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MINI-REVIEW

Bacillus subtilis, an ideal probiotic bacterium to shrimp and fish aquaculture that increase feed digestibility, prevent microbial diseases, and avoid water pollution

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Abstract
Beneficial microorganisms maintain the ecosystems, plants, animals and humans working in healthy conditions. In nature, around 95% of all microorganisms produce beneficial effects by increasing nutrients digestion and assimilation, preventing pathogens development and by improving environmental parameters. However, increase in human population and indiscriminate uses of antibiotics have been exerting a great pressure on agriculture, livestock, aquaculture, and also to the environment. This pressure has induced the decomposition of environmental parameters and the development of pathogenic strains resistant to most antibiotics. Therefore, all antibiotics have been restricted by corresponding authorities; hence, new and healthy alternatives to prevent or eliminate these pathogens need to be identified. Thus, probiotic bacteria utilization in aquaculture systems has emerged as a solution to prevent pathogens development, to enhance nutrients assimilation and to improve environmental parameters. In this sense, *B. subtilis* is an ideal multifunctional probiotic bacterium, with the capacity to solve these problems and also to increase aquaculture profitability.

Keywords Probiotics · *Bacillus subtilis* · Pathogenic bacteria · Aquaculture · Profitability

Overview

Seafood always has been an excellent source of proteins, lipids, carbohydrates, vitamins, minerals, and essential micronutrients; however, fisheries capture has been decreasing for the last 20 years (FAO 2006). In this sense, aquaculture systems are gaining social, environmental, and economic importance around the world. However, aquaculture diets contain fish protein and fish oil inducing oceans fish imbalance, increase animals feeding cost and generate ponds contamination (Tacon and Metian, 2008; Olmos et al. 2015). Ponds contamination produces an imbalance in environmental parameters that immunocompromise the animals, induce pathogens proliferation and consequently the organism's death (Merrifield et al. 2010; Stentiford et al. 2012; Tran et al. 2013). Thus, proteins, carbohydrates and complex lipids (PCL) from grains (soybean, corn, wheat and sorghum) are increasingly common in shrimp and fish aquaculture to reduce feeding cost and to prevent ponds contamination (Olmos et al. 2011; Lopez et al. 2016). However, neither shrimp nor marine fishes produce enzymes to digest complex PCL contained in grains, which increase feed losses, ponds contamination and enhance viral and bacterial diseases (Olmos and Paniagua 2014; Olmos 2017). In this sense, *B. subtilis* can grow in almost any carbon and nitrogen source, because its enzymes break down proteins, carbohydrates and complex lipids from animal and vegetable origin (Sonnenschein et al. 1993; Arellano and Olmos 2002; Ochoa and Olmos 2006; Cui et al. 2018). *B. subtilis* enzymes could prevent diseases by improving water quality through ponds bioremediation (Olmos et al. 2011; Zorriehzahra et al. 2016). In addition, good relationship has been established by a long period of time between *B. subtilis* and the animal's immune system (Cutting, 2011; Huang et al.

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2013; Lazado et al. 2015). For this reason, *B. subtilis* is generally recognized as safe (GRAS) for animals and humans consumption by the Food and Drug Administration (Olmos and Contreras 2003; Chen et al. 2017). Furthermore, *B. subtilis* antimicrobial activity is achieved by their ability to produce several kind of antibiotics (Stein 2005; Ongena and Jacques 2008; Abriouel et al. 2010). In this sense, *B. subtilis* utilization as a probiotic bacterium in shrimp and fish aquaculture could solve several problems because it (a) improves growth and weight gain in animals by increasing food conversion ratio, (b) reduce feeding cost using high concentration of less-expensive nutrients, (c) produce safe seafood by preventing pathogens' development due its ability to synthesize antimicrobial compounds, (d) clean ponds water and sediments due its aerobic and anaerobic bioremediation capacities, (e) can be supply in aquaculture feeds due to spores tolerance production process, (f) is FDA approved (Shen et al. 2010; Olmos and Paniagua 2014; Zorriehzahra et al. 2016; Banerjee and Ray 2017). In summary, production yields, profits and safety could be increased using *B. subtilis* in shrimp and fish aquaculture (Olmos et al. 2011; Lopez et al. 2016).

