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Review Articles

Research progress on the application of *Clostridium butyricum* in shrimp aquaculture

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In recent years, with the continuous expansion of aquaculture and the vigorous development of the aquaculture industry, the breeding environment and diseases have become increasingly prominent, and the quality and output of aquaculture products have been affected to a certain extent, which has become one of the important factors restricting the development of aquaculture industry. *Clostridium butyricum* is a kind of clostridium bacillus, a gram-positive bacterium, strictly anaerobic, which can form spores and produce short-chain fatty acids, biofuel compounds, and biomaterial precursors such as H_2, butanol, and 1,3-propanediol in the process of fermenting carbohydrates and sugars. This article reviews the isolation, identification, main biological functions, and mechanism of *C. butyricum* in the gastrointestinal tract of aquatic animals and summarizes the role of *C. butyricum* in improving the growth performance, digestibility, survival, immune response, disease resistance, and intestinal structure, as well as regulating the intestinal symbiotic microbiota and metabolic disorders of aquatic animals. To provide insights into the key research directions of *C. butyricum* in shrimp aquaculture in the future, including research on the relationship between *C. butyricum* and the host immune system and the possibility of using *C. butyricum* as an antibiotic substitute for disease prevention and treatment, this study aims to provide a reference for the comprehensive utilization of *C. butyricum* in shrimp aquaculture and promote the high-quality development of the shrimp aquaculture industry.

INTRODUCTION

Shrimp farming is an important component of aquaculture, which plays a crucial role in ensuring sustainable development and global food security.[1] Due to improvements in breeding and aquaculture techniques, global shrimp farming production has grown rapidly. Currently, shrimp farming is moving towards intensification and diversification, but this has led to increased environmental pressure to some extent.[2] Therefore, it is necessary to pay comprehensive attention to the quality of feed, feed utilization, and aquaculture methods to improve the growth performance, feed efficiency, and disease resistance of cultured shrimp. In this context, the development of natural and friendly drug substitutes and growth promoters has become very important and urgent for healthy shrimp aquaculture.

Probiotics are live microbial feed supplements that have beneficial effects on host animals by improving their gut microbiota balance.[3,4] *Clostridium butyricum* is one type of probiotic, which is Gram-positive, anaerobic and widely present in the intestines of humans and animals. It has a positive glycolytic effect and produces a large amount of gas in a culture medium containing fermentable carbohydrates.[5-7] Short-chain fatty acids(SCFA) are the main products of *C. butyricum* fermentation and play an important role in improving the growth and immunity of aquatic animals. *C. butyricum* can also ferment sugars and glycerol into biofuel compounds and biomaterial precursors, such as H_2, butanol, butyric acid, and 1,3-propanediol.[8,9] In aquatic animals, *C. butyricum* is widely used as a probiotic to improve growth performance, feed efficiency, antioxidant capacity, and immune responses. So far, there has been no de-

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tailed review on the application of *C. butyricum* as a feed additive in shrimp farming. This article reviews the isolation, identification, safety, pathogenicity, mechanism of action, and its impact on the growth and health of aquatic animals such as shrimp, with the aim of providing a theoretical reference for the large-scale development and utilization of *C. butyricum* in shrimp farming and to promote the healthy, green, and sustainable development of the shrimp farming industry.

BIOLOGICAL CHARACTERISTICS AND ISOLATION IDENTIFICATION OF *CLOSTRIDIUM BUTYRICUM*

BIOLOGICAL CHARACTERISTICS OF *CLOSTRIDIUM BUTYRICUM*

BIOLOGICAL CHARACTERISTICS

Clostridium butyricum, also known as butyric acid bacteria or *Bacillus butyricum*, this type of fungus appears in a straight or slightly curved shape, existing alone or forming short chains, and sometimes forming filamentous structures. The colony appears as slightly convex, white, or cream colored with a diameter of 1-3 mm on agar medium, with irregular edges. As a strict anaerobic, Gram-positive bacterium, *C. butyricum* can produce acid by fermenting monosaccharides and disaccharides such as glucose and lactose but does not decompose gelatin or serum proteins. It can produce amylase, butyric acid, acetic acid and lactic acid, as well as small amounts of formic acid and propionic acid. This strain is widely distributed and can grow in environments ranging from 25 ℃ to 37 ℃ and pH 4.0 to 9.8, resisting harsh environments by producing spores.

