

Probiotic characterization of a novel *Bacillus cereus* strain fkW8-1-2 isolated from intestine of white crucian carp (*Carassius cuvieri*)

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ABSTRACT

Microbial infection can pose a great threat to health of farmed fish. This study aimed to investigate the probiotic characteristics of *Bacillus cereus* strain fkW8-1-2 isolated from intestine of WCC and its anti-biofilm activity against pathogenic bacteria. The strain fkW8-1-2 showed a high resistance to low pH, bile salt and lysozyme during long-term incubation. The strain fkW8-1-2 displayed a gradual increase of cell surface hydrophobicity with various forms of hydrocarbons. The strain fkW8-1-2 showed the remarkable autoaggregation and coaggregation activity with *A. hydrophila* L3-3 and *E. tarda* 11-4. The culture medium (CM) and cell-free supernatants (CFS) of strain fkW8-1-2 at 48 h cultivation could attenuate biofilm formation of *A. hydrophila* and *E. tarda* with the maximum inhibition rates. In addition, the intact cells of strain fkW8-1-2 exhibited the strong antioxidant activity for DPPH removal and superoxide anion clearance. These results indicated that strain fkW8-1-2 possessing probiotic characteristics can pose a protective role in health of farmed fish.

1. Introduction

Water contamination can exhibit a direct immune suppression in aqueous livings, which may elevate frequency of infectious diseases during aquaculture [1]. Although antibiotics administration may effectively eliminate pathogenic bacteria, its overuse may easily enable the challenge of microbe population to enhance emergence of resistant pathogens in natural environment [2]. In addition, increased use of antibiotics may also disrupt gut microbiota, which may pose a great threat to health of aquatic animals [3].

Current findings reveal that probiotics administration may be an ideal alternative to antibiotics use in animal nutrition [4]. In general, bacterial antagonism is a common phenomenon in natural environment, thus microbial interaction exhibits a pivotal role in the equilibrium between competing probiotics and potential pathogens [5]. As is well known, the population of endogenous microbiota may be joint action of heredity, nutrition and environmental factors, thus gut microbiota in aquatic animals is mainly constituted by indigenous microbiota and high levels of foreign microorganisms from surrounding water, which may exert a great impact on the health status of aquatic animals than those of terrestrial animals [6]. The predominant indigenous microbiota in

marine fish is composed of gram-negative facultative anaerobic bacteria, including *Vibrio* sp. and *Pseudomonas* sp. [7], while representatives of the family *Aeromonas* sp., *Plesiomonas* sp., *Enterobacteriaceae* sp. and other obligate anaerobic bacteria may constitute the predominant indigenous microbiota in freshwater fish [8]. The crucial characteristics of a potential probiotics used in aquaculture may include modulation of intestinal immunity, improvement of nutrient absorption, antagonist against invading pathogens as well as direct degradation of organic and toxic substances in water [9].

The reciprocal interaction of gut, gut flora and liver constitutes a major immune microenvironment in teleost fish [10,11]. Gut-associated lymphoid tissue (GALT) exhibits a crucial role in mucosal immune surveillance and pathogenic elimination, while gut flora composed of a dynamic community of bacteria, fungi, archaea and protozoans can mediate gut mucosal barrier function, including production of secretory immunoglobulins, lymphocyte recruitment as well as antagonism against pathogenic colonization [12]. However, some invasive pathogens are able to effectively avoid pathogenic killing mechanism in host cells and then breach mucosal barrier for deeper infection in inflammatory foci [13]. Crucian carp (*Carassius auratus*) is one of popular fish species in China, which is due to its delicious taste and high stress

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resistance, but dispersal of infectious diseases exerts a deleterious impact on its farming [14]. Our previous studies indicated that *A. hydrophila* can disturb epithelial permeability in midgut, increase bacterial burdens as well as dysregulate immune response in gut-liver axis of WCC [15]. However, few reports have been conducted on the probiotic performance of *Bacillus cereus* from WCC.

Therefore, the aims of this study were to characterize the novel probiotic properties of *Bacillus cereus* strain fkW8-1-2 isolated from intestine of WCC. The stress tolerance, cell surface hydrophobicity and antioxidant property were evaluated. Then, autoaggregation, coaggregation and biofilm forming ability were studied, which may provide a novel insight into the understanding of probiotic feature from WCC.

