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Probiotic spore-based antigen delivery: a novel oral vaccine strategy against *Vibrio* infections in aquaculture

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Abstract

Background Vibriosis is a deadly illness caused by various species of the *Vibrio* genus. Due to its high incidence in aquaculture plants, vibriosis is responsible for significant economic losses. Currently, anti-vibriosis treatments rely on antibiotics. However, the global rise in antibiotic resistance necessitates the development of alternative approaches. Novel vaccines and effective probiotics have been proposed as potential alternative to antibiotics in fighting bacterial infections. Here we propose a combined vaccine/probiotic strategy based on the use of probiotic bacterial spores for the oral delivery of *Vibrio* antigens. Spores of various species of the *Bacillus* genus are widely used as probiotics and have been shown to efficiently display antigens in a non-recombinant way.

Results Spores of various probiotic strains were analyzed to assess their effectiveness in displaying a heterologous model protein, and *B. megaterium* MV30 was identified as the most efficient strain. MV30 spores were then used to display two antigens of *Vibrio harveyi*, the entire Hsp33 protein of 33 kDa and a 239 amino acids fragment of OmpK (OmpK$^{21/260}$), identified as the most immunogenic part of the protein. While Hsp33 is a stable protein, OmpK$^{21/260}$ is unstable at conditions mimicking those encountered in an aquaculture plant and the interaction with MV30 spores reduced such instability. The protective ability of the combined probiotic/vaccination strategy was assayed on Medaka fish (*Oryzias latipes*), as a model. In a challenge experiment with a virulent strain of *Vibrio harveyi*, a protective effect was observed with MV30 spores alone and such effect was significantly increased when the same spores displayed either one of the two antigens.

Conclusion Our results support the use of probiotics and oral vaccines as a valid alternative to antibiotics and point to the application of probiotic spore-based antigen delivery as a novel strategy to fight pathogenic infections.

Keywords Mucosal vaccine, Mucosal adjuvant, *Bacillus*, Gut, Delivery system

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Introduction

Vibriosis is an infection caused by non-cholera *Vibrio* species, such as *V. harveyi*, *V. vulnificus*, *V. parahaemolyticus*, *V. mimicus*, *V. anguillarum*, and *V. alginolyticus* [1, 2]. Such infections are harmful to several aquatic organisms in marine or freshwater environments and can cause severe and life-threatening gastroenteritis in humans exposed to those habitats or consuming raw or undercooked infected seafoods [1, 2]. The potential epidemic/pandemic risk associated with the transmission of these infections to humans has significantly increased over the past few decades due to climate warming resulting in the expansion of *Vibrio* species into geographical regions previously considered inhospitable for these microorganisms [3, 4]. An additional factor that has increased the disease risk associated with *Vibrio* species is the global consumption of seafood, which has grown at an average rate of 3% per year in the last 60 years [4]. *Vibrio* species are also harmful for most farmed aquatic animals such as fishes, shrimps and shellfishes, making vibriosis one of the primary concerns of aquaculture plants and the cause of serious economic losses [5]. It is recognized that a critical phase in aquaculture is larval rearing, the initial developmental stage of aquatic organisms. Low larvae survival rate is common for many species and among the different possible causes, vibriosis and alterations in larval microbiota play significant roles [5]. Current therapies against vibriosis rely on antibiotics [6]. However, due to the increase of antibiotic-resistant bacterial populations in nature, these treatments are expected to become ineffective in the near future [6]. Moreover, antibiotics cause alterations in the animal microbiota which in turn are believed to cause a reduction of the efficiency of larval rearing [5]. In this context, the use of vaccines and probiotics has been proposed as possible alternatives to antibiotics to fight bacterial infections [7–10]. However, the use of most vaccines has been, so far, limited in aquaculture plants due to either their administration route or their low efficacy. Indeed, while the highly immunogenic intraperitoneal route requires that each individual animal is injected, an expensive and stressful procedure that affects fish growth performances [2, 5], oral vaccines are poorly immunogenic, mainly due to the lack of appropriate adjuvants and delivery systems [9, 10]. On the other end, the efficacy of probiotics to fight pathogens in aquaculture plants is still under evaluation, although it is commonly accepted that the oral administration of live bacteria stimulates the immune system [11] and reduces pathogen infections [12].

This study explored a novel anti-infection strategy based on the use of probiotics as mucosal vaccine delivery vehicles. The strategy combines the health beneficial effects of probiotics with the induction of an antigen-specific immune response. In a murine model, a commercial probiotic, based on *Bacillus cereus* spores, increased the immune response to an orally administered anti-tetanus vaccine [13]. Here, probiotic bacterial spores were used to deliver and immunize animals.

The use of bacterial spores to display antigens has long been proposed [14] and extensively reviewed [15–17]. Spore-based vaccines for marine organisms, such as *Cambarus clarkii* [18], *Litopenaeus vannamei* [19, 20], *Penaeus monodon* [21], *Danio rerio* and *Dicentrarchus labrax* [9] have also been proposed. In all these cases, spores were genetically modified to display on their surface the selected antigen, an approach that poses safety concerns because of the use and environmental release of recombinant spores. An alternative non-recombinant spore display system, based on the tight adhesion of a heterologous antigen to the spore surface by a combination of electrostatic and hydrophobic interactions, has been proposed and tested in murine models [17, 22].

In the present study, spores of *Bacillus megaterium* (recently renamed as *Priesta megaterium*) MV30 [23] were selected to display in a non-recombinant way two previously identified antigens of *V. harveyi* [2, 9, 24]. The model Medaka fish (*Oryzias latipes*) was used to evaluate the efficacy of the proposed vaccination/probiotic strategy.

Results

Probiotic MV30 spores efficiently adsorb mRFP

A screening of spore forming bacteria isolated from intestinal samples allowed the selection of four strains with in vitro probiotic potentials belonging to the *Bacillus subtilis* (MV24), *B. velenzensis* (MV4 and MV11) and *B. megaterium* (MV30) species [23]. Here, these strains were analyzed for their efficiency in adsorbing a model heterologous protein, the monomeric Red Fluorescent Protein (mRFP), previously used for spore adsorption [25, 26]. The same number of purified spores (1.0×10^9) of each strain was reacted with 2 µg of purified mRFP and analyzed by fluorescence microscopy. All four strains adsorbed mRFP more efficiently than spores of the PY79 strain, a laboratory collection strain of *B. subtilis* previously used for spore display experiments [25, 26] (Fig. 1A). A quantitative analysis of the obtained auto fluorescence signals was performed by measuring the total corrected cellular fluorescence (TCCF). Considering the TCCF as proportional to the quantity of protein on the spore surface [25], the data confirmed that all strains were more efficient than PY79 and indicated spores of MV24 and MV30 strains as those with the highest binding efficiency ($p < 0.0001$) (Fig. 1B). Spores of *B. megaterium* MV30 were selected and used for all further experiments.

