of *B. subtilis* E20 (10^4, 10^6 and 10^8 CFU g^{-1}) on gorwth performacne parameters of orange-spotted grouper, and revealed that dietary probiotic significantly increased feeding efficiency and weight gain. The authors suggested that this improvement can be attributed to provision of nutrient and exogenous enzymes e.g. protease and lipase by *B. subtilis*. It has been reported that *Bacillus* sp. can supply essential nutrients, such as amino acid, and vitamins K and B_{12} for host organism which can have beneficial effects on growth performance (Sun *et al.*, 2012; Liu *et al.*, 2012). Wu *et al.* (2012) used another strain (*B. subtilis* Ch9) in grass carp diet. Fish were fed with different levels (1.0×10^9, 3.0×10^9 and 5.0×10^9 CFU kg^{-1}) of probitoic for 56 days, and at the end of feeding trial, probitoic fed fish had significantly higher weight gain, SGR and FCR. The best results achieved when fish fed with 3.0×10^9 CFU kg^{-1} of *B. subtilis.* Besides, the authors noticed signficant increase of digestive enztmes activiy and improved intestinal morphology in probitoic fed fish. Similarly, dietary administration of *B. Subtilis* (1×10^{10} CFU g^{-1}) significantly increased final weight, FCR and protein efficiency ratio of olive flounder compared to those fish fed basal diet (Cha *et al.*, 2013). Furthermore, supplementation of Nile tilapia diet with 5×10^6 CFU g^{-1} level of *B. subtilis* significantly improved growth performance paameters (Telli *et al*., 2014). The survival rate and weight gain of Nile tilapia (65 5 g) were significantly increased when fish were fed with *B. subtilis* at 1 × 10^7 /g for two months (Aly *et al*. 2008). Recently, Liu *et al*., (2017) revealed in a 8-week experiment that dietary administration of *B. subtilis* HAINUP40 (10^8 cfu/g^{-1}) effectively enhances growth performance, intestinal probiotic recovery, digestive enzyme activities in Nile tilapia (95 ± 8 g). Dietary administration of *B. subtilis* (0.42 -1.35× 10^7 cfu g^{-1}) in juvenile large yellow croaker (*Larimichthys crocea*) (7.82 g) in floating sea cages for 10 weeks provided a better growth performance at the higher dosage of probiotic than lower dosage, suggesting a significant effect by probiotic dosage optimization (Ai *et al*., 2011) as similar results were revealed when Nile tilapia was orally fed h high dosage of *B. amyloliquefaciens* for two months (Reda *et al*., 2015). Reda *et al*. (2015) reveald that high dosage of the bacilli resulted in a increase of intestinal villi heights, higher numbers of goblet cells and intraepithelial lymphocytes in fish intestine vs. control and/or low supplemented levels. Further, higher levels of protein and lipid contents were measured in fish carcass fed higher level of the bacilli. However, Gobi *et al*. (2016) in a study with catfish (*Pangasius hypophthalmus*) reveald that application of *B. licheniformis* Dahb1 (10^5 cfu mL^{-1}) was more effective on growth than higher dosage indicating of host and probiotic specific species. Further, juvnile Asian sea bass (*Lates calcalifier*) fed diets supplemented with different dosages of the combination of *B. licheniformis* and *B. subtilis* showed significantly a better growth at 1 × 10^6 CFU g^{-1} than higher or lower levels of the probiotics after being fed for 2 months (Adorian *et al*., 2018). Also, a better performance in term of body composition, total protein and digestive enzymes (protease, lipase, and amylase) were seen in this dosage than others suggesting the dosage optimization could play a significant role on the fish grwoth status.

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

Beside the oral administration of *Bacillus*, a few studies attemped to assess the effect of bath application of *Baciliius* on fish growth. For instance, in a study with Nile tilapia, Zhou *et al.* (2010) used *B. coagulans* as water probiotic. The culture water was treated with a single dose (10^7 CFU ml^{-1}) of probiont for 40 days, the authors stated that fish in probiotic treated group had significantly higher final weight and SGR compared to the control one. Also, 56-days application of *B. subtilis* (10^7 CFU ml^{-1} with 2 days interval) as water additive caused a remarkable increase in final weight and specific growth weight in the probiotic treated fish (Zhou *et al.*, 2010). However, the mode of action of water probiotic on fish grwoth perfomance warranted further research works.

