Communication

Colonization of *Lactobacillus rhamnosus* GG in *Cirrhinus molitorella* (Mud Carp) Fingerling: Evidence for Improving Disease Resistance and Growth Performance

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Abstract: The use of probiotic bacteria can not only enhance the nutritional utilization of fish feeds to produce more biomass but can also provide a practically "safer" alternative to the fish farming industry to reduce the abuse of antibiotics and drugs. This study investigated the possibility of colonizing *Lactobacillus rhamnosus* strain GG (LGG) to the intestine of *Cirrhinus molitorella* (mud carp) fingerling. Colonization of LGG was observed in gut tissue after 14 days of administration with a diet supplemented with 1×10^8 CFU/mL LGG. Moreover, growth performance parameters of the LGG-supplemented diet group, including relative weight gain, feed conversion ratio and feed efficiency, were found about two-fold higher than the control group after 60 days. In addition, fish fed with an LGG-supplemented diet for 60 days showed substantial resistance against the infection of pathogenic bacterial *Aeromonas hydrophila*, with a relative survival rate of up to 57% compared to the control group. In summary, the results indicated that LGG as dietary supplement for mud carp fingerling can enhance nutrition utilization and better protect fish against the infection of *Aeromonas hydrophila*. The results provide an insight to the fish farming industry, encouraging a reduction in the use of antibiotics and drugs and the production of "safer" mud carp for the market at a manageable cost.

Keywords: *Cirrhinus molitorella*; *Lactobacillus rhamnosus* GG; colonization of probiotics; *Aeromonas hydrophila*; disease resistance

1. Introduction

Cirrhinus molitorella (Mud carp), a typical native freshwater species in Asia, has a long history in fish farming since the Tang Dynasty (618–904 A.D.) [1]. Due to its high meat quality and nutritional value, it is one of the most popular inland fish species cultivated in southern China and can be supplied either fresh or in a can. Mud carp is a slow growing species, smaller than other carps such as grass, bighead, common and silver carps. They take about 2 years to reach a marketable size (about 200 g), much slower than common carp, which usually only require one season to reach a body weight of 1.0 kg [1].

Among the various factors hindering the growth of the industry, disease of aquaculture is a primary concern in many countries [2,3], as it would lead to serious losses in production. Bacterial infection accounts for about 20% loss in aquaculture [4]. Mud carp, a bottom dweller in freshwater fishponds, is also susceptible to bacterial infection, such as *Aeromonas hydrophila* (*A. hydrophila*) [1]. Outbreaks of *A. hydrophila* have also been reported by accidental abrasion [5], which are common in intensive fish farming of mud carp. Current practices rely on the extensive use of various chemicals and drugs, however this affects the food safety and introduces further environmental issues, such as using malachite green, a carcinogenic industrial dye, in the treatment of fish parasites and protozoan diseases [6].

The use of antimicrobial agents has increased dramatically [7]; an estimated 10,259 tonnes antimicrobial agents were used in aquaculture in 2017 [8]. The non-therapeutic use of antimicrobials is common, especially in intensified production [9]. The abuse of antimicrobials has become a global concern due to the development of antibiotic resistance genes (ARGs) in pathogenic bacteria [10,11]. Some ARGs could be transferred to other bacteria that have never been exposed to antibiotics [12], which could eventually render the drugs ineffective. Thus, there is an urgent need to replace conventional antimicrobials in aquaculture with sustainable alternatives to maintain productivity in aquaculture.

One of the possible approaches for disease management in aquaculture is strengthening the defense mechanism via the dietary/oral administration of probiotics [13,14]. The term "probiotics" was first proposed by Parker [15], and can positively contribute to the host by enhancing its intestinal balance. A modified definition was suggested by Verschuere et al. [16], referring to living micro-organisms that confer health benefits when consumed adequately, which have been proven to regulate the adaptive immune responses of the hosts. By the nature of regulating adaptive immune responses, probiotics can be used in aquaculture to reduce the use of antimicrobial agents and drugs. Many studies revealed that probiotics can enhance immune responses in aquaculture through various pathways. Korkea-aho et al. [17] demonstrated the use of *Pseudomonas* M162 stimulated lysozyme activity and total serum immunoglobulin levels of rainbow trout, which protect against fry syndrome. Another probiotic, *Bacillus subtilis* AB1, obtained from fish intestine, can stimulate gut lysozyme and lymphocyte in rainbow trout and was found effective in preventing disease caused by highly virulent *Aeromonas* sp. [18]. Apart from stimulating antimicrobial agents such as lysozyme and lymphocyte, *Lactobacillus* spp. JK-8 and JK-11 can produce organic acids to inactivate pathogenic bacteria [19]. Moreover, these two species can remove NH_4^+, NO_2^- and NO_3^- from contaminated shrimp farms to improve water quality.

The mechanism of probiotics supplement may be species-specific and the probiotic effects may be varied by the hosts [20]. Numerous studies have suggested that administrating probiotic bacteria to fish could improve growth performance, either directly by upregulation of growth factors [21], or indirectly through enhancing digestibility of feeds [22]. For example, significantly better feed conversion and specific growth rates (SGR) were observed in marine fish such as olive flounder (*Paralichthys olivaceus*), fed with *Lactococcus lactis* WFLU12 [23], as some enzymes such as phosphohydrolase and glycosidase were found increased and thus enhancing the dietary energy extraction and metabolism. *Labeo rohita*, fed with a diet supplemented with *Lactobacillus plantarum* VSG3, resulted in significantly higher SGR and feed utilization efficiency [24], with an improvement in digestibility by stimulating the level of vitamins and enzymatic activity. Bäckhed et al. [25] suggested that microbiota in the gut is an important environmental factor for the host in regulating energy uptake from diet. However, not all probiotic bacteria species positively effect the growth of aquaculture; a study conducted by Ahire et al. [26] showed no significant improvement for feed supplemented with *Lactobacillus helveticus* CD6 in goldfish (*Carassius auratus*).

Among different probiotic candidates, lactic acid bacteria (LAB) are widely used probiotic genera that are extensively used on livestock and humans with various beneficial effects; several terrestrial origin LAB have been proven to be effective in fish [27], whereas *Lactobacillus rhamnosus* GG (LGG) has been considered as one of the most universally studied and commercialized probiotic LAB strains for humans [28,29]. Several functional and health-related characteristics of LGG have been demonstrated by in vitro and in vivo studies in humans, including but not limited to the stimulation of immune responses against pathogens. A recent study demonstrated that LGG may have potential in perverting helper T (Th) response towards Th1-direction [30]. Moreover, it has also been shown to have immunotherapeutic potential in ameliorating and preventing the development of Th2-mediated inflammatory diseases (e.g., allergies and atopic eczema) [30]. Some studies have proven LGG can adhere to the gut mucosa of zebrafish [31] and protect juvenile red tilapia against *Aeromonas veronii* [32].

Since there is a lack of studies relating to the use of probiotics in mud carp aquaculture, LGG was administrated to mud carp fingerling in this study to determine whether LGG can colonize in their gut, as the colonization of probiotic bacteria is critical in helping fish to fight pathogens [33]. The enhancement of growth and disease resistance against *A. hydrophila* through a diet supplemented with LGG has been studied. If successful, the aquaculture of mud carp will be improved, and productivity will be increased.

