Aquaculture Reports 37 (2024) 102253

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

The oral delivery of *Bacillus subtilis* spores modulates skin and intestinal mucus responses in rainbow trout (*Oncorhynchus mykiss*)

Samuel Vicente-Gil [a], Silvia Nogales-Mérida [a], Gabriela Gonçalves [b,c], Cláudia R. Serra [b,c], M. Camino Ordás [a,1], Carolina Tafalla [a,*], Patricia Díaz-Rosales [a,*]

[a] Fish Immunology and Pathology Group, Animal Health Research Centre (CISA-INIA-CSIC), Valdeolmos-Alalpardo, Madrid 28130, Spain
[b] Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto, Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos, S/N, Matosinhos 4450-208, Portugal
[c] Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre, Edifício FC4, Porto 4169-007, Portugal

ARTICLE INFO

Keywords:
Aquacultured fish
Probiotics
Sporeformes
Mucosal immunity
Enzymatic activities
Oxidative status

ABSTRACT

Bacillus subtilis is a spore-forming microorganism, recognized as a safe probiotic strain. Its endospores are easily produced at a large scale, can be dehydrated and maintain their characteristics after long-term storage, providing great advantages for their application in aquafeeds. Nonetheless, knowledge on the effects that *B. subtilis* provokes on fish mucosal immunity is still scarce. In this context, the aim of this work was to explore the effects of the oral administration of *B. subtilis* spores to rainbow trout (*Oncorhynchus mykiss*) for 30 days on the intestinal and skin mucus, focusing on a range of immune and enzymatic parameters. Thus, fish supplemented with *B. subtilis* spores showed increased levels of total immunoglobulin (Ig) in intestinal and skin mucus, as well as IgM levels in both mucus and serum. *B. subtilis* spores induced an increase of peroxidase activities in mucus from both sources. Although superoxide dismutase (SOD) activity was not affected by the spores, NO production significantly decreased in skin mucus. Finally, the bactericidal activity of the intestinal and skin mucus was significantly higher in fish fed the probiotic spores. The results obtained demonstrate that the dietary supplementation with *B. subtilis* spores enhances mucosal defense mechanisms by increasing immunological parameters of the intestinal and skin mucus.

1. Introduction

Aquaculture has grown quickly in the last 30 years in a context of increasing food demand and competition for natural resources. This rapid expansion of farmed fish production has consequently increased the risk of outbreaks provoked by infectious agents that generate important economic losses. Although vaccination is from all points of view the most adequate method to prevent infectious diseases, not all fish vaccines commercially available are capable of avoiding mortalities in the field provoked by these pathogens. Furthermore, there are still many fish pathogens for which commercial vaccines are not available (Du et al., 2022). Hence, traditionally, antimicrobials have been used by farmers to control infectious diseases and minimize economic damage (Done et al., 2015). However, their use involves a high risk of environmental consequences due to the possible release of antibiotics/chemotherapeutic compounds to the aquatic environment (Cabello, 2006). Additionally, the use of antibiotics favors the appearance of resistant bacterial strains and the transference of resistance genes from bacteria in aquatic animals to bacteria potentially dangerous for humans (Done et al., 2015; Santos and Ramos, 2018). In this context, the use of feed additives or supplements that may act as immunomodulators is considered a sustainable way to replace the use of antibiotics or chemotherapeutics and is currently a research area that has attracted great interest (Banerjee and Ray, 2017; Encarnação, 2016; Vallejos-Vidal et al., 2016). The use of these functional feeds would not only decrease the impact of disease outbreaks but would also contribute to increase animal welfare and promote a healthy environment.

Among the potential feed immunostimulants that could be incorporated in feeds, probiotics are considered an excellent alternative for immunoprophylactic control in fish farms (El-Saadony et al., 2021; Gatesoupe, 1999; Newaj-Fyzul et al., 2014). Probiotics are defined as microbes that have a beneficial effect in the host organism, mainly

* Corresponding authors.
E-mail addresses: tafalla@inia.csic.es (C. Tafalla), pdiazrosales@inia.csic.com (P. Díaz-Rosales).
[1] Present address: Departamento de Biología y Geología, Física y Química Inorgánica, Universidad Rey Juan Carlos, 28933 Móstoles, Spain.

https://doi.org/10.1016/j.aqrep.2024.102253
Received 23 April 2024; Received in revised form 9 July 2024; Accepted 10 July 2024
Available online 13 July 2024

through the modulation of the intestinal microbiota (Domínguez-Maqueda et al., 2021; El-Saadony et al., 2021), but also having direct beneficial effects on the immune system. The main requirements that have to be met to consider a microorganism a probiotic are not being pathogenic for fish, having the capacity to colonize the intestinal surface and the ability to modulate the immune system (Alonso et al., 2019; Gatesoupe, 1999; Simón et al., 2021). In fact, most probiotics have been shown to modulate different innate and adaptive immune functions of fish (Azad et al., 2018; El-Saadony et al., 2021; Lazado and Caipang, 2014; Nayak, 2010), consequently improving disease resistance (Simón et al., 2021; Verschuere et al., 2000).

Bacillus subtilis has been widely recognized as a safe and reliable probiotic microorganism. This Gram-positive bacterium is able to colonize the gastrointestinal tract by adhering to epithelial cells, secreting antimicrobial compounds and enhancing the immune status and disease resistance by regulating the fish immune system (Di et al., 2019; Docando et al., 2022a; Galagarza et al., 2018; Han et al., 2020; Nayak, 2021; Olmos et al., 2020; Santos et al., 2021; Won et al., 2020; Zhang et al., 2014). Importantly, the fact that *B. subtilis* is a spore-forming microorganism and its endospores are easily produced at a large scale provide a critical advantage for its application in aquafeeds. These spores can be dehydrated thus allowing their long-term storage without losing their characteristics (Setlow, 2014). Interestingly, the potential of *B. subtilis* spores as protein display platforms (Bartels et al., 2018) to express bacterial (Gonçalves et al., 2022) or viral (Aps et al., 2015; de Souza et al., 2014; Docando et al., 2022b; Gao et al., 2021; Sun et al., 2020) antigens has also been recently explored, pointing to this probiotic as a suitable vehicle for delivery of oral vaccines.