2. Materials and methods

2.1. Fish sampling and *Bacillus* sp isolation

Healthy WCCs (approximately 38.91 ± 3.25 g) were obtained from a fishing base in Changsha, China. The intestinal samples were isolated and homogenized in sterile PBS. After incubation at 70 °C for 30 min, intestinal homogenates were cultured onto Luria-Bertani (LB) agar plates at 30 °C under aerobic condition for 24 h. Then, the single colonies of suspected *Bacillus* sp were selected and identified by using the methods described previously [16]. After colony purification, extracted bacterial DNA was used to amplify 16S rRNA sequence by using universal primers 27F and 1492R and was subjected to for sequencing confirmation (Tsingke, China). After that, the resulting 16S rRNA sequence of isolated *B. cereus* strain fkW8-1-2 was deposited to GenBank with the accession number OR553396. Then, the phylogenetic tree analysis was performed by using MEGA 6.0 software and ITOL program with neighbor-joining (N-J) method.

2.2. Determination of hemolytic activity

Bacterial culture were incubated on 5% (v/v) goat blood agar plate at 30 °C for 48 h. Hemolytic type of *B. cereus* strain fkW8-1-2 was determined according to the formation of hemolytic zone around the colony. The experiment was repeated in triplicate.

2.3. Growth curve detection of *B. cereus* strain fkW8-1-2

Bacterial strain fkW8-1-2 was inoculated into Luria-Bertani (LB) liquid medium (2% v/v), brain-heart infusion (BHI) liquid medium (2% v/v) and beef extract peptone (BEP) liquid medium (2% v/v) at 180 rpm/min for 48 h (30 °C). Then, the absorbances were detected at 600 nm every 6 h. The experiment was repeated in triplicate.

2.4. Tolerance determination

Bacterial strain fkW8-1-2 was inoculated into LB liquid medium at 2% inoculum and incubated at 180 rpm/min for 24 h (30 °C). Then, bacterial broth with 2% (v/v) was added to LB liquid medium with different pH values (pH 2.5, pH 3.5 and pH 4.5), different concentration of bile salt (0.1%, 0.3% and 0.5% bile salt) and lysozyme (50 µg/mL, 100 µg/mL and 200 µg/mL) for 12 h and 24 h of cultivation. LB broth without treatment was used as control group. OD₆₀₀ values of experimental group, control group and blank group were recorded as A₁, A_c and A₀, respectively. The experiment was repeated in triplicate. The survival rate was determined as below: Survival rate (%) = $(A_1 - A_0)/(A_c - A_0) \times 100\%$

2.5. Autoaggregation and coaggregation assays

The autoaggregation and coaggregation assays were performed as previously described [17]. In brief, overnight bacterial culture was centrifuged at 5000 rpm/min for 10 min at room temperature. The

obtained pellets were washed three times and resuspended in PBS. The absorbance at 600 nm was recorded as A₀. For autoaggregation assay, 4 mL of strain fkW8-1-2 suspension was vortexed and incubated at 30 °C for different incubation times (6 h, 12 h, 24 h and 48 h), then The absorbance at 600 nm was recorded as A₁. The experiment was repeated in triplicate. Autoaggregation (%) = $1 - A_1/A_0 \times 100\%$

For coaggregation assay, *Aeromonas hydrophila* L3-3 and *Edwardsiella tarda* 11-4 was used as pathogenic bacteria. Autoaggregation of pathogenic bacteria were confirmed as above described and OD₆₀₀ values were recorded as A_a and A_b, respectively. After that, 2 mL of strain fkW8-1-2 suspension was vortexed and incubated with equivalent volume of pathogenic bacteria, then at 30 °C for different incubation times (6 h, 12 h, 24 h and 48 h), then The absorbance at 600 nm was recorded as A₃. The experiment was repeated in triplicate. Coaggregation (%) = $(1 - A_3)/[(A_a(b) + A_0)/2] \times 100\%$