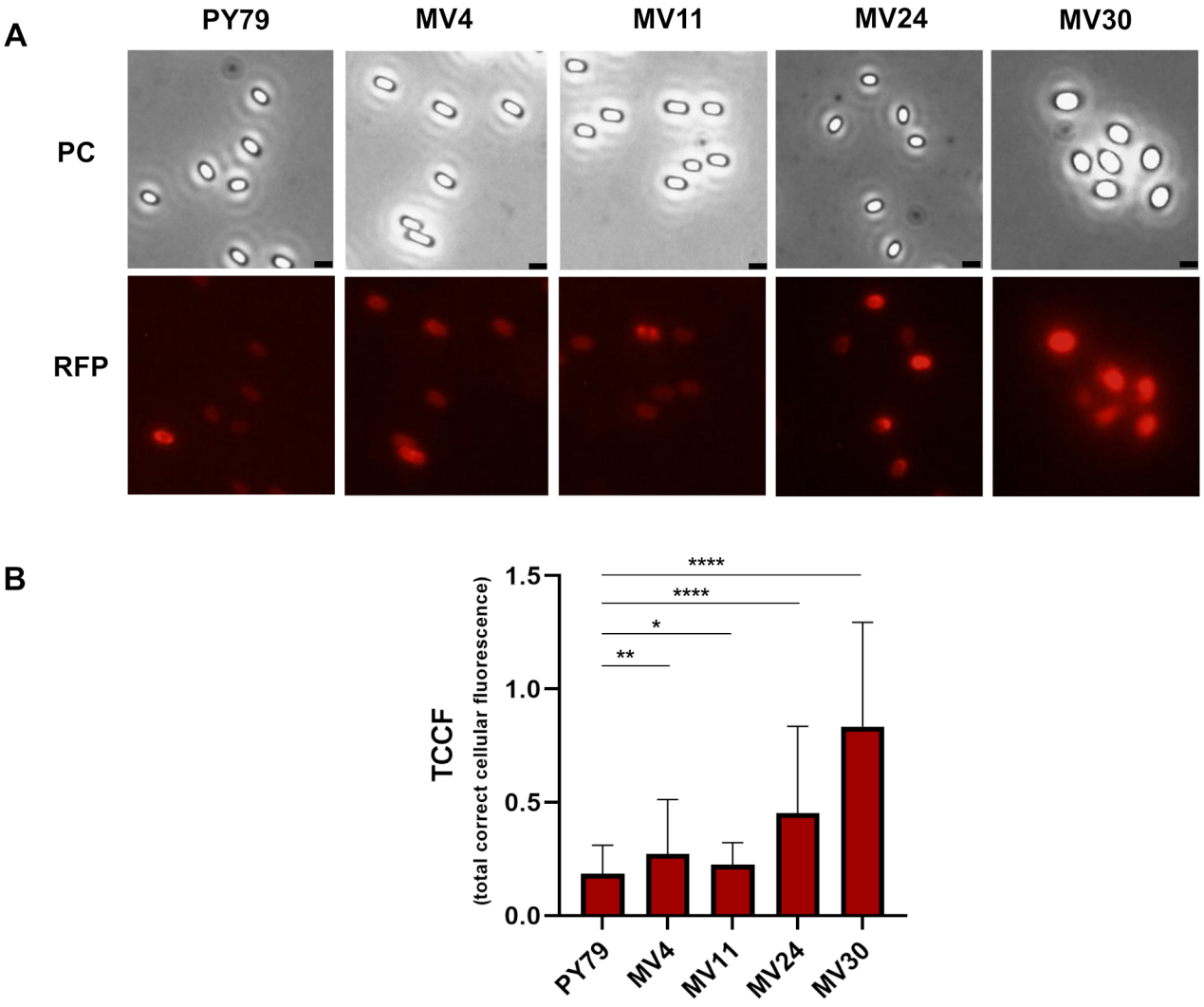

Fig. 1 Efficiency of mRFP adsorption by spores of four probiotic strains. **(A)** Fluorescence microscopy analysis of spores adsorbed with 2 µg of mRFP in sodium citrate buffer at pH 3.5. Phase Contrast (PC) and fluorescence (RFP) images of spores of PY79, MV4, MV11, MV24 and MV30 strains are shown. Exposure time 500 ms, Scale bar 1 µm. **(B)** Quantitative analysis of fluorescence of 80 spores for each strain by ImageJ software (Metods). The X- axis indicates the analyzed strains, the Y-axis shows the total corrected cellular fluorescent (TCCF) value. Dunnett's multiple comparisons test was used for statistical significance, ****$P < 0.0001$

Selection of *V. harveyi* antigens

The outer membrane protein OmpK and the heat-shock protein Hsp33 have been previously proposed as potentially effective antigens in controlling pathogenic species of the *Vibro* genus, such as *V. harveyi*, *V. parahaemolyticus*, *V. anguillarum*, *V. alginolyticus* [2, 9, 24]. Genes coding for these two antigens were identified in the genome of *V. harveyi* 1173, a virulent strain isolated from *Solea solea* with splenomegaly (Methods) and characterized by an Average Nucleotide Identity (ANI) value of 98.73% when compared with the reference strain *V. harveyi* ATCC 33,843.

Genes coding for the two antigens (*ompK* and *hsp33*), were analyzed to identify both linear and conformational epitopes and predict their immunogenic regions by using the Antibody Epitope Prediction software BepiPred-3.0 (https://services.healthtech.dtu.dk/services/BepiPred-3.0 /) [27]. The in silico prediction indicated that the antigenic epitopes were distributed throughout the sequence of Hsp33 (Suppl. Mat. Fig. S1A) and located between positions + 21 and + 260 for OmpK (Suppl. Mat. Fig. S1B). The biochemical properties of Hsp33 and $OmpK^{21/260}$ were predicted in silico (Suppl. Mat Table S1) and showed high homology with genes present in *Vibrio* species other than *V. harveyi*, suggesting that could induce protection against various *Vibrio* pathogenic species (Suppl. Mat Table S2). Therefore, the entire Hsp33 and the region of OmpK between positions 21 and 260 ($OmpK^{21/260}$) were considered as antigens.

Adsorption of Hsp33 and OmpK$^{21/260}$ on MV30 spores

DNA fragments coding for Hsp33 and OmpK$^{21/260}$ were amplified using *V. harveyi* 1173 chromosomal DNA as a template and oligonucleotide pairs Omp ompK_F/ompK_R and hsp33_F/hsp33_R (Suppl. Mat Table S3) to prime the amplification reactions. Amplified DNA fragments were cloned in frame with a poly-His tag, overexpressed in *Escherichia coli* BL21(DE3-Codon plus RIL) and the expression products purified by affinity chromatography (Methods). The purified proteins were recognized by anti-6xHis antibody in western blotting experiments (Suppl. Mat. Fig. S2).

Pure Hsp33 and OmpK$^{21/260}$ were then independently used for adsorption on purified spores of strain MV30. To set up the adsorption conditions, the reaction was performed using 2 µg of purified antigens and 1.0×10^9 purified spores at pH 3.5 in different buffer conditions. The adsorption reactions were fractionated by centrifugation. The pellet fraction, containing the spore-adsorbed antigens, was used to extract the spore surface proteins that were then analyzed by western blotting, while the supernatant fraction, containing the unbound antigens, was serially diluted and quantified by dot blotting. For Hps33 the strong signal observed by western blotting (Suppl. Mat. Fig. S3A), together with the absence of signal observed by dot blotting (Suppl. Mat. Fig. S3B), clearly indicated the acetate buffer (NaAc) as the best adsorption condition. OmpK$^{21/260}$ was similarly adsorbed in all tested buffers (not shown). When 5 and 10 µg of purified OmpK$^{21/260}$ were used, the adsorption was similarly efficient in acetate (NaAc) and citrate (NaCit) buffers and less efficient in phosphate buffer (PBS) (Suppl. Mat. Fig. S3C-H). Based on these results all further reactions of both antigens with MV30 spores were performed with sodium acetate buffer at pH 3.5.

To define the maximal quantity of antigen adsorbed by 1.0×10^9 spores of MV30, up to 20 µg of each antigen was independently reacted with spores and the adsorbed amount quantified by dot blotting. As shown in Fig. 2, most of the reacted antigens remained adsorbed on the spore surface and only a limited amount of each antigen was left unbound in the supernatant fractions. The densitometric analysis of the signals derived from the dot blottings of Fig. 2 indicated that when 20 µg of each antigen were reacted less than 3% of Hsp33 and less than 1% of OmpK$^{21/260}$ remained unbound in the supernatant fractions (Suppl. Mat. Tables S4 and S5).

Figure 2. Adsorption of Hsp33 or OmpK$^{21/260}$ on MV30 spores. Western Blot analysis of MV30 spore surface proteins extracted after adsorption with 15 µg (lanes 1) or 20 µg (lanes 2) of Hsp33 (A) or OmpK$^{21/260}$ (B). Dot blot analysis performed with the serial dilutions of the supernatant fraction of the adsorption reaction with 15 µg (lanes 1) or 20 µg (lanes 2) of Hsp33 (C) or OmpK$^{21/260}$ (D). ST = pure Hsp33 (C) or OmpK$^{21/260}$ (D). Immune reactions were performed using anti-6xHis primary antibody and peroxidase conjugated secondary antibody.