PHYSIOLOGICAL CHARACTERISTICS

HEAT RESISTANCE

Research has shown that the heat tolerance of *C. butyricum* varies depending on its strain. Some studies have found some strains can survive treatment at 100 ℃ for 5 minutes. When other strains are treated at 80 ℃ for 30 min or 90 ℃ for 20 min, the survival rate can exceed 90%. For example, after treatment at 90 ℃ for 30 min, spore activity of strain DL-01 was almost unaffected, and the germination rate was 100%. However, after treating the spore fermentation broth of strain B1 at 80 ℃ for 30 min, the survival rate was only 6.48%.In addition, the heat resistance evaluation after mixing *C. butyricum* with the feed showed a survival rate of 70.43% after 2.5 min of treatment at 85 ℃, indicating that the longer the high-temperature treatment time, the lower the activity of the strain. These findings are of great significance for the use of *C. butyricum* in pelletized feed, suggesting that the temperature should be controlled during feed processing to maintain the activity of the strains.

DRUG RESISTANCE

C. butyricum can be used alone or in combination with antibiotics, and its sensitivity to different antibiotics varies depending on the strain. For example, a study found that a certain medicinal strain of *C. butyricum* showed sensitivity to multiple antibiotics while showing tolerance to other antibiotics. Another study compared the antibiotic resistance of different strains of *C. butyricum* and found that they were resistant to certain aminoglycoside antibiotics but more sensitive to other commonly used antibiotics. This indicates that the antibiotic resistance characteristics of *C. butyricum* may support its potential for combined use with antibiotics in specific situations, particularly when ensuring that there is no risk of drug resistance gene transfer.

ACID RESISTANCE

Research has shown that *C. butyricum* can survive under strongly acidic conditions, as observed in animal gastrointestinal environments. Its survival pH range is between 1.0 and 5.0, and it can grow at extreme pH values, with an optimal growth pH of 4.6 to 10.6. In addition, *C. butyricum* C1-6 showed a high survival rate in acidic environments, with survival rates of 72.5% and 91.8% after 3 h of incubation at pH 2 and 3 conditions, respectively. Compared with other probiotics, such as Lactic acid bacteria, Enterococcus, and Bifidobacterium, *C. butyricum* has a stronger tolerance to acidity and bile salts.

ISOLATION OF CLOSTRIDIUM BUTYRICUM

C. butyricum is often present in the intestines and environment of humans and animals[10] , with some non-toxic strains used as probiotics, while others are pathogens of infants and premature newborns.[11] *C. butyricum* can be isolated from various environments such as soil, yogurt, cheese, human feces, and blood.[12,13] Li *et al*. isolated *C. butyricum* from fish meal containing fish meal, and the specific method was as follows: 0.1g of fish meal was dispersed in 1 mL of homogeneous PBS and centrifuged at 500 rpm for 3 min. The supernatant was heated in an 80 ℃ water bath for 10 min, and then spread on a solid plate (containing 0.3% yeast extract, 1% tryptone, 1% glucose, 0.5% sodium chloride, 0.3% sodium acetate trihydrate, 0.05% cysteine, and 1.5% agar). Under ventilated conditions (containing 5% hydrogen, 10% carbon dioxide, and 85% nitrogen), the plate was anaerobically cultured at 37 ℃ for 48 h. Individual colonies were selected based on their color, size, and shape, and cultivated in liquid medium.[14] *C. butyricum* can also be isolated from the intestines of freshwater shrimp. Dilute the stock solution ten times with peptone water and inoculate 0.1mL of the stock solution into Enhanced Clostridium Culture Medium (ECCM) containing bacterial agar. After anaerobic cultivation at 37 ℃ for 48 h, select white, crystal clear, root-like protrusions with small branches on the edge, and identify them using the PCR method.[15,16] In addition, *C. butyricum* can also be isolated from the intestines of piglets, and piglet intestinal waste (1 g) was added to 250 mL of liquid Lactic Acid Decom-

position Medium (LADM) for cultivation, shaken at 30℃, and regularly monitored for lactic acid consumption. After rapid degradation of lactic acid, the bacteria were diluted 10 times and laid on a LADM solid medium placed in an anaerobic chamber. Anaerobic cultivation is carried out at 37℃ for 3-5 days. Single colonies were screened and purified using the stripe inoculation method in LADM solid culture medium, and then the isolated strains were inoculated into anaerobic tubes containing 10 milliliters of reinforced clostridium medium (RCM) liquid culture medium. The strains were hot pressed at 80 ℃ for 10 min, and then cooled with water. Cultivate the culture tube at 37 ℃ for 4 days and regularly monitor lactic acid consumption. This method can select strains with high lactic acid utilization and butyric acid conversion rates.[17]

IDENTIFICATION OF CLOSTRIDIUM BUTYRICUM

C. butyricum is active in glycolysis and produces a large amount of gas in a culture medium containing fermentable carbohydrates.[5-7] The colony diameter of *C. butyricum* is 1-4 millimeters, and it grows well on PY agar. The colony is white, round, or slightly irregular in shape, with a dark bump in the middle. The surface is moist and shiny. Through electron microscopy observation, the fungus (0.6-0.8 μ m x 3.0-7.0 μ m) is straight or slightly curved, single or in pairs, surrounded by flagella, blunt at the end, and slightly enlarged in the middle. It is reported to be a γ-hemolytic bacterium that is positive for amylase activity, but negative for cellulase, protease, lecithin, lipase, and catalase. Physiological and biochemical identification confirmed that the strain could also decompose alpha lactose, oligofructose, D-galactose, xylooligosaccharides, D-mannose, resistant starch, D-fructose, sucrose, D-glucose, starch, D-maltose, inulin, D-fibro disaccharide, D-mannitol, and glycerol, but cannot decompose L-sorbitol, D-sorbitol, and xylitol.[18]

Previously, the genomes of *C. butyricum* strains isolated from different sources, such as human feces and pit mud, have been sequenced.[19,20] For example, the genome sequence of *C. butyricum* isolated from a case of botulism was based on Sanger shotgun sequencing, which provides a genome sequence divided into 123 scaffolds, including 3827 putative protein coding genes and 259 putative pseudogenes.[21] Two key enzyme genes, acetyl CoA transferase and butyrate kinase, were identified in the butyric acid production pathway of *C. butyricum* CG-3. Obviously, it has been found that the bacteria can produce butyric acid when cultured under anaerobic conditions. Butyric acid is an important energy source for intestinal cells and has a proliferative effect on them.[22]

BIOLOGICAL FUNCTIONS OF CLOSTRIDIUM BUTYRICUM

C. butyricum belongs to Gram-positive probiotics, which have the characteristic of producing butyric acid and can grow endophytic spores in anaerobic environments. It is part of the normal gut microbiota.[15,23,24] C. butyricum can generate trace elements such as vitamin B and vitamin K, and can also produce enzymes such as amylase, lipase, and protease.[25] These enzymes can generate beneficial nutrients such as glucose and maltose through the fermentation process of C. butyricum, which aids digestion and absorption in the human body.[26] In addition, C. butyricum can also produce many enzymes that can break down polysaccharides in the feed, which is beneficial for the digestion and absorption of feed.

IMPROVE INTESTINAL STRUCTURE AND ENHANCE DIGESTIVE SYSTEM FUNCTION

As an essential short-chain fatty acid, butyric acid, which is a metabolic product of *C. butyricum*, is capable of stimulating the growth of intestinal epithelial cells. Furthermore, it can enhance the villus height of intestinal epithelial cells in aquatic animals, thereby enlarging the intestinal surface area and facilitating intestinal absorption of nutrients.[27] Meanwhile, the various short chain fatty acids produced by *C. butyricum* during its metabolic process can lower intestinal pH, increase digestive enzyme activity, and promote digestion and absorption of nutrients in the intestine.[28]In addition, butyric acid serves as the main source of energy for the repair of intestinal epithelial cells, reducing the harm caused by the intestinal absorption of toxins by repairing damage to the intestinal mucosa. *C. butyricum* can also produce various digestive enzymes, promoting the digestion and utilization of nutrients in aquatic animal bodies.[29]

Feeding *Penaeus monodon* with *C. butyricum* as a mixed feed can increase the villus height of intestinal epithelial cells, enhance the content of short chain fatty acids in the intestine of *P. monodon*, and enhance its anti-ammonia stress ability and immune function.[30,31] In addition, Poolsawat et al.'s research has shown that *C. butyricum* can significantly increase the height of tilapia foregut villi and reduce the number of *Escherichia coli* in the intestine.[32] These studies indicate that supplementation with *C. butyricum* can significantly alter the intestinal structure of aquatic animals. However, further research is needed to clarify whether changes in intestinal morphology are beneficial to host metabolism and health.