2. Materials and Methods

2.1. Experimental Setup and Feeding Trial

A total of 120 tails of mud carp fingerling, with a size 6–7 cm, were obtained from a local fish market (Mong Kok, Hong Kong). All fish were bathed in 3% salt water before being introduced into two glass fish tanks (120 L). Water temperature was maintained at 26 ± 2 °C with continuous aeration. The tanks were connected to an in-house external filter with flow rate at 250–300 mL/min for mechanical and biological filtration. Fish were acclimatized for at least 2 weeks. Fish were fed twice per day with a commercial fish feed powder, according to 3% body weight of fish. Fish appetite, behavior and mortality were checked daily. After the acclimatization period, a total of 60 mud carp tails (~2.6 g in weight, 6–7 cm in length) were randomly divided into each glass tank (120 L) ($n = 30$, in duplicate), with the tank conditions the same as the one during the acclimatization period. Both experimental diet with LGG and control diet were tested; each diet group was tested in duplicate. All the treatment groups were fed regularly for 60 days according to 3% of the body weight of fish (w/w) per day, where the amount of feeds taken by each group was recorded daily. Throughout the experimental period, fish tanks were kept under 12 h light: 12 h dark cycle. Water temperature was maintained at 26 ± 2 °C. Other water quality parameters such as pH, total dissolved solids, ammonium nitrogen, nitrite, nitrate and total phosphorus were monitored weekly using handheld testers (PCTestr 35, Eutech Instruments Pte Ltd., Singapore) and DR1900 Portable Spectrophotometer (Hach, CO, USA). Fish swimming behavior, feed consuming ability, and color of body were also monitored to confirm the health status of fish during the experimental period. Table 1 shows the physical and chemical parameters of water quality in the water tanks of LGG supplemented diet and control groups.

Table 1. Physical and chemical parameters of water quality.

Parameters	LGG Treatment Group	Control Group
Water temperature (°C)	26.6 ± 0.4	26.8 ± 0.3
Total ammonium nitrogen (mg/L)	<0.1	<0.1
Nitrite (mg/L)	0.008 ± 0.003	0.014 ± 0.003
Nitrate (mg/L)	0.5 ± 0.1	0.7 ± 0.2
Total Phosphorous (mg/L)	<0.1	<0.1
pH	7.65 ± 0.20	7.87 ± 0.07
Total dissolved solids (mg/L)	<0.1	<0.1

2.2. Preparation of Experimental Diets and Supplementation

Lyophilized LGG was obtained from Valio Ltd. (Helsinki, Finland) and used as the probiotic strain in this experiment. Pure culture of LGG was reactivated using De Man, Rogosa and Sharpe (MRS) broth (LAB M Limited, Lancashire, UK) at 37 °C. After incubation for 18–24 h, bacterial cells were harvested by centrifugation at $3000 \times g$ for 3 min, and the pellets were washed twice with sterile 0.85% (w/v) saline. The cell densities of LGG were determined by spectrophotometry. The LGG-supplemented diet was prepared by spiking 10^8 CFU/mL of LGG into the commercial fish diet, with a feed to cell culture ratio of 1:1 ($w:v$). On the other hand, the control diet was prepared by mixing sterile 0.85% saline with the commercial fish diet (1:1) ($w:v$). All diets were freshly prepared and used within the same day. Unconsumed diet was removed after 30 min and dried in an oven to determine the actual feeding amount.

Weight of fish was measured in triplicate using an electrical balance (Mettler Toledo, Greifensee, Switzerland) for every 2 weeks. Growth performance and diet nutrient utilization were analyzed in terms of relative weight gain (RWG), feed conversion ratio (FCR), specific growth rate (SGR), feed efficiency (FE), and average feed intake (AFI), which were calculated using the following equations [34]:

$$\text{RWG }(\%) = \frac{(\text{Average final body mass} - \text{Average Initial body mass })}{\text{Average initial body mass}} \times 100\% \quad (1)$$

$$\text{FCR} = \frac{\text{Feed intake (g)}}{(\text{Final biomass} - \text{Initial biomass})(\text{g})} \quad (2)$$

$$\text{SGR (\% per day)} = \frac{(\ln \text{Final weight (g)} - \ln \text{Initial weight (g)})}{\text{Feeding period (day)}} \times 100\% \quad (3)$$

$$\text{FE }(\%) = \frac{\text{Weight gained (g)}}{\text{Feed intake (g)}} \times 100\% \quad (4)$$

$$\text{AFI (g per fish per day)} = \frac{\text{Total feed intake (g)}}{(\text{number of fish} \times \text{feeding period (day)})} \quad (5)$$