OmpK$^{21/260}$ and Hsp33 are efficiently and homogeneously adsorbed on MV30 spores

A flow cytometry analysis was performed to assess whether the adsorption of the antigen was uniform on the MV30 spore population. After the adsorption with 20 µg of Hsp33 or OmpK$^{21/260}$, the reaction was fractionated by centrifugation and the pellet fraction fixed with formaldehyde. Fixed samples were then reacted with anti-6xHis primary antibody and fluorescent-conjugated secondary antibody (Methods) and run on a flow cytometer (Methods). As shown in Fig. 3, the vast majority of the reacted spores carried the adsorbed antigens (over 84% and over 89% of the spores reacted with Hsp33 or OmpK$^{21/260}$, respectively), indicating a homogeneous distribution of the antigens in the spore population.

Adsorption on MV30 spores increases the stability of OmpK$^{21/260}$

The interaction with the spore has been previously shown to stabilize and protect the adsorbed molecules against high temperatures and low pH conditions [28]. To verify whether Hsp33 and OmpK$^{21/260}$ were stabilized when adsorbed on MV30 spores, the same amount of free and spore-adsorbed proteins was incubated in water, a condition mimicking that encountered in aquaculture plants. After various times of incubation, proteins were extracted from spores and compared with free proteins treated in parallel. Proteins were analyzed by western blotting and signals used for a densitometric analysis. While Hsp33 appeared stable at the tested condition and no differences were observed between free and spore-adsorbed Hsp33 (not shown), over 60% of OmpK$^{21/260}$ was degraded after 2 h of incubation in water and a total degradation was observed after 6 h (Fig. 4). When adsorbed on spores OmpK$^{21/260}$ was significantly more stable than the free antigen and over 60% of the spore-adsorbed OmpK$^{21/260}$ was still extractable from spores after 6 h of incubation (Fig. 4, Suppl. Mat. Tables S6).

Immunization of *Oryzias latipes* and infection with *V. harveyi* 1173

The efficacy of probiotic spores, antigens and spore-adsorbed antigens was tested in *vivo* using larvae of Medaka fish (*Oryzias latipes*) [29] as a model. As schematically reported in Fig. 5, Medaka larvae were randomly split into seven groups ($n > 30$). Five groups were treated with either spores (1.0×10^9), OmpK$^{21/260}$ (20 µg), Hsp33 (20 µg), spore-adsorbed OmpK$^{21/260}$ (1.0×10^9 spores loaded with 20 µg), or spore-adsorbed Hsp33 (1.0×10^9 spores loaded with 20 µg). Concordantly with

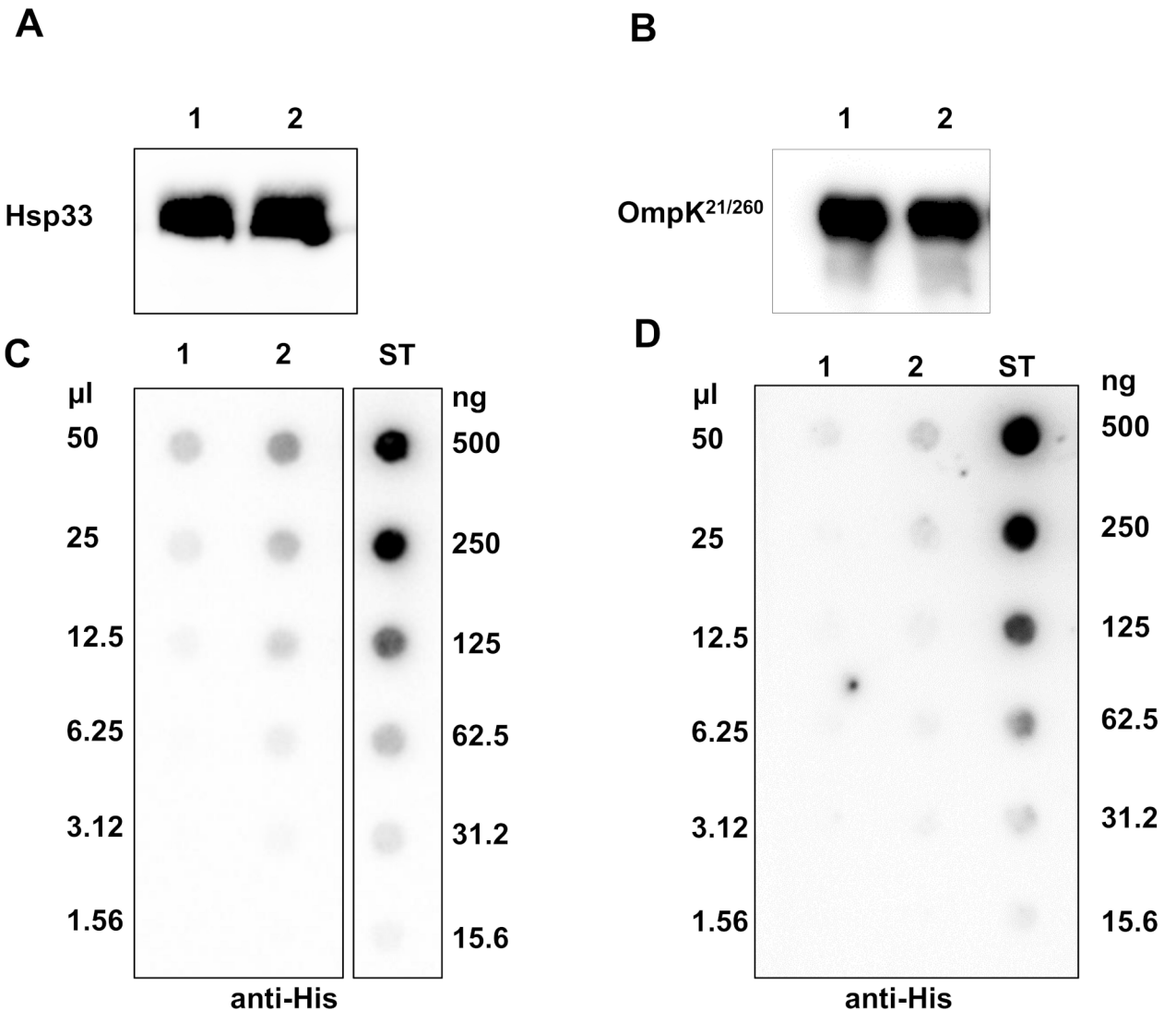

Fig. 2 Adsorption of Hsp33 or OmpK$^{21/260}$ on MV30 spores. Western Blot analysis of MV30 spore surface proteins extracted after adsorption with 15 µg (lanes 1) or 20 µg (lanes 2) of Hsp33 **(A)** or OmpK$^{21/260}$**(B)**. Dot blot analysis performed with the serial dilutions of the supernatant fraction of the adsorption reaction with 15 µg (lanes 1) or 20 µg (lanes 2) of Hsp33 **(C)** or OmpK$^{21/260}$**(D)**. ST = pure Hsp33 **(C)** or OmpK$^{21/260}$ (D). Immune reactions were performed using anti-6xHis primary antibody and peroxidase conjugated secondary antibody

the previously assessed adsorption efficiency, the amount of spore-adsorbed antigen was similar to the amount of free antigen administered to the larvae. After the treatment, larvae of the five groups and of one of the two not treated groups were challenged with the pathogenic strain *V. harveyi* 1173 (Fig. 5).

In the used experimental conditions, the infection with the pathogen caused a statistically significant (Control vs. *Vibriop* = 0.0000312, Table 1) mortality of the larvae with less than 40% of survivals (Fig. 6). The treatment with the spores alone or the free antigens (yellow and orange lines respectively in Fig. 6) caused an increase of the survival percentage up to 50–60% even if these variations were not statistically significant. Instead, a strong statistically significant protection (Spores + OmpK$^{21/260}$ vs. *Vibriop* = 0.0045 and Spores + Hsp33 vs. *Vibriop* = 0.0098, Table 1) was observed when the larvae were treated with both spore-adsorbed antigens (green lines), showing over 80% of larvae survival in both cases (Fig. 6). Altogether these data support the use of probiotic spore-based antigen delivery as a novel strategy to protect fish from pathogenic infections.

