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Bacillus velezensis LG37 absorbs and utilizes ammonia nitrogen from aquaculture water and enhances the toxicity tolerance of grass carp (*Ctenopharyngodon idella*) to ammonia (non-ionic ammonium)

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Abstract

High concentrations of ammonia nitrogen lead to multi-organ damage, decreased immunity, and metabolic disorders in aquaculture animals, which cause disease outbreaks. This study mainly screened probiotic strains for good control of the concentration of ammonia nitrogen and identified strain LG37 as *Bacillus velezensis* according to its morphological, 16S rDNA, phylogenetic, and genomic data analysis. This LG37 strain achieved efficiently purifies aquaculture water and enhances the toxicity tolerance of grass carp to ammonia. The experimental on growth characteristics showed that LG37 exhibited a satisfactory growth trend with 10 types of sugar, 17 types of amino acids, and 6 types of organic nitrogen as the carbon or nitrogen source. Under the conditions of initial dissolved oxygen at 6±1 mg/L, the temperature at 27±2°C, and pH 7±0.3. Through wastewater treatment equipment, which degraded ammonia nitrogen and the feed protein in grass carp culture wastewater reached up to 55.5% and 73.6% within one week, respectively. Moreover, in the acute toxicity test, when feed without LG37 was fed to grass carp, the safe concentration of ammonia nitrogen (non-ionic ammonium) was 3.37 mg/L (0.2 mg/L). When feed with LG37 was fed to grass carp, the safe concentration of ammonia nitrogen (non-ionic ammonium) was 5.11 mg/L (0.31 mg/L). Compared to feed without LG37, the feed with LG37 increased the toxicity tolerance of grass carp to ammonia nitrogen (non-ionic ammonium) by 51.63% (55%).

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Introduction

Aquaculture is one of the most rapidly growing food production industries in the world. Over the past 30 years, with the continuously increasing human demand for aquatic protein, aquaculture has gradually developed from a small-scale model to a high-density, intensive recirculating water culture model (Schumann et al., 2020). According to food chain efficiency theory, the high-density culture model is accompanied by a large amount of feed protein input during the first nitrogen cycle, and only a small portion of the total amount of feed protein nitrogen is absorbed by aquacultured animals. The remainder of the feed protein nitrogen is recirculated in the aquaculture water by means of residual feed and excreta (Xiao et al., 2019). Feed protein nitrogen eventually exists in aquaculture water in the form of protein, amino acids, and inorganic nitrogen (Hlordzi et al., 2020; Liu et al., 2020).

Under the action of microbial decomposition, the organic nitrogen in the aquaculture water is further transformed into inorganic nitrogen, resulting in the accumulation of inorganic nitrogen (Gao et al., 2019; Deng et al., 2021). Inorganic nitrogen mainly includes ammonia nitrogen, nitrite nitrogen, and nitrate nitrogen, and ammonia nitrogen exists in the form of ionic ammonium and non-ionic ammonium. Ionic ammonium exists in a hydrated form and is less toxic to cultured animals. Non-ionic ammonium is a fat-soluble substance that can penetrate biofilms and enter the body, and it is highly toxic to animals (Solanki et al., 2022; Xu et al., 2021)

Ammonia nitrogen stress can promote the proliferation of fish silk tissue, thickening of mucous cells, decreased oxygen-carrying capacity, liver tissue edema, and decreased antioxidant capacity, which affect growth and survival. Nitrite nitrogen is an intermediate product of ammonia nitrogen conversion to nitrate nitrogen in an aquaculture environment. Nitrate nitrogen is considered less toxic to aquaculture animals (Yun et al., 2019). When the total amount of inorganic nitrogen in aquaculture water is maintained, there is a dynamic balance between the thr.ee types of inorganic nitrogen (ammonia, nitrite, and nitrate). However, because nitrite nitrogen is poorly absorbed and utilized by microorganisms, this study aimed to decrease the concentration of inorganic nitrogen by rapidly reducing ammonia nitrogen concentration.

Bacillus is the main probiotic used for purifying aquaculture water. It can rapidly absorb and utilize inorganic nitrogen, which can improve intestinal microflora and increase the immunity of aquacultured animals (Hlordzi et al., 2020; Khan et al., 2021). *Bacillus* can also secrete various extracellular hydrolases to efficiently decompose organic matter. It decomposes organic matter efficiently, and it produces a series of antibacterial peptides to widely inhibit the growth and proliferation of harmful microorganisms such as bacteria, fungi, and viruses in aquaculture water. *Bacillus* has the advantages of high efficiency, low cost, no toxicity, and safety in purifying aquaculture water, and this bacterial genus can produce dormant spores that are convenient for product processing, storage, and transportation. Therefore, the application prospects for *Bacillus* are very broad (Hlordzi et al., 2020).

