

Bacillus subtilis A Potential Probiotic Bacterium to Formulate Functional Feeds for Aquaculture

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Abstract

In shrimp/fish aquaculture, feed represents the most expensive production cost. The quantity and quality of diets are primary factors influencing shrimp/fish growth, health status, disease prevention, pound contamination and expenses. Utilization of probiotic bacteria has emerged as a solution with enormous applications in the aquaculture feeding industry. *Bacillus* species principally *B. subtilis* are one of the most investigated bacteria for animal probiotic development due to: a) versatility of growth nutrients utilization, b) high level of enzymes production, c) secretion of antimicrobial compounds, d) spore producers, e) develops in aerobic and anaerobic conditions, and f) *B. subtilis* is Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA). Functional feeds development with alternative-economic nutrient vegetable sources of proteins, carbohydrates, lipids and *Bacillus subtilis* probiotic strains, must be considered in shrimp/fish aquaculture production systems; as an option to eliminate animal feed ingredients, improves digestion-assimilation, reduce water pollution and diseases, and to increase yields and profits.

Keywords: *Bacillus subtilis*; Probiotics; Functional Feed; Vegetable ingredients

Bacillus subtilis Properties

Bacteria of the *Bacillus* genus are among the most widespread microorganisms in nature, they can be found in soil, water and air. *Bacillus* constitutes a diverse group of rod-shaped, Gram-positive bacteria, characterized by their ability to produce a robust spore [1-5]. The *Bacillus subtilis* genome is totally sequenced, leading to generation of a great amount of basic knowledge in this bacterium [6]. Additionally, developments of molecular and genetic methodologies are well established in *B. subtilis* [7-9]. *B. subtilis* is not harmful to mammals, including humans, and is commercially important as producer of a high and diverse amount of secondary metabolites like antibiotics, fine chemicals and enzymes, as well as heterologous proteins, antigens and vaccines [1,10-15]. *B. subtilis* grow efficiently with low-cost carbon and nitrogen sources, because its enzymes are very efficient breaking down a great variety of proteins, carbohydrates and lipids from animal and vegetable origin, into their constituent units [1,16-18]. The enzymes also degrade organic accumulated debris from shrimp/fish cultures inducing ponds bioremediation and consequently the prevention of viral and bacterial diseases [19-22]. On the other hand, the antimicrobial activity of *Bacillus* is greatly determined by their ability to produce antibiotics, principally from peptide origin [23]. There were identified 795 antibiotics from *Bacillus* species [11]. *B. subtilis* is the genus most productive species devoting 4-5% of its genome to their synthesis, producing 66 antibiotics [11]. Furthermore, *Bacillus subtilis* is Generally Recognized as Safe (GRAS) by the FDA, meaning this bacterium is not harmful to animals or humans (Figure 1). Taking into account beneficial properties of *B. subtilis*, this bacterium is a potential probiotic candidate to be considered in "Functional Feeds" of crustaceans and fishes [16-19,24].

Bacillus subtilis Probiotic Capacities

B. subtilis is between the oldies species on earth, reason why animals and humans since the beginning of their existence have been in contact with this bacterium [25,26]. In this sense, recognition of *B. subtilis* for

animal and human immune system is well established and a symbiotic relationship had been developed for a long period of time [27]. On the other hand, million years of evolution has created an amazing quorum sensing communication-recognition mechanism between beneficial and pathogenic bacteria [1,28,29]. However, animals and humans only sense pathogens when the disease had been developed, due equilibrium between beneficial and pathogenic bacteria had been broken because; environmental, nutritional and/or metabolic changes has occurred favoring pathogens proliferation. Synthetic antibiotics was the first option to control pathogens overgrowth in humans and animals, however the unregulated use of these compounds induced a multi-resistant mechanisms preferentially developed by the pathogenic bacteria [30]. Today, antibiotics utilization is well regulated in humans and also in animals including aquaculture. In this sense, the utilization of beneficial bacteria (probiotics) has emerge as an alternative due good results are been obtained in both; animals and humans [27].