Discussion

The main conclusions of this work are twofold. A first relevant conclusion is that spores of the probiotic *B. megaterium* strain MV30 efficiently adsorbed both selected antigens, Hsp33 and OmpK$^{21/260}$. When 20 µg of purified

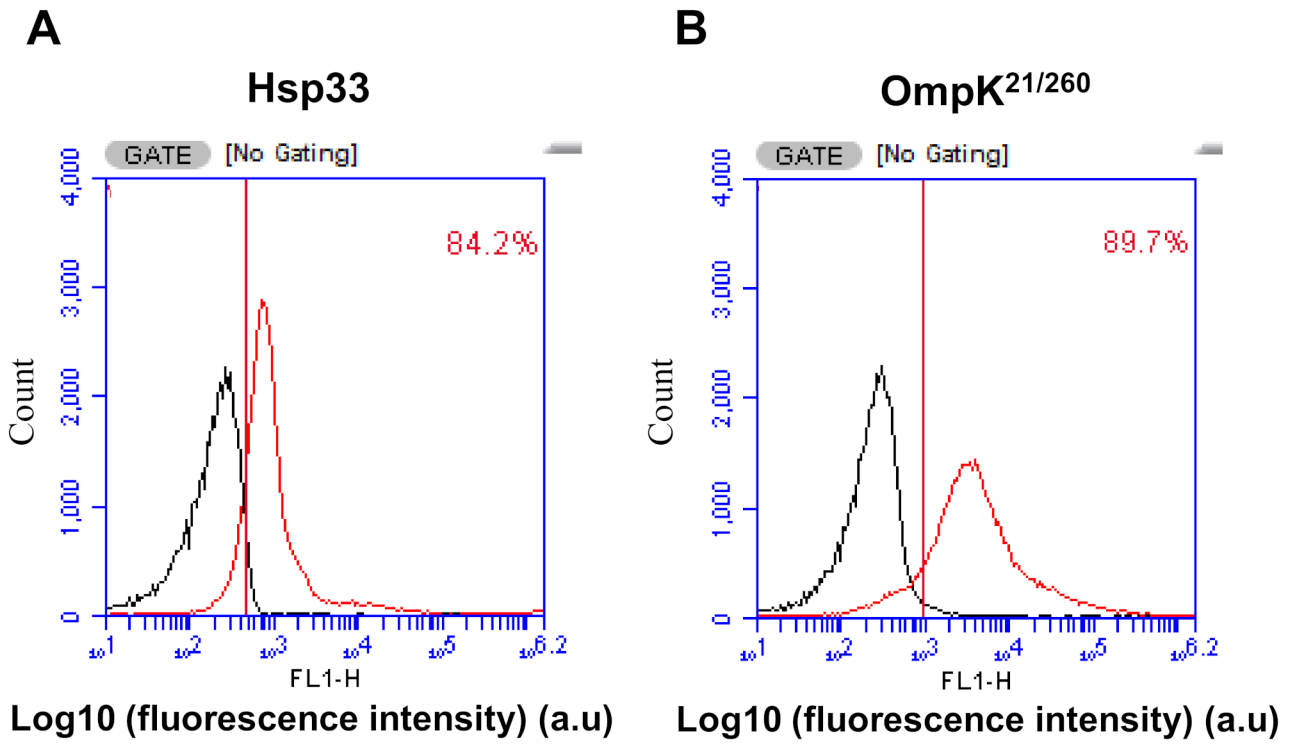

Fig. 3 Flow cytometry analysis of MV30 spores adsorbed with Hsp33 or OmpK$^{21/260}$. 50.000 spores were analyzed for each sample. The percentage of MV30 spores that adsorbed Hsp33 or OmpK $^{21/260}$ is indicated. Red lines indicate the fluorescence signals of MV30 spores adsorbed with Hsp33 **(A)** or OmpK$^{21/260}$**(B)** and reacted with anti-6xHis primary antibody and fluorescently conjugated secondary antibody. Black curves indicate the background fluorescence signals of MV30 spores not adsorbed with any of the antigens. The red line represents the threshold level based on the negative control

Hsp33 or OmpK$^{21/260}$ were reacted with 1.0×10^9 spores, over 95% of each antigen was tightly attached to the spores. This is a relevant point since previous studies on spore-based immunization strategies have been mainly performed with laboratory strains of *Bacillus*. The possibility of using probiotic strains as antigen delivery vehicles could increase the efficiency of the immunization both for the probiotic effects of the used spores, as discussed below, and also for a probiotic-induced increase of the immune response [13].

A second relevant conclusion of this work is that the oral immunization of larvae of Medaka fish with spore-adsorbed antigens induced a strong protection. Over 80% of the immunized larvae survived the challenge with the pathogenic strain of *V. harveyi*, while under the same experimental conditions, only less than 40% of the not-immunized larvae survived. When larvae were immunized with free antigens only a limited and not statistically significant protection was observed, thus pointing to the spore-based immunization as a valuable antigen delivery strategy. Interestingly, immunization with spore-adsorbed OmpK$^{21/260}$ appeared more protective than that with spore-adsorbed Hsp33. Indeed, only when larvae were immunized with spore-adsorbed OmpK$^{21/260}$ the percentage of survival was not statistically different ($p > 0.05$) with respect to that observed with not challenged larvae.

Bacterial spores have been previously proposed as vehicles to immunize marine organisms [18–21]. However, in all these previous studies recombinant spores carrying an antigen genetically fused to a spore surface protein were used. These genetically modified spores raise safety concerns and have, therefore, limited the applications of the spore-based vaccination strategy. In this study the selected antigens were displayed in a non-recombinant way (spore-adsorption [22]) on probiotic spores. The proposed vaccine, therefore, does not pose any safety concern and makes this non-recombinant spore vaccination strategy ready for field tests in aquaculture plants.

At conditions mimicking those encountered during the challenge experiment Hsp33 remained stable while OmpK$^{21/260}$ was relatively unstable and was stabilized by the interaction with the spores (Fig. 4). Although the stabilization of the carried antigen is certainly a relevant effect of the interaction with the spores, it does not seem the major determinant of the different survival percentages observed between free and spore-adsorbed antigens in the challenge experiments. Indeed, both the stable (Hsp33) and unstable (OmpK$^{21/260}$) antigens showed a