In the current study, a probiotic named LG7 was isolated from an aquaculture wastewater pond, and it was determined that this bacterial strain can efficiently absorb and utilize ammonia nitrogen and residual feed protein already present in water used for aquaculture of grass carp (*Ctenopharyngodon idella*). The growth curve of LG37 was determined, and circulating water body treatment equipment was designed based on the characteristics of aquaculture wastewater to decrease the amount of inorganic nitrogen and residual feed protein. According to the aquatic toxicity method, the role of LG37 in decreasing the toxicity experienced by grass carp from ammonia nitrogen was investigated. This study provides an experimental and theoretical basis for applying LG37 in aquaculture.

Materials and Methods

Isolation and identification of LG37

The spore-forming *Bacillus* species used in this study was isolated from a local fish pond in Wuhan, China, based on its growth in inorganic salt minimal medium with inorganic nitrogen (ammonium, nitrite, and nitrate) as the sole nitrogen source after heating at 90°C for 10 min (Liu et al., 2020). The colony morphology was observed by streak plates using Luria-Bertani (LB) solid medium, and the LG37 spore formation was observed by simple staining.

The 16S rRNA gene was amplified, and the 16S rRNA gene PCR product was purified and sequenced (Shanghai Oebiotech Co., Ltd., Shanghai, China) (Patel, 2001). Sequences were aligned using the ClustalW program. The phylogenetic tree was generated by the neighbor-joining method using the software package MEGA6 (He et al., 2021).

Determining the growth characteristics of LG37

The LG37 strain was grown in a 250-mL Erlenmeyer flask with 100 ml LB medium and an agitation speed of 160 rpm. In batch tests, the operations were conducted at a different temperature, initial pH, initial salinity, and initial dissolved oxygen in LB medium, and these experimental parameters and the results obtained from their use have been previously published (Liu et al., 2020).

A single bacterial colony was inoculated into LB liquid medium, cultured overnight at 32°C (160 rpm), centrifuged for 5 min at 6000 × *g*, washed twice with sterile phosphate-buffered saline (PBS) solution, and then resuspended in inorganic salt minimal medium. (1) The resuspended LG37 was inoculated into a minimal medium, in which the carbon sources were 4-carbon sugars (suritose), 5-carbon sugars (arabinose, xylose), or 6-carbon sugars (glucose, mannose, galactose, or fructose); the monosaccharide concentration was 0.1 mol/L. Disaccharides (sucrose, maltose, or lactose) were also used at a concentration of 0.05 mol/L. (2) Different amino acids were the only source of nitrogen in the minimal medium; the 20 amino acids used were tryptophan (Trp), tyrosine (Tyr), arginine (Arg), phenylalanine (Phe), histidine (His), methionine (Met), glutamate (Glu), glutamine (Gln), lysine (Lys), aspartate (Asp), asparagine (Asn), isoleucine (Ile), leucine (Leu), cysteine (Cys), threonine (Thr), valine (Val), proline (Pro), serine (Ser), alanine (Ala), or glycine (Gly). The final concentration of amino acids-N was 1.85 mmol/L. (3) The resuspended LG37 was inoculated into fresh inorganic salt minimal medium. Its nitrogen was derived from sources of organic nitrogen commonly used in aquatic feed, including fish meal, blood meal, silkworm pupa powder, potato powder , yeast powder, peanut powder, soybean meal powder, peptone, corn starch, and wheat bran, at 1 g/L. (4) The LG37 suspension was inoculated into medium with 1% grass carp feed as the sole source of nitrogen. The initial OD_{600} was 0.02, and sampling was performed once every 4 hr., with 3 repetitions for each gradient. To determine the OD_{600}, 2 ml bacterial solution was added to a cuvette and then measured with a spectrophotometer. The feed in the culture medium was filtered, dried, and weighed. The amount of protein was subsequently determined by the Kjeldahl method, and the degradation rate was calculated (Trikilidou et al., 2020).

Cultivation of biofilms

The biofilm was cultured in two groups, with flax silk as a carrier in a minimal medium, which contained 10 mmol/L ammonia as the sole nitrogen source. The LG37 bacterial biofilm was cultured with an initial OD_{600} of 0.01, 27±2°C, initial pH 7±0.3, continuous aeration, and static culture for 24 hr. The OD_{600} of the minimal medium was measured every 12 hr. When the OD_{600} reached 1.0, 1/3 of the minimal medium was exchanged. Afterward, 1/3 of the minimal medium was removed and replaced with fresh medium every 12 hr. for continuous culture of the biofilm for 2 weeks.