Mucosal surfaces are in constant exposure to the external medium and therefore constitute an initial barrier to infection, blocking the pathogens at the entry site. The main fish mucosal surfaces are the skin, the gills and the intestine, although other mucosal tissues such as those associated to the nasal or the buccopharyngeal cavities have been recently described (Garcia et al., 2022; Salinas, 2015; Yu et al., 2019). One of the distinctive features of fish mucosal tissues is the presence of an external mucus. The mucus is a physical, chemical and biological barrier, considered the first line of defense against infection (Benhamed et al., 2014). The main molecules present in the mucus are the mucins that act as a filter for pathogens. In addition, a wide variety of both innate and adaptive immune components such as lysozyme, immunoglobulins (Igs), complement, cytokines, lectins, agglutinin, calmodulin, interferon, C-reactive protein, antimicrobial peptides and proteolytic enzymes are secreted into the mucus and contribute to block pathogen entry (Brinchmann, 2016; Guardiola et al., 2014; Lazado and Caipang, 2014; Nigam et al., 2012; Sanahuja et al., 2019; Subramanian et al., 2007, 2008).

Several studies have previously assessed the effects of probiotics on some immune parameters of the mucus. For example, in gilthead seabream (*Sparus aurata*), *Shewanella putrefaciens* (Pdp11) and *Bacillus* sp. up-regulated IgM levels, peroxidase and protease activities in the skin mucus (Cerezuela et al., 2016). In goldfish (*Carassius auratus*), the effects of the dietary administration of the lactic-acid bacteria *Lactobacillus acidophilus* on the skin mucus protein pattern was studied by SDS-PAGE (Hosseini et al., 2016). Modanloo et al. (2017) also reported increased serum and skin mucus total Ig levels, as well as skin mucus lysozyme activity in carp (*Cyprinus carpio*) fed diets supplemented with galactooligosaccharide and *Pediococcus acidilactici*. Similarly, Van Doan et al. (2021) reported increased lysozyme and peroxidase activities on skin mucus of Nile tilapia (*Oreochromis niloticus*) fed watermelon rind powder and *Lactobacillus plantarum* administered individually or in combination. Positive effects of *Bacillus cereus* QSI-1 administration on some parameters of the skin mucus were also observed in crucian carp (*Carassius auratus gibelio*), a study in which an increased resistance to *Aeromonas hydrophila* was also reported in response to the probiotic (Jiang et al., 2019). In rainbow trout (*Onchorhynchus mykiss*), supplementing diets with galactooligosaccharides (GOS), *P. acidilactici* or a combination of both, all increased the bactericidal activity and the protein content of the skin mucus (Hoseinifar et al., 2015). Yet, these studies mostly focused on the effects observed on the skin mucus, and the effects on the intestinal mucus were rarely investigated, even though it would be presumed that important effects would also be provoked locally by the fed probiotics. In the case of Nile tilapia, the effects of *Bacillus velezensis*, *B. subtilis* and *Bacillus amyloliquefaciens* in the diet were studied in intestinal samples, although not in mucus itself, observing that intestinal antioxidant enzymes (catalase and superoxide dismutase) as well as intestinal lipase activity were increased in the groups fed with probiotics (Kuebutornye et al., 2020a).

In this context, in the current study, we wanted to determine the effects that *B. subtilis* supplementation could have on the mucus activity of rainbow trout, this time focusing on both skin and intestinal mucus. Hence, several enzymatic activities of both mucus obtained from rainbow trout fed for 30 days with a diet supplemented with *B. subtilis* were determined, and compared to those of fish fed a control diet. Additionally, the concentration of IgM on both mucus samples and serum was assessed. Our results demonstrate the immunostimulating effects of *B. subtilis* on both skin and intestinal mucus, providing additional evidence of the beneficial effects of *B. subtilis* on the immune status of aquacultured fish.

2. Materials and methods

2.1. Fish care and maintenance

Rainbow trout (*O. mykiss*) with a mean body weight of approximately 24 g were obtained from the Cifuentes fish farm (Cifuentes, Guadalajara, Spain). Fish were maintained at the animal facilities of the Animal Health Research Centre (CISA-INIA-CSIC) in an aerated recirculating system at 15°C, with a photoperiod of 12:12 h light/dark. Fish were fed once a day with a commercial diet (Skretting, Norway) and acclimatized to laboratory conditions for at least two weeks prior to the experimental procedure. During this period, no mortalities were experienced and no pathological signs were ever observed. Ammonia and nitrite levels were measured daily and maintained below 0.025 and 0.3 mg/l, respectively. The experiments agreed with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals and were approved by the INIA Ethics Committee (Code PROEX 064.2/21).