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

Among different probiotic strains, there are some reports on beneficial effects of *Bacilus* sp. on the immune functions of fish. The first study on using of *B. subtilis* as immunostimulant was performed by (Newaj-Fyzul *et al.*, 2007). The authors isolated *B. subtilis* AB1 from the digestive tract of rainbow trout and supplemented rainbow trout diet with a single dose (10^7 g kg^{-1}) for 14 days. Interestingly, probiotic fed fish showed significant increase of immune parameters including respiratory burst, serum and gut lysozyme, peroxidase, phagocytic killing, total and a1-antiprotease activities. Furthermore, probiotic fed fish demonstrated higher resistance against *Aeromonas* sp. Also, Liu *et al.* (2012) studied protective effects of *B. subtilis* E20 isolated from fermented boiled soybeans, and reveald a significant increase in some immune parameters including lysozyme, phagocytosis, respiratory burst and complement activities. When treated fish weere chanllenge with *Streptococcus* sp an increase resistance was seen indicating a positive correlation between immune responses and survival. Also, the most beneficial effects was noticed in fish fed with higher levels of the probiotic. Similarly, dietary *B. subtilis* protected olive flounder against *Streptococcus iniae* (Cha *et al.*, 2013). This was attributed to positived effects of *B. subtilis* on immune system, as the authors observed significantly higher responses of respiratory burst, lysozyme, superoxide dismutase and myeloperoxidase activities in probiotic fed fish. In two seperate studies with Nile tilapia, Zhou *et al.* (2010) and Telli *et al.* (2014) studied possible effects of *B. subtilis* on some immune parameters. However, the studies were different both in terms of administration dose (10^7 CFU ml^{-1} or 5×10^6 CFU g^{-1}) and rout (oral or bath). Interestingly, both studies showed that this probiont significantly increased the fish immune responses including lysozyme, phagocytosis, catalase and_myeloperoxidase activities. Nile tilapia fed *B. subtilits* demonstrated an enhancement in some immune parameters including nitroblue tetrazoliume assay, neutrophil adherence and lysozyme activity compared with the untreated control group (Aly et al. 2008). When yellow croaker was fed with *B. subtilis*, a significant enhancement was seen in fish immune responses and survival after challenging with *V. harveyi*. It was interesting that the addition of fructooligosaccharide as a feed source of probiotic in fish diet did not significantly affect immune responses, disease resistance and growth performance of treated fish (Ai *et al*., 2011). *B. subtilis* LR1 isolated from the intestine of rohu could effectively reduce bacterial pathogenicity in Indian major carp (Banerjee *et al*., 2017). In a study by Liu *et al*. (2017) oral use of *B. subtilis* HAINUP40 enhanced some immune responses and disease resistance of Nile tilapia challenged with *Str. agalactiae* (Table 7).

Apart from *B. subtilis*, the immune responses of fish treated with other bacilli have been also demonstrated by some researchers. Sun *et al.* (2010) fed orange spotted grouper with previously isolated *B. clausii* and *B. pumilus* from this fish gut at a single dose (10^8 CFU g^{-1}) for 60 days. The results revealed that both *Bacillus* bacteria were able to significantly increase phagocytosis, and complement C3, but no significant effect on lysozyme and superoxide dismutase activities. Such results suggest a beneficial effect of endigenous *Bacilli* on fish immune responses. However, more details works are requeited to judge such resluts. For example, a correlcation of efficacy of the probiotic on immue status with the survival of target animal after being challenged with a virulent pathogen is essential to approve the efficacy of the probiotic. The immunomodulatory and disease controling effects of *B. amyloliquefaciens* have been studied in two seprate studies on *C. catla* (Das *et al.*, 2013) and Nile tilapia (Selim & Reda, 2015). In both trials the probiotics increased immune parameters including serum and mucus lysozyme, serum and mucus myeloperoxidase, respiratory burst, nitric oxide, serum bactericidal activity and some immune genes expression. Intrestigly, *B. amyloliquefaciens* increased mocusal immunity of catla as well as resistance against *E. tarda* (Das *et al.*, 2013). Further, the possible effects of two *Bacillus* species (*B. coagulans* MTCC 9872, and *B. licheniformis* MTCC 6824) on non-specific immunity of common carp and fish protection against *A. hydrophila* infection was evaluated by Gupta *et al.* (2014). After 80-days application of a single dose (10^9 CFU g^{-1}), both probionts significanltly increased lysozyme, myeloperoxidase and respiratory burst activities. This increase in immune parameters also resulted in noticably protection against *A. hydrophila.* A 14-days oral application of *B. amyloliquefaciens* G1 (3 × 10^7 and 3 × 10^9 cfu/g feed) in eel (*Anguilla anguilla*) resulted in cumulative mortalities of 69.24% and 30.76% lower than in the high and low probiotic-cell-density groups, respectively compared to control groups, when fish were challenged with *A. hydrophila* at 1 × 10^9 cfu/cells (Lu *et al*., 2011). Use of *B. amyloliquefaciens* in diet of tilapia increased the erythrocyte and leukocyte counts, hemoglobin content and hematocrit, but higher level of bacilli provided a better effect on serum total protein and globulin levels (Reda *et al*., 2015). When turbot was orally subjected to *B. amyloliquefaciens*, significantly a higher activity of sera superoxide dismutase and total protein content as well as a higher relative per cent of survival (62.7%) were obtained after challenging fish with *V. anguillarim* (Chen *et al*., 2016b). Again, oral administration of trout with 1:1 mixture of *B. subtilis* and *B. licheniformis* spores (BioPlus2B) could improve the survival and immune responses (antibody titer, lymphocyte and total protein) of fish against *Yersinia ruckeri* challenge (Raida *et al.*, 2003), suggesting that *Bacillus* and *Yersinia* may share antigens with some similarity to expand certain B- or T-lymphocyte clones induced by the *Bacillus* that could confer some protection against yersiniosis. Asian sea bass fed combined bacilli probiotics (*B. licheniformis* and *B. subtilis*) demonstrated an enhancement in immunocompetent cell populations (leukocytes) (Adorian *et al*., 2018).