Establishment of wastewater treatment equipment

The water circulation for this experiment was provided by a closed-loop aquaculture system at the Aquatic Teaching Base of Huazhong Agricultural University, China. The aquaculture wastewater treatment equipment was divided into two parts: physical filtration (sponge filters, volcanic stones, and activated carbon) and microbiological degradation (biofilm system with flax silk as a carrier).

Degradation of ammonia nitrogen and residual protein by wastewater treatment equipment

The parts of the necessary wastewater treatment equipment were placed in the wastewater treatment pond. The efficiency of the wastewater treatment equipment in degrading ammonia nitrogen and residual feed protein was determined using grass carp culture wastewater. The wastewater that did not pass through the treatment system was used as the control group. The amount of ammonia nitrogen and residual feed protein in the aquaculture water was measured every other day. The experimental water sampling points were set at fixed positions. The ammonia nitrogen and residual feed were measured by Nessler's reagent spectrophotometry and the Kjeldahl method, respectively (Gao et al., 2018).

Effects of LG37 on acute toxicity tolerance of ammonia nitrogen by grass carp

The experimental juvenile grass carp were purchased from Hubei Bairong Aquatic Breed Co., Ltd., China. The full length and average weight of the grass carp was 9.67±2 cm and 20-30 g. The grass carp were divided into two groups, A and B, which were temporarily cultured in 250-L plastic buckets designed to accommodate circulating water for aquaculture. During the temporary culture for four weeks, the water was continuously aerated, and was maintained at 27.0±2°C, pH 7.0±0.3. The grass carp of the A and B groups were fed 4-9 g feed every morning at 9:00 a.m., and LG37 was mixed into the feed of the B group at 10±1 billion bacteria per kilogram. After cessation of feeding for 24 hr., fish with strong vitality were selected for the acute toxicity experiment, and those with an intact body surface, and no parasites or other visible diseases were chosen.

The experimental fish selected from the A and B groups were randomly distributed into 30-L glass containers, with 10 fishes each. According to the aquatic toxicity determination method, the water was not aerated during the experiments. Analytically pure $(NH_4)_2SO_4$ was used in the prepared experiments with grass carp to obtain its 24-hr. absolute lethal concentration and maximum tolerated concentration. According to this concentration range, grades were designed by equal space between logarithm values. The experimental group of grass carp was treated with ammonia nitrogen (non-ionic ammonium) at 30-100 mg/L (1.82-6.06 mg/L), and the control group received 0 mg/L. Each concentration was set to thr.ee parallels, and the test solution was changed every 12 hr. The temperature was 27±2°C, and the pH was 7.0±0.3. The activity of the fish was observed every 12 hr., and the number of dead fish at 24 hr., 48 hr., and 96 hr. was recorded. Dead fish were promptly removed from the containers during the experiment, which lasted for 96 hr.

Data processing

For the probit regression analysis, the experimental data were analyzed by SPSS software to obtain the half lethal concentration (LC_{50}) of ammonia nitrogen (non-ionic ammonium) for grass carp:

Safe concentration (SC) = 96 hr. $LC_{50} \times 0.1$ Eq. 1

The 96 hr. LC_{50} is the 96 hr. half lethal concentration for the experimental animals. Non-ionic ammonium concentration = ammonia nitrogen concentration/$[10^{(pKa-pH)}+1]$; pKa = $0.09018 + 2729.92 / T$ Eq. 2

where T denotes the degrees Kelvin, T = 273 + t, and t denotes the experimental water temperature (Frias-Espericueta et al., 2000).

Results

The spore-forming LG37 bacteria were isolated by heating at 90°C for 10 min. In this study, the spore-forming strains were isolated using inorganic nitrogen as the sole nitrogen source in the minimal medium for culture, to obtain a stronger capacity by LG37 to absorb and utilize inorganic nitrogen. Afterward, LG37 was cultured on LB solid medium at 30°C for 24 hr., and it was observed that the colony morphology was irregular, translucent white in color, 2-7 mm in diameter, bulging, glossy, and contained a great deal of water (**Figure 1A**). When cultured for 48 hr., there was a great deal of water loss and irregular folds in the colonies, which were white and opaque, and were adhered to the plate (**Figure 1B**). LG37 was cultured in a spore medium for 48 hr., and spores were found by simple staining. The spores were located at both ends or in the middle of the body, and did not swell; the number of spores was 1 (**Figure 1C**).

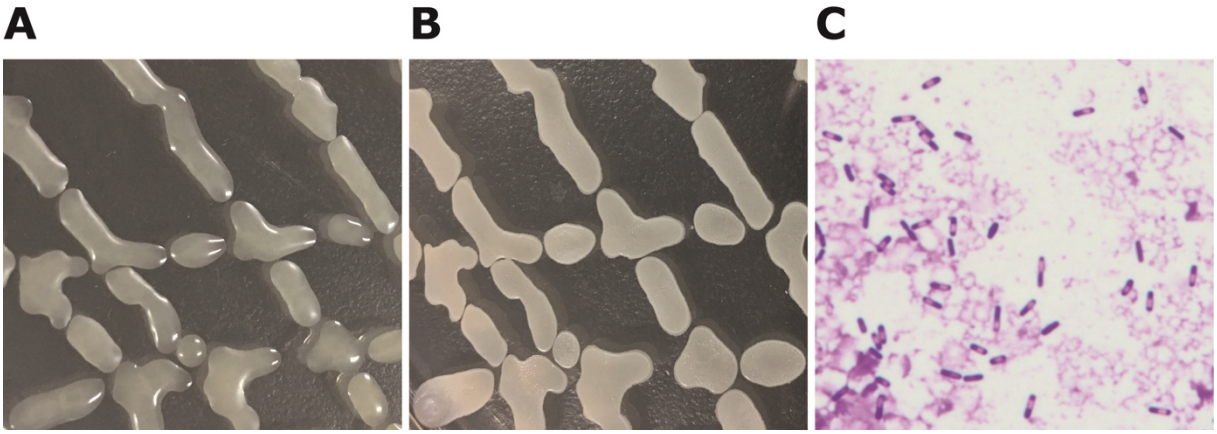

Figure 1 Morphological analysis of *Bacillus*. (A) 30 ºC culture for 24 hr. (B) 30 ºC culture for 48 hr. (C) Simple staining.

The LG37 16S rRNA gene sequence was 99% like that of *Bacillus velezensis* FZB42, and the phylogenetic tree analysis was also clustered into *B. velezensis* (**Figure 2**). These data combined with the genome sequencing data indicated that the bacteria should be named *B. velezensis* LG37.

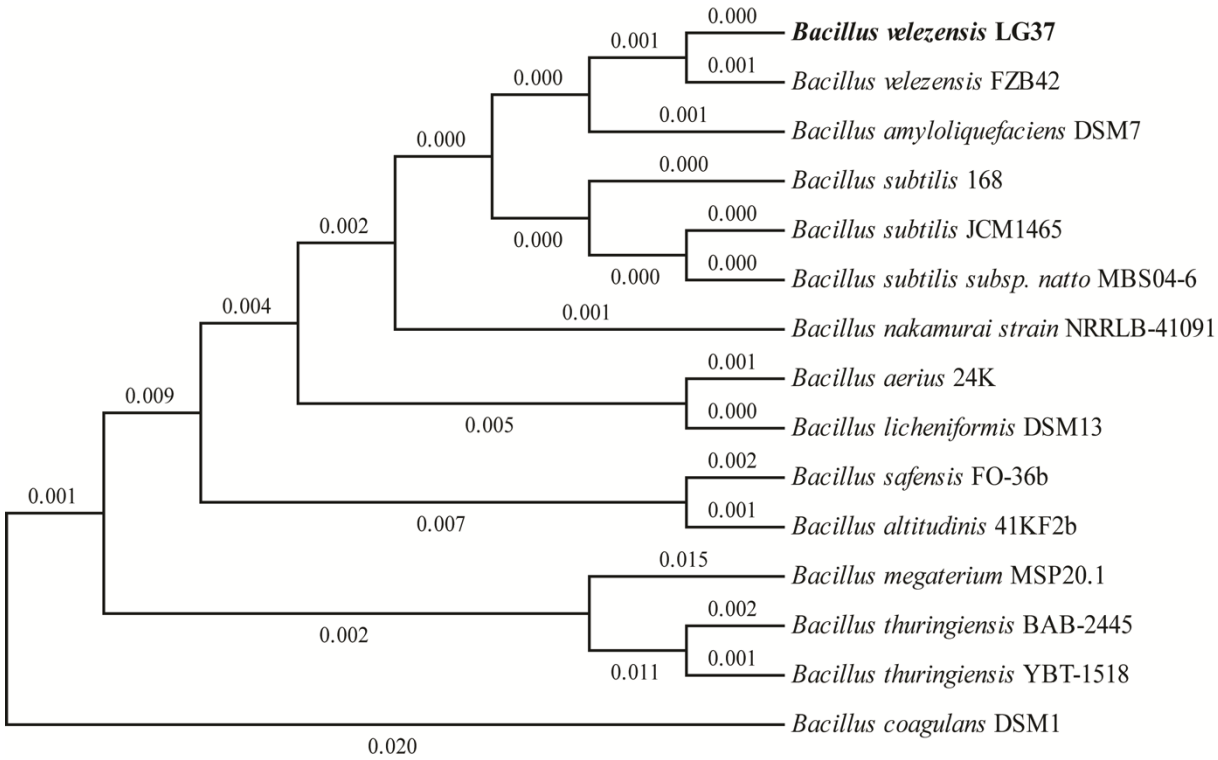

Figure 2 The neighbor-joining tree of LG37 based on 16S rDNA sequences.