2.2. Diets and experimental design

A commercial diet (Skretting, Spain) was used as the basic feed. The experimental diet was prepared adding the lyophilized spores of *B. subtilis* dissolved in 0.9 % NaCl to the basic feed pellets, mixing thoroughly. The *B. subtilis* strain used was the ABP1, known to exert high immunomodulatory effects as previously described by our group (Docando et al., 2022a). The spores were obtained by nutrient exhaustion in DSM (Difco Sporulation Medium) and were purified and quantified as described before (Bartels et al., 2018; Gonçalves et al., 2022; Serra et al., 2014). The final concentration of the spores in the experimental diet was 1×10^{10} spores/kg. The control diet used in the experiment was obtained adding the same volume of 0.9 % NaCl to the basic feed pellets. Diets were allowed to dry, kept in a light protected environment and stored at 4°C during all the experimental trial. At this temperature, the spores have been shown to remain unaltered in the feed during the entire period of the trial (Cappellozza et al., 2023; Rangel et al., 2024). Fish were randomly assigned to two identical tanks (10 fish per group) and received either the control or the *B. subtilis*-supplemented diet. The fish were fed once a day with each corresponding diet during one month at a rate of 1 % body weight per day. Once this experiment was over, an identical experiment was carried out with another stock of fish to confirm the results obtained. This new experiment was performed in exactly the same conditions, with 10

control and 10 *B. subtilis*-supplemented fish.

2.3. Sample collection

After 30 days of feeding, fish from each group were euthanized with benzocaine (Sigma-Aldrich) overdose administered by immersion (50 mg/ml) following the recommendation of Zahl et al. (2012). Skin mucus was first collected avoiding contamination with blood, urinogenital and intestinal excretions. Thus, skin mucus was gently collected from each fish by scraping the whole-body skin surface with a glass slide, adding 15 ml of protease inhibitor buffer, following the protocol previously standardized by Kelly and collaborators (Kelly et al., 2017). This buffer consists in phosphate buffer saline (PBS) (Sigma-Aldrich) containing 1X protease inhibitor cocktail (Roche), 1 mM phenylmehtylsulfonyl fluoride (PMSF, Sigma-Aldrich), 0.5 % bovine serum albumin (BSA, Sigma-Aldrich) and 100 μg/ml trypsin inhibitor (Sigma-Aldrich). Thereafter, blood was extracted from the caudal vein and let to clot at 4°C overnight. Serum extraction was performed by centrifugation at 4000 × *g* for 10 min at 4°C and the supernatant was centrifuged again at 10,000 x *g* for 10 min at 4°C. Serum was stored at −80°C until use. Finally, fish were opened to collect the intestine. The adipose tissue was removed from the intestine, which was cut and then opened longitudinally. Then, the intestinal mucus was collected scraping with a glass slide, adding 5 ml of the protease inhibitor buffer described above. Both skin and intestinal mucus were kept on ice during the entire isolation procedure. In both cases, the mucus was then vortexed to homogenize the samples and centrifuged at 40 × *g* during 10 min at 4°C to eliminate debris. The supernatants were transferred to a clean tube and centrifuged again at 400 × *g* for 10 min at 4°C. After this centrifugation, the mucus samples were aliquoted and stored at −80°C until further analysis.

2.4. Mucus total protein content

Total protein concentration in the mucus was determined by the dye binding method of Bradford (Bradford, 1976) in a 96-well plate, using bovine serum albumin (BSA, Sigma-Aldrich) as a protein standard. For this, 2 mg/ml of BSA solution were serially diluted in PBS. Serial dilutions of skin and intestinal mucus were also prepared in PBS. At this point, 200 μl of Bradford reagent (Sigma-Aldrich) were added to each well and incubated for 30 min at 37°C. The absorbance of each sample was measured at 562 nm and the results obtained were extrapolated onto the standard curve to obtain protein content of skin and intestinal mucus.

2.5. Total immunoglobulin (Ig) content in intestinal and skin mucus

Total Ig content in the mucus was estimated following the method described by Soltis and Hasz (1983) with slight modifications. Briefly, equal volumes of mucus and 12 % polyethylene glycol (PEG, Sigma-Aldrich) in Milli-Q water were incubated for 10 min at room temperature (RT) with constant shaking. The mix was then centrifuged at 10,000 × g for 5 min at 16°C, and the supernatant collected to determine protein concentration. The difference in total protein content prior and after precipitation of the Ig component was calculated and used to estimate the Ig content expressed as mg/ml. Triplicates of all mucus samples were assayed.

2.6. Determination of total IgM levels

Total serum and mucus IgM levels were determined using the enzyme-linked immunosorbent assay (ELISA) as described by Martín--Martín et al. (2020) with slight modifications. Thus, plates were coated with 2 μg/ml of an anti-trout IgM in bicarbonate buffer. Thereafter, non-specific binding sites were blocked by incubation with 5 % BSA in PBS for 1 h at RT. Plates were washed with 0.05 % Tween 20 (PBS-T) (Sigma-Aldrich) and dilutions of each sample (mucus and serum) added in PBS with 1 % BSA and incubated for 1 h at RT. The plates were washed again three times in PBS-T and 100 μl of 1 μg/ml biotinylated anti-trout IgM mAb (clone 4C10) diluted in PBS containing 1 % BSA added. After 1 h at RT, the plates were washed again three times with PBS-T and 100 ng/ml of HRP-streptavidin (Thermo Fisher Scientific) in PBS with 1 % BSA added. After 1 h of incubation at RT, 100 μl of o-phenylenediamine dihydrochloride substrate reagent (Sigma-Aldrich) were added to each well. The reaction was stopped after 15 min by adding 50 μl of 2 M sulfuric acid (H_2SO_4, Sigma-Aldrich). Absorbance at 490 nm was recorded using a FLUOstar Omega plate reader (BMG Labtech). Internal positive and negative control samples were included in all assays. Triplicates of all samples were analyzed.

2.7. Evaluation of enzymatic activities in intestinal and skin mucus

2.7.1. Ceruloplasmin oxidase activity

The ceruloplasmin oxidase activity was measured following the method described by Dunier and Siwicki (1994). Briefly, equal volumes of mucus, 0.1 % *p*-phenylenediamine (Sigma-Aldrich) in acetate buffer (pH 5.2) and 0.02 % sodium azide (Sigma-Aldrich) were incubated. The kinetic increase of absorbance at 550 nm was followed for 15 min and 1 unit was defined as an increase of OD of 0.001 min^{-1}. Triplicates of all mucus samples were tested.

2.7.2. Peroxidase activity

The peroxidase activity was measured as previously described by Quade and Roth (1997) with slight modifications. For this, 30 μl of mucus were added (in triplicate) to a flat-bottomed 96-well plated containing 120 μl of Hanks buffer (HBSS, Thermo Fisher Scientific) without Ca^{+2} or Mg^{+2}. Afterwards, 50 μl of 10 mM tetramethylbenzidine (TMB, Sigma-Aldrich) and 50 μl of 5 mM hydrogen peroxide (H_2O_2, Sigma-Aldrich) were added to each well as substrates. After a 2 min incubation, the reaction was stopped with 50 μl of 2 M H_2SO_4. The OD at 490 nm was measured in a FLUOstar Omega plate reader. As negative controls, samples without mucus were included. One unit was defined as optical density change in comparison with negative samples, and were expressed as U/ml.

2.7.3. Superoxide dismutase activity

The superoxide dismutase activity (SOD) was measured by a spectrophotometric method based on the inhibition of xanthine oxidase-induced reduction of cytochrome c (Sigma-Aldrich) in 50 mM phosphate buffer (pH 7.0) at 25°C as previously described (Peskin and Winterbourn, 2000). Thus, the substrate solution consists in 50 mM potassium phosphate buffer, 2 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich), 100 μM xanthine (Sigma-Aldrich) and 12 μM cytochrome c (Sigma-Aldrich). Briefly, 10 μl of mucus 1:20 diluted was mixed with 90 μl of the substrate solution, and changes in absorbance at 550 nm recorded each second for 3 min on a FLUOstar Omega microplate reader in triplicate. One unit of activity was defined as the amount of protein required to inhibit the cytochrome c reduction under the experimental conditions. Triplicates of all mucus samples were analyzed.

2.7.4. Nitric oxide production

The nitrite production was analyzed using the Griess reaction (Green et al., 1982) and following a modification of the method described by McCord and Fridovich (1969). The Griess reaction quantifies nitrite content in samples, since nitric oxide (NO) is an unstable molecule and degrades to nitrite (NO_2^-) and nitrate (NO_3^-). For this, 50 μl of mucus (in triplicate) were transferred to a 96-well plate to which 100 μl of 1 % sulfanilamide in 2.5 % phosphoric acid (H_3PO_4, Sigma-Aldrich) were added followed by 100 μl of 0.1 % N-naphthyl-ethylenediamine (Sigma-Aldrich) in 2.5 % H_3PO_4. The plates were then incubated for 5 min at RT in darkness. At this point, the absorbance at 540 nm was recorded

using a FLUOstar Omega plate reader. Additionally, a standard curve was obtained with known concentrations of sodium nitrite ($NaNO_2$, Sigma-Aldrich). The data was expressed in nitrite concentrations (μM).

2.8. Bactericidal activity in intestinal and skin mucus

The Gram-negative fish pathogen *Aeromonas salmonicida* (CECT4237) was used to establish the bactericidal activity of the mucus. The bacteria were aerobically grown in Luria-Bertani (LB) agar (Sigma-Aldrich) at 25°C overnight. At this point, a fresh single colony was cultured in 10 ml of LB liquid culture medium and incubated at 25°C with agitation (100 rpm) in an orbital incubator for 18 h. The antimicrobial activity of the skin and intestinal mucus was determined by evaluating the effect of mucus on bacterial viability. Briefly, 25 μl of mucus were placed in each well of a 96-well plate in triplicate. Then, 75 μl of *A. salmonicida* culture were added and incubated for 3 h at RT. After the incubation, the plate was centrifuged for 10 min at 200 × *g* and the supernatant discharged. Then 100 μl of 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) were added (0.5 mg/ml in LB) to each well. After 15 min in the dark at RT, absorbance was measured at 600 nm and the bactericidal activity calculated by comparing the bacterial culture density with and without mucus (Ordás et al., 2000).

2.9. Statistical analysis

Data handling, statistical analyses and graphic representation were performed using GraphPad Prism version 8 (GraphPad Software). Before the analyses, data were checked for normality and homoscedasticity using the Shapiro–Wilk and Levene tests. The statistical analyses of the normalized data were carried out using an unpaired Student's *t* test. The differences between the mean values were considered significant on different degrees, where * means $p \leq 0.05$, ** means $p \leq 0.01$, *** means $p \leq 0.001$ and **** means $p \leq 0.0001$.

3. Results

3.1. Total protein, total Ig and IgM content in mucus and serum

We chose to study the effects of *B. subtilis* spores after 30 days of feeding because we performed a preliminary experiment in which some mucus parameters where determined at earlier time points (7 or 14 days of feeding), and the results were not as conclusive (Figure S1). For this reason, we decided to conduct all posterior analysis described in this work after 30 days of feeding.

The total protein content of both the intestinal and the skin mucus was significantly higher in fish fed the *B. subtilis*-supplemented diet than that of fish fed the control diet (Figure S2). Similarly, dietary supplementation with *B. subtilis* spores provoked a significant increase of the concentration of total Igs in both intestinal and skin mucus (Fig. 1), results that were confirmed in a second experiment (Figure S3). Additionally, the IgM concentration was determined with an ELISA, not only in intestinal and skin mucus, but also serum. Fish fed the diet supplemented with *B. subtilis* spores showed significantly increased IgM levels in mucus from both sources (Fig. 2A) and serum (Fig. 2B) when compared to fish fed the control diet. Again, these effects were confirmed in a second trial (Figure S3 and S4). Interestingly, the increase in IgM levels in response to *B. subtilis* was higher in skin mucus (3.77-fold increase when compared to control fish) than in the intestinal mucus (1.29-fold increase when compared to control fish) or serum (1.5-fold increase when compared to control fish) (Fig. 2).

3.2. Evaluation of enzymatic responses of the intestinal and skin mucus

The oxidative status of the mucus was evaluated analyzing the activity of the enzymes ceruloplasmin, peroxidase and SOD, as well as

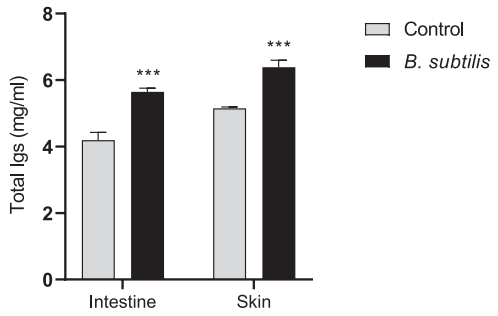

Fig. 1. Detection of total Igs in both intestinal and skin mucus isolated from rainbow trout fed (1 % daily body weight) a control diet (Control) or a diet supplemented with *B. subtilis* spores (1×10^{10} spores/kg) for 30 days. Total Igs are shown in mg/ml. Bars represent means + SEM (n = 10) and asterisks denote significantly different values in the mucus of fish fed the *B. subtilis*-supplemented diet when compared to those of fish fed the control diet. Asterisks denote significantly different values in the mucus of fish fed the *B. subtilis*-supplemented diet when compared to those of fish fed the control diet (***$p \leq 0.001$).

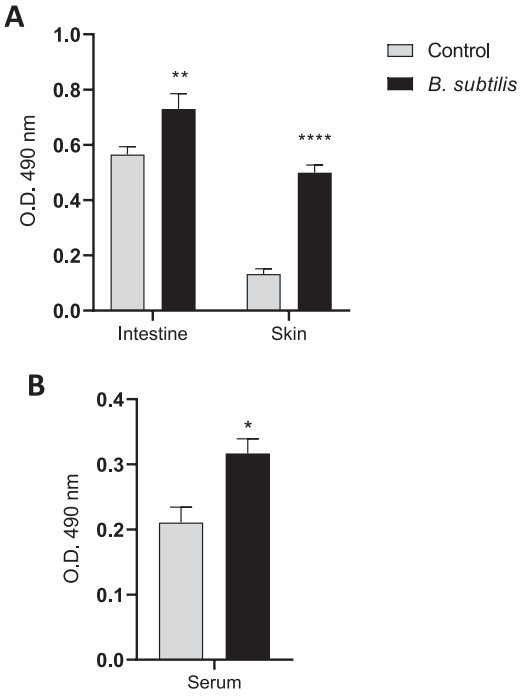

Fig. 2. Detection of total IgM in intestinal and skin mucus **(A)** and in serum **(B)** obtained from rainbow trout fed (1 % daily body weight) a control diet (Control) or a diet supplemented with *B. subtilis* spores (1×10^{10} spores/kg) for 30 days. Results are shown as mean values of absorbance at 490 nm. Bars represent means + SEM (n = 10) and asterisks denote significantly different values in samples from fish fed the *B. subtilis*-supplemented diet when compared to those of fish fed the control diet (*$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$).

determining the NO content. Ceruloplasmin levels were not significantly affected in the intestinal or skin mucus of fish fed the diet supplemented with *B. subtilis* spores (Fig. 3A). No significant effects were detected when the experiment was repeated either (Figure S3). In contrast, peroxidase activity of the mucus strongly increased in both intestinal and skin mucus of fish fed the diet supplemented with *B. subtilis* spores when compared to levels obtained in fish fed the control diet (Fig. 3B). This up-regulation of mucus peroxidase activity was confirmed in the second experiment (Figure S3).

Non-significant changes of the SOD activity were detected in the mucus of fish fed with the *B. subtilis*-supplemented diet when compared

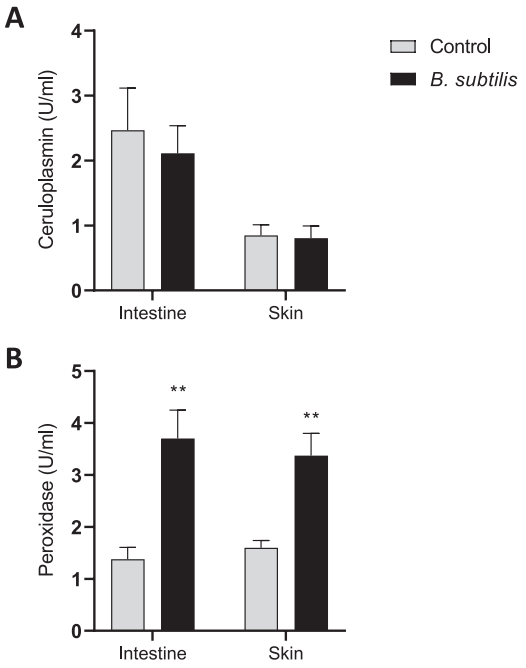

Fig. 3. Ceruloplasmin (U/ml) **(A)** and peroxidase (U/ml) **(B)** activities analyzed in intestinal and skin mucus isolated from rainbow trout fed (1 % daily body weight) a control diet (Control) or a diet supplemented with *B. subtilis* spores (1×10^{10} spores/kg) for 30 days. Bars represent means + SEM (n = 10) and asterisks denote significantly different values in the mucus of fish fed the *B. subtilis*-supplemented diet when compared to those of fish fed the control diet (**$p \leq 0.01$).

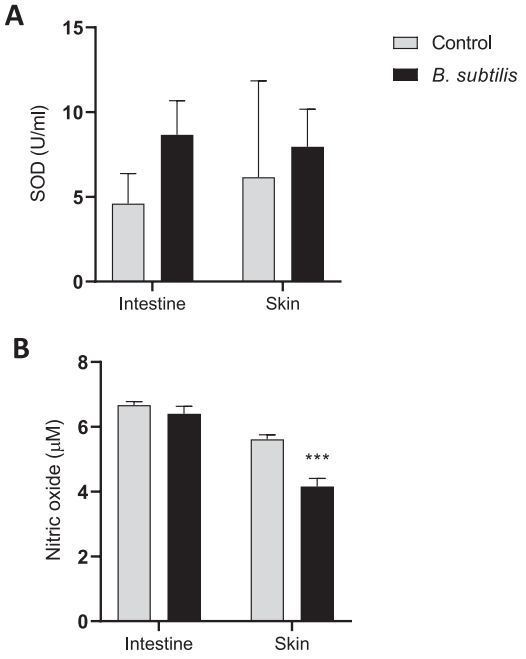

Fig. 4. Superoxide dismutase (SOD, U/ml) **(A)** and NO content (μM) **(B)** detected in intestinal and skin mucus isolated from rainbow trout fed (1 % daily body weight) a control diet (Control) or a diet supplemented with *B. subtilis* spores (1×10^{10} spores/kg) for 30 days. Bars represent means + SEM (n = 10) and asterisks denote significantly different values in the mucus of fish fed the spores from *B. subtilis* supplemented diet when compared to those fish fed the control diet (***$p \leq 0.001$).

to fish fed the control diet (Fig. 4A; Figure S3). Finally, NO production was not significantly influenced by *B. subtilis* spores in intestinal mucus, but significantly decreased in the skin mucus of fish fed the diet supplemented with *B. subtilis* spores (Fig. 4B). This specific effect on the skin mucus was confirmed in the second experiment (Figure S3).

3.3. Bactericidal activity of the intestinal and skin mucus

The bactericidal activity of intestinal and skin mucus was established using *A. salmonicida* and significantly increased in fish fed *B. subtilis* spores in comparison to that of fish fed the control diet (Fig. 5). In this case, in the second experiment, the increased bactericidal activity of the mucus of fish fed *B. subtilis* spores was confirmed for the intestinal mucus but not for the skin mucus (Figure S5).

4. Discussion

The mucus layer plays a key role in the response to pathogens, acting as a first barrier, also maintaining the homeostasis between autochthonous host microorganisms and those potentially pathogenic (Lazado and Caipang, 2014). Both adaptive and innate immune factors are present in the fish skin and intestinal mucus to block the entry of pathogens inside the host, including antimicrobial peptides, lysozyme, complement proteins or Igs (Cabillon and Lazado, 2019).

In the recent past, several studies have reported the beneficial effects of probiotics on fish performance and health, not only improving growth performance, but also conferring resistance to pathogens (El-Saadony et al., 2021; Rohani et al., 2022; Simón et al., 2021). Among these effects, some authors have reported the effect of probiotics on immune parameters of the skin mucus such as enzymatic activities (alkaline phosphatase, esterase, ceruloplasmin, proteases, antiproteases, peroxidases, lysozyme, mieloperoxidase), IgM levels, bactericidal activity and/or NO production (Cerezuela et al., 2016; Chen et al., 2020; Das et al., 2013; Guardiola et al., 2017; Tarkhani et al., 2020; Van Doan et al., 2018), but to our knowledge, no studies have evaluated the effects of probiotics on the intestinal mucus, despite the fact that it seems probable that the local effects of an immunostimulant administered through the diet would be higher than those exerted on a distal mucosa.

The mucus of fish has been shown to contain all three Ig isotypes found in fish, namely IgM, IgD and IgT (Salinas et al., 2021). IgT has been shown to have an important role in mucosal immunity and

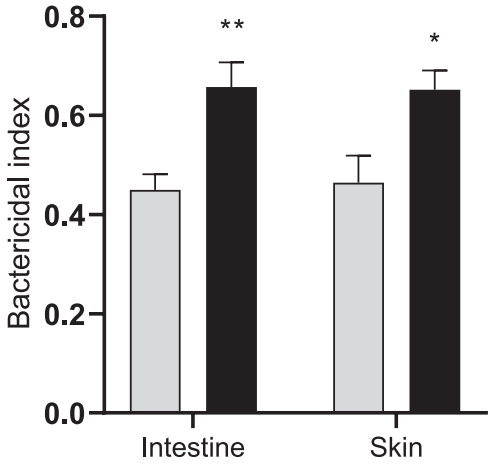

Fig. 5. Bactericidal activity against *Aeromonas salmonicida* detected in intestinal and skin mucus isolated from rainbow trout fed (1 % daily body weight) a control diet (Control) or a diet supplemented with *B. subtilis* spores (1×10^{10} spores/kg) for 30 days. Results are shown as mean values of absorbance at 600 nm + SEM (n = 10). Asterisks denote significantly different values in the mucus of fish fed the *B. subtilis*-supplemented diet when compared to those of fish fed the control diet (*$p \leq 0.05$ and **$p \leq 0.01$).

microbiota homeostasis (Xu et al., 2013, 2016, 2020; Zhang et al., 2010, 2017, 2021), but an antibody to quantify its production in rainbow trout mucus is not commercially available. IgD, on the other hand, is a greatly unexplored Ig that is usually co-expressed on the surface of naïve B cells along with IgM, but also secreted. Its role in the secreted form is still unknown, although recent studies point to a potential role in mucosal immunity (Perdiguero et al., 2019). Finally, IgM is the most common Ig in serum and mucus and plays a key role in systemic immune responses, but it is also known to contribute to the blocking of pathogens in mucosal surfaces (Salinas et al., 2021). IgM participates in the opsonization of pathogens by facilitating their phagocytosis and activates complement (Sunyer, 2012). IgM, together with the other two Igs found in fish (IgD and IgT) are highly specialized recognition glycoproteins that can recognize a great variety of antigens from bacteria, viruses, and other disease-causing organisms and recruit other cells and molecules to destroy these pathogens (Mantis et al., 2019; Yu et al., 2020). Therefore, in the current study, both total Ig levels and IgM production were detected. Our results demonstrated that fish fed with *B. subtilis* spores had significantly higher levels of both total Igs and IgM in both the intestinal and skin mucus. These results are in accordance with several authors that reported an increase in the total content of IgM in skin mucus in fish fed with supplemented diets with a combination of plant extract and probiotics (Bahi et al., 2017; Guardiola et al., 2017), including the probiotic *Bacillus* sp. (Abarike et al., 2018). This increased IgM level in fish fed *B. subtilis* was also visible in the serum of rainbow trout. Similarly, increased total serum IgM levels were observed in fish fed with yeast probiotic *Yarrowia lipolytica* and the probiotic bacteria *Lactobacillus plantarum* (Reyes-Becerril et al., 2021: Soltani et al., 2019).

Numerous studies have demonstrated the antimicrobial activity of the fish skin mucus (Balasubramanian et al., 2012; Dash et al., 2018; Dhanaraj et al., 2009; Fekih-Zaghbib et al., 2023; Guardiola et al., 2014). This antimicrobial activity usually is a result of antimicrobial peptides and factors such as lysozyme. In our study, we tested the antimicrobial activity using a well-known rainbow trout pathogen, *A. salmonicida*, and established that the *B. subtilis* spores increased the bactericidal activity of the intestinal and skin mucus. However, although all other results obtained in this work were confirmed in a second experiment, the increased bactericidal activity of the skin mucus of fish fed the *B. subtilis* spores was not observed in the second experiment. The reason for this weaker effect on skin mucus is unknown, but it has to be taken into account that *Bacillus* sp. are known to produce compounds such as bacteriocins that inhibit the growth of other microorganisms (Banerjee et al., 2017; Cao et al., 2019; Kuebutornye et al., 2020b; Paul and Rahman, 2022; Yi et al., 2018), therefore it might be possible that the increased antibacterial activity of the intestinal mucus is due to these bacterial factors rather than to molecules released by the host. Additionally, it might be possible that the immunomodulatory effects of *B. subtilis* are stronger locally than in a distal mucosal surface such as the skin.

Enzymatic activities related to oxidative status were also evaluated in this study. Ceruloplasmin is a multicopper oxidase, considered a type of plasma antioxidant (Floris et al., 2000), due to its ability to react with and scavenge oxygen species such as SOD does (Das and Sahoo, 2018). Ceruloplasmin is also considered an acute-phase protein, involved in several immune functions which include limiting the dispersion of infectious agents, contributing to tissue damage repair, pathogen killing and having an anti-inflammatory function (Das and Sahoo, 2018; Dautremepuits et al., 2004; Gitlin, 1988). Consequently, ceruloplasmin expression has been reported in mucosal tissues after parasitic and bacterial infection in fish (Chang et al., 2005; Lu et al., 2012). The other two enzymes related to the oxidative response that were evaluated included SOD and peroxidase. SOD catalyses the dismutation of superoxide anion (O^{2-}) to H_2O_2 (Abarike et al., 2018). This H_2O_2 production due to the activity of SOD is fixed by peroxidases, hence producing hypochlorous acid to kill pathogens, and maintaining the redox homeostasis of the immune system (Chen et al., 2020; Nayak, 2010). In the present study, ceruloplasmin and SOD activity were not significantly affected in the mucus of fish fed *B. subtilis* spores, whereas peroxidase activity increased in both cases. In serum, it has been reported that SOD increased in rainbow trout fed with a commercial probiotic mixture containing two *Bacillus* species (Abarike et al., 2018). Surprisingly, our findings contrast those found in tilapia (*O. niloticus*) and red seabream (*Pagrus major*) where SOD activity significantly increased in the serum in tilapia treated with probiotics (*B. subtilis*, *B. coagulans* and *Rhodopseudomonas palustris*) as water additives (Zhou et al., 2010) and red seabream fed with a diet supplemented with *Lactobacillus rhamnosus* and *Lactococcus lactis* (Dawood et al., 2016). In correlation with our results, Wang et al. (2022) reported an increase in the serum peroxidase activity in Nile tilapia after probiotic supplementation in the water. Nevertheless, Zaineldin et al. (2018) reported a decrease in the peroxidase activity in red sea bream after dietary administration of *B. subtilis*. In gilthead seabream fed with inactivated *B. subtilis*, peroxidase activity was not affected in head-kidney leucocytes, however, in serum the activity increased in those fish fed with *B. subtilis* diet (Salinas et al., 2008).

NO is produced mainly by fish granulocytes and macrophages interfering in immune response (Grayfer et al., 2018; Yin et al., 1997). Although NO is a reactive free radical and potentially toxic, it is being known to act as an intercellular messenger helping to defend against pathogens. The production of NO by NO synthase (iNOS) is an important component of host immune responses. The classically activated M1 macrophages possess high levels of the inducible nitric oxide synthase enzyme (iNOS/NOS2). Therefore, iNOS expression serves as a marker of M1 macrophage activation. The parallel production of superoxide and NO can result in the formation of peroxynitrite which is a potent antiparasitic/antimicrobial agent. The fish macrophage iNOS gene is induce by antimicrobial and inflammatory stimuli such as PAMPs, pro-inflammatory cytokines and cleaved transferrin products (Grayfer et al., 2018; Yin et al., 1997). NO has been shown to have potent antimicrobial effects against a relevant number of fish pathogens (Campos-Pérez et al., 2000; Forlenza et al., 2009; Tafalla et al., 1999; Wiegertjes and Forlenza, 2010). Thus, the NO content in skin and intestinal mucus was assessed. In the present study, a downward trend has been found in fish fed with the diet supplemented with probiotic spores when compared to control fish, yet this decrease was only significant in the case of the skin mucus. These results contrast those reported in catla (*Catla catla*) and European sea bass (*Dicentrarchus labrax*) in which the NO content in serum was higher in fish fed with different strains of *Bacillus* species (Das et al., 2013; Monzón-Atienza et al., 2022).

5. Conclusions

In the current study, we have established that the dietary supplementation of rainbow trout for 30 days with *B. subtilis* spores increases protein content, total Ig content, IgM levels, peroxidase and bactericidal activities of both the intestinal and the skin mucus. Our studies provide novel information regarding the effects of *B. subtilis* on the host mucosal immune response, which support the benefits of supplementing the feeds given to farmed fish with *B. subtilis* as a way to increase their immunological status.

Funding

This work was supported by the Spanish Ministry of Science and Innovation [grants number PID2020-113268RB-I00] funded by MCNI/AEI/10.13039/501100011033 and VAPC202240I184.

CRediT authorship contribution statement

Samuel Vicente-Gil: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Silvia Nogales-Merida:** Methodology, Investigation. **Gabriela Gonçalves:** Resources, Methodology, Investigation. **Cláudia R. Serra:** Writing – review &

editing, Resources, Methodology, Conceptualization. **Maria del Camino Ordás:** Methodology, Investigation, Data curation. **Carolina Tafalla:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Patricia Diáz-Rosales:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgements

The authors want to thank Lucia González for technical help.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2024.102253.

References

Abarike, E.D., Cai, J., Lu, Y., Yu, H., Chen, L., Jian, J., Tang, J., Jun, L., Kuebutornye, F. K., 2018. Effects of a commercial probiotic BS containing *Bacillus subtilis* and *Bacillus licheniformis* on growth, immune response and disease resistance in Nile tilapia, *Oreochromis niloticus*. Fish. Shellfish Immunol. 82, 229–238. https://doi.org/10.1016/j.fsi.2018.08.037.

Alonso, S., Castro, M.C., Berdasco, M., de la Banda, I.G., Moreno-Ventas, X., de Rojas, A. H., 2019. Isolation and partial characterization of lactic acid bacteria from the gut microbiota of marine fishes for potential application as probiotics in aquaculture. Probiotics Antimicrob. Proteins 11 (2), 569–579. https://doi.org/10.1007/s12602-018-9439-2.

Aps, L.R., Diniz, M.O., Porchia, B.F., Sales, N.S., Moreno, A.C.R., Ferreira, L.C., 2015. *Bacillus subtilis* spores as adjuvants for DNA vaccines. Vaccine 33 (20), 2328–2334. https://doi.org/10.1016/j.vaccine.2015.03.043.

Azad, M.A.K., Sarker, M., Wan, D., 2018. Immunomodulatory effects of probiotics on cytokine profiles. BioMed. Res. Int., 8063647 https://doi.org/10.1155/2018/8063647.

Bahi, A., Guardiola, F.A., Messina, C., Mahdhi, A., Cerezuela, R., Santulli, A., Bakhrouf, A., Esteban, M.A., 2017. Effects of dietary administration of fenugreek seeds, alone or in combination with probiotics, on growth performance parameters, humoral immune response and gene expression of gilthead seabream (*Sparus aurata* L. Fish. Shellfish Immunol. 60, 50–58. https://doi.org/10.1016/j.fsi.2016.11.039.

Balasubramanian, S., Baby Rani, P., Arul Prakash, A., Prakash, M., Senthilraja, P., Gunasekaran, G., 2012. Antimicrobial properties of skin mucus from four freshwater cultivable fishes (*Catla catla*, *Hypophthalmichthys molitrix*, *Labeo rohita* and *Ctenopharyngodon idella*). Afr. J. Microbiol. Res. 6, 5110–5120. https://doi.org/10.5897/AJMR11.532.

Banerjee, G., Ray, A.K., 2017. The advancement of probiotics research and its application in fish farming industries. Res. Vet. Sci. 115, 66–77. https://doi.org/10.1016/j.rvsc.2017.01.016.

Banerjee, G., Nandi, A., Ray, A.K., 2017. Assessment of hemolytic activity, enzyme production and bacteriocin characterization of *Bacillus subtilis* LR1 isolated from the gastrointestinal tract of fish. Arch. Microbiol. 199 (1), 115–124. https://doi.org/10.1007/s00203-016-1283-8.

Bartels, J., Lopez Castellanos, S., Radeck, J., Mascher, T., 2018. Sporobeads: the utilization of the *Bacillus subtilis* endospore crust as a protein display platform. ACS Synth. Biol. 7 (2), 452–461. https://doi.org/10.1021/acssynbio.7b00285.

Benhamed, S., Guardiola, F.A., Mars, M., Esteban, M.Á., 2014. Pathogen bacteria adhesion to skin mucus of fishes, 25 Vet. Microbiol. 171 (1-2), 1–12. https://doi.org/10.1016/j.vetmic.2014.03.008.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, 7 Anal. Biochem 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Brinchmann, M.F., 2016. Immune relevant molecules identified in the skin mucus of fish using -omics technologies, 21 Mol. Biosyst. 12 (7), 2056–2063. https://doi.org/10.1039/C5MB00890E.

Cabello, F.C., 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ. Microbiol. 8 (7), 1137–1144. https://doi.org/10.1111/j.1462-2920.2006.01054.x.

Cabillon, N.A.R., Lazado, C.C., 2019. Mucosal barrier functions of fish under changing environmