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Enhanced bath immersion vaccination through microbubble treatment in the cyprinid loach

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A B S T R A C T

Immunization by bath immersion is likely the simplest method of fish vaccination. Although the route of immunogenicity has not been fully identified, immersion vaccination is clearly a useful labor-saving technique. In this study, microbubble (MB) treatment was assessed for its ability to improve the efficacy of bath immersion vaccination in the cyprinid loach. MBs are commonly defined as minute particles of gas with a diameter of less than 100 μm, which generated free radicals. Here, the efficacy of MB treatment for vaccination enhancement in the cyprinid loach was assessed in direct challenge experiments using the virulent *Aeromonas hydrophila* JUNAH strain; assessments comprised agglutination titer assay and non-specific parameter analysis. Agglutination titers were high in loaches that were immunized via injection with inactivated cells (FKC group); however, non-specific immune activation parameters (e.g., lysozyme, superoxide dismutase, and phagocytic activity) were more increased in loaches that were immunized via bath immersion with MB treatment. Moreover, MB-treated loaches showed comparable survival rates, relative to loaches immunized via injection with formalin inactivated cells. Thus, higher levels of non-specific immune parameters suggest increased efficacy of this vaccine approach. Improving the effectiveness of bath immersion vaccine will increase its affordability and ease of application in aquaculture.

1. Introduction

Aquaculture is an important component of a fishery for food, according to the Food and Agriculture Organization of the United Nations. Loss reduction is critical for maintaining stable food production. A variety of factors affect aquaculture, such as optimal husbandry, biosecurity, nutrition genetics, system management, and water quality; however, disease outbreaks are a critical factor in aquaculture industry losses [1–4].

For bacterial and parasitic diseases, there are many available chemotherapeutants. These antibiotics and parasiticides are effective for disease treatments; however, they can cause damage to the aquatic environment and may lead to problems associated with antibiotic resistance. Vaccination is gradually gaining acceptance as an important component of aquaculture, based on its effectiveness in controlling disease outbreaks [5]. A few aspects must be considered to overcome the disadvantages involved in vaccination. First, high-priced vaccines are difficult to be applied economically, compared to those used in other farming industries, such as pig or cattle rearing. Second, the administration method influences the use of the vaccine; the syringe injection method is an effective immunostimulation technique compared to oral and immersion routes, but is labor-intensive, thus requiring a greater financial investment. The route of immunogenicity of the immersion vaccine has not been fully identified, and host antigen absorption is affected by various conditions (e.g., vaccine concentration, immersion time, animal size, adjuvant use, antigenic form, and water

Abbreviations: CFU, Colony-forming units; MBs, Microbubbles; PBS, Phosphate-buffered saline; PCR, Polymerase chain reaction; RPS, Relative percent survival; wpv, weeks post-vaccination

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temperature) [6]. However, immersion vaccination is clearly a useful labor-saving technique.

Microbubbles (MBs) are commonly defined as minute particles of gas with a diameter of less than 100 μm [7]. MBs exhibit characteristics that differ from those of macro bubbles, which are greater than 100 μm in size. MBs have been used in aquaculture to improve the seafloor environment and water quality, as well as to reduce the rate of mortality and promote growth by enhancing available oxygen in shellfish farms [8–10]. These bubbles rise slowly to the surface and remain in solution for an extended period; moreover, they can aid in gas dissolution into solution [11]. In MBs, surface tension causes high internal pressure, which can cause the gas to dissolve in the surrounding water, resulting in condensation and collapse [7]; free radicals are generated when the bubbles are collapsed, and these are useful for decomposition of organic chemicals and for wastewater treatment processes [12].