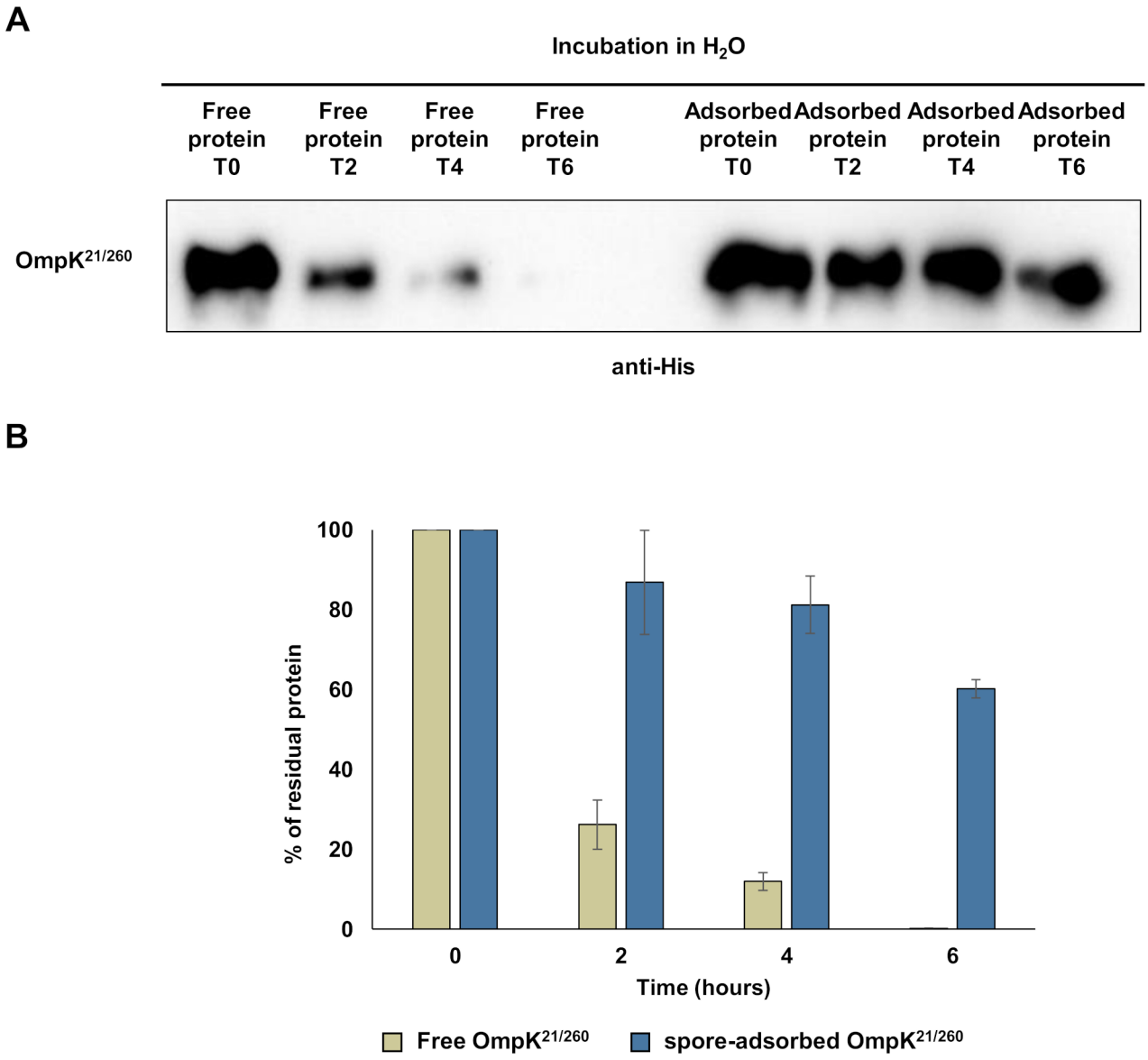

Fig. 4 Stability of free and spore-adsorbed OmpK$^{+21/+260}$. **(A)** Western Blot analysis of free OmpK$^{+21/+260}$ and of spore surface protein extracted after adsorption of purified OmpK$^{+21/+260}$ incubated in H_2O in water for various times (from 0 to 6 h). **(B)** Densitometric analysis of the western blots

modest and not statistically significant protection against the pathogen.

The treatment of Medaka larvae with probiotic spores not displaying any antigen also increased the survival percentage against the pathogen, although the protective effect was not statistically significant (Fig. 6). Although the effect was limited, these results suggest that probiotic spores partially reduce the virulence of *V. harveyi* 1173. Since no signs of spore germination were observed during the challenge experiments, the reduced virulence could be due to either a competitive exclusion effect of the spores against the pathogen or to an antigen-independent immune response. This aspect, together with the characterization of the immune response induced by the spore-adsorbed antigens will be essential future research goals. Altogether, results reported in this study point to the use of probiotic spores delivering antigens in a non-recombinant way as an effective strategy to fight infectious diseases in aquaculture plants.

Methods

Bacterial strains and molecular procedures

Bacterial strains used in this work are listened in Suppl. Mat. Table S7. *E. coli* strains DH5α and BL21 (DE3-Codon plus RIL) (Invitrogen, Agawam, MA, USA) were used for cloning and overexpression of OmpK$^{21/260}$ and Hsp33, respectively. *E. coli* strains were cultured in Luria-Bertani (LB) broth (for 1 L: 10 g Bacto-Tryptone, 5 g

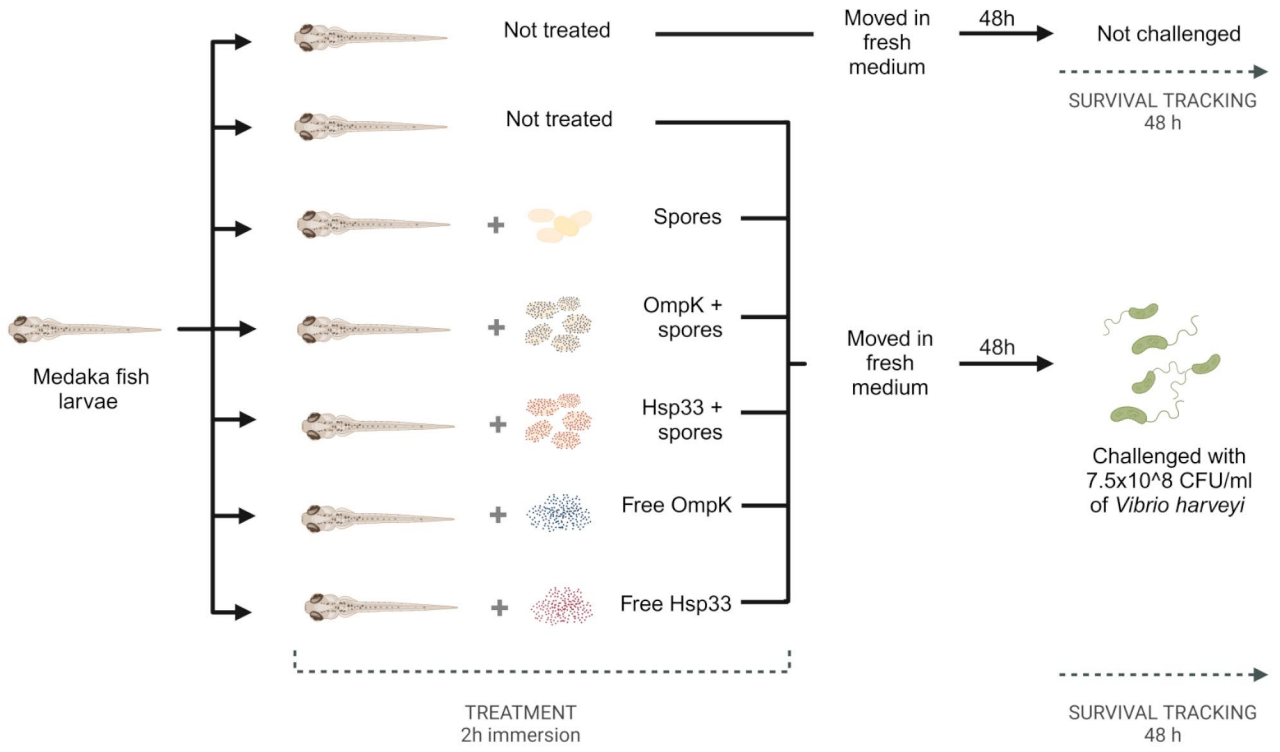

Fig. 5 Design of the challenge experiment. Medaka fish larvae were splitted into 7 groups ($n > 30$). Larvae of five groups were treated (immersion for 2 h) with either spores, free Hsp33, free OmpK$^{21/260}$, spore adsorbed Hsp33 or spore adsorbed OmpK$^{21/260}$. After 48 h of incubation in fresh media, the five treated groups and one of the two not treated were challenged with the pathogen and the survival rate monitored for additional 48 h

Table 1 Statistical analysis of the challenge experiments

Comparison	*p*-value	
Vibrio vs. Control	0.0000312	****
Vibrio vs. Spores	0.170	ns
*Vibrio*vs. OmpK21/260	0.170	ns
Vibrio vs. Hsp33	0.580	ns
Vibrio vs. Spores + OmpK$^{21/260}$	0.00145	**
Vibrio vs. Spores + Hsp33	0.00980	**

Bacto-yeast extract, 10 g NaCl, pH 7.0) and were transformed by previously described procedures [30]. DNA ligation, isolation of plasmids, and restriction digestions were performed by using standard methods [30].

V. harveyi 1173 is a pathogenic strain isolated from *Solea solea* with splenomegaly at the Experimental Zooprophylactic Institute (Naples, Italy). *V. harveji* was cultured in LB medium supplemented with NaCl 2% at 25 °C overnight with vigorous shaking. Chromosomal DNA extraction was performed as previously described [31] with an additional pre-treatment step involving homogenization using FAST-PREP FP120 (Qbiogene, Inc). The strains *B. subtilis* PY79, *B. velezensis* MV4 and MV11, *B. subtilis* MV24 and *B. megaterium* MV30 were cultured in LB medium at 37 °C.

Spore production and purification

Spores of probiotic strains were prepared by the exhaustion method [32] using Difco Sporulation Medium (DSM) (for 1 L: 8 g Nutrient Broth, 1 g KCl, 1 mM $MgSO_4$, 1 mM $Ca(NO_3)_2$, 10 µM $MnCl_2$, 1 µM $FeSO_4$, Sigma-Aldrich, Germany) and incubated at 37 °C for 30 h. Spores were analyzed under the light microscope, collected by centrifugation, washed four times with distilled water, and purified by water-washing using overnight incubation in H_2O at 4 °C to lyse residual sporangial cells [32]. Spores were considered pure when less than 10% of vegetative cells were observed under the light microscope. Pure spores were quantified at the light microscope using a Burker chamber (Sigma-Aldrich) and stored at -20 °C.