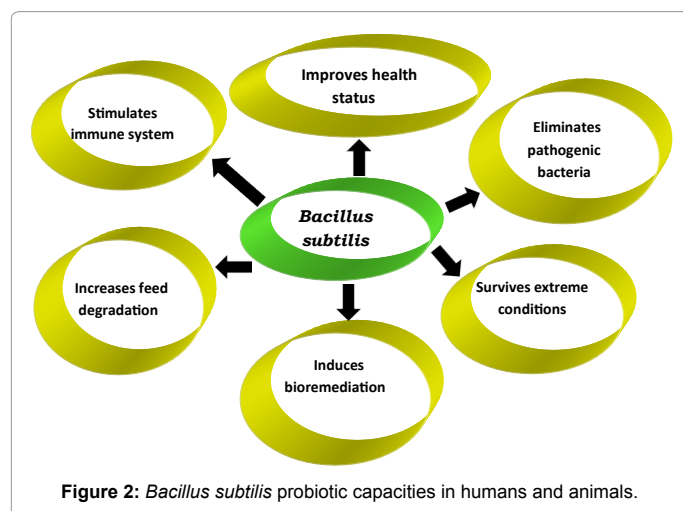
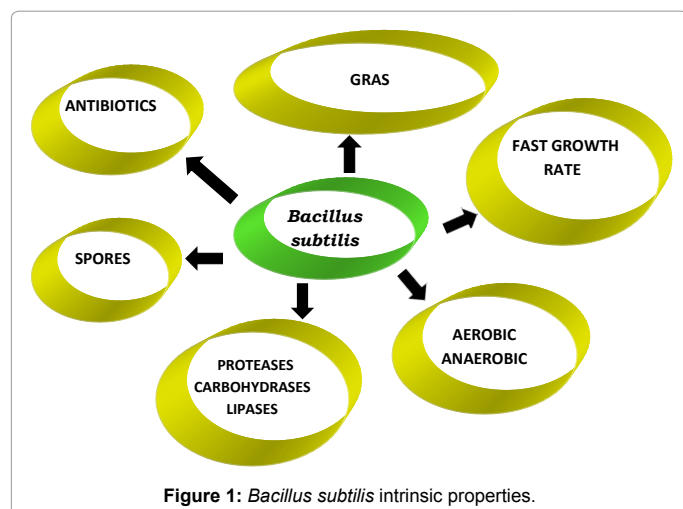
The term "probiotics" was derived from the Greek word, meaning "for life" [31]. According to the currently adopted definition by FAO/WHO, probiotics are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host [32]. Since 50 year ago exists a scientific and commercial interest in the use of beneficial bacteria for the prevention and treatment of diseases [27,33]. *Lactobacillus* and *Bifidobacterium* genera has been used almost exclusively for the competitive exclusion of pathogenic bacteria from the

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gut of humans, farmed animals and most recently aquaculture species [23,27,33,34]. However, the utilization of probiotic to application others than pathogenic bacterial exclusion are now been develop in several areas like; immune system stimulation, vaccine production, bioremediation and functional feed development [15-19,24,35]. In this sense, and based in our own experiences, we redefined probiotics as a living microbial supplement that: (a) positively affects host health status by modifying the host-associated microbial community and its immune system; (b) secrete a variety of enzymes to increases feed degradation-assimilation enhancing its nutritional values; (c) improves quality of environmental parameters by the bioremediation of waste products and (d) support extreme changing environmental and physical parameters (Figure 2).

However, to accomplish all these objectives the utilization of “*Bacillus subtilis* spore producing multifunctional probiotic bacterium” it is recommended, to support environmental, nutritional and metabolic changing conditions where probiotic will be involve and most importantly, to take advantage of them [17-19,24,35-37]. In this sense, *B. subtilis* spores have the capacity to resist extreme pH conditions, UV irradiation, high temperatures, solvents and long time periods of storage without refrigeration [1] In summary, *B. subtilis* has a) versatility of growth nutrients utilization, b) high level of enzymes production, c) secretion of antimicrobial compounds, d) spore

producer, e) develops in aerobic and anaerobic conditions, and f) *B. subtilis* is Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA) (Figure 1). In this sense, *B. subtilis* in “theory” could be considered as a perfect multifunctional probiotic bacterium for humans and animals [1,16-19,23,24,27,33,38,39].