Use of a lower dosage (10^5 cfu/ml) of *B. licheniformis* provided a better efficacy on catfish immune parameters and antioxidant than higher dosage (10^7 cfu/ml) (Table 7) (Gobi *et al*., 2016). Again, a better protective effect was seen when fish were challenged with *V. parahaemolyticus.* Such a promising result via bath administration of bacilli raised various issues including effectiveness of different administration methods, dosage of bacilli and duration of its application as well as the species of target fish. Innate immunological variables including lysozyme activity, respiratory burst assay, myeloperoxidase content, catalase and superoxidase dismutase activities were improved when common carp were treated by *P. polymyxa* as water additive probiotic for 8 weeks at 29.5ºC (Table 7) (Gupta *et al*., 2016). Such enhancement in immune variables was confirmed by an increase resistance in the treated fish challenged with *A. hydrophia*. Atlantic salmon fed either with single *B. velezensis* or in combination with *R. mucilaginosa* demonstrated an enhancement in immune responses (Table 7) and disease resistance after fish being challenged with *A. salmonicida* (Wang *et al*., 2018). Further, application of *B. velezensis* either in a single form or in combination with *L. plantarum* significantly enhanced various innate immune responses and resistance against *S. agalactiae* infection in Nile tilapia compared to control fish (Doan *et al*., 2018). Giri *et al*. (2014) reveald that application of *B. subtilis* as single probiotic or in combination with *P. aeruginosa* + *L. plantarum* enhanced some immune responses and diseases resistance against A. hydrophial in of rohu compared to control one.

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

Table 7 here

7.2. *Bacillus* as probiotics in shellfish culture

Among the several probiotic candidates in shrimp aquaculture, *Bacillus* has brought encouraging results (Zokaeifar *et al*., 2012b). *Bacillus* have been used to improve the growth performance, enhance immune response and disease management (e.g. Shen *et al*., 2010; Keysami *et al*., 2012) . In addition, it is also well documented that *Bacillus* species are able to secrete a wide range of extracellular substances and antimicrobial peptides, which improves the water quality, feed digestion and absorption, boost shrimp health and immunity, promotes growth and reproduction and survival against pathogenic microorganism (Zokaeifar *et al*., 2012b; NavinChandran *et al*., 2014; Chai *et al*., 2016; Laranja *et al*., 2017). Detail of effects of *Bacillus* as probiotics on growth performance, immune responses and disease resistances in shrimp culture are presented in Table 8.