LG37 exhibited a satisfactory growth trend with different types of monosaccharides and disaccharides as carbon sources, and the OD_{600} of LG37 exceeded 7%. For using the 10 common organic nitrogen, the utilization capacity of LG37 for soybean meal powder, wheat bran, corn starch, and potato powder was weak (**Figure 3A**). But the remaining six organic nitrogen absorption ability is strong. It can be well used as a source of organic nitrogen for LG37 to absorb and use for LG37 growth and reproduction needs (**Figure 3B**).

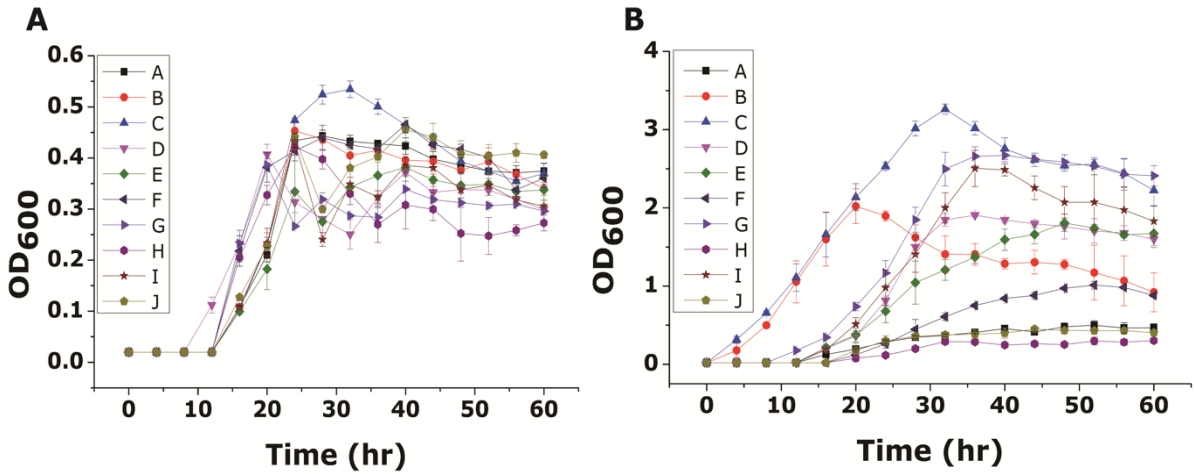

Figure 3 Growth curve of LG37. (A) Growth curve of 10 types of sugar as the carbon source of LG37 (A, galactose; B, maltose; C, arabinose; D, xylose; E, mannose; F, sucrose; G, glucose; H, suritose; I, fructose; J, lactose).(B) Growth curve of 10 types of organic nitrogen as the nitrogen source of LG37 (A, soybean meal powder; B, yeast powder; C, peptone; D, blood meal; E, silkworm pupa powder; F, wheat bran; G, peanut powder; H, corn starch; I, fish meal; J, potato powder).

When 20 different types of amino acids were tested as the sole nitrogen source in a minimal medium, all were absorbed and utilized by LG37 except glycine (Gly), tyrosine (Tyr), and tryptophan (Trp). The utilization of 6 types of amino acids (phenylalanine (Phe), methionine (Met), leucine (Leu), cysteine (Cys), serine (Ser), and lysine (Lys)) was relatively slow in the early stage. Still, with the extension of time, the number of strains of LG37 also gradually increased (**Figure 4**).