2.3. Disease Resistance of Mud Carp against A. hydrophila

A. hydrophila (ATCC 7966) was obtained from ATCC (Virginia, USA) and was cultured using lysogeny broth (LB). Bacterial cells were harvested by centrifugation at $3000 \times g$ for 3 min, and the pellets were washed twice with sterile saline (0.85% w/v). Cells were resuspended in sterile 0.85% (w/v) saline and the cell densities were determined by spectrophotometry. The cell concentrations were adjusted to 1×10^6 CFU/mL and 0.5×10^6 CFU/mL using sterile 0.85% saline. Fish, after treatment with probiotic and control diets, were anaesthetized with 50 mg/L tricaine methanesulfonate (MS222) and, subsequently, 100 μL of adjusted bacterial suspension was injected intra-muscularly to both treatment groups (5 fish from each of the duplicate tank per treatment group and a total of 10 fish per treatment group; dosage of treatment groups equivalent to 1×10^5 CFU for high dose group and 0.5×10^5 CFU for low dose group). Mortalities were immediately removed and recorded. Challenged fish were monitored for 14 days. All dead fish were examined for external infection signs. Relative percentage survival (RPS) was calculated using the following formula:

$$\text{RPS }(\%) = \left(1 - \frac{\text{\% mortality in LGG supplemented diet group}}{\text{\% mortality in control group}}\right) \times 100 \quad (6)$$

2.4. Verification of LGG Colonization at the Fish Gut Tissue

Mud carp fingerling samples ($n = 2$) from each group were dissected 7, 14, 30 and 60 days after treatment with LGG to evaluate any colonization of probiotic bacteria in gut tissue. Prior to sacrifices, fish were fasted for 24 h to ensure emptying of the digestive tract. Fish surface was disinfected with 70% ethanol. Fish gut tissue from both treatment groups was collected by dissection using sterile scalpel and forceps. About 0.25 g of dissected fish gut tissue was homogenized in a sterile mortar and pestle(Fisher Scientific International Inc., Pittsburgh, United States) with the addition of 1 mL sterile 0.85% (w/v) saline. Zero-point one mL of the resulting tissue suspension solution was streaked onto MRS agar, and were incubated at 37 °C for 48 h. After incubation, representative colonies presented on the MRS agar plates were picked for identification by molecular method, where DNA of isolated bacterial colonies were extracted and purified using QIAamp DNA mini kit (QIAGEN GmbH, Hilden, Germany), according to the manufactures' instructions. The DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA). Amplification of the 16S rRNA gene using bacterial universal primers (27F 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R

5′-GGTTACCTTGTTACGACTT-3′) [35]. PCR conditions were as follows: (i) denaturation at 95 °C for 10 min, (ii) followed by 35 cycles of denaturation at 95 °C for 30 s, (iii) annealing at 50 °C for 30 s and (iv) extension at 72 °C for 1 min, and (v) with a final elongation step at 72 °C for 10 min. Sequencing was performed on an Applied Biosystems 3730xl DNA Analyzer (Life Technology Corporation, Carlsbad, CA, USA) conducted by Tech Dragon Limited.

2.5. Statistical Analysis

The statistical p values between growth performance and water quality parameters were calculated by one-way ANOVA (SPSS Statistics, version 26, IBM). Parameters with p value less than 0.05 were considered significant difference.

3. Results

Table 1 shows physical and chemical parameters of water quality in the water tanks of LGG-supplemented diet and control groups. The pH value, total suspended solid, total ammonium nitrogen, nitrite, nitrate and phosphorous of the LGG-supplemented diet group were found to be 7.65 ± 0.20, <0.1 mg/L, <0.1 mg/L, 0.008 ± 0.003 mg/L, 0.5 ± 0.1 mg/L, <0.1 mg/L, respectively; all parameters did not present any significant differences from the control group ($p > 0.05$).