Pathogen bacteria development in aquaculture ponds

E. coli, *Salmonella*, *Pseudomonas*, *Streptococcus* and *Vibrio*, are more common each day in shrimp and fish aquaculture because (a) anthropogenic activities have been increasing near farms, (b) ponds poor management and (c) non-safe animal ingredients utilization in diets (Balcazar et al. 2007; Lee et al. 2014; Liu et al. 2015). In addition, pathogens could also come from animal stocks or could be induced during Bioflocs development (Kasan et al. 2017). These pathogens could take ponds control when growing conditions are appropriate for its development (Fernandez et al. 2017). In this sense, aforementioned pathogens have evolved under low oxygen conditions, high nitrogen, phosphate, and sulfate levels and also at extreme pH. Furthermore, pathogen species are adapted and produce toxic compounds under these unfavorable growing conditions (Saravanan et al. 2007; Roy et al. 2013; Kayansamruaj et al. 2017). Pathogen bacteria are resistant to most antibiotics, making their prevention and elimination difficult (Romero et al. 2012; Albuquerque et al. 2015; Su et al. 2018). These bacteria are capable of producing animals and humans unsafety metabolites, like toxins and virulent enzymes (Roy et al. 2013; Tran et al. 2013; Liu et al. 2015). Opportunistic pathogens grow, control ecological niches and induce animal's death, when ponds' conditions begin to deteriorate (Stentiford et al. 2012; Fernandez et al. 2017; Kayansamruaj et al. 2017). Therefore, probiotic bacteria utilization in shrimp and fish cultures could be a solution to prevent these aquaculture problems (Ninawe and Selvin 2009; Olmos and Paniagua 2014; Lazado et al. 2015; Liu et al. 2015; Olmos et al. 2015).

Criteria for selection of Bacillus probiotic strains

According to currently adopted definition, probiotics are: "Live microorganisms which when administered in adequate amounts confer health benefits to the host [FAO/WHO]. In this sense probiotic bacteria must (a) improve animal's immune system to eliminate pathogens, (b) increase growth and weight gain by enhancing feed digestion and nutrients assimilation, (c) avoid water pollution inducing ponds bioremediation and (d) not induce health problems neither in animals nor in humans (Fig. 1) (Olmos and Paniagua 2014; Zorriehzahra et al. 2016). Therefore, probiotics selection and production must be carefully evaluated since farmed animals, including those produced by aquaculture, are for human consumption. In this sense, GRAS denomination is indispensable to probiotic species; however, some *Vibrio*, *Pseudomonas* and other *proteobacteria* are being used to improve aquaculture system conditions, without considering the health problems they could induce in humans (Cardona et al. 2016; Kasan et al. 2017). Furthermore, even some *Bacillus* species could also produce toxic metabolites for animals and humans (John et al. 2013; Driehuis et al. 2018). Therefore, molecular identification as well as toxin genes detection are indispensable to select probiotic species (Arellano and Olmos 2002; Lee et al. 2014). In addition to molecular tests, field trials must be carried out exhaustively to rule out toxic effects in animals before probiotics commercialization (Hernandez and Olmos 2006; Garcia and Olmos 2007). *Bacillus thuringiensis* an entomopathogenic bacterium is regularly used in aquaculture to kill parasites and to inhibit pathogens growth (Mendoza et al. 2016); however, its differentiation from *B. cereus* and *B. anthracis* is complicated

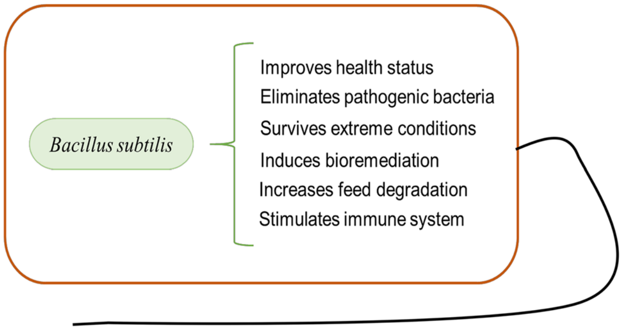

Fig. 1 *Bacillus subtilis* probiotic capacities to improve aquaculture development

using 16S rDNA technology (Vilas-Boas et al. 2007). With respect to *Vibrio* species 16S rDNA analysis revealed high sequence similarities between them, making its differentiation difficult (Hernandez and Olmos 2004; Hoffmann et al. 2010; Fernandez et al. 2017).