Whole-genome sequencing and in silico analysis of *V. harveyi* 1173

Whole-Genome sequencing of *V. harveyi* 1173 was conducted by GenProBio srl (Parma, Italy) using Illumina MiSeq Sequencing System. The genome assembly was performed by using SPAdes v3.14.0 via the MEGAnnotator pipeline [33].

The genome has been deposited in GenBank as BioProject PRJNA1230127 (accession number JBNAZB000000000). Amino acid sequences of OmpK$^{21/260}$ and Hsp33 were obtained from the annotated

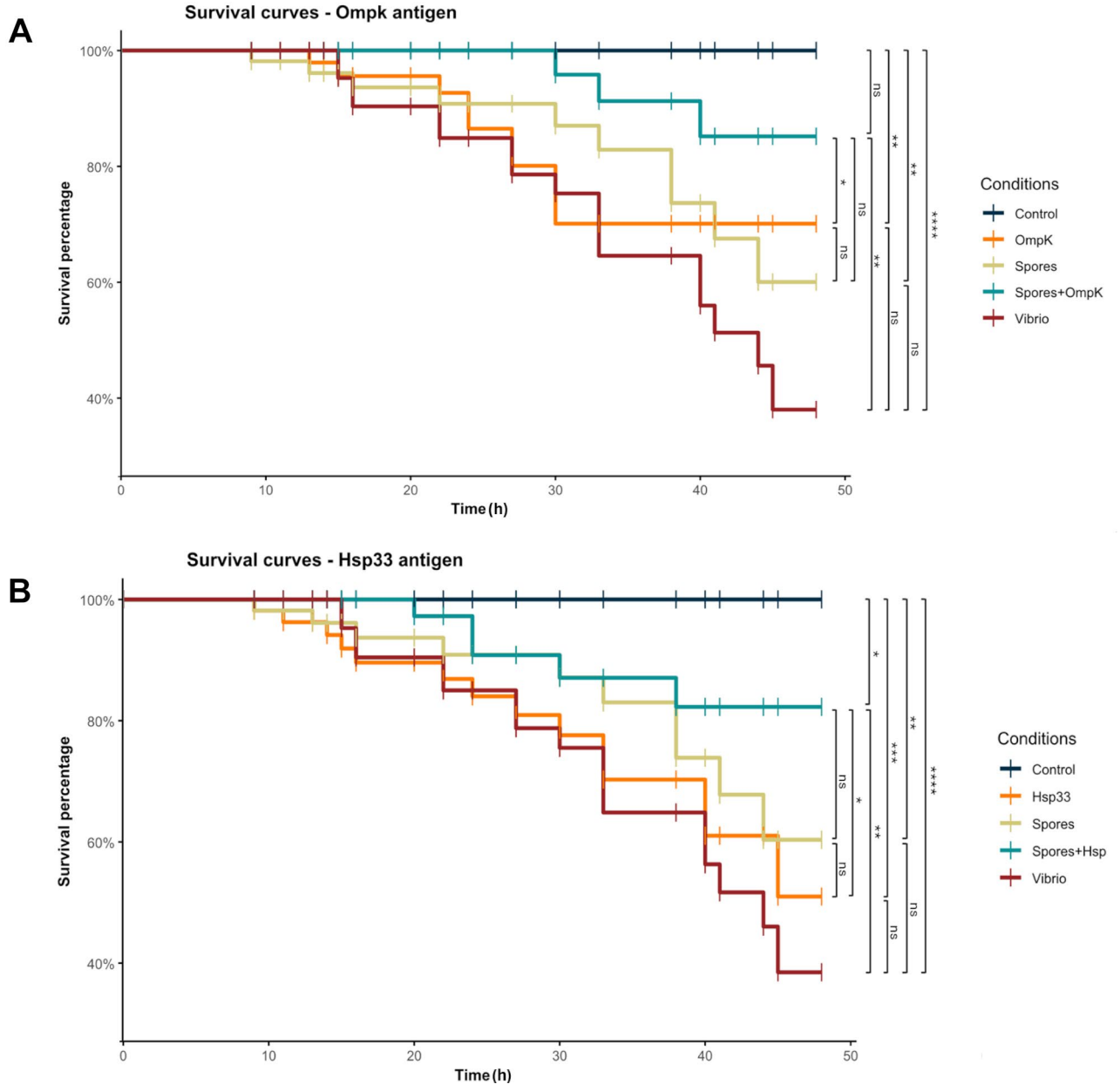

Fig. 6 Kaplan-Meier survival curves of Medaka fish larvae challenged with *Vibrio harveyi* 1173, 48 h post-vaccination. Larvae were vaccinated with spores from MV30 alone (Spores), spores displaying the OmpK$^{21/260}$ (Spores + OmpK) or free OmpK$^{21/260}$ (OmpK) **(A)** or spores displaying the Hsp33 (Spores + Hsp33), or free Hsp33 (Hsp33) **(B)**. Non-vaccinated larvae exposed to the pathogen (Vibrio) and non-challenged larvae (Control) served as positive and negative control groups, respectively. Significant differences between groups were assessed using the log-rank test as indicated with asterisks: * = $p < 0.05$; ** = $p < 0.005$; *** = $p < 0.0005$; ****= $p < 0.00005$. ns = not statistically significant

genome sequence of *Vibrio harveyi* 1173. Biochemical properties (GRAVY value, protein length, molecular weight kDa, isoelectric point) of both proteins were obtained by using protparam software (https://web.expasy.org/protparam/). Conformational and linear epitope predictions were evaluated by using the Antibody Epitope Prediction software BepiPred-3.0 (https://services.healthtech.dtu.dk/services/BepiPred-3.0/) with default settings. Multiple sequence alignment between OmpK$^{21/260}$ and Hsp33 from different species were performed using ClustalW (https://www.ebi.ac.uk/Tools/msa/clustalo/) with default settings.

Protein over-production and purification

For the over-expression of OmpK$^{21/260}$ and Hsp33 the respective coding sequences were amplified using *V. harveyi* 1173 chromosomal DNA as a template and specific oligonucleotides pairs: OmpK_F and OmpK_R

or Hsp33_F and Hsp33_R (Suppl. Mat. Table S3) to prime the reactions. Two amplified DNA of 720 bp (for *ompK*$^{21/260}$) and 876 bp (for *hsp33*) were cloned into the pGEMT-easy vector (Promega, Madison, Wisconsin, USA) obtaining pGEM-*ompK* and pGEM-*hsp33* respectively. The pGEM-*ompK* vector was digested using *Bam*HI/*Hind*III restriction enzymes (inserted with the amplification primers), whereas pGEM-*hsp33 w*as digested with *Nco*I/*Hind*III (*Nco*I site contained in the polylinker while *Hind*III contained in the amplification primer). These digested fragments were then respectively inserted in-frame into the sequence encoding an N-terminal polyhistidine-tag present in the expression vector pRSET-A (Invitrogen), which had been previously digested with the same enzymes. The resulting recombinant plasmids were utilized to transform competent cells of BL21 (DE3 codon plus-RIL), resulting in the recombinant strains pRSETA_*ompK* EC and pRSETA_*hsp33* EC (Suppl. Mat. Table S7). These strains were independently cultured overnight in LB medium supplemented with ampicillin 50 µg/mL and chloramphenicol 20 µg/mL. The cultures were diluted 1:100 in the same medium, grown up to an optical density of 0.6 OD_{600nm} and used to induce the expression of the heterologous proteins by IPTG (Isopropyl β-D-Thiogalactopyranoside, Sigma-Aldrich) addition. The His-tagged OmpK$^{21/260}$ and Hsp33 proteins were solubilized from inclusion bodies utilizing 8 M Urea and purified using a His-Trap column (eluted with 250 mM imidazole) following the manufacturer's recommendations (GE Healthcare Life Science, Merk, Darmstadt, Germany). The purified proteins were concentrated, desalted using a Centricon cutoff 10 kDa (Merck, Millipore, Darmstadt, Germany) and the purity assessed by 12.5% SDS-PAGE and Western blot with an anti-His antibody (1:7000, Sigma-Aldrich). mRFP was over-produced and purified as previously described [25].