Immune responses induced by immersion vaccination are generally less robust and have shorter durations than those obtained by injection [13–15]. To increase the uptake of vaccines by fish mucosal tissues, thereby maximizing the efficacy of immersion vaccination, hyperosmotic immersion and ultrasound-mediated immersion have been adopted [16–20]. The free radicals produced by microbubbles could be able to stimulate the mucosal surfaces of fish, such as in the preceding methods, to increase the uptake of antigens [21,22]. In the present study, MB treatment was used to enhance the efficacy of bath immersion vaccination; the efficacy of MB-enhanced vaccination was evaluated in a cyprinid loach model through the direct challenge with the virulent *Aeromonas hydrophila* JUNAH strain. The immunogenicity of the vaccines was assessed by agglutination test and the activities of lysozyme (LZM), superoxide dismutase (SOD), and phagocytosis.

2. Materials and methods

2.1. Ethics statement

All experimental protocols were performed in accordance with the Guidelines on the Regulation of Scientific Experiments on Animals, issued by Seoul National University (SNU) Institutional Animal Care and Use Committee (SNU, Republic of Korea). The anesthesia procedure for the sampling of blood and organs and euthanasia of the fish were performed using tricaine methanesulfonate (MS-222).

2.2. Animals

A total of 700 cyprinid loaches (*Misgurnus anguillicaudatus*, mean body weight ± standard deviation: 8.28 ± 1.85 g) were purchased from commercial fish farms in Gyeonggi province, Republic of Korea. The fish were acclimatized in the laboratory of the College of Veterinary Medicine of SNU (Seoul, Republic of Korea) for 30 days before commencing the experiment. Fish were kept in 200-L fiberglass tanks at 25 ± 2 °C and fed twice per day with commercial feed (Tetra Bits Complete, Tetra, USA). Approximately 30% of the water in each tank was changed daily.

2.3. Vaccine preparation

Aeromonas hydrophila JUNAH strain, stored in the lyophilized condition in our laboratory was used for this study. The bacterial strain was isolated from a cyprinid loach in 2009, in the Republic of Korea [23]. The bacteria were recovered from the lyophilized condition and subcultured in tryptic soy broth (Difco, Detroit, MI, USA) at 25 °C for 24 h. The cultured bacterial cells were harvested and suspended in phosphate buffered saline (PBS), then treated with 0.5% formalin (v/v) and maintained at 25 °C for 48 h. The cells were washed twice and resuspended in PBS.

2.4. Vaccination

The 700 loaches were randomly divided into 10 experimental groups, immunized with 3 different methods. Immersion-vaccinated groups (IM1, IM2, and IM3) were bath immersion-vaccinated for 1 h with formalin-inactivated *A. hydrophila* cells (1×10^9 colony-forming units (CFU)/mL). The groups, immersion-vaccinated with MB-treatment (MB1, MB2, MB3, MB4, and MB5), were immersion-vaccinated for 1 h with formalin-inactivated *A. hydrophila* cells (1×10^9 CFU/mL); concurrent treatment with MBs was performed. The MBs were generated by using a homogenizer (HG-15D; DAIHAN Scientific, Republic of Korea) at a rate of 6000 rpm, and continuously supplied into vaccinated water tanks. The formalin-inactivated cells group (FKC) was immunized with 0.1 mL of formalin-inactivated *A. hydrophila* cells (2×10^8 CFU/fish), through intraperitoneal injection. Control fish were treated with microbubbles for 1 h. Vaccinated fish were immediately returned to their rearing tanks.

IM1 and MB1 groups were immersion-vaccinated only 1 time. IM2 and MB2 groups were booster-vaccinated once, 1 week after initial vaccination. IM3 and MB3 groups were booster-vaccinated twice, at 1-week intervals. The MB4 group was booster-vaccinated 3 times, at 1-week intervals. The MB5 group was booster-vaccinated twice, at 1-week intervals, then booster-vaccinated again at 6 weeks post-vaccination (wpv). Experimental groups and corresponding schedules of vaccination are listed in Table 1.