***Bacillus subtilis* Recombinant Probiotic Development**

The global sales of probiotic supplements were predicted to rise 48% from \$2.7 bn in 2011 to \$4 bn in 2016. In US, per capita spending on probiotic supplements is expected to nearly double by 2016 and overtakes Japan [34]. In this sense, the utilization of non-recombinant probiotic strains in humans, farmed animals and aquaculture systems is well established, principally for the competitive exclusion of pathogenic bacteria (Table 1) [27,33,40-42].

On the other hand, utilization of recombinant probiotic strains in humans and animals it is not accepted due Genetically Modified Organism (GMO) regulations [27,43]. *B. subtilis* strains with overproduction of antimicrobial peptides or enzymes already exist scientifically and commercially, but only their products are being sold not the strains [12,23,44,45]. Overproduction of antimicrobial peptides and/or enzymes by recombinant probiotic strains could induce health problems in animals and consequently in the consumers, as the unregulated utilization of synthetic antibiotics did. Human and animal market “supposedly” consumes non recombinant *Bacillus* probiotic strains, due health FDA regulation and restrictions that must be solved, before acceptance of GMO products commercialization.

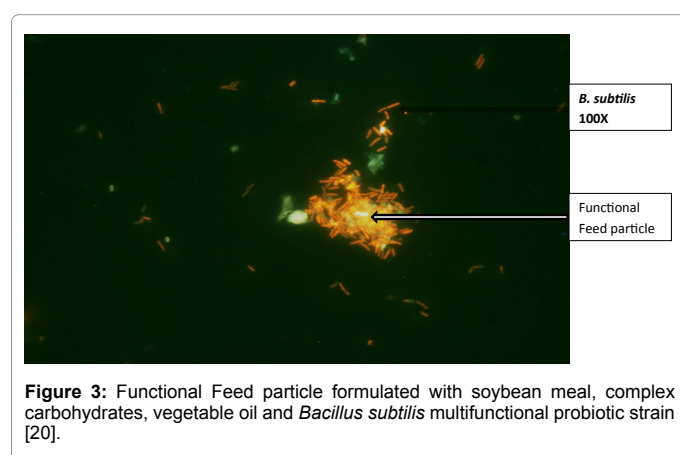
However, diseases like the one induced by White Spot Syndrome Virus (WSSV) in shrimps, cannot be controlled at the moment by any non-recombinant bacterial probiotic strain. In this sense, production of vaccines using *B. subtilis* probiotic recombinant strains, are starting to be develops to prevent disease impacts on animals and humans [15,46-48]. Environmental contamination and utilization of strong stressful agents are inducing development of new bacterial and virus diseases. In this sense, recombinant probiotic utilization could be the only effective way to fight versus pathogen agent’s proliferation. However, even when promising results have been obtained inducing immunization with recombinant *B. subtilis* probiotic strains, several FDA tests must be carried out in animal models before a massive food/feed inclusion and commercialization of recombinant probiotics can be done.

Aquaculture Development Restrictions

The United Nations Food and Agriculture Organization (FAO) define aquaculture as the farming of aquatic organisms including mollusks, crustaceans, fish and plants. Marine capture fisheries and aquaculture, supplied the world with about 154 million tons of marine products in 2011 [49]. However, global population demanding for marine food products is increasing around the world, but fisheries capture production has leveled off, due principal fishing areas have reached their maximum potential and some have started to go down [24,49,50]. In this sense, aquaculture the multibillion dollar fastest growing industry of the world food economy, is increasing by more than 10% per year and currently accounts for more than 50% of all shrimp/ fish consumed [49]. For this reason, prices of fishmeal and oil utilized precisely to produce aquaculture feeds have increased considerably last years, making the industry unprofitable in many countries [24,50]. Worry about negative impacts of fishmeal and oil overproduction in ecology of global fisheries and environmental alterations have arisen [51,52]. Aquaculture represents the greatest potential to meet demands for aquatic food products; however, instead of helping to ease the crisis