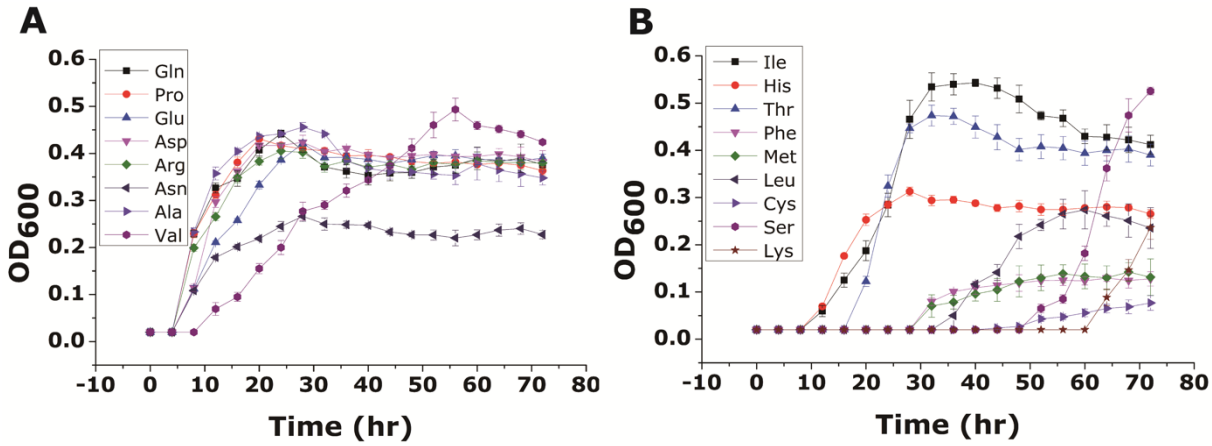

Figure 4 Growth curve of 20 types of types of amino acids as the sole nitrogen source of LG37.

The cultured LG37 biofilm with the carrier was placed in the wastewater treatment equipment, as shown in **Figure 5**. This equipment was used to treat the ammonia nitrogen and feed protein present in the aquaculture wastewater, and the results are shown in **Figure 6**. The initial concentration of ammonia nitrogen in the grass carp culture wastewater was 2.11 mg/L. After one week of continuous degradation, the fastest degradation rate for ammonia nitrogen in the grass carp culture water was 34.6% in three days, and the total degradation rate was 55.5% in one week. However, the fastest degradation of feed protein intended for grass carp occurred on the first day, at 26.5%. The removal rate of grass carp feed protein reached up to 73.6% in a week.

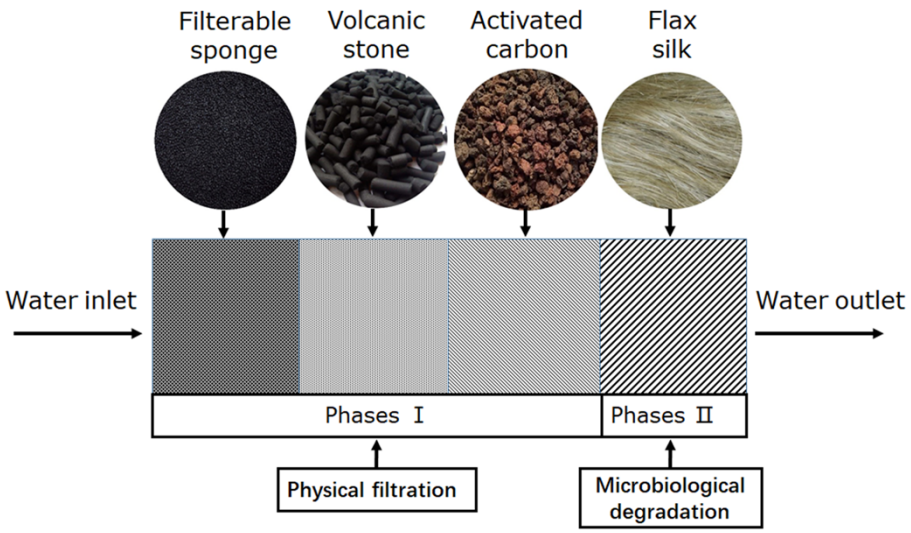

Figure 5 The wastewater treatment equipment of physical filtration and microbiological degradation.

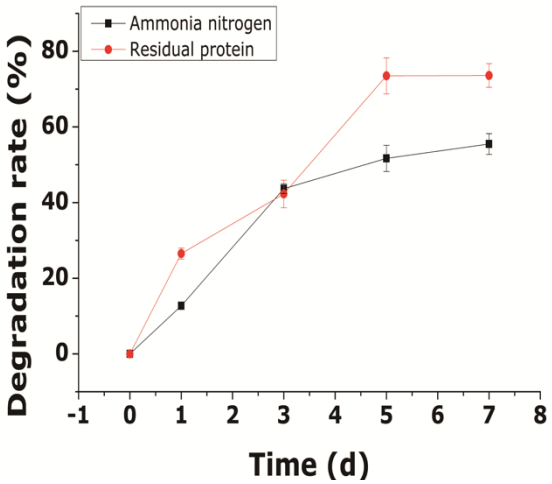

Figure 6 Degradation rate of ammonia nitrogen and residual protein by wastewater treatment equipment.