2.5. Sample collection

Blood specimens for agglutination titer, LZM, and SOD analysis were collected from the caudal vein of three randomly chosen fish in each group (IM1, IM2, IM3, MB1, MB2, MB3, MB4, MB5, FKC, and control) after they were anesthetized with MS-222 (300 ppm). For measurement of phagocytic activity, a syringe with anticoagulant was used to collect specimens. For the agglutination assay, samples were collected at 2, 4, 6, 8, and 10 wpv; for the immune response assessment, samples were collected at 1, 2, 3, and 4 wpv. Blood specimens for agglutination, LZM activity, and SOD activity assays were transferred to microcentrifuge tubes (Eppendorf, Hamburg, Germany); serum was collected after centrifugation at 6500 g for 10 min at 4 °C. Then, the collected serum was stored at −20 °C until use. Leukocytes from whole blood were separated using the Ficoll gradient method described by Noble et al. [24]. Cell viability was evaluated using the trypan blue exclusion test and cell concentration was determined using a

Table 1
Experimental groups and immunization schedule.

Experimental groups	Methods	Vaccination (weeks)						
		0	1	2	3	4	5	6
FKC	IP[a]	Treated						
IM1	IM[b]	Treated						
IM2	IM	Treated	Treated					
IM3	IM	Treated	Treated	Treated				
MB1	MB[c]	Treated						
MB2	MB	Treated	Treated					
MB3	MB	Treated	Treated	Treated				
MB4	MB	Treated	Treated	Treated	Treated			
MB5	MB	Treated	Treated	Treated				Treated

Formalin-inactivated cells (FKC) group was vaccinated with 2×10^8 CFU/fish of *Aeromonas hydrophila* inactivated cells by intraperitoneal injection.
Immersion-vaccinated groups (IM1, IM2 and IM3) and Immersion-vaccinated with microbubble-treated groups (MB1, MB2, MB3, MB4 and MB5) groups were bath-immersed with 1×10^9 CFU/L of *A. hydrophila* inactivated cells.
[a] IP, Intraperitoneal injection.
[b] IM, immersion.
[c] MB, immersion with microbubble treatment.

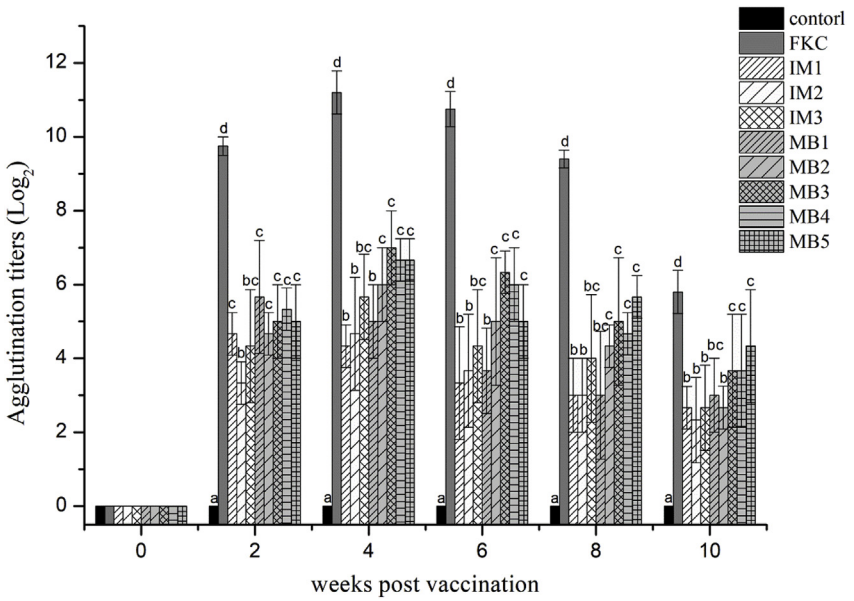

Fig. 1. Serum agglutination titers of cyprinid loach vaccinated formalin killed cells of *Aeromonas hydrophila* JUNAH strain (FKC) by intraperitoneal injection, and bath immersion of *A. hydrophila* JUNAH strain (IM1, IM2, and IM3), bath immersion of *A. hydrophila* JUNAH strain with microbubble treatment (MB1, MB2, MB3, MB4, and MB5), or microbubble treatment without antigens (Control). Bars represent mean ± standard error of the mean (n = 3). Different letters above the bars represent the statistical significance ($p < 0.05$) among different groups at the same time point.

hemocytometer. Harvested cells were adjusted to 1×10^6 cells/mL for use in the assay.