In modern aquaculture, high concentration of animals per square meter is common, this condition induces pond's rapid deterioration because; (a) the great amounts of feed used, (b) non-digestible and highly contaminating feed formulations utilized (c) huge animal's feces production and (d) low water exchanges in ponds. All these factors induce diseases proliferation, animal's death and economic losses (Liu et al. 2010; Olmos et al. 2015). Therefore, probiotics utilization is a great opportunity to improve pond's conditions and make aquaculture profitable (Ninawe and Selvin 2009; Liu et al. 2015; Zorriehzahra et al. 2016; Banerjee and Ray 2017). In this sense, microbial consortiums (Bioflocs) are being applied to improve aquaculture parameters with great results (Pilotto et al. 2018); however, sometimes induced species belong to non-safety groups (Saravanan et al. 2007; Tran et al. 2013; Kasan et al. 2017). Hence, some opportunistic pathogens could induce toxic effects in animals or in people who consume them (Kasan et al. 2017). Bioflocs are being utilized taking into account positive effects, without considering negative effects that could be produced by opportunistic pathogens (De Schryver et al. 2008; Cardona et al. 2016). It is important to point out that both; probiotic and pathogenic bacterial species contained in ponds could be induced with Bioflocs technology.

On the other hand, lactic acid bacteria (LAB) from *Lactobacillus* and *Bifidobacterium* genera have been applied for pathogens exclusion in humans, farmed animals and most recently, aquaculture species (Douillard and De Vos 2014; Qin et al. 2018; Ringo et al. 2018). Unlike Bioflocs, LAB is a group of well-studied and GRAS-recognized species that have been used for many years to prevent diseases in human (Zhong et al. 2014). One LAB advantage is their capacity to tolerate acid environments like those found in animals and humans digestive tract, which allow them to survive and grow (Alonso et al. 2018). Simple sugars' and small peptides' degradation is another LAB property; however, its enzymes present limited capacities to degrade carbohydrates, proteins and complex lipids, restricting LAB inclusion in animals feeds. Nevertheless, host protection against bacterial diseases is indeed LAB main characteristic (Li et al. 2011). In this sense, Surfer–Layer–Proteins (SLPs) with great antibacterial activity has been reported recently in LAB (Fagan and Fairweather, 2014). In addition, SLPs have the capacity to prevent viral diseases by stimulating dendritic cells from animal and human immune systems (Martínez et al. 2012). However, spores' absence in LAB is a major restriction to include them in shrimp and fish feeds, due high temperatures used in their production process. In this sense, *Bacillus* species producing SLPs have been recently found to opening new possibilities for aquaculture probiotics development (Grin'ko et al. 2009; Saggese et al. 2018). Nevertheless, animal tests must be carried out to rule out possible toxic effects and to know if they are capable to: (1) prevent viral and bacterial diseases, (2) improve feeds digestion and nutrients assimilation and (3) enhance pond's bioremediation (Fig. 1).