7.2.1. Bacillus probiotics as growth promoter

In shrimp aquaculture, manipulation of microbiota using probiotics have been reported as a worthy practice to control or inhibits the pathogenic bacteria, improves digestive enzyme activity and growth performance and enhance immune response of host against pathogenic infection or physical stress (Balcázar *et al*., 2006; Pérez *et al*., 2010). One of the expected advantage of *Bacillus* as probiotics is a direct growth-promoting effect by induction of digestive enzymes, including protease and amylase, which consequently stimulates the natural digestive enzyme activity of the host (Liu *et al*., 2009; Wang, 2007). In shrimps, it has been reported that *Bacillus* sp. have contributes to host nutrition, especially by supplying fatty acids and vitamins and improves growth and survival in *P. monodon* postlarvae without water exchange (Devaraja *et al*., 2013; NavinChandran *et al*., 2014; Kumar *et al*., 2016). Zokaeifar *et al*. (2012b, 2014) demonstrated that *Bacillus* probiotic enhances the digestive enzyme activity, growth performance in shrimps. The results further revealed that adminsitartion of *B. subtilis* to *L. vannamei* increased the activity of protease and amylase digestive enzyme and subsequently improves the growth of shrimp juveniles i.e., final weight and weight gain(Zokaeifar et al., 2012b; Zokaeifar *et al*., 2014). Nimrat *et al*. (2012) and Sadat Hoseini Madani *et al.* (2018) assessed the effect of commercial *Bacillus* probiotics application in growth performance, bacterial number, feed efficiency, body composition during rearing of *L. vannamei*. The study pointed out that *L. vannamei* with *Bacillus* probiotics added to their experimental tanks had significantly higher effect on their length gain %, weight gain %, average daily gain, SGR % and FCR than with the control. The probiotic bacteria also improves feed utilization of *L. vannamei* postlarvae. Bachruddin *et al*. (2018) reported that probiotics treatment which includes *Bacillus* into the culture water white shrimp, *L. vannamei* significantly improves weight gain, total length, FCR of shrimp species. Kongnum & Hongpattarakere (2012) and Chai *et al*. (2016) investigated the effects of *Bacillus* probiotic bacteria isolated from intestine of a healthy, wild shrimps on the growth rate in *L. vannamei*. The results of these studies show that *Bacillus* probiotics reduced shrimp culture risks becuase of stressful conditons or facotors and improved the growth performance, i.e., total weight, relative growth rate %, FCR, digestion and nutrient absorption of shrimp. In a study by Olmos *et al.* (2011) best growth performance in term of weight gain and food-conversion ratio was observed in juvenile *L. vannamei* orally fed with *B. subtilis* compare to both control and the fish fed with *B. megaterium* suggesting probiotic species selection is an important factor. Dietary application of *B. licheniformis* either LS-1 or *B. flexus* LD-1, or in a combined form demonstrated an enhancement in the weight gain and SGR of *L. vannamei* and improve water quality suggesting of dual beneficial effects of *Bacillus*, both to the host and to the rearing water (Cai *et al*., 2019).

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

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

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

Earlier studies with *Bacillus* suggested that the probiotic bacteria were able to enhance provide resistance of shrimp species (e.g. *P. monodon*) when challenged with *Vibrio harveyi* (Rengpipat *et al*., 1998a; Rengpipat *et al*., 1998b). However, the mode of action was only understood after few authors started describing the underlying mechanism including immune response, whether invertebrates including shrimps are able to mount an immune response with some of the attributes of the vertebrate`s immune system. Rengpipat *et al* (2000) observed that *Bacillus* probiotics (e.g. *Bacillus* S11) stimulates the immune response by activating phenoloxidase, phagocytosis and antimicrobial activity in hemolymph of *P. monodon* and improves survival and disease resistance against *V. harveyi* (Rengpipat *et al*., 2000). It has been also shown that feeding *Bacillus* probiotics can increase the survival of shrimp species against bacterial and viral pathogens through immune modification, e.g, *B. subtilis* E20, isolated from fermented soyabean have been reported to increase resistance to white shrimp (*L. vannamei*) against *V. alginolyticus* through increase in phenoloxidase, and phagocytic activity, whereas, feeding *Bacillus* OJ, to white leg shrimp enhance the immune response (phenoloxidase, phagocytosis, etc.) and provide protection against white spot syndrome virus (WSSV) (Tseng *et al*., 2009; Li *et al*., 2009). Feeding of *L. vannamei* with *Bacillus* probiotics as a supplemented diet deceased the total viable counts of bacteria and the *Vibrio* count in the shrimp intestine (Li *et al*. 2009). Bath administration of *B. fusiformis* (10^5 cfu mL^{-1}) added either as daily or as interval day increased survival in both treatments in the larvi-culture system of *L. vannamei* (Guo et al., 2009).

B. cereus enhanced various immunological variables including phenoloxidase, lysozyme, respiratory burst, bactericidal activity in *P. monodon* (Chandran *et al*., 2014). Such enhancement of immune status was confirmed by higher a survival seen in the treated shrimp after being challenged with *V. harveyi.*

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

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

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

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

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

Table 8 here

8. Pathogenic *Bacillus*

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

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

In an experimental study by Sineva *et al.* (2009) expression of *B. cereus* hemolysin II in *B. subtilis* rendered the bacterium being pathogenic for the crustacean *Daphnia magna* when was challenged with the expressed *B. subtilis* at 0^4-10^6 cfu L^{-1} at water tmeprature 20 ± 5˚C. The lethal concentrations 50% (LC_{50}) on the fifth day of the experiment for the expressed *B. subtilis* and *B. cereus* were 5.4×10^5 and 4.5×10^5 cfu mL^{-1}, respectively.