2.6. Serum agglutination assay

The assay was conducted in microtiter plates with U-shaped wells. Serum samples (n = 3) were serially two-fold diluted in PBS and homologous heat-killed *A. hydrophila* (10^7 cells/mL) was added. Serum agglutination activity was determined on the basis of the lowest dilution without agglutination and is expressed as the reciprocal of that dilution. This test was performed once.

2.7. Non-specific immune parameters

LZM and SOD activities were measured using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). One unit of lysozyme activity was defined as the amount of lysozyme that caused a reduction in absorbance of 0.001 unit per min at 530 nm; one unit of SOD was defined as the amount required to reduce the rate of xanthine reduction by 50% in a 1-mL reaction system. The optical density for SOD activity was measured at 550 nm. Assessment of phagocytic activity in whole blood was performed using a commercial kit (CytoSelect™ 96-Well Phagocytosis Assay, Cell Biolabs, San Diego, CA, USA). *Escherichia coli* was used as a substrate and the absorbance was measured at 450 nm.

2.8. Challenge experiment

The experimental groups (n = 30) were challenged with *A. hydrophila* JUNAH stain at 4 wpv with the median lethal dose by intraperitoneal injection. Fish were anesthetized using MS-222 (300 ppm) before the challenge experiment. Cumulative mortalities and clinical signs were monitored twice per day for 2 weeks. The head kidneys of dead fish were streaked onto tryptic soy agar, and isolated bacteria were identified by polymerase chain reaction (PCR) method as previously described [25]. Vaccine efficacy was assessed by relative percent survival (RPS) using the following formula:

RPS = [1 – (cumulative mortality of vaccinated group/cumulative mortality of control group)] × 100%

2.9. Statistical analysis

All data were analyzed using SPSS version 22.0 (IBM Corp., Armonk, NY, USA). A one-way analysis of variance (ANOVA) was used to analyze the data, followed by Duncan's multiple range test, to compare variations in immune parameters for differences at a significance level of 0.05. The mean ± standard error of the mean of assayed parameters was calculated for each group.

3. Results

3.1. Safety of microbubble treatment

MB treatment was performed for 3 h in a group of fish (n = 10) that were observed for 2 weeks to verify the safety of the free radicals generated by the collapse of MBs; notably, no symptoms or mortality occurred in those fish. These findings suggested that no safety issues would be encountered in the present study because 1 h of vaccination was used in this safety assay.

3.2. Antibody titers

The MB groups showed tendencies for higher titers than the IM groups. The MB1 group showed significantly higher titers than the IM1 group at 2 and 4 wpv. A higher number of vaccinations was associated with higher antibody titer in both IM groups and MB groups. However, the MB3 group, which underwent booster-vaccinated 3 times, and the MB4 group, which underwent booster-vaccinated 4 times, showed similar antibody titers. The MB5 group was designed to ensure that antibody titer is induced again upon booster-vaccination following a delay after initial vaccination. The antibody titer of the MB5 group peaked at 4 wpv and then decreased; however, it increased again at 8 wpv after vaccination at 6 wpv. Throughout the experimental period, the FKC group showed higher titers than the other groups. Agglutination titers in the control groups remained at zero throughout the experimental period, as shown in Fig. 1. Serum titers indicated no detectable antibodies prior to vaccination in all groups.