Bacillus subtilis **probiotic capacities**

Bacillus species are among the most widespread bacteria worldwide; can be found in soil, fresh and sea water, air and different kind of foods (Ferrari et al. 1993). *Bacillus* species produce heat, solvent, UV-light and cold-resistant spores that keep them dormant for many years (Nicholson, 2004). *B. subtilis* is the best characterized species of the genus, its genome is totally sequenced and a great number of methodologies have been developed to manipulate this bacterium (Harwood and Cutting 1990; Kunst et al. 1997). *B. subtilis* spores tolerate high temperatures and other extreme conditions (Fig. 2). Recently, *B. subtilis* spores have been used to produce vaccines against shrimp and fish pathogens (Valdez et al. 2014; Tang et al. 2017). This bacterium is FDA-approved because it is not toxic to animals neither humans. Most *B. subtilis* enzymes are secreted to the culture medium facilitating its purification and feed application (Ferrari et al. 1993; Olmos et al. 1997; Gu et al. 2018a, b). Since subtilisin commercial production, *B. subtilis* has become one of the most utilized bacteria in biotechnology industry (Sonnenschein et al. 1993; Cui et al. 2018). *B. subtilis* enzymes break down proteins, carbohydrates and complex lipids from any source allowing it to grow in almost any carbon and nitrogen sources (Ochoa and Olmos 2006; Olmos et al. 2011). This bacterium can grow in aerobic and anaerobic conditions and at extreme pHs. *B. subtilis* produces a great

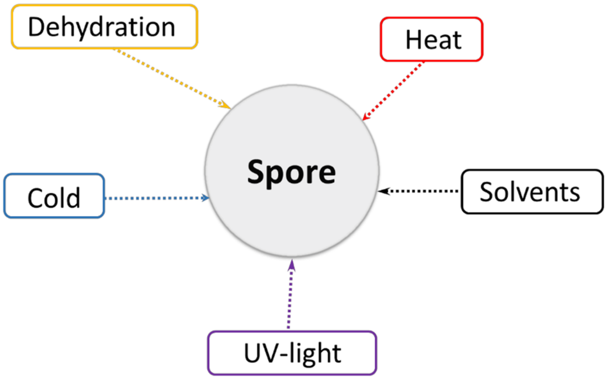

Fig. 2 *Bacillus subtilis* spores tolerate high temperature and other extreme conditions

variety of peptide antibiotics that inhibit pathogens development (Stein 2005; Ongena and Jacques 2008; Abriouel et al. 2010). Additionally, this bacterium can produce high levels of secondary metabolites, fine chemicals and heterologous proteins (Olmos and Contreras 2003; Harwood et al. 2018). In this sense, *B. subtilis* is an ideal probiotic bacterium for functional feeds formulation, however, not even the most capable probiotic can work alone in aquaculture ponds, in this sense, prebiotics and eubiotics must also be supplemented in functional feeds (Fig. 3). Therefore, suitable concentrations and formulations must be found to help *B. subtilis* become a successful probiotic for aquaculture (Merrifield et al. 2010; Harikrishnan et al. 2011; Akhter et al. 2015; Nawaz et al. 2018). In addition, a multifunctional and non-toxic ecotype, easy to manipulate and non-expensive to grow bacterial strain must be found (Olmos et al. 2015).

Improvement of aquaculture production using functional feeds with *B. subtilis*

Feeding represents the most expensive activity in shrimp and fish aquaculture; however, nutrition is also the most important factor for aquaculture development (Tacon and Metian 2008; Naylor et al. 2009). In this sense, suitable formulations could induce growth and weight gain in animals, stimulate their defense system, prevent ponds' contamination and decrease opportunistic pathogens occurrence (Oliva 2012; Olmos and Paniagua 2014). Therefore, proper formulations can lead to a successful and profitable aquaculture and be friendly with the environment (Bostock et al. 2010; Olmos et al. 2015). On the other hand, non-proper formulations could inhibit growth and weight gain by reducing nutrients' assimilation. Consequently, contamination and diseases proliferation will increase in culture ponds. Therefore, non-proper formulations will decrease production yields, profits and will not be friendly with the environment (Olmos and Paniagua 2014).