MAINTAINING THE BALANCE OF GUT MICROBIOTA AND INHIBITING THE GROWTH OF PATHOGENIC BACTERIA

C. butyricum can promote the growth of gut probiotics such as bifidobacteria and lactobacilli, and regulate the balance of gut microbiota. *C. butyricum* possesses the ability to colonize the intestines of aquatic animals via antagonistic mechanisms. It competes with pathogenic bacteria for colonization sites and establishes adhesive competitive inhibition against pathogenic bacteria. This effectively curbs the adherence of harmful bacteria to the intestinal mucosal surfaces of aquatic animals, thereby suppressing the proliferation of pathogenic bacteria and fostering the growth of beneficial bacteria. Thus, *C. butyricum* plays a pivotal role in maintaining equilibrium of the intestinal microbiota.[33, 34] Meanwhile, *C. butyricum* can inhibit the growth of harmful bacteria by competing for nutrients required for micro-

bial growth. *C. butyricum* is highly tolerant to low pH and relatively high bile concentration and temperature environments, and has been used as a good feed additive.[35] *C. butyricum* can also produce many prebiotics, including bacteriocins, fatty acids, and hydrogen, which help regulate animal antioxidant and antibacterial functions.[36-38] Research has shown that *C. butyricum* and its lipoteichoic acid components can inhibit the growth of pathogens, including *E. coli*, *Salmonella enteritidis*, and *Vibrio parahaemolyticus*. The metabolic production of bacteriocins can inhibit the growth of harmful bacteria such as *Pseudomonas aeruginosa* and *Pseudomonas aeruginosa*.[39]

ENHANCE THE IMMUNE SYSTEM

C. butyricum has immunomodulatory function and can act as an immune active factor in the intestinal mucosa, generating secreted immunoglobulin M (lg M) and immunoglobulin A (Ig A), or stimulating lymphoid tissue, thereby affecting cytokines involved in immune regulation and white blood globulins in the blood.Simultaneously, by promoting the development of immune organs in the body, immune function can be improved.[40] In the modulation of inflammatory responses, *C. butyricum* effectively regulates the immune signalling pathway through the precise modulation of the expression of specific receptors. This regulation enhances the overall immune function of the organism. After adding *C. butyricum* to the feed, lysozyme activity in the serum and surface mucus of American red fish (*Sciaenops ocellatus*) increased, accompanied by an enhancement in the activities of acid phosphatase (ACP) and phenolic oxidase (PO). At the same time, the concentration of lgM in the blood and the level of complement C3 in the serum increased and macrophage activity was significantly enhanced. In summary, adding *C. butyricum* to the feed improved the immune ability of American red fish.[41]

PROGRESS IN THE APPLICATION OF C. BUTYRICUM IN AQUACULTURE

APPLICATION METHODS OF C. BUTYRICUM

C. butyricum can act in aquaculture through three pathways: immersion in medium (water), oral administration, and injection.[8,31,42] *C. butyricum* added to water can grow by absorbing nutrients from the water.[43] By competing to utilize nutrients, pathogenic bacteria remain in a state of long-term hunger, leading to malnutrition, and beneficial bacteria dominate the water body.[44] One drawback of this method is that it cannot guarantee the absorption and utilization of *C. butyricum* by aquatic animals in the water.

In addition, *C. butyricum* can also be added to artificial feed and fed to aquatic animals to increase the beneficial microbial community in the intestine or by feeding halogenated insects or microalgae species rich in *C. butyricum* to improve the growth performance and survival rate of aquatic animals during the aquaculture stage.[45] Microencapsulation is another method of administering *C. butyricum*, which can directly affect the water quality, physical parameters, and health of aquatic animals. One advantage of this method is that aquatic animals can obtain various types of *C. butyricum* that their bodies need, but this method requires continuous testing of the survival ability of *C. butyricum* to ensure its effectiveness.[46]

Finally, direct injection can be used to ensure that *C. butyricum* enters the bodies of aquatic animals.[47] The disadvantage of this method is that it requires high time and cost to inject each shrimp, making it difficult to apply in large quantities. Additionally, there are also requirements for operational techniques and shrimp size.

ENHANCING THE GROWTH PERFORMANCE OF AQUATIC ANIMALS

Multiple reports have demonstrated the positive effects of *C. butyricum* addition to feed on various aquatic animals.[48-50] Compared with the control group, the addition of *C. butyricum* to the feed of *Miichthys miiuy* improved the growth performance of the fish and reduced the feed coefficient. The modification variable was related to the amount of *C. butyricum* added, and the optimal dosage was 1.0×10^9 cfu g^{-1}.[49] Supplementing *C. butyricum*, *Lactobacillus plantarum*, or *Bacillus subtilis* 1.0×10^8 cfu g^{-1} respectively in the feed of *Pampus argenteus* showed that after 30 and 60 days of feeding with *C. butyricum*, the final body weight, relative weight gain, and specific growth rate were significantly higher than those of the control group. In addition, the feed conversion rate (FCR) was significantly higher than that in the control group.[50] Studies have shown that feeding feed containing *C. butyricum* can enhance the digestive enzyme activity of silver pomfret, improve feed utilization, and thus, improve its growth performance.[51]In another recent study, adding 1.0×10^5 cfu of *C. butyricum* per gram of diet increased the specific growth rate and feed intake of tilapia (*Oreochromis mossambicus*), improving feed efficiency.[51] The above research indicates that supplementation with *C. butyricum* is of great significance for the growth performance and digestive ability of aquaculture species.