3.3. Non-specific immune response

In the analysis of LZM activity, MB groups (MB1, MB2, and MB3) showed significantly higher activity than IM groups (IM1, IM2, and

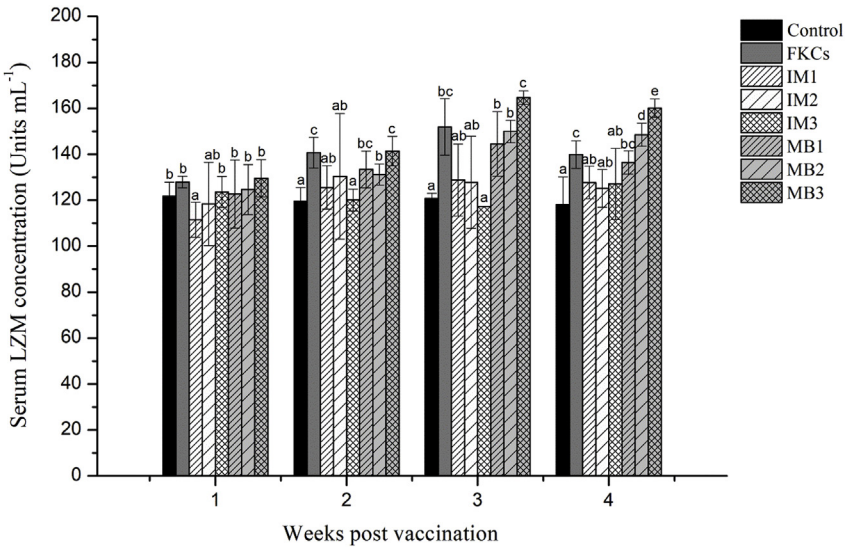

Fig. 2. The activity of lysozyme in the blood of cyprinid loach, intraperitoneally administered formalin killed cells of *Aeromonas hydrophila* JUNAH strain (FKC), vaccinated by bath immersion method (IM1, IM2 and IM3), vaccinated by bath immersion method with microbubble treatment (MB1, MB2 and MB3), or microbubble treatment without antigens (Control). Bars represent mean ± standard error of the mean (n = 3). Different letters above the bars represent the statistical significance ($p < 0.05$) among different groups at the same time point.

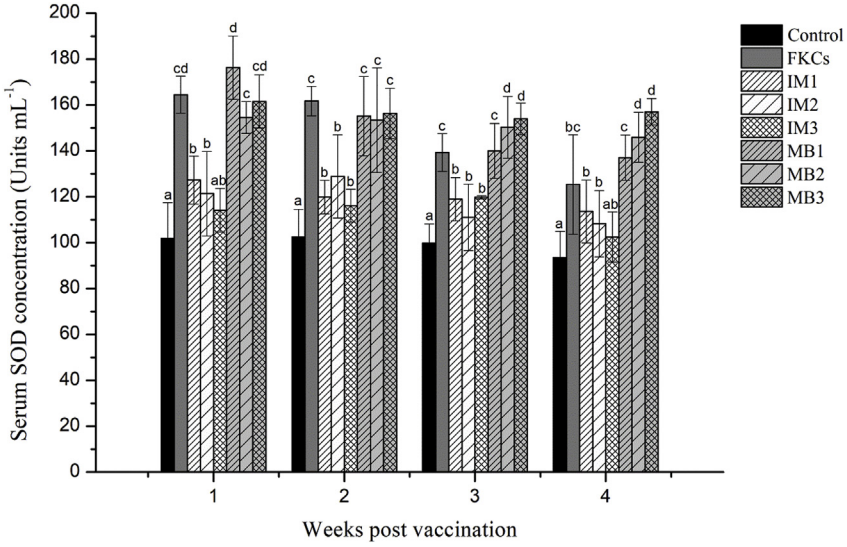

Fig. 3. The activity of superoxide dismutase in the blood of cyprinid loach, intraperitoneally administered formalin killed cells of *Aeromonas hydrophila* JUNAH strain (FKC), vaccinated by bath immersion method (IM1, IM2 and IM3), vaccinated by bath immersion method with microbubble treatment (MB1, MB2 and MB3), or microbubble treatment without antigens (Control). Bars represent mean ± standard error of the mean (n = 3). Different letters above the bars represent the statistical significance ($p < 0.05$) among different groups at the same time point.