The acute tolerance of the toxicity of ammonia nitrogen (non-ionic ammonium) by grass carp was tested, and the experimental results are shown in **Figure 7**. During the experiment, no fish died in the A/B control group. With the increase in ammonia nitrogen (non-ionic ammonium) concentration and the extension of the stress time, the toxicity to grass carp in the A/B group significantly increased, and the number of dead grass carp significantly increased.

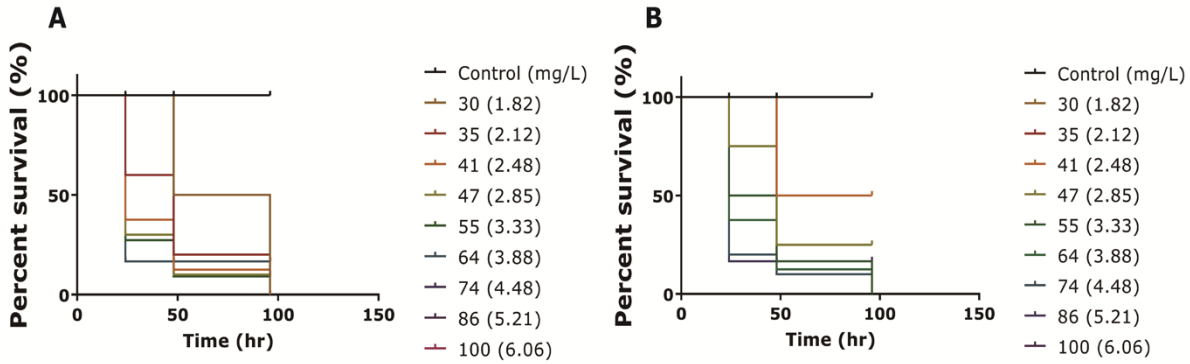

Figure 7 The percent survival of acute toxicity of ammonia (non-ionic ammonium) nitrogen to grass carp. (A) Feed without LG37. (B) Feed with LG37.

In grass carp group A (without LG37), the half lethal concentration of ammonia (non-ionic ammonium) at 24, 48, and 96 hr. was 42.34 (2.57), 35.9 (2.18), and 33.68 (2.04) mg/L, respectively. The safe ammonia (non-ionic ammonium) concentrations for grass carp were 3.37 (0.2) mg/L. In grass carp group B (with LG37), the half lethal concentration of ammonia (non-ionic ammonium) at 24, 48, and 96 hr. was 60.18 (3.65), 54.9 (3.33), and 51.11 (3.1) mg/L, respectively. The safe ammonia concentration (non-ionic ammonium) for grass carp was 5.11 (0.31) mg/L. These results showed that the tolerance of grass carp to ammonia nitrogen toxicity was greatly enhanced by LG37 compared with the experimental group without LG37. The concentration of ammonia nitrogen (non-ionic ammonium) that grass carp could withstand and be considered safe was increased by 51.63% (55%) (**Table1**, **Table2**). Through acute toxicity tolerance experiments, it was discovered that LG37 significantly increased the tolerance to ammonia nitrogen (non-ionic ammonium) in herbivorous grass carp.

Table 1 Group A (feed without LG37), the half lethal concentration (LC_{50}) and safe concentration (*SC*) of ammonia (non-ionic ammonium) nitrogen to grass carp at 24, 48 and 96 hr.

Grass carp	*24hr. LC_{50}*	*48hr. LC_{50}*	*96hr. LC_{50}*	*SC*
Ammonia nitrogen (mg/L)	42.34	35.9	33.68	3.37
Non-ionic ammonium (mg/L)	2.57	2.18	2.04	0.2

Table 2 Group B (feed with LG37), the half lethal concentration (LC_{50}) and safe concentration (*SC*) of ammonia (non-ionic ammonium) nitrogen to grass carp at 24, 48 and 96 hr.

Grass carp	*24hr. LC_{50}*	*48hr. LC_{50}*	*96hr. LC_{50}*	*SC*
Ammonia nitrogen (mg/L)	60.18	54.9	51.11	5.11
Non-ionic ammonium (mg/L)	3.65	3.33	3.1	0.31

Discussion

In recent years, with the continuous break throughs in aquaculture breeding modes and breeding technology, the breeding density and yield have also increased (Geng et al., 2022). However, a high feed input is required to achieve a high yield. The continuous accumulation of feed residues and excreta during the breeding process directly leads to a continuous increase in the concentration of nitrogen sources and nutrients in the water. In contrast, the decomposition of organic nitrogen increases the concentration of toxic ammonia nitrogen in the water, which may limit breeding (Kuebutornye et al., 2019). As a probiotic for purifying water quality, *Bacillus* spp. has been widely used in aquaculture, and its effects have been verified by many scientists and recognized by farmers (Kuebutornye et al., 2020).