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

9. Safety of *Bacillus*

The possibile use of *Bacillus* as probiotics in aquatic animal feed or supplementation to the rearing water entering the human food chain should be considered as an important public health issue. However, there is no data available related to the risk of human food contamination with *Bacillus* used in aquatic animals. Transfer of an antibiotic resistance via the presence of transmissible antibiotic resistance genes in some probiotic bacteria is one of the critical risk issues. Also, infections from the probiotics and existing of enterotoxins and emetic toxins in probiotic bacteria is another important risk requires serious attention in aquaculture industry. However, Almost alll published data relating to *Bacillus* probiotics in aquaculture are associated with their efficacy rather than the safety. It is also worth to say that safety evaluation and data on a specific *Bacillus* strain probiotic must not be considered common to similar probiotic strain because the safety and risk assessment of each probiotic should be concerned based on a case-by-case basis. The severity of a negative effect by a particular probiotic is associated with the level of susceptibility of immune-physiological conditions of the target aquatic animal e.g. early hatched fish larvae. Therefore, it is possible that a *Bacillus* probiotic strain is considered as a safe under particular conditions but it is unsafe in other conditions. Like antibiotics, it is feasible to say that there is no specific probiotic to be regarded as 100% safe. Also, the existence of unwanted/contaminated bacteria or their toxic substances in the form of a probiotic can be also considered as another important safety and quality issue. Sometimes such contaminants may be a more significant issue than the specific quality of the probiotics. At the present time probiotics such as *Bacillus* species used in aquatic animal feed or in their water cultures are generally considered as safe. However, some of the bacterial probiotic species potentiate some risks of transmission of antibiotic resistance to some pathogenic bacteria or production of some their enterotoxins (Anadón *et al*., 2006).

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

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

9.2. Risk assessment

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

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

10. Conclusions

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

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

Use of *Bacillus* bacteria as probiotics in feed or as biodegrading bacteria in the ponds rearing water and soil is now a sustainable motion to reduce the environmental impact induced by aquaculture industry. There are at least ten species of *Bacillus* used as the main components of commercial probiotic (bioremediation) products for improvement of water quality of aquatic animals. Bioremediation by some *Bacillus* strains in finfish and shellfish pond waters have revealed not only an increase in the bioremediation efficacy, but also improved survival of the cultured animals. These probiotic *Bacillus* species are a well tool by maintaining a higher density of beneficial bacteria and a lower load of pathogenic agent in the ponds. As probiotics, *Bacillus* play a significant role in maintaining optimum water quality parameters particularly toxic gases including ammonia, nitrite, nitrate, hydrogen sulphide and carbon dioxide throughout the growing period with a significant reduction of stress level. Elimination of stressors, thus lead to an optimum immuno-physiological balance in the target animal with a better growth performance and survival rate as the final consequence of the aquaculture activity. Therefore, the application of *Bacillus* as probiotics in aquaculture is growing rapidly, particularly in the regions where intensive aquaculture systems have been developed. *Bacillus* probiotics as components of biocontrol products either in feed or in water column and sediment are often used as a mixture of different species to provide a range of beneficial effects on aquaculture systems. *Bacillus* as the ubiquitous bacteria in sediments are also naturally ingested by aquatic animals. Also, study about the effects of a particular *Bacillus* species or strains on different fish and shellfish species, age, growth condition, water quality condition and diet types can identify the condition in which the probiotics could work well. *Bacillus* spp. are not generally involved in horizontal gene transfer processes with Gram-negative bacteria e.g. *Vibrio* and *Aeromonas*. Thus, obtaining antibiotic resistance genes from these *Bacillus* probiotics is doubtful. *Bacillus* probiotics are able to rapidly replicate, tolerate a multitude of environmental conditions giving a wide range of beneficial effects in aquaculture sector. Also, the sporulation process by *Bacillus* probiotics enables them for the simple process and formulation as well shelf-stable probiotic spore products. However, some *Bacillus* species used as probiotics e.g. *B. subtilis* produce cytotoxic and emetic toxins. Therefore, detailed safety studies are recommended for these bacterial strains before to be used as the safe probiotics.

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