IMPROVING THE DISEASE RESISTANCE OF AQUATIC ANIMALS

C. butyricum has a positive effect on improving the immune system of aquatic animals. Pan et al. studied the effect of the *C. butyricum* CB2 strain on its antibacterial activity in the intestinal epithelial cells of *M. miiuy*. The results showed that on agar plates and cell models, the *C. butyricum* CB2 strain had high antagonistic activity against *Aeromonas hydrophila* and Eel bacteria. In addition, this strain can also inhibit cell apoptosis induced by *S. enteritidis* and *V. parahaemolyticus*.[52] As a probiotic, *C. butyricum* can prevent fish from being infected with *Enterococcus* and *V. parahaemolyticus*, thereby promoting fish health and improving disease resistance.[39] In tilapia, adding different concentrations of *C. butyricum* to the diet can significantly reduce the cumulative mortality rate of fish after 14 consecutive days of stress caused by *Streptococcus lactis*.[53] *C. butyricum* has the capability to enhance the survival rate of crucian carp when subjected to herpesvirus (CaHV) stress.

It effectively diminishes the replication and dissemination of CaHV within fish, while concurrently activating the expression of crucial immune-related genes, including interleukin-11, interferon regulatory factor 7, and protein kinase R. Thus, *C. butyricum* has a promoting effect on the innate immune response of early viral infections in fish, thereby contributing positively to the resistance of fish to viral infections.[36] He et al. showed that the addition of live *C. butyricum* and its fermentation broth can improve the growth performance, digestive enzyme activity, and non-specific immune response of pearl gentian grouper (*Epinephelus fuscoguttatus*♀×*E. lanceolatus*♂).[54]

At the same time, *C. butyricum* can produce a large amount of short chain fatty acids such as butyric acid, forming an acidic environment, reducing pH value, making it difficult for other microorganisms to survive, thereby effectively inhibiting the growth of pathogenic bacteria in the intestinal tract of fish and playing a role in disease resistance.[55,56]

IMPROVING THE AQUACULTURE ENVIRONMENT

Adding *C. butyricum* to aquaculture can affect water quality.[57,58] Adding external bacteria such as *C. butyricum* or nitrifying bacteria, lactobacilli, and spores can affect the dissolved oxygen, pH, ammonia nitrogen, and alkalinity concentrations in water, as these bacteria oxidize ammonia nitrogen to nitrite and convert it into nitrate.[59-61] Ammonia in aquaculture may originate from unfinished feed, feces, dead or decaying plankton, and debris in the air.[62-64] *C. butyricum* in water can also compete with pathogenic bacteria for survival space, thereby reducing the number of pathogenic bacteria.[65-67]

Adding *C. butyricum* to aquaculture water may result in a decrease in dissolved oxygen content, yet this reduction remains within the acceptable and safe range necessary for the survival and well-being of aquatic animals.[68] This may be the result of an increase in the number of bacteria in water after the addition of *C. butyricum*. The increase in bacteria in water intensifies competition for oxygen.[69] Therefore, it is necessary to apply an appropriate dosage of *C. butyricum* and monitor indicators such as dissolved oxygen in aquaculture water to avoid excessive consumption of dissolved oxygen in the water.

C. butyricum is also used in biological systems for aquatic animal production and has been proven to maintain good water quality in these systems. *C. butyricum* promotes water quality through nitrification and denitrificationand induces the formation of biofilms.[70] Bioflocculation is a collection of various organisms and organic matter, such as bacteria, fungi, algae, protozoa, and worms. When integrated into flocculation, shrimp can the feed on these flocs, reduce the amount of feed used in farming, and improve feed conversion rate.[71]