2.6. Hydrophobicity assay

Hydrophobicity detection of *B. cereus* strain fkW8-1-2 was performed as previously described [18]. In brief, bacterial culture at different culture times (24 h, 48 h and 72 h) was collected and centrifuged at 5000 rpm/min for 10 min at room temperature. The obtained pellets were washed three times and resuspended in PBS. The absorbance at 600 nm was recorded as A₀. Then, 3 mL of strain fkW8-1-2 suspension was incubated with 1 mL chloroform or xylene for 20 min. After removing organic phase, the mixed liquid was diluted with PBS and measured at OD₆₀₀ nm as A_t. The experiment was repeated in triplicate. Hydrophobicity (%) = $(A_0 - A_t)/A_0 \times 100\%$

2.7. Biofilm formation assay

Biofilm formation ability was evaluated by crystal violet staining method [19]. In brief, strain fkW8-1-2 was inoculated into LB liquid medium. After bacterial adjustment to 1×10^8 CFU/mL, 200 µL of diluted bacterial culture was added into 96-well plate. After another 24 h of incubation at 30 °C, strain fkW8-1-2 was fixed in dark, washed with PBS solution and stained with crystal violet. After dissolving with ethanol, the mixed liquid was measured at OD₅₇₀ nm as A_t. Measurement value of LB culture medium alone was used as the control and recorded as A₀. Ratio of Biofilm formation ability = A_t/A_0 . If the ratio >1, the detected bacteria was considered as biofilm-positive strain.

Inhibitory effects of probiotics on pathogenic biofilm formation were performed [20]. Culture medium (CM) of strain fkW8-1-2 was harvested at 24 h and 48 h of incubation. The above CM was sterilized by 0.22 µm filter (Millipore) and used as strain fkW8-1-2 cell-free supernatants (CFS). *A. hydrophila* L3-3 and *E. tarda* 11-4 was used as pathogenic bacteria. When OD₆₀₀ value attained 0.6, pathogenic counts were adjusted to 1×10^7 CFU mL⁻¹ in PBS. Then, adjusted pathogens were treated with above CM or CFS at 30 °C for 24 h. OD₅₇₀ values of bacterial biofilm treated with CFS or CM were recorded as A_{CFS} or A_{CM} by crystal violet staining method, respectively. OD₅₇₀ values of bacterial biofilm without treatment were recorded as A₀. The experiment was repeated in triplicate. Biofilm inhibition activity (%) = $(1 - A_{CFS(CM)})/A_0 \times 100\%$

2.8. Free radical scavenging assay

Bacterial strain fkW8-1-2 was cultured in LB medium at 30 °C. In brief, bacteria at stationary growth phase were collected and centrifuged at 7000 rpm/min for 10 min at room temperature. The obtained pellets were washed three times and resuspended in PBS. After bacterial adjustment to 1×10^8 CFU/mL, superoxide anion radical scavenging activity and DPPH radical scavenging activity of strain fkW8-1-2 were detected by using superoxide anion radical detection kit (Solarbio, China) and DPPH radical activity kit (Jiancheng Bioengineering institute, China). The absorbances were measured at 530 nm and 517 nm, respectively. The intact cells scavenging activity (%) = $[(1 - A_t/A_c)] \times$

100%. Wherein, A_t and A_c represented the absorbance values of tested samples and the controls, respectively. The experiment was repeated in triplicate.

2.9. Statistical analyses

SPSS program was used for data calculation, which is subjected to one-way ANOVA. If the analytical levels reach less-than 0.05 P-value, results were statistically significant.

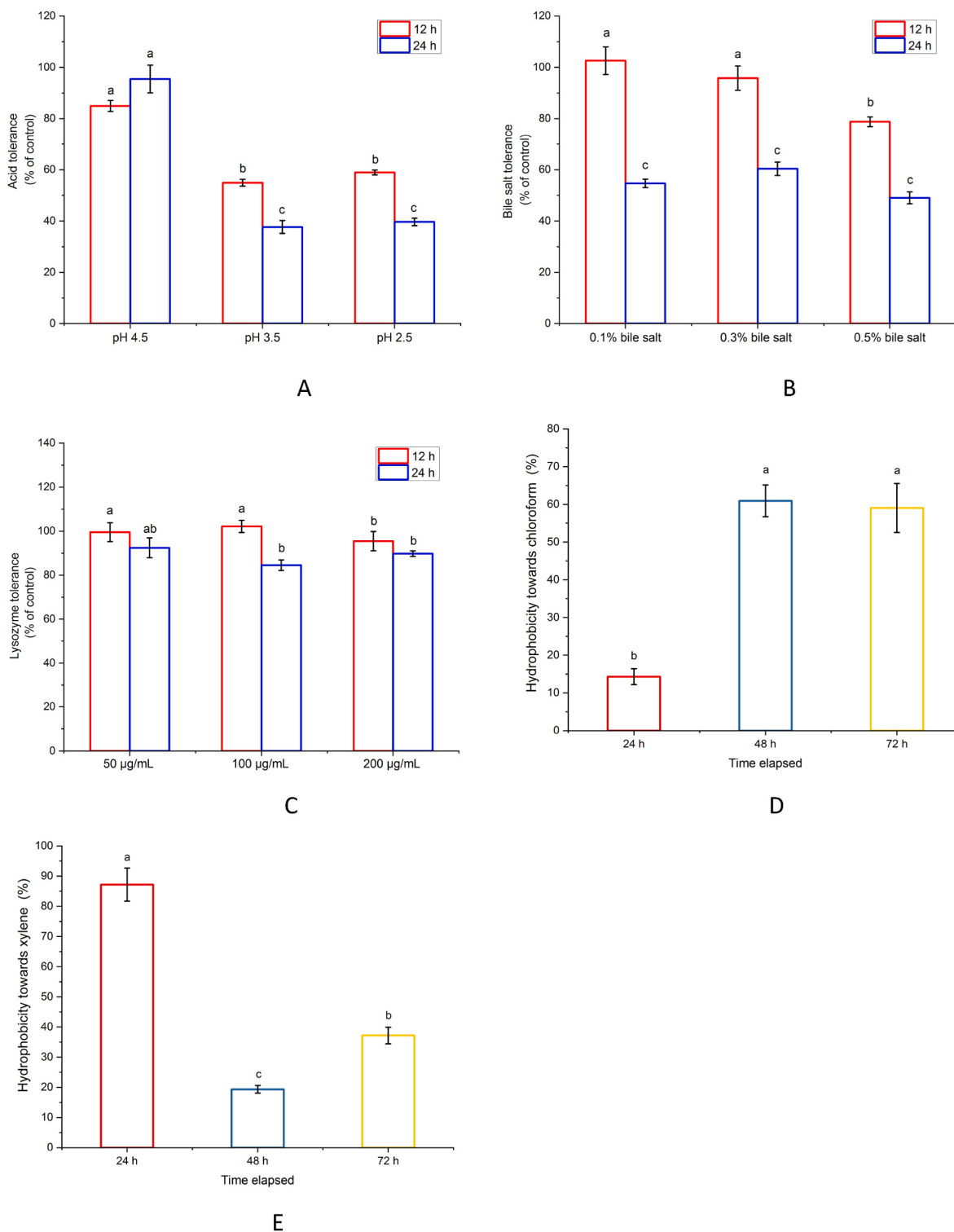


Fig. 1. Tolerance analysis and Cell surface hydrophobicity of strain fkW8-1-2. (A) Effect of acidic condition (pH 4.5, pH 3.5 and pH 2.5) on the survival rate of strain fkW8-1-2. (B) Effect of bile salt (0.1%, 0.3 and 0.5% bile salt) on the survival rate of strain fkW8-1-2. (C) Effect of lysozyme (50 µg/mL, 100 µg/mL and 200 µg/mL) on the survival rate of strain fkW8-1-2. Cell surface hydrophobicity of strain fkW8-1-2 towards chloroform (D) and xylene (E). The calculated data (mean ± SD) with different letters were significantly different ($P < 0.05$) among the groups. The experiments were performed in triplicate.