Table 2 shows the growth performance of mud carp fingerlings fed with and without LGG. The groups fed with the LGG-supplemented diet showed significantly ($p < 0.05$) higher RWG (47.6%) than the control group (21.9%). The average feed intake was not significantly differed between the two groups ($p > 0.05$) and thus no adverse effect to the appetite by LGG supplement. The mud carp fingerlings with the LGG-supplemented diet had a specific growth rate of 0.649% per day; 95% faster than the control group ($p < 0.05$). The FCR value of the LGG-supplemented diet group (1.80) was found much smaller than the control group (3.99) ($p < 0.05$), where a decrease of 45% in FCR value indicated that the feed supplemented with LGG had a higher quality [36]. Compared with FE, over 55.7% of the LGG supplemented-diet consumed was converted to the fish biomass, which was more than double to the control diet.

Table 2. Growth performance of mud carp fingerling after feeding with basal diet with and without LGG for 60 days. Asterisks (*) represent a significant difference (α = 0.05) of the LGG treatment from the control groups by two-sample *t*-test.

Parameters	LGG Treatment Group	Control Group
Average initial weight (g)	2.69 ± 0.254	2.63 ± 0.218
Average final weight (g)	3.98 ± 0.288 *	3.21 ± 0.276
Relative weight gain (RWG) (%)	47.6 ± 3.27 *	21.9 ± 0.76
Average feed intake (AFI) (g/fish per day)	0.0378 ± 0.0073	0.0377 ± 0.0059
Feed conversion ratio (FCR)	1.80 ± 0.27 *	3.99 ± 0.04
Feed efficiency (FE) (%)	55.7 ± 8.28 *	25.1 ± 0.26
Specific growth rate (SGR) (%/day)	0.649 ± 0.016*	0.332 ± 0.014

3.1. Disease Resistance of Mud Carp against A. hydrophila

To investigate the effect of LGG on the resistance of pathogens in mud carp fingerling, after feeding for 60 days, fish samples from both the LGG-supplemented diet and control groups were injected intramuscularly with two levels of *A. hydrophila* (high dose at 1×10^5 CFU and low dose at 5×10^4 CFU). A negative control was conducted by injecting 0.85% (*w/v*) saline and no mortality was observed for the negative control throughout the experiment. Figure 1 shows the results of relative survival rate (%) after 14 days monitoring period.
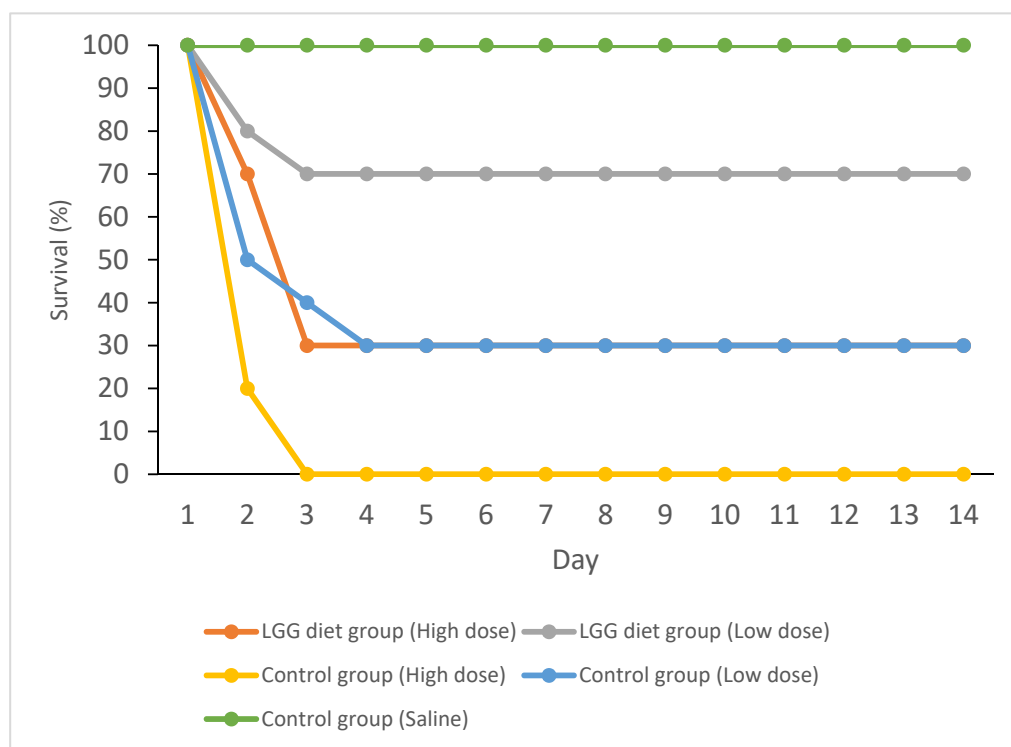
Figure 1. Survival rate of the challenged mud carp fingerlings after administration of LGG supplemented and control diets. Mud carp fingerlings were intramuscularly injected with low dose (5×10^4 CFU) and high dose (1×10^5 CFU) of *A. hydrophila* for 14 days. A set of negative control was conducted by injecting 0.85% saline instead of A. *hydrophila*.