BACILLUS PROBIOTIC FOR HUMAN USE			
Product	Manufacturer		Comments
Bio-Kult®	Probiotics International Ltd (Protexin), UK. http://www.bio-kult.com		<i>B. subtilis</i> is one component of 14 strains carried in this probiotic supplement (minimum 2x10 ⁹ CFU/capsule).
Medilac-Vita®	Hanmi Pharmaceutical Co. Ltd., Seoul, Korea http://www.hanmipharm.com		<i>B. subtilis</i> in combination with <i>Streptococcus faecium</i> (at 10 ⁸ /g).
Primal Defense®	Garden of Life®, Florida, USA. http://www.gardenoflife.com		<i>B. subtilis</i> is one of the 12 bacteria species in this probiotic.
Biobaby®	Ildong Pharmaceutical Ho Chi Minh, Vietnam http://www.thuocbo.com.vn/Danh-muc-thuoc-bo/men-vi-sinh/biobaby.html		Granules for solution with <i>B. subtilis</i> 3 mg/g (3x10 ⁶ CFU/g), in combination with <i>Lactobacillus sporogenes</i> and <i>Clostridium butyricum</i> (1x10 ⁷ CFU/g).
Totalflora	Roux-Ocefa Laboratorios Buenos Aires, Argentina http://www.roux-ocefa.com		Each capsule contains: <i>Lactobacillus acidophilus</i> , <i>Bifidobacterium bifidum</i> , <i>Bacillus subtilis</i> , <i>Saccharomyces cerevisiae</i> and <i>Bacteriophages</i> .
BACILLUS PROBIOTIC FOR AQUACULTURE			
Brand	Manufacturer		Comments
Naturalle <i>Bacillus subtilis</i>	Wuhan Nature's Favour Bioengineering Co., Ltd, Wuhan City, China http://www.wuhannature.com		<i>Bacillus subtilis</i> (2x10 ¹⁰ CFU/g).
Biozyme	Bio-Pharmachemie Joint-Venture Company, Ho Chi Minh, Vietnam http://www.biopharmachemie.com		<i>Bacillus subtilis</i> and <i>Saccaromyces cerevisiae</i> .
Fubon <i>Bacillus subtilis</i>	Angel Yeast Co., Ltd. Hubei, China http://www.angelyeast.com		<i>Bacillus subtilis</i> (≥ 20 billion CFU/g).
Bioron	American Pharma International, India http://www.americanpharmainternational.com		Each kg contains: <i>Bacillus subtilis</i> (4.5x10 ⁸ CFU), <i>B. licheniformis</i> , <i>B. megaterium</i> , <i>Lactobacillus lactis</i> , <i>L. helveticus</i> , <i>Nitrosomonas sp.</i> , <i>Nitrobacter sp.</i> , <i>Saccharomyces cerevisiae</i> and <i>Aspergillus oryzae</i> .
Lactomin	American Pharma International, India http://www.americanpharmainternational.com		Each kg contains: <i>Bacillus subtilis</i> (45,000 million CFU), <i>B. licheniformis</i> , <i>Lactobacillus acidophilus</i> , <i>L. sporogenes</i> and <i>Saccharomyces cerevisiae</i> .
BACILLUS PROBIOTIC FOR VETERINARY USE			
Brand	Animal	Manufacturer	Comments
BioGrow®	Poultry, calves and swine	Provita Eurotech Ltd., Omagh, Northern Ireland, UK http://www.provita.co.uk	Listed as containing spores of <i>B. licheniformis</i> (1.6x10 ⁹ CFU/g) and <i>B. subtilis</i> (1.6x10 ⁹ CFU/g)
BioPlus® 2B	Piglets, chickens, turkeys for fattening	Christian Hansen Hoersholm, Denmark http://www.chr-hansen.com	Mixture (1/1) of <i>B. licheniformis</i> (DSM 5749) and <i>B. subtilis</i> (DSM 5750) at 1.6x10 ⁹ CFU/g of each bacterium, EU approved
Biozyme	Poultry, swine, shrimp, fish	Bio-Pharmachemie Joint-Venture Company Ho Chi Minh, Vietnam http://www.biopharmachemie.com	Formulation: <i>B. subtilis</i> and <i>Saccharomyces cerevisiae</i> .
Proflora	Poultry	Zoetis, New Jersey, USA http://www.zoetis.com	Proflora is a unique combination product with a Live DFM <i>B. subtilis</i> strain QST 713 plus Beta Mos®, a yeast-extract prebiotic.
Vime-Baciflor	Pigs, poultry	Vemedin Corporation, Can Tho, Vietnam http://www.vemedim.vn	<i>Bacillus subtilis</i> .