3. Results and discussions

3.1. Characterization of *B. cereus* strain fw8-1-2

The *Bacillus* strains were isolated from the intestine of WCC by using heat stimulation procedure. As is well known, most of the isolated *Bacillus* strains showed the β -hemolytic activity [21]. To obtain non-hemolytic bacterial strains for further utilization in food processing purposes, the single bacterial colonies were firstly spot-planted onto goat blood agar plates after enrichment. Then, at least 6 isolates showed no hemolytic zones on goat blood agar plate after 48 h incubation. In comparison to other strains, strain fw8-1-2 exhibited a higher activity of autoaggregation and coaggregation with pathogens. Thus, the probiotic characteristic of *B. cereus* strain fw8-1-2 was investigated *in vitro*.

As shown in Fig. S1A, strain fw8-1-2 showed the major characteristics of large and milk-white colony with the smooth and moist surface. In Fig. S1B, no clear hemolytic zone was observed around bacterial colony of strain fw8-1-2 on goat blood agar plate after 48 h incubation. These results indicated that strain fw8-1-2 exhibited γ -hemolysis ability and belonged to non-hemolytic *Bacillus* strains. Then, strain fw8-1-2 was genotypically identified by 16S rRNA sequencing. The 16S rRNA sequence of strain fw8-1-2 showed a 96.95% identity to *B. cereus* strain C-LM-2 (OL913798.1) by using blastn analysis. As shown in Fig. S1C, the phylogenetic tree was constructed by using N-J method, which depicted its position among various *Bacillus* species.

3.2. Growth curve analysis of strain fw8-1-2

In this study, growth curves of strain fw8-1-2 were investigated after continuous cultivation with various commercial culture media. As shown in Fig. S2A-B, strain fw8-1-2 rapidly entered logarithmic growth phases after 6 h of cultivation in LB and BHI liquid medium, and then maintained the plateau phase after 30 h of cultivation. In contrast, strain fw8-1-2 began its logarithmic growth rate at 12 h of cultivation in BEP liquid medium and attained the highest OD₆₀₀ value after 42 h of cultivation, with which the growth rate and bacterial density were lower than those of LB and BHI liquid medium (Fig. S2C). These results indicated that LB and BHI medium were more suitable for growth and proliferation of strain fw8-1-2.

3.3. Tolerance to acid, bile salt and lysozyme

In general, acidic condition of stomach may ranges from pH 1.5 to 4.5, which can enable the food digestion within 3 h [22]. Thus, isolated probiotics should show a high tolerance against highly acidity. In this study, strain fw8-1-2 was investigated on its remarkable tolerance at various acidic condition and its growth at pH 6.5 was considered as control group. As shown in Fig. 1A, the maximum survival rates of *B. cereus* strain fw8-1-2 were approximately 84.97% and 95.46% at 12 h and 24 h after challenge exposure at pH 4.5, respectively. When pH of the broth dropped to pH 3.5 and pH 2.5, survival rates of strain fw8-1-2 were closed to 60% and 40% at 12 h and 24 h, respectively, suggesting that strain fw8-1-2 was found to endure the highly acidic condition with a high survival rate during long-term incubation.

As is well known, bile salt can injury bacterial cells and vandalize the structural coherence of cell membranes via dissolution of surface proteins [23]. In this study, strain fw8-1-2 was investigated on its bile tolerance following exposure to various concentration of bile salt. In Fig. 1B, high survival rates of strain fw8-1-2 were observed at 12 h after the exposure to 0.1% and 0.3% bile salt, while the survival rate decreased to 78.80% under 12 h-exposure to 0.5% bile salt. In contrast, survival rates of strain fw8-1-2 ranged from 49.01% to 60.34% at 24 h following challenge exposure to various concentration of bile salt.

Lysozyme is a group of widely distributed antimicrobial peptides in body surface, skin and gut tract of teleost fish, which may play a regulatory role in fish immunity and possess lytic activity against bacteria by

specifically cleaving glycosidic bonds [24]. Thus, tolerance to lysozyme should be considered as a potential criterion for probiotic selection. Current findings indicate that *B. coagulans* GBI-30 is a commercial probiotic strain isolated from canned evaporated milk could exhibit a 32.34% decreased survival rate under the exposure to 100 μ g/mL lysozyme. In Fig. 1C, high survival rates of strain fw8-1-2 were above 84.51% at 12 h and 24 h following the exposure to different concentration of lysozyme (50 μ g/mL, 100 μ g/mL and 200 μ g/mL). Our results implied that *B. cereus* strain fw8-1-2 should be a highly resistant bacteria that may show its probiotic function in complex condition of gut tract.

3.4. Assessment of cell surface hydrophobicity

Cell surface hydrophobicity is one of pivotal parameters in determination of bacterial adhesive capacity to epithelial cells. In general, various factors, such as cultivation time, composition of cultivation medium, solvent types and presence of some acids, may exhibit a great impact on cell surface hydrophobicity of isolated probiotics [25]. In this study, xylene and chloroform were selected as solvents for the cell surface hydrophobicity of strain fw8-1-2 during the continuous cultivation. As shown in Fig. 1D, the hydrophobicity of *B. cereus* strain fw8-1-2 with chloroform was about 14.30% after 24 h of cultivation, while it dramatically increased to 60.92% and 59.03% at 48 h and 72 h, respectively. In Fig. 1E, *B. cereus* strain fw8-1-2 showed a stronger hydrophobic capacity towards xylene with the highest value of 87.21% at 24 h of cultivation, followed by a sharp decrease at 48 h and 72 h, respectively. Previous studies reported that cell surface hydrophobicity of isolated *Bacillus* strains and *Lactobacillus* strains varied from 6.1% to 87.4% [26]. In addition, hydrophobicity values of potential probiotics should be close to 40%, which were indispensable for their probiotic characteristics [27]. Thus, taken together, these results suggested that strain fw8-1-2 should be an agreeable probiotic bacteria for further study.

3.5. Evaluation of autoaggregation and coaggregation

Isolated probiotics may require high levels of autoaggregation ability for strict attachment to gut epithelial cell and thereby encourage health benefits, which is widely considered as one of potential characteristics for the probiotic evaluation of its gut colonization [28]. The autoaggregation ability of isolated *L. rhamnosus* strains gradually increased from around 10% to above 50% within 24 h of cultivation, suggesting that probiotic autoaggregation may increase as incubation time elapsed [29]. In our findings, autoaggregation ability of *B. cereus* fw8-1-2 strain significantly increased at 12 h and then achieved the strongest autoaggregation ability of 34.49% after 48 h of incubation (Fig. 2A).

In this study, *A. hydrophila* strain L3-3 and *E. tarda* strain 11-4 were used as pathogenic bacteria in coaggregation assay. As shown in Fig. 2B, the coaggregation of *B. cereus* strain fw8-1-2 with *A. hydrophila* strain L3-3 increased dramatically at 24 h and peaked at 48 h with the highest coaggregation ability of 57.45%. In Fig. 2C, a gradual increased coaggregation of *B. cereus* strain fw8-1-2 with *E. tarda* strain 11-4 was observed and the highest coaggregation ability was 58.04% at 48 h of incubation. Previous studies indicated that coaggregation abilities of *Bacillus* strains with different strains of pathogenic bacteria may varied from 9% to 48% [30]. Our results suggested that *B. cereus* strain fw8-1-2 may be a promising probiotic bacterium with a exceptional aggregation property.

3.6. Analysis of strain fw8-1-2 biofilm formation

Biofilm formation is the growing process of microbe-derived sessile community embedded in a self-produced extracellular matrix and exhibit an altered phenotype with respect to bacterial growth and gene expression. In general, biofilm formation by probiotic bacteria is broadly