The accumulative mortality rate of LGG supplemented diet group (30% at 5×10^4 CFU and 70% at 1×10^5 CFU) was found significantly lower than the control group (70% at 5×10^4 CFU and 100% at 1×10^5 CFU) ($p < 0.05$). Relative percentage survival (RPS) of mud carp fed supplemented with LGG decreased when the dose of *A. hydrophila* increased. All dead fish exhibited hyperaemia on the body with ragged fin, and enlarged eyes (exophthalmos), which are typical symptoms to the infection of *A. hydrophila* [1]. Table 3 summarized the results of the challenging test.

Table 3. Accumulated mortality rate and relative percentage survival (RPS) of A. *hydrophila* challenging study. Asterisks (*) represent a significant difference ($\alpha = 0.05$) of the LGG treatment from the control groups by two-sample *t*-test.

Amount of *A. hydrophila* Injected	Accumulated Mortality Rate (%)		RPS (%)
	LGG Treatment Group	Control Group	
5×10^4 CFU (low dose)	30 *	70	57
1×10^5 CFU (high dose)	70 *	100	30

3.2. Colonization of LGG in Gut of Mud Carp

Bacterial colonies with morphological characteristics similar to LGG were observed in the dish cultured with suspension of gut tissue after 14-, 30- and 60-day treatment of LGG-supplemented diet. Colonies with similar morphological characteristics were picked and identified to be *Lactobacillus rhamnosus* species by DNA sequencing (similarity 100%). No colony was found on the MRS culture dishes of the control group. Water samples from the fish tanks were also checked and no LGG was found in both the LGG diet and control groups.

4. Discussion

Mud carp is one of the major freshwater aquaculture species in traditional polyculture ponds in southern China. As mud carp grows slowly and is easily infected by bacterial diseases such as *A. hydrophila* [1], antibiotics and drugs are commonly used in disease control of intensive culture. Up until now, little information has been available on the use of probiotic species as feed supplements to mud carp. This study tried using probiotics as an alternative feed supplement in the culturing of mud carp, not only to reduce to the use of antibiotics and drugs, but to also improve the growth performance of this slowly growing species.

In the growth performance study (Table 2), a significant increase in body mass of mud carp fingerling was observed in the LGG-supplemented diet group. The magnitude of improvement in growth performance parameters (i.e., RWG%, FCR, FE and SGR) was found to be much better than a similar study where juvenile red tilapia (*Oreochromis* spp.) were fed with a symbiotic composed of Jerusalem artichoke and LGG (10^8 CFU/g) for 4 four weeks [32]. However, in their study, a control diet with Jerusalem artichoke only produced a similar effect (about 41%) in SGR, indicating that the growth enhancement by the symbiotic supplement may be contributed mainly by the components in prebiotics such as inulin, fructooligosaccharides, carbohydrate, protein, Vitamin C and minerals. Results in our study demonstrated that LGG supplemented in the feed alone can enhance the growth rate and feed conversion ratio of relatively slow growing mud carp.