THE PROBIOTIC EFFECT OF C. BUTYRICUM IN SHRIMP FARMING

CLOSTRIDIUM BUTYRICUM PROMOTES THE GROWTH OF SHRIMP

In shrimp farming, feeding a diet supplemented with 11.0×10^9 cfu g^{-1} *C. butyricum* can improve the growth performance and crude protein content of shrimp, reduce the feed coefficient,and maintain a normal survival rate after 56 days. This indicates that *C. butyricum* can improve the growth performance and feed utilization of shrimp.[27] Other studies have shown that compared with the control group, adding 0.25%, 0.5%, and 1.0% *C. butyricum* can increase the intestinal amylase and protease activity of *Litopenaeus vannamei*, whereas adding 0.25% and 0.5% *C. butyricum* can affect lipase activity.[27]

The digestive enzyme activity of crustaceans plays a central role in nutritional physiology by directly or indirectly regulating growth, molting cycles, and complex dietary formulas. The increasing trend observed in digestive enzyme activity can potentially be attributed to the symbiotic behavior exhibited by *C. butyricum*. This behavior stimulates the establishment of healthier gut microbiota, subsequently leading to modifications in the selection of bacterial enzymes. Nimrat et al.showed that *C. butyricum* not only promoted shrimp growth, but also greatly enhanced the activity of enzymes in shrimp bodies.[59]

Duan et al.'s research showed that compared with the control group, adding 1.0×10^8 cfu of *C. butyricum* per gram of feed significantly improved the growth performance of *L. vannamei* and reduced the feed coefficient. This indicates that *C. butyricum* can effectively improve the feed efficiency of *L. vannamei*, harvest more shrimp products, and achieve greater economic benefits by using less feed.[72] Another study has found that the effect of *C. butyricum* on the digestive activity, metabolic capacity, and SCFA content in the intestine of *L. vannamei* is related to the amount of *C. butyricum* added. The incorporation of *C. butyricum* into feed formulations has been shown to enhance pepsin activity, trypsin gene expression, serotonin concentration, and SCFAs content. Furthermore, supplementation of dietary intake with *C. butyricum* has been shown to augment amylase and lipase activity, along with alpha-amylase gene expression.[28]

Adding 1.0×10^{10} and 2.0×10^{10}cfu of *C. butyricum* per kilogram of diet can improve the growth rate of *L. vannamei*, increase the survival rate of shrimp after 24 and 48 h of exposure to nitrite stress, and improve the feed conversion rate.[73] *C. butyricum* can increase the content of SCFAs such as acetic acid, propionic acid, and butyric acid in the shrimp intestine. Recent research has demonstrated that incorporating various forms of *C. butyricum* strains, including live cells, cell-free extracts, spray-dried spores, or a combination of live cells and supernatants, into the feed formulation can significantly enhance the growth performance of *L. vannamei*. This supplementation is effective in augmenting the final weight and specific growth rate of the

species as well as optimizing feed efficiency by improving the feed conversion rate.[51]

CLOSTRIDIUM BUTYRICUM AFFECTS THE EXPRESSION OF RELATED GENES

In shrimp farming, the addition of *C. butyricum* to feed increases the total antioxidant capacity (T-AOC), lysozyme (LZM), and relative expression levels of Toll and immunodeficiency genes in *L. vannamei*. However, the activity of inducible nitric oxide synthase (iNOS) increased in the 0.5% and 1.0% groups, while the expression of the heat shock protein 70 (HSP70) gene increased in the 1.0% group. The nitric oxide (NO) was not affected.[27] When exposed to ammonia stress, the intestinal immune biochemical indicators, such as T-AOC, LZM, iNOS, and NO, as well as the expression levels of HSP70, hydrogen peroxide, and immunodeficiency genes in the group supplemented with *C. butyricum*, were higher than those in the control group. This indicates that *C. butyricum* may affect the intestinal immune response of *L. vannamei*, thereby protecting intestinal health against environmental stress by stimulating its antioxidant activity and immune response.[27]

In addition, feeding *C. butyricum* can enhance the relative expression of immune related genes, such as propofol oxidase (proPO), lipopolysaccharide, and β -1,3-glucan binding protein, LZM, chitin, and superoxide dismutase (SOD) in *L. vannamei*.[74] Spraying feed supplemented with spores or a mixture of live cells and supernatant can stimulate the expression of SOD, LZM, proPO, Toll, immune deficiency, Relish, TOR, 4E-BP, eIF4E1, and eIF4E2 genes in the serum of *L. vannamei*.[51] However, the addition of *C. butyricum* fermentation supernatant did not significantly affect the expression of proPO, SOD, Toll, immune deficiency, Relish, elF4E1, or elF4E2 genes in the body of *L. vannamei*. These results indicate that the mixture of spores and live cells of *C. butyricum* with the supernatant exhibited better probiotic effects in regulating the immune response of *L. vannamei*. In addition, adding *C. butyricum* (1.0×10^{11} or 1×10^{12} cfu kg^{-1}) to feed can enhance the immune response of *L. vannamei*, while stimulating the activity of alkaline phosphatase (AKP), ACP, LZM, and total nitric oxide synthase. *C. butyricum* promotes respiratory activity and stimulates the expression of Toll, immune deficiency, and Relish genes in the lymphoid organs of shrimp.[51]

The enhancement of serum and hemolymph immunity of *L. vannamei* by *C. butyricum* was also related to the dosage added. When the dosage was 1.0×10^{11} cfu·kg^{-1} and 1.0×10^{12} cfu·kg^{-1}, immunity was significantly enhanced. In addition, it has been reported that feeding *C. butyricum* at a concentration of 1.0×10^{9}-10^{12} cfu·kg^{-1} can increase the expression level of immune genes in the lymphoid organs of *L. vannamei*. After 1-2 hours of injection of inactivated *V. parahaemolyticus*, the expression of the Relish gene in shrimp fed with *C. butyricum* was significantly up-regulated, and the immune stress response of *L. vannamei* was accelerated, proving that *C. butyricum* is beneficial for the immune system's resistance to pathogens.[51]

CLOSTRIDIUM BUTYRICUM ENHANCES DISEASE RESISTANCE

In shrimp farming, adding a fermentation supernatant of *C. butyricum* strain CBG01 at a concentration of 120 mL/kg of feed to the diet improved the survival rate of *L. vannamei* under *V. parahaemolyticus* stress. In addition, compared with the control group, the survival rate of *L. vannamei* with added *C. butyricum* CBG01 (1.0×10^{8}-1.0×10^{12} cfu kg^{-1}) was significantly improved after being challenged with *V. parahaemolyticus*. The improvement in survival rate is related to the dosage, and the highest survival rate was observed in shrimp fed *C. butyricum* CBG01 at a concentration of 1.0×10^{12} cfu kg^{-1}.[75] In summary, *C. butyricum* helps inhibit the growth of pathogens and improve survival rates, indicating its beneficial effects on the health of aquatic animals.

C. butyricum can also promote the secretion of digestive enzymes. In *P. monodon*, an increase or decrease in the number of digestive enzymes can be used to evaluate the effect of *C. butyricum* on its digestive and immune abilities. For example, enzymes such as alpha-amylase, lipase, and trypsin play important roles in nutrient digestion.[42] In crustaceans such as *Macrobrachium rosenbergii*, feeding *C. butyricum* additive has a significant positive effect on inhibiting the growth of *Vibrio harveyi* and increasing the growth performance and digestive enzyme activity of shrimp.[15,76]

Duan et al. investigated the effects of the dietary content of *C. butyricum* on the growth, intestinal digestive enzyme activity, antioxidant capacity, and resistance to nitrite stress in *L. vannamei*. By feeding diets containing different levels of *C. butyricum* 0% (control), 0.5% (CB1), 1.0% (CB2), and 2.0% (CB3), results showed that CB2 and CB3 can effectively promote shrimp growth. After 24 and 48 h of nitrite stress, the survival rate of shrimp increased compared with that of the control group. The intestinal amylase and trypsin activities of the three *C. butyricum* groups were all improved, whereas lipase activity was only affected in the CB3 group. After 56 days of cultivation under normal conditions, the activity of SOD and the expression levels of hsp70 and ferritin genes in the shrimp intestines increased. The activity of glutathione peroxidase (GPx) in the CB2 and CB3 groups also increased. After 24 and 48 h of exposure to nitrite stress, the activity of intestinal antioxidant enzymes (SOD, CAT, and GPx), as well as the expression levels of hsp70 and ferritin in the three groups of *C. butyricum* were higher than those in the control group. These findings suggest that *C. butyricum* has the potential to enhance the growth of *L. vannamei*. Additionally, it is capable of elevating the activity of intestinal digestive enzymes and antioxidant enzymes, subsequently increasing the immune ability against nitrite stress. This signifies that *C. butyricum*, serving as an effective probiotic in shrimp aquaculture, possesses the capability to augment the disease resistance of shrimp.[77]

Other studies have shown that dietary supplementation with *C. butyricum* can significantly increase intestinal SCFA content, provide nutrition for intestinal epithelial cells, and maintain the structural integrity of the immune system.