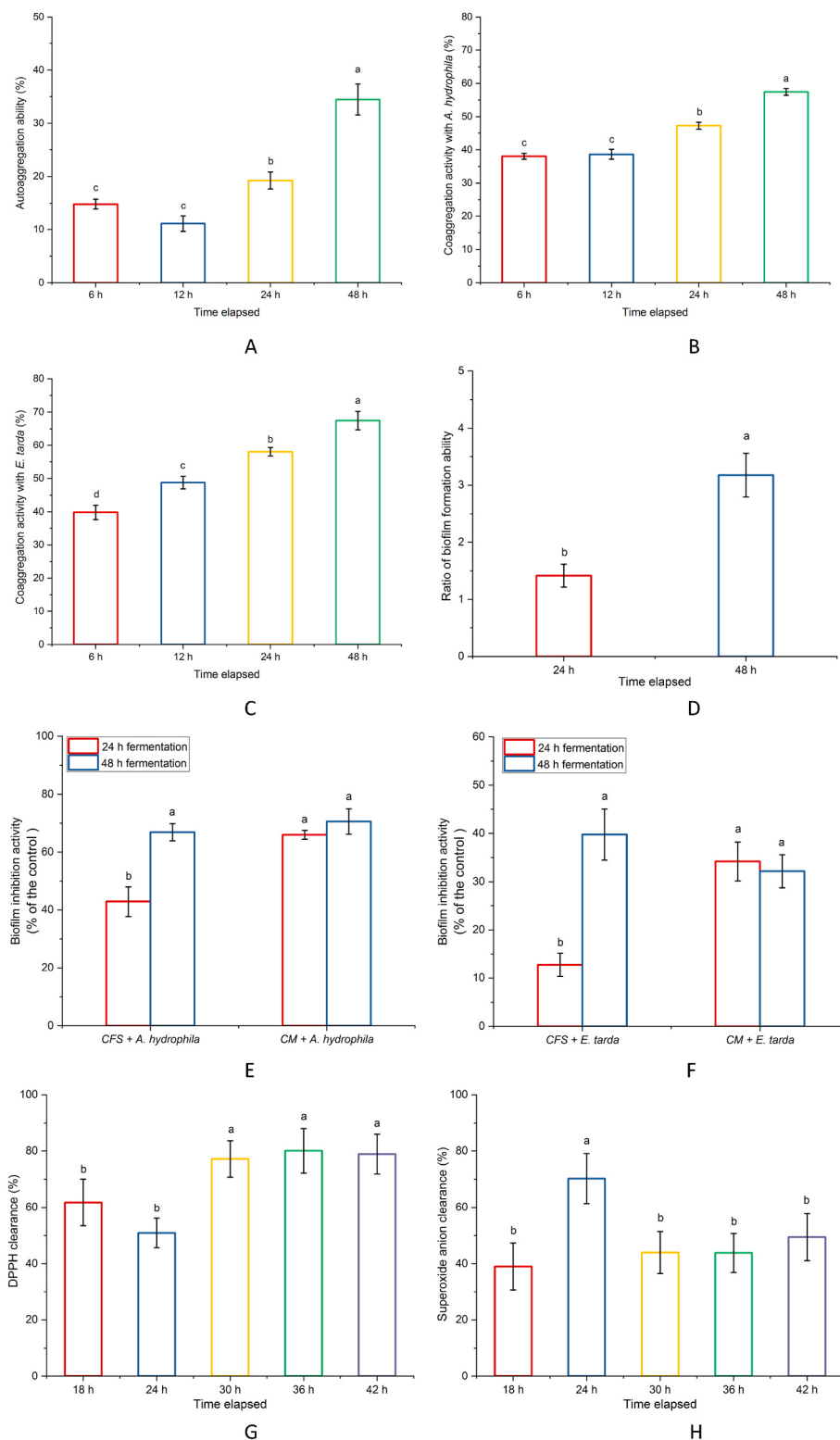


Fig. 2. Analysis of aggregation, Biofilm formation and Antioxidant capacity. (A) Autoaggregation ability of strain fkW8-1-2. (B–C) Coaggregation with *A. hydrophila* strain L3-3 and *E. tarda* strain 11-4. (D) Biofilm formation ratio of strain fkW8-1-2. (E–F) CM and CFS of strain fkW8-1-2 exhibit an inhibitory effect on the biofilm formation of *A. hydrophila* strain L3-3 and *E. tarda* strain 11-4. (G) DPPH clearance. (H) Superoxide anion clearance. The calculated data (mean $\pm$ SD) with different letters were significantly different ($P < 0.05$) among the groups. The experiments were performed in triplicate.