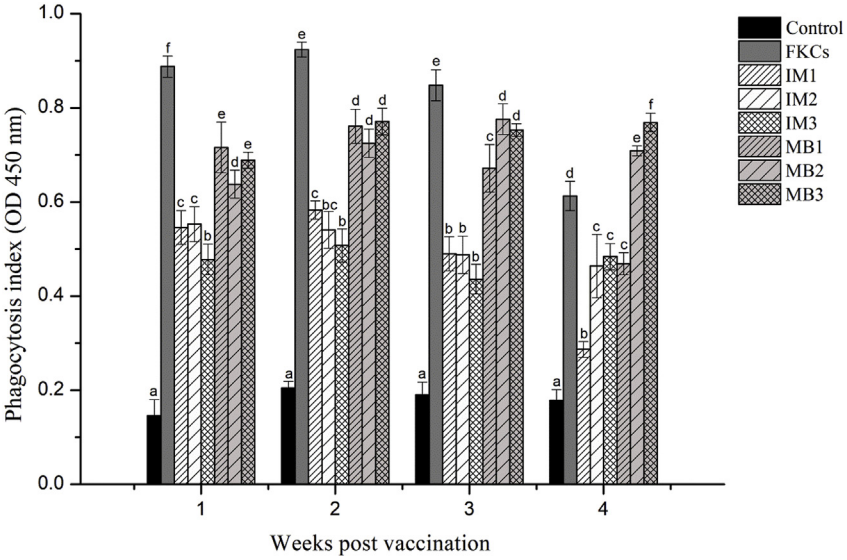

Fig. 4. The activity of phagocytosis in the blood of cyprinid loach, intraperitoneally administered formalin killed cells of *Aeromonas hydrophila* JUNAH strain (FKC), vaccinated by bath immersion method (IM1, IM2 and IM3), vaccinated by bath immersion method with microbubble treatment (MB1, MB2 and MB3), or microbubble treatment without antigens (Control). Bars represent mean ± standard error of the mean (n = 3). Different letters above the bars represent the statistical significance ($p < 0.05$) among different groups at the same time point.

IM3) at 2, 3, and 4 wpv. IM and MB groups showed no significant differences in the first week; however, the MB groups showed increased LZM activity after 2 wpv, which peaked at 3 wpv and then decreased. Group MB3 showed a significantly higher level of LZM activity than group FKC at 3 and 4 wpv. LZM activity in Group MB2 also consistently increased and was higher than that of group FKC at 4 wpv (Fig. 2). The serum SOD activity of the MB groups was significantly greater than that of the IM groups throughout the experimental period; it peaked at 1 wpv, then slowly decreased. The MB groups showed similar levels of SOD activity to those of the FKC group during the first week. Thereafter, the MB groups showed significantly greater levels of SOD activity; after 3 wpv, the levels were maintained in a stable manner (Fig. 3). The phagocytosis indexes of the MB groups were higher than those of IM groups throughout the experiment. The phagocytosis index of the MB1 group peaked at 2 wpv, then slowly decreased; however, the phagocytosis index of the MB2 and MB3 groups were sustained throughout the experiment. These groups showed lower phagocytosis indexes than that of the FKC group at 1, 2, and 3 wpv; However, the MB2 and MB3 groups showed higher phagocytosis indexes than that of the FKC group at 4 wpv, because the phagocytosis index of the FKC group decreased (Fig. 4).