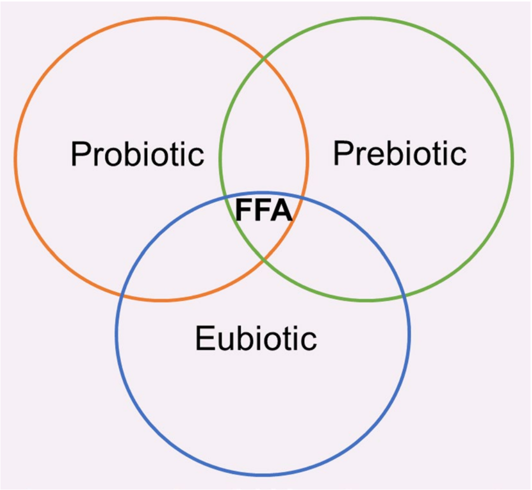

Fig. 3 Functional feeds formulations for aquaculture must include probiotics, prebiotics and eubiotics

Fishmeal and fish oil contain high protein and lipid levels that could be easily assimilated, inducing growth and good health in cultivated animals (Tacon and Metian 2008; Naylor et al. 2009). Thus, both ingredients have become the most important in shrimp and fish formulations through the years (Oliva 2012). Unfortunately, these ingredients have increased their prices lately and could be highly polluting if they are not managed properly. Additionally, fish ingredients are inducing an ecological imbalance in oceans due to pelagic overfishing (Bostock et al. 2010). Thus, in coming years there will not be enough fish captures to sustain human consumption neither for aquaculture needs, therefore, it is necessary to find alternative ingredients to replace fishmeal and fish oil. In this sense, shrimp requires 20 and 30% of carbohydrates and proteins, respectively, and, between 5 and 10% of lipids (Rosas et al. 2010; Kureshy and David 2002). On the other hand, marine fishes require between 40 and 60% of protein, 10–20% of lipids and do not tolerate more than 10% of carbohydrates (Polakof et al. 2012; Lopez et al. 2016; Kamalama et al. 2017). Taking into account these animal needs; carbohydrates, proteins and lipids from grains could be a possible alternative to replace fishmeal and fish oil (Gatlin III et al. 2007; Hardy 2010; Olmos et al. 2015). Nevertheless, most plant ingredients contain complex macromolecules and anti-nutritional compounds that inhibit nutrients assimilation and produce illness (Gu et al. 2018a, b; Pan et al. 2018). These animal inconveniences are induced principally by the absence of proteases, carbohydrases and appropriate lipases (Olmos 2017). In this sense, *B. subtilis* ecotypes producing these enzymes could be added in shrimp and fish formulations to improve plant ingredients digestion and assimilation (Ochoa and Olmos 2006; Olmos et al. 2011; Lopez et al. 2016). Corn, wheat, soybean and sorghum contain enough nutrients to replace fishmeal and fish oil in aquaculture diets (Table 1). However, proper enzymatic machinery to digest these plant ingredients is required by the animals (Olmos 2017). In this sense, Olmos and coworkers have being formulating shrimp and fish feeds with plant ingredients and *B. subtilis*, to improve aquaculture profitability and sustainability (Ochoa and Olmos 2006; Olmos et al. 2011; Olmos and Paniagua 2014; Lopez et al. 2016). In those assays animals grew and gained weight more than controls, not developed diseases and pond water remained in good condition. Furthermore, nor essential amino acids neither high levels of polyunsaturated fatty acids were needed, when fish products were replaced by plant ingredients (Swick 1998; Lopez et al. 2009; Oliva 2012; Gu et al. 2017). Nevertheless, great results were obtained in shrimp and fish assays using plant ingredients and *B. subtilis*, demonstrating

Table 1 Plant ingredients with potential application in functional feeds for aquaculture

Grains	Molecule	%	Price/ton US Dlls indexmundi.com	Fish needs or tolerance % without and with B. subtilis	Shrimp needs or tolerance % without and with *B. subtilis*
Soy			400–450	~20 and ???	~20 and >50 Olmos et al. (2011)
	Protein	40			
	Cho	30			
	Lipids	20			
	Others	10			
Corn			200–250		
	Protein	10			
	Cho	70		~10 and >20 López et al. (2016)	~30 and ~50 Olmos et al. (2011)
	Lipids	10			
	Others	10			
Wheat			200–250		
	Protein	16			
	Cho	72			
	Lipids	2			
	Others	10			
Sorghum			150–200		
	Protein	12			
	Cho	72			
	Lipids	4			
	Others	12			
Fish					
	Fishmeal	25	1500–2000	~50 and ???	~35 and ~25
	Fish oil	5	1500–2000	~20 and ~10 López et al. (2016)	~10 and ≤5 Olmos et al. (2011)