considered as a beneficial property that can enable its long-term permanence on the surface of gut epithelial cells within the host and then restrict gut colonization of invading pathogens [31]. Our findings suggested that the OD₅₇₀ absorbance of *B. cereus* strain fkW8-1-2 was

approximately 0.14 at 24 h and it significantly increased to 0.34 at 48 h. As shown in Fig. 2D, biofilm formation ratio of *B. cereus* strain fkW8-1-2 increased significantly and peaked at 48 h with a value of 3.18-fold increase by comparing with the control. Similarly, *B. subtilis* strain B-1

can form a robust floating biofilm and its OD₅₇₀ value may ranged from 0.1 to 0.3 [32]. These results implied that strain fw8-1-2 belonged to biofilm-positive strains.

3.7. Inhibitory effect of *B. cereus* strain fw8-1-2 on pathogenic biofilm formation

In this study, to investigate the inhibitory effect of *B. cereus* strain fw8-1-2 on pathogenic biofilm formation, CM and CFS were collected after continuous incubation. In Fig. 2E, 24 h-CFS of *B. cereus* strain fw8-1-2 could inhibit biofilm formation of *A. hydrophila* L3-3 by 42.88%, while the inhibitory ratio of 48 h-CFS achieved 66.86%. In addition, the inhibitory ratios of 24 h- and 48 h-CM on biofilm formation of *A. hydrophila* L3-3 maintained the high levels of 65.87% and 70.54%, respectively. In Fig. 2F and 24 h-CFS of *B. cereus* strain fw8-1-2 could reduce biofilm formation ability of *E. tarda* 11-4 by 12.76%, while the inhibitory ratio of 48 h-CFS attained 39.76%. In addition, biofilm formation ability of *E. tarda* 11-4 could be inhibited by 24 h- and 48 h-CM with the value of 34.19% and 32.18%, respectively. These results implied that *B. cereus* strain fw8-1-2 could possess the forming ability of robust biofilm and exhibit anti-biofilm effort against *A. hydrophila* and *E. tarda*.

3.8. Determination of antioxidant capacity

Redox homeostasis is playing a pivotal role in cellular determination of life and death events, but its aberration may cause oxidative stress and promote ROS accumulation [33]. In this study, percentages of DPPH removal and superoxide anion clearance were evaluated in strain fw8-1-2 during the stationary growth phase. As shown in Fig. 2G-H, the intact cells of strain fw8-1-2 exhibit the abilities of DPPH and superoxide anion removal with the highest values of 80.02% and 70.25%, respectively, which is much higher than that of *E. faecium* strain MC-5 [34]. These results indicated that strain fw8-1-2 exhibit a strong antioxidant capacity.

4. Conclusion

In summary, we characterized the probiotic feature of strain fw8-1-2 isolated from intestine of WCC. Strain fw8-1-2 showed high levels of acid, bile salt and lysozyme tolerance and displayed an increase of cell surface hydrophobicity towards chloroform and xylene. Strain fw8-1-2 displayed a gradual increase of cell surface hydrophobicity with chloroform or xylene. Strain fw8-1-2 showed the remarkable autoaggregation activity, dramatically coaggregated with pathogenic bacteria as well as attenuated pathogenic biofilm formation. In addition, Strain fw8-1-2 exhibited a strong antioxidant activity for DPPH clearance and superoxide anion clearance. Our results suggested that the strain fw8-1-2 may act as potential probiotics for aquaculture. Future studies on *in vivo* application are required to prove its effectiveness on immune regulation and growth rate of farmed fish.

Ethics approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. We followed the laboratory animal guideline for the ethical review of the animal welfare of China (GB/T 35892–2018).

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Consent to participate

The authors of this manuscript are all aware of the journal to which the manuscript was submitted, and all agree to continue to support the follow-up work.

Consent to publish

Not applicable.

Conflict of interest statement

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Xu-Ying Kuang: Validation, Methodology, Investigation, Formal analysis, Data curation. **Zi-Xuan Fang:** Methodology, Investigation, Formal analysis, Data curation. **Ning-Xia Xiong:** Supervision, Conceptualization. **Jie Ou:** Supervision. **Fei Wang:** Supervision. **Sheng-Wei Luo:** Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.repbre.2024.03.003>.

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