3.4. Survival analysis

The cumulative mortality was observed after the challenge experiment at 4 wpv (Fig. 5). In all groups, mortality began at 6 h post-infection and continued through 72 h after challenge. The observation was performed for 2 weeks, and the surviving fish remained alive for the rest of the experiment; they showed no symptoms after 72 h. The FKC group showed higher RPS (43.48%), relative to that of the control group. The survival rates of both IM and MB groups also tended to increase as the number of vaccinations increased. The MB1 group showed a higher survival rate (26.09%) than the IM1 group (13.04%); two or more vaccinations led to higher survival (MB2 (52.17%), MB3 (56.52%) and MB4 (52.17%)) than that observed in the FKC group. There were no notable differences among the groups (MB2, MB3, and MB4) that showed higher survival rates than the FKC group (Fig. 5). All fish that died during the challenge experiment exhibited typical clinical signs of *A. hydrophila* infection. The isolated bacteria were confirmed to be *A. hydrophila* by using the PCR method (data not shown).

4. Discussion

Currently, injection of inactivated vaccines is used in the mainstream aquaculture industry, as inactivated vaccines can be produced inexpensively, and are generally recognized to induce stronger immunity than other vaccination methods (e.g., oral vaccines and immersion vaccines) [26,27]. The immersion method is not widely used because its effects are weaker than those of injection vaccines. However, the immersion method, unlike the injection vaccine approach, can greatly reduce the manpower required for the vaccination process. This provides a competitive advantage in that it allows easy vaccination without the aid of experts. The purpose of this study was to improve the effectiveness of the bath immersion technique using MB treatment. Notably, MB treatment is used as a clean and efficient method to purify drinking water and wastewater, and has attracted increasing research interest in recent years [26]; moreover, it has physical and chemical properties that can modify fish skin. The size of MBs is gradually reduced as it rises and subsequently collapses because of the dissolution of interior gases into the surrounding water [7]. The temperature inside the bubbles at the time of collapse is $>$ 5000 K; thus, free radicals (e.g., superoxide and hydroxyl) are created [12,28–31]. These may be involved in the initiation of free radical chain reactions, which may damage cells [32]. Cell membrane-dependent functions are affected by membrane fluidity and physical state, both of which are determined by the membrane lipid acyl chain profile; notably, acyl chains are transformed by free radicals [33]. External free radicals could affect the mucous cell membrane of the fish in a manner similar to that of hyperosmotic pretreatment, which affects osmotic pressure in the cell membrane, as well as in a manner similar to that of ultrasound treatment, which widens the intercellular space [34,35]. Using these features of MB, this study was intended to confirm the possibility of improving the effectiveness of the bath immersion technique. Further studies are needed to reveal the mechanism by which MB treatment affects the absorption of antigens.

After immunization, fish that were injection-vaccinated with inactivated bacteria showed increased agglutination titer against *A. hydrophila*. The MB groups showed higher antibody titers than IM groups. These results are similar to those of prior studies that used hyperosmotic pretreatment to accelerate the uptake of antigens [36]. During all experimental periods, the FKC group showed higher titer than that of all other groups, peaking at 4 wpv then gradually decreasing; this was similar to the findings of our previous study [37]. In general, high antibody titers are considered to have high protection capabilities.