Table 1: *Bacillus subtilis* commercial products from humans and animals.



in wild fisheries, unsustainable aquaculture development may aggravate the problems and create new ones, damaging our important and already-stressed coastal areas [53]. In order to solve these aquaculture restrictions, the development of Functional Feeds using alternative-economical vegetable protein, carbohydrates, lipids and “*Bacillus subtilis* spore producing multifunctional probiotic bacterium” are recommended, to increase aquaculture profitability and environmental and ecological sustainability [16-19,24].

Importance of Functional Feed Development in Aquaculture

The rapid development of aquaculture from last years has increased feed ingredients demand and its prices, for this reason feeding represent 40-60% of the total production costs in shrimp/fish farming. In this sense, aquaculture industry development has become a great challenge for future generations; not only for feeding expenses but also from availability of fishmeal and oil ingredients. Reduced inclusion of fishmeal and oil in formulations is a tendency for almost a decade. It is estimated that these ingredients will be minimal or null in diets by the year 2020 [50]. Consequently, alternative feed formulation ingredients development is urgent and they must meet the animal nutritional requirements, be less expensive and sustainable with the environment [17,18,35,51,52,54-57]. Partial or complete fishmeal and oil substitution by alternative-economic vegetable protein, carbohydrates and lipids has been the goal to the field for more than 10 years, without satisfactory results due toxic effects and anti-nutritional nature of some vegetable molecules [58-63]. However, feeds evolution with inclusion of vegetable ingredients inducing; growth, health, environmental and economic benefits beyond traditional feeds, is a great opportunity to assure the future of a profitable and sustainable aquaculture [16-19,24]. Nevertheless, proteases, carbohydrases and lipases limited expression in shrimp/fish is the major obstacle to digestion and assimilation of vegetable ingredients, regulating its high level inclusion in feed

formulations [58-63]. Thus, the utilization of specifically selected “*Bacillus subtilis* spore producing multifunctional probiotic bacterium” has emerged as a solution with huge applications in the aquaculture feeding industry (Figures 1 and 2). In this sense, excellent results have been obtained in *Litopenaeus vannamei*, *Oreochromis niloticus* and *Atractoscion nobilis*; increasing digestion-assimilation of vegetable ingredients, improving Food Conversion Ratio (FCR) and inducing optimal growth on cultivated animals, when a *B. subtilis* probiotic strain have been added to feeds. Additionally, inhibition of pathogens proliferation and increased health status of animals was also improved. Finally, bioremediation of pond contaminated water was also achieved efficiently by *B. subtilis* probiotic bacteria used [16-19,24]. These results open great opportunities to new Functional Feeds production for shrimp and fish aquaculture (Figure 3).

Complex carbohydrates degradation, proteases inhibitors inactivation and lipids assimilation, were not a problem to animals when “*Bacillus subtilis* spore producing multifunctional probiotic bacterium” was added to vegetable based-feed formulations [16-19,24].

Conclusion

The world market for probiotics supplements has been growing for the last 50 years; today most dairy human products contain probiotic bacteria. Lactobacillus and Bifidobacterium are the most used genera principally for the exclusion of pathogens in humans and animals. However, species of these genera do not present multifunctional probiotic capacities as *B. subtilis* does [15-19,24]. In this sense, animal feed preparation processes usually require high temperatures and only spore producer bacteria are optimal to support these conditions. Other *Bacillus* than *B. subtilis* can also be used for feed preparation, however *B. subtilis* is one of the few recognized as safe by the FDA. *B. subtilis* enzymes support extreme environmental conditions increasing digestive human and animal capabilities, to assimilate ingredients from animal and plant origin [16-19,24]. The capacity of *B. subtilis* to grow aerobically and anaerobically allows this bacterium to survive and growth adequately inside and outside humans and animals, and in aquaculture water systems. All these properties give *B. subtilis* the potential capacity to become one of the “most perfect multifunctional probiotic bacteria” to formulate Functional Feeds for aquaculture. However, *B. subtilis* probiotic properties are strain-specific meaning bioprospection and selection methods needs to be well designed.

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