that fish products can be replaced with less-expensive, less-contaminating and safer plant ingredients; however, *B. subtilis* probiotic strains must be added to these feeds (Olmos et al. 2011; Lopez et al. 2016). In this sense, shrimp diets containing high levels of starch, soybean meal and *B. subtilis* were formulated to evaluate plant proteins inclusion, in this sense, higher levels of glucose, cholesterol, lactate and protein were found in their hemolymph with respect to control diets (Olmos et al. 2011). Additionally, better growth, greater survival and higher tolerance to ammonium and oxygen levels, were found in *L. vannamei* fed with plant ingredients and *B. subtilis* (Olmos et al. 2011). These results indicate *B. subtilis* enzymes efficiently digested plant ingredients and induced a better assimilation and utilization of them (Arellano and Olmos 2002; Ochoa and Olmos 2006).

With respect to carnivorous fishes' main focus was to increase 20% or more starch concentration to decrease fish oil levels in diets, because this ingredient is the most expensive in aquaculture formulations (Tacon and Metian 2008; Lopez et al. 2016). In addition, complex carbohydrates utilization as energy source instead of fish protein could improve grow and weight gain. However, carnivorous fishes cannot tolerate more than 10% of complex carbohydrates; therefore, these compounds are highly restricted in its formulations. Nevertheless, formulations containing lower levels of fish oil, higher levels of carbohydrates and *B. subtilis,* duplicated growth and weight gain and, improved all the analyzed parameters (Lopez et al. 2016).

In these works, authors demonstrated both shrimp and carnivorous fishes can assimilate plant ingredients and use them to grow and as energy sources, as long as *B. subtilis* is included. In addition to nutritional benefits, no pathogens were observed and water parameters remained in good conditions (Olmos et al. 2015).

B. subtilis probiotic effects on shrimp and fish performance

Probiotics have the capacity to increase the sustainability of shrimp and fish farming by improving feed utilization and growth, activate immune system responses to protect against diseases and improving the water quality in aquaculture ponds through bioremediation (Table 2). However,

Table 2 *B. subtilis* effects on shrimp and fish aquaculture

Strain	Included	Host	Immune response	Enzyme increased	Survival rate and growth	References
B. subtilis	Diet	White shrimp	Pen-3a, Lec-3, Trx proPO	Amylases Proteases	Increased	Chai et al. (2016)
B. subtilis	Diet	White shrimp	SOD, GSH-Px	Lysozyme Phenoloxidase	Increased	Chen et al. (2017)
B. subtilis	Diet	White shrimp		Amylases Proteases Lipase	Increased	Olmos et al. 2011
B. subtilis	Diet	Tilapia	T-AOC SOD	Amylases Proteases	Increased	Liu et al. (2017)
B. subtilis	Diet	White seabass		Amylases Proteases Lipase	Increased	Lopez et al. (2016)
B. subtilis	Diet	Tilapia		Proteases amylases	Increased	Efendi and Yusra (2014)

further regulation and management are required to normalize the production and usage of aquatic probiotics (Kuebutornye et al. 2019). In this sense, *Bacillus* species are among the most used as probiotics in shrimp and fish aquaculture, because some of them are recognized as GRAS by the FDA (Wang et al. 2019). In this respect, it is important to mention that most of the aquaculture farmed species are consumed by humans, for this reason, the selection of certified bacterial species as *B. subtilis* will help to increase the confidence in this activity.

Conclusion

B. subtilis utilization in shrimp and fish aquaculture can improve feed digestion and assimilation, enhance water bioremediation and prevent diseases development. In addition, production yields and profits must be increased, as well as the safety of food. In this sense, probiotic strains isolation, identification, production and selling, implicates a great responsibility to improve aquaculture development, and human and environmental health.

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