Adsorption reaction, efficiency and protein stability

Adsorption reactions were carried out as previously described [25] with some modifications: 2 µg of purified mRFP were added to 1.0×10^9 spores of PY79, MV4, MV11, MV24, or MV30 strains in Sodium Citrate 50 mM at pH 3.5 and incubated at 25 °C in a final volume of 200 µL for 1 h. Similarly, various amounts (2, 5, 10, or 20 µg) of purified OmpK$^{21/260}$ or Hsp33 were added to a suspension of 1.0×10^9 MV30 spores in Sodium Acetate 50 mM, Sodium Citrate 50 mM, or PBS 1x at pH 3.5. The efficiency of spore adsorption was evaluated by fractionation of spore-bound OmpK$^{21/260}$ or Hsp33 from unbound proteins by centrifugation and analysis of both fractions by western- and dot-blotting analyses with anti-6xHis antibody. The stability of spore-bound OmpK$^{21/260}$ or Hsp33 was evaluated by suspending the purified and the spore-adsorbed proteins in water. At different times aliquots were fractioned by centrifugation and analyzed independently.

Western- and dot-blotting

Pure proteins or 1.0×10^9 spores with antigen adsorbed on the surface were suspended in extraction buffer 2x [34] incubated at 100 °C for 10 min and loaded onto a 12.5% SDS-PAGE gel. The proteins were then electro transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Milano, Italy) and used for Western blot analysis using anti-His antibody (Sigma-Aldrich) at dilutions of 1:7000. A quantitative determination of the amount of OmpK$^{21/260}$ or Hsp33 was obtained by dot blot experiments analyzing serial dilutions of purified OmpK$^{21/260}$ and Hsp33, binding assay supernatant and OmpK- and Hsp33-adsorbed spores using anti-His antibody (Sigma-Aldrich) at dilutions of 1:5000.

Quantification of proteins on the dot-blots was accomplished by exposing the membrane to Clarity Western ECL (Bio-Rad, Segrate, Milano, Italy), acquiring the images via Quantity One 1-D Analysis Software (Bio-Rad, Segrate, Milano, Italy) and subsequently analyzing them by densitometry.

Fluorescence microscopy and signal quantification

The display of mRFP on spores was monitored by posing 6 µL of a spore suspension onto a microscope slide and covering it with a coverslip pre-treated with a 0.1% poly-L-lysine solution (Sigma-Aldrich). The slides were then observed under an Olympus BX51 microscope equipped with a 100x UplanF1 objective and U-WIBA filters (with excitation filters ranging between 460 and 490 nm and barrier filters between 515 and 550 nm). Exposure times were set at 500 ms. Captured images were processed using analySIS software (SIS) and subsequently analyzed using ImageJ software (https://imagej.nih.gov/ij/) to cal culate the TCCF (corrected total-per-cell fluorescence). Signal quantification and immunofluorescence experiments were performed as previously described [34] and [13], respectively. Adsorbed or free MV30 spores were fixed for 15 min using 4% formaldehyde at pH 7.4. Subsequently, the samples were centrifuged and washed three times with PBS 1x, followed by a 1 h blocking step with 1% BSA-PBS 1x. The samples were then incubated with Anti-His antibody (diluted 1:10, Sigma-Aldrich) for 2 h at 4 °C, washed three times in PBS 1x, and further incubated with a 1:50 dilution of anti-mouse antibody conjugated with FITC (Bethyl Laboratories, Inc) for 1 h at 25 °C. Afterward, the samples were washed three times in PBS 1x and analyzed using a flow cytometer (BD Accuri C6; BD Biosciences, San Jose, CA) with detection performed using FL1, utilizing the standard C6 filter configuration (FL1 = 530/30 BP). Specific fluorescence attributed to His-tagged proteins adsorbed on spores was

differentiated from nonspecific spore fluorescence by comparing the signal from spores adsorbing proteins versus untreated spores.

Medaka fish stocks

Cab-strain of wild-type Medaka fish (*Oryzias latipes*) was used throughout the study and maintained under standard conditions (12 h/12 h dark/light cycle at 27 °C), as previously described [29]. The developmental stages of the embryos were monitored using a stereomicroscope (Olympus, SZX7) for ten days post-fertilization, until stage 40, corresponding to the first fry stage, according to the method proposed by Iwamatsu [35]. Larvae were randomly divided into seven group ($n = 30$). Five groups were immersed for two hours in a Yamamoto medium containing either spores (1.0×10^9), $OmpK^{21/260}$ (20 µg), Hsp33 (20 µg), spore-adsorbed $OmpK^{21/260}$ (1.0×10^9 spores loaded with 20 µg), or spore-adsorbed Hsp33 (1.0×10^9 spores loaded with 20 µg). After the treatment, larvae of the five groups and of one of the two not treated groups were challenged with 7.5×10^8 CFU/ml of the pathogenic strain *V. harveyi* 1173 and checked for 48 h. Ethical approval was not required for this study involving medaka larvae at stage 40 in strict accordance with the Institutional Guidelines for animal research and as indicated by D.Lgs. 26/2014 on animal experimentation of the Italian Ministry of Health; Department of Public Health, Animal Health, Nutrition, and Food Safety (D.Lgs. 26/2014).

Immunization of Medaka fish and challenge experiments with *Vibrio harveyi 1773*

To evaluate the potential protective effect of spore-adsorbed $OmpK^{21/260}$ and spore-adsorbed Hsp33, Medaka larvae ($n > 30$) at stage 40 were distributed into 6 mm petri dish containing 10 mL of Yamamoto medium (for 1 L: 7.5 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$, 0.02 g $NaHCO_3$) for every condition. Larvae were treated for 2 h in a Yamamoto medium containing either *B. megaterium* spores (1.0×10^9), spore-adsorbed $OmpK^{21/260}$ (1.0×10^9 spores loaded with 20 µg of antigen), spore-adsorbed Hsp33 (1.0×10^9 spores loaded with 20 µg of antigen), the free antigen $OmpK^{21/260}$ (20 µg) or Hsp33 (20 µg). After 2 h of incubation the medium containing spores or pure proteins was removed, and larvae were allowed to grow for an additional 72 h. Subsequently, the larvae were divided into 24-well plates, with 1 larva for each well, and submerged in a final volume of 800 µL of Yamamoto broth containing 7.5×10^8 CFU / ml of the pathogenic strain *V. harveyi* (OD_{600} 4.5). The resulting cumulative mortalities were registered during 48 h. Two control groups included: (i) non-challenged larvae as negative control, (ii) non-vaccinated larvae exposed to the pathogen as positive control. The experiment was independently carried out 3 times.

Statistical analysis

Survival data were analyzed using Kaplan-Meier estimations and the log-rank test in R (*survival*, *survminer* packages). Survival curves were generated with *survfit*(), and differences between groups were assessed using pairwise log-rank tests based on Chi-square statistics. Graphical visualization was performed with *ggsurvplot*(), including survival percentages and risk tables. A significance threshold of $p < 0.05$ was applied.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-025-02725-w.

Supplementary Material 1

Supplementary Material 2

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Author contributions

MDS and GDGB: performed most of the experiments and contributed to experiment design; SM and IC: contributed to the animal experiments; YDL and CB: contributed to the preparation of spores and pathogen cells for the animal experiments; LB, GDV, KP and IC: contributed to the experiment design and manuscript writing; ER: contributed to the experiment design and wrote most of the manuscript; AS: coordinated the experimental work, contributed to experiment design and manuscript writing. All authors read and approved the final manuscript.

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Data availability

All data generated or analysed during this study are included in this published article [and its supplementary information files]. The accession number for the genome sequence of Vibrio harveyi 1173 has been requested to the NCBI and will be provided as soon as it is available.