LG37 exhibited satisfactory adaptability to temperature, dissolved oxygen, pH value, and salinity, and thus can be widely used in various types of aquaculture water (Liu et al., 2020). In aquaculture, numerous sources of nitrogen from residual bait and excreta rapidly accumulate in the water body (Robles-Porchas et al., 2020). Generally, the concentration of nitrogen sources in the water is high, while the low concentration of carbon sources will result in carbon becoming a limiting factor for the proliferation of probiotics (Roleda et al., 2019; Shang et al., 2018). Thus, the rapid proliferation of probiotics is currently accomplished by adding quick-acting sugar to water. LG37 can efficiently absorb and use 10 common monosaccharides or disaccharides for rapid proliferation, and there was no significant difference in the utilization rate by LG37 when a variety of sugars was tested. Therefore, combined with the relationship between supply and demand and market price, the rapid proliferation of probiotics can be realized by adding inexpensive industrial sugar so that it can be used as a quick-acting carbon source. This process greatly reduces the input cost and significantly increases the utilization of microbial preparations added to water.

The main components of organic nitrogen in the feed are animal sources and plant sources. Within 60 hr., there was low utilization by LG37 of organic nitrogen from four plant sources with high cellulose content, but the experimental results showed a slow-growth trend. The degradation cycle for plant-derived organic nitrogen in water is significantly longer than that of animal-derived organic nitrogen. The consumption of dissolved oxygen and the change in the ammonia nitrogen concentration from plant-derived organic nitrogen is also slow. After organic nitrogen from animals is discharged into the water body in the form of residual bait and excreta, it is decomposed into amino acids and ammonia nitrogen through microbial degradation and denitrification. Various

amino acids are further decomposed into ammonia nitrogen, which eventually leads to a rapid increase in the ammonia nitrogen concentration in the water body (Heerthana et al., 2019; Hoseini et al., 2019).

LG37 rapidly utilizes organic nitrogen from animals, and also satisfactorily utilizes 20 different amino acids. By rapid utilization, it prevents amino acids from further decomposing into ammonia nitrogen in water and thus prevents a rapid increase in ammonia nitrogen concentration. When the concentration of molecular ammonia in water reaches 0.20 mg/L in fish breeding, it is deemed to be a toxic effect. When the pH value is 7.0 and the NH_3 concentration is 0.2 mg/L, the total ammonia nitrogen is 33.33 mg/L (1.85 mmol/L), and therefore, the concentration of amino acid-N is 1.85 mmol/L. The monosaccharide concentration of 0.1 mol/L and disaccharide concentration of 0.05 mmol/L are set according to the amount of carbon in the minimal medium with only one nitrogen source.

Within one week, the ammonia nitrogen in water was reduced by 55.5% through the LG37 biofilm device. During the first thr.ee days, its utilization rate was the highest, reaching 34.6%, which may have occurred because LG37 was reintroduced to all the aquaculture water through the circulation system. Because a large amount of protein remained in the aquaculture water, it sufficed as a source of organic nitrogen for LG37 and resulted in its rapid proliferation. In the process of proliferation, LG37 can simultaneously absorb and utilize ammonia nitrogen as an inorganic nitrogen source. Over time, the ammonia nitrogen concentration continued to decrease, resulting in a gradual decrease in the ability of LG37 to absorb and utilize ammonia nitrogen. The fastest degradation rate for grass carp feed protein was 26.5% on the first day, which may have occurred due to the removal of insoluble particles of feed protein by physical filtration.

The organic protein in the culture water is mainly composed of animal protein and plant protein. LG37 is more inclined to absorb easily decomposed animal protein to meet the needs of its early rapid growth and proliferation. With the extension of time, plant protein was transformed into the main component, and the growth rate of LG37 gradually decreased, resulting in a decrease in the degradation rate of residual protein, but the grass carp protein removal rate reached 73.6% in one week. Through the acute toxicity tolerance test, it was determined that LG37 increased the concentration of ammonia nitrogen (non-ionic ammonium), which is safe for grass carp, by 51.63% (55%). By analyzing the reasons, it is possible that LG37 is fixed in the intestines of grass carp as a probiotic, which can increase the immunity and tolerance of fish to ammonia (non-ionic ammonium) by changing the population structure of the intestinal microflora.

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