SCFA can increase the activity of digestive enzymes and inhibit pathogens by reducing intestinal pH. In addition, *C. butyricum* can induce an increase in intestinal immune biochemical indicators and gene expression levels, thereby enhancing immune function against ammonia stress. Therefore, integrity of the intestinal structure, digestive ability, and immune ability jointly improve the intestinal health status, ultimately leading to improved disease resistance in shrimp. These research indicate that adding *C. butyricum* to shrimp diet has potential benefits in improving shrimp yield, intestinal health, and crude protein content in the shrimp body.[78]

With increasing *C. butyricum* content in the feed, the concentrations of LZM, ACP, and AKP in *L. vannamei* exhibited a notable increase, whereas the glutathione transaminase content decreased. This finding suggests that substituting C. butyricum with FM in feed containing concentrated cottonseed protein can enhance the non-specific immune response of *L. vannamei* the same time, while the secretion of *C. butyricum* has the effect of repairing intestinal damage.[79] The addition of 1% and 5% *C. butyricum* can significantly improve the growth rate of *Macrobrachium nipponense* and increase the activity of SOD and CAT, leading to tolerance and resistance to nitrite.[80]

Sumon et al. measured the antagonistic effects of *V. harveyi* and *C. butyricum* on the growth performance of freshwater shrimp,as well as their probiotic effects. After the addition of *C. butyricum*, the abundance of *V. harveyi* showed a significant decrease. When *C. butyricum* was added to the feed, the growth rate of shrimp was significantly higher than that of the shrimp in control group. This study showed that a diet supplemented with *C. butyricum* is beneficial for the cultivation of *M. rosenbergii*. It effectively enhances the growth performance of shrimp, augments protease and amylase activities, and suppresses the propagation of pathogens, thereby bolstering the disease resilience of shrimp. The results of this study will contribute to improving the aquaculture of freshwater shrimp.[81]

IMPROVING INTESTINAL STRUCTURE

Little research has been conducted on changes in the gut structure of aquatic animals under the influence of *C. butyricum*. A previous study reported that adding 0.25%, 0.5%, or 1.0% *C. butyricum* to the diet of *L. vannamei* can increase the tight junctions of intestinal epithelial cells, neat arrangement and density of microvilli, and height of intestinal epithelial cells, but no signs of intestinal cell necrosis or cell damage were observed.[27] In addition, spraying live cells, dried spores, or a mixture of live cells and the supernatant of *C. butyricum* significantly increased the survival rate of shrimp compared to the control group. In addition, supplementation with different concentrations of *C. butyricum* improve the villus height and intestinal wall thickness of *L. vannamei*. *C. butyricum* can increase the connectivity and height of intestinal epithelial cells in *P. monodon*, making microvilli denser and more organized. The intestine exhibited no indications of intestinal cell necrosis or cellular damage, suggesting that *C. butyricum* effectively enhances the shrimp's capacity to digest and assimilate nutrients from the feed.

EXPECTATION

In the past few decades, pathogenic bacteria and viruses have become the main causes of disease in shrimp farming. Antibiotics can improve these diseases to a certain extent; howere, in the long run, they can develop resistance and endanger host organisms and consumers. Therefore, the need for more friendly alternatives is increasing.[82,83] *C. butyricum* is a candidate product served as a dietary supplement, capable of enhancing the resistance against pathogenic microorganisms within aquaculture systems. Additionally, it has been demonstrated to enhance immune parameters while maintaining the well-being of shrimp. *C. butyricum* is a better alternative to antibiotics and similar products for protecting and maintaining environmental stability.[84]

Although *C. butyricum* has many benefits, its improper use can lead to excessive nutrient production and microbial disruption. In summary, there is an urgent need to gain a deeper understanding of the genetic composition, transcriptome, and proteome profile of *C. butyricum* in order to improve the methods and comprehensive practical applications of C. butyricum in shrimp farming.[85] Compared to antibiotics, *C. butyricum* has a higher efficacy in increasing the yield of aquatic organisms. However, further studies are needed to investigate the effects of the addition of *C. butyricum* on the regulation of gut health and the physiological and biochemical response mechanisms of aquatic organisms.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

AUTHORS' CONTRIBUTION

Conceptualization: Jingyan Li (Equal), Jieyi Wang (Equal), Falin Zhou (Equal), Keng Yang (Equal), Song Jiang (Equal). Writing – review & editing: Jingyan Li (Equal), Jieyi Wang (Equal), Dewei Kong (Equal), Falin Zhou (Equal), Jianzhi Shi

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ETHICAL APPROVAL

The whole experiment was conducted according to the guidelines established by the National Institutes of Health.

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