Declarations

Ethics approval and consent to participate

Ethical approval was not required for this study involving medaka larvae at stage 40 in strict accordance with the Institutional Guidelines for animal research and as indicated by D.Lgs. 26/2014 on animal experimentation of the Italian Ministry of Health; Department of Public Health, Animal Health, Nutrition, and Food Safety (D.Lgs. 26/2014).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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References

1. Bell A, Bott M, Vibriosis. What you and your patients need to know. Dela J Public Health. 2021;7:14–21.
2. Xu K, Wang Y, Yang W, Cai H, Zhang Y, Huang L. Strategies for prevention and control of vibriosis in Asian fish culture. Vaccines. 2023;11:98.
3. Trinanes J, Martinez-Urtaza J. Future scenarios of risk of *Vibrio* infections in a warming planet: a global study. Lancet Planet Health. 2021;5:e515–20.
4. Baker-Austin C, Archer E, Hartnell R, Trinanes J, Martinez-Urtaza J. Stemming the rising tide of *Vibrio* disease. Lancet Planet Health. 2024;8:e426–35.
5. Vadstein O, Attramadal KJK, Bakke I, Olsen Y. K-selection as microbial community management strategy: a method for improved viability of larvae in aquaculture. Front Microbiol. 2018;9:2730.
6. Sanches-Fernandes GMM, Sá-Correia I, Costa R. Vibriosis outbreaks in aquaculture: addressing environmental and public health concerns and preventive therapies using Gilthead seabream farming as a model system. Front Microbiol. 2022;13:904815.
7. Rello J, Parisella FR, Perez A. Alternative to antibiotics in an era of difficult-to-threat resistance: new insights. Expert Rev Clin Pharmacol. 2019;12:635–42.
8. Romario Silva D, de Cassia Orlandi Sardi J, de Souza Pitangui N, Magri Roque S, Barbosa da Silva AC, Rosalen PL. Probiotics as an alternative antimicrobial therapy: current reality and future directions. J Funct Foods. 2020;73:104080.
9. Gonçalves G, Santos RA, Coutinho F, Pedrosa N, Curado M, Machado M, Costas B, Bonneville L, Serrano M, Carvalho AP, Díaz-Rosales P, Oliva-Teles A, Couto A, Serra CR. Oral vaccination of fish against vibriosis using spore-display technology. Front Immunol. 2022;13:1012301.
10. Kwong KW-Y, Xin Y, Lai NC-Y, Sung JC-C, Wu K-C, Hamied YK, Sze ET-P, Lam DM-K. Oral vaccines: a better future of immunization. Vaccines. 2023;11:1232.
11. Yan F, Polk DB. Probiotics and immune health. Curr Opin Gastroenterol. 2011;27:496–501.
12. D'Accolti M, Soffritti I, Bini F, Mazziga E, Caselli E. Tackling transmission of infectious diseases: A probiotic-based system as a remedy for the spread of pathogenic and resistant microbes. Microb Biotechnol. 2024;17:e14529.
13. Santos FDS, Mazzoli A, Maia AR, Saggese A, Isticato R, Leite F, Iossa S, Ricca E, Baccigalupi L. A probiotic treatment increases the immune response induced by the nasal delivery of spore-adsorbed TTFC. Microb Cell Fact. 2020;19:42.
14. Potot S, Serra CR, Henriques AO, Schyns G. Display of Recombinant proteins on *Bacillus subtilis* spores, using a Coat-Associated enzyme as the carrier. Appl Environ Microbiol. 2010;76(17):5926–33.
15. Reljic L, Sibley JM, Huang I, Pepponi A, Hoppe HA, Hong SM. Cutting. Mucosal vaccination against tuberculosis using inert bioparticles. Infect&Immun. 2013;81:4071–80.
16. Guoyan Z, Yingfeng A, Zabed H, Qi G, Yang M, Jiao Y, Li W, Wenjing S, Xianghui Q. *Bacillus subtilis* spore surface display technology: a review of its development and applications. J Microbiol Biotechnol. 2019;29:179–90.
17. Saggese A, Baccigalupi L, Donadio G, Ricca E, Isticato R. The bacterial spore as a mucosal vaccine delivery system. Int J Mol Sci. 2023;24:10880.
18. Ning D, Leng X, Li Q, Xu W. Surface-displayed VP28 on *Bacillus subtilis* spores induce protection against white spot syndrome virus in crayfish by oral administration. J Appl Microbiol. 2011;111:1327–36.
19. Valdez A, Yepiz G, Ricca E, Olmos-Soto J. First Litopenaeus vannamei WSSV 100% oral vaccination protection using CotC::Vp26 fusion protein displayed on Bacillus subtilis spores surface. J Appl Microbiol. 2014;117:347–57.
20. Nguyen AT, Pham CK, Pham HT, Pham HL, Nguyen AH, Dang LT, Huynh HA, Cutting SM, Phan TN. *Bacillus subtilis* spores expressing the VP28 antigen: a potential oral treatment to protect *Litopenaeus vannamei* against white spot syndrome. FEMS Microbiol Lett. 2014;358:202–8.
21. Pham K-C, Tran HTT, Van Doan C, Le PH, Van Nguyen AT, Nguyen HA, Hong HA, Cutting SM, Phan T-N. Protection of *Penaeus monodon* against white spot syndrome by continuous oral administration of a low concentration of *Bacillus subtilis* spores expressing the VP28 antigen. Lett Appl Microbiol. 2014;64:184–91.
22. Ricca E, Baccigalupi L, Isticato R. Spore-adsorption: mechanism and application of a non-recombinant display system. Biotechnol Adv. 2021;47:107693.
23. Vittoria M, Saggese A, Isticato R, Baccigalupi L, Ricca E. Probiotics as an alternative to antibiotics: genomic and physiological characterization of aerobic spore formers from the human intestine. Microorganisms. 2023;11:1978.
24. Xing J, Li P, Tang X, Zhan W. Recombinant Hsp33 and OmpC protein can serve as promising divalent vaccine with protection against *Vibrio anguillarum* and *Edwardsiella tarda* in flounder (Paralichthys olivaceus). Fish Shellfish Immunol. 2018;74:341–8.
25. Donadio G, Lanzilli M, Sirec T, Ricca E, Isticato R. Localization of a red fluorescence protein adsorbed on wild type and mutant spores of *Bacillus subtilis*. Microb Cell Fact. 2016;15:153.
26. Lanzilli M, Donadio G, Addevico R, Saggese A, Cangiano G, Baccigalupi L, Christie G, Ricca E, Isticato R. The exosporium of *Bacillus megaterium* QM B1551 is permeable to the red fluorescence protein of the coral Discosoma Sp. Front Microbiol. 2016;7:1752.
27. Clifford JN, Høie MH, Deleuran S, Peters B, Nielsen M, Marcatili P. BepiPred-3.0: improved B-cell epitope prediction using protein Language models. Protein Sci. 2022;31:e4497.
28. Sirec T, Strazzulli A, Isticato R, De Felice M, Moracci M, Ricca E. Adsorption of beta-galactosidase of *Alicyclobacillus acidocaldarius* on wild type and mutants spores of *Bacillus subtilis*. Microb Cell Fact. 2012;11:100.
29. Zappa F, Intartaglia D, Guarino AM, Cegli RD, Wilson C, Salierno FG, Polishchuk E, Sorrentino NC, Conte I, Matteis MAD. Role of trafficking protein particle complex 2 in medaka development. *BioRxiv* 2023; https://doi.org/10.1101/2023.07.14.548998
30. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.
31. Cutting S, Vander-Horn PB. Genetic analysis. In: Harwood CR, Cutting SM, editors. Molecular biological methods for Bacillus. Ltd: Wiley; 1990. pp. 27–74.
32. Nicholson WL, Setlow P. Sporulation, germination, and outgrowth. In: Harwood CR, Cutting SM, editors. Molecular biological methods for Bacillus. New York, NY, USA: Wiley; 1990. pp. 391–450.
33. Lugli GA, Milani C, Mancabelli L, Van Sinderen D, Ventura M. MEGAnnotator: A user-friendly pipeline for microbial genomes assembly and annotation. FEMS Microbiol Lett. 2016;363:049. https://doi.org/10.1093/femsle/fnw049.
34. Isticato R, Ricca E, Baccigalupi L. Spore adsorption as a nonrecombinant display system for enzymes and antigens. J Vis Exp. 2019;19(145). https://doi.org/10.3791/59102.
35. Iwamatsu T. Stages of normal development in the Medaka Oryzias latipes. Mech Dev. 2004;121(7–8):605–18. https://doi.org/10.1016/j.mod.2004.03.012.

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