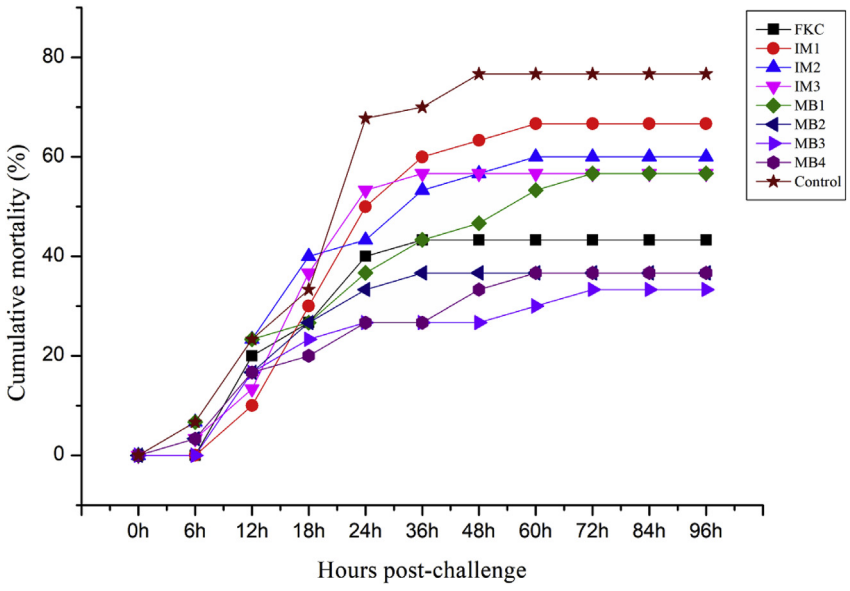

Fig. 5. Cumulative mortality curve of challenge experiment on cyprinid loach, intraperitoneally administered formalin killed cells of *Aeomonas hydrophila* JUNAH strain (FKC), vaccinated by bath immersion method (IM1, IM2 and IM3), and vaccinated by bath immersion method with microbubble treatment (MB1, MB2, MB3, and MB4).

However, specific serum antibody titers are not always related to the level of protection [38,39]. Thus, further research is needed to investigate the underlying mechanism.

The levels of important immunological parameters, such as LZM, SOD, and phagocytic activities in fish were measured, compared to control fish. LZM is an enzyme that splits peptidoglycan in the bacterial cell wall and plays an important role in natural defense mechanisms [40]. In higher vertebrates, this enzyme is speculated to contribute to opsonization, immune response potentiation, and antiviral activity [41]. SOD is a primary antioxidant defense enzyme generated in response to oxidative stress. Phagocytic cells also play an important role in antibacterial defense mechanisms [42]. LZM activity was significantly higher in immunized groups, indicating that the vaccine elicited a non-specific immune response [43]. Wang et al. reported that SOD activities also significantly increased in Chinese breams immunized with the Omp38 recombinant protein [44]. In assessments of all parameters, the FKC group showed the highest levels in the first week, but MB2 and MB3 groups both showed the highest levels at 4 wpv. The method of MB treatment is expected to induce non-specific immune responses, as MB-treated groups showed higher levels than all IM groups in assessments of LZM, SOD, and phagocytic activities. Superoxide anion, a type of free radical generated by MBs, is converted rapidly into H_2O_2, which freely cross cell membranes [45] and reacts with thiols to form disulfide bonds [46]. In addition, H_2O_2 has been reported to participate in many cellular processes, such as cell growth, stem cell renewal, cell death, cell senescence, and cell migration, as well as immune responses [47–51]. This bath immersion vaccine enables vaccination with multiple treatments at low cost, such that it is possible to induce greater non-specific immune responses than with the original FKC vaccine.

Agglutination titers of the MB2 and MB3 groups were lower than those of the FKC group during this experimental period, but non-specific immune parameters were significantly higher. These non-specific immune parameters, as well as antibody titers, are critical to protection from diseases [52,53]. Immunization by immersion is likely to be the simplest method of vaccination [54]. Moreover, if the effectiveness of bath immersion vaccine can be improved through a variety of approaches, it could be more broadly applied in the prevention of fish diseases.

In this study, MBs were used to enhance the efficacy of bath immersion vaccine against *A. hydrophila* infection. The MB-treated groups, MB3 and MB4, showed higher survival rates that were comparable to those of the FKC group; those groups also showed high levels of non-specific parameters. This suggests that MB-treated bath immersion vaccination groups may be effective than an immersion vaccinated only groups, and in some respects, it may be equally or more effective to FKC injection vaccination. Therefore, consideration of its application in a fish farm may be useful, according to the results of this study. Through research involving more diverse techniques, the efficacy of the bath immersion vaccine may be improved, which could lead to the provision of an affordable and easy to apply vaccine for aquaculture.

Conflicts of interest

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

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