The study revealed that LGG cells may bound to the mucosa zone of the gut wall [31]. Upon colonization, more energy can be supplied to the host cells in the form of short-chain fatty acids through the metabolism of the LGG [37] and, thus, can improve the feed utilization of the fish. Moreover, the height and width of villus in the digestive tract may be increased to enhance the absorption of nutrients [32]. Zhou et al. [38] observed that the adhesiveness of lactobacillus species to the intestinal cell varied between strains. Further study to the adhesiveness of LGG to the mud carp fingerling's intestine wall may be required.

As fish feed used in the fish farming industry can cause significant water pollution [39], water quality parameters were analyzed weekly to determine any detrimental effect to the introduction of LGG to the basal diet. From the results obtained (Table 1), it was found that an addition of LGG to the feed would not cause any additional pollution issues to the aquaculture ecosystem. Moreover, all water quality parameters between the LGG diet and control groups were found within the recommended levels as specified by Boyd and Tucker [40].

In the challenge study (Table 3), the accumulated mortalities in both the LGG- supplemented and control diet groups showed a dose dependent relationship to the amount of *A. hydrophila* injected. An increased amount of *A. hydrophila* injected would decrease the RPS of the challenged group from 57% to 30%. It has been suggested that supplemented diet with LGG may enhance the immune response of mud carp by increasing lysozyme activity [41]. A similar effect with increased levels of lysozyme activity was observed when juvenile red tilapia was fed with LGG-supplemented diets [32]. The mechanism may be a result of immunomodulation of LGG by secreting soluble factors to promote pro-inflammatory type-1 cytokines (e.g., TNFα) [42]. In addition, the LGG-supplemented diet group exhibited faster healing to the wound of injection than the control group (data not shown), which may be caused by stimulation of migration of keratinocytes and induction of re-epithelialization [43]. Further study into the mechanisms of antipathogenic properties is necessary in order to provide more detailed information concerning the immunity enhancement of LGG to mud carp aquaculture.

Apart from technological factors, the costs associated with acquiring LGG is one of the most important considerations to the practicality of using LGG as a feed supplement. Archaka et al. [44] demonstrated the use of corn flour as a medium for preparation of lactobacillus probiotic strain; the selling price of the resulting probiotics preparation (containing 5×10^8 CFU g^{-1}) can be controlled as low as USD 5.00 per kg. When culturing probiotics in

situ, extensive drying processes for the preparation is not necessary and the probiotics can be fortified to the feed in wet form. The increase in running cost of the feed supplemented with LGG is thus manageable and can be outweighed by the growth performance, feed efficiency and the anti-pathogenic infection to *A. hydrophila*.

5. Conclusions

All in all, the work in this study demonstrated the beneficial effects of using LGG as an effective supplement in diet on both the growth performance and antipathogenic properties of mud carp fingerling, which can be colonized in the fingerling stage of mud carp. After a 60-day supplemented diet treatment, LGG can enhance not only the nutritional utilization of fish feeds to produce more biomass, but also demonstrate substantial resistance against the infection of pathogenic bacterial *A. hydrophila*, with an increase in relative survival rate of up to 57% over the control group. The results provide an insight into a more economic and safer alternative to mud carp aquaculture industry through a reduction in the use of antibiotics and drugs in combating pathogenic diseases.

Author Contributions: Conceptualization, I.W.-Y.M. and E.T.-P.S.; methodology, Y.-M.Y. and I.W.-Y.M.; software, Y.-M.Y. and E.T.-P.S.; validation, Y.-M.Y. and E.T.-P.S.; formal analysis, Y.-M.Y., A.A.S. and P.M.-Y.P.; investigation, Y.-M.Y. and I.W.-Y.M.; resources, F.W.-F.L. and E.T.-P.S.; data curation, Y.-M.Y.; writing—original draft preparation, Y.-M.Y., S.M.-N.C. and I.W.-Y.M.; writing—review and editing, F.W.-F.L. and E.T.-P.S.; visualization, Y.-M.Y. and E.T.-P.S.; supervision, E.T.-P.S.; project administration, E.T.-P.S.; funding acquisition, F.W.-F.L. and E.T.-P.S. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: The study was approved by the Institutional Review Board of Hong Kong Metropolitan University (Reference No. AE-PACRD2020/1.17; Date of approval: 13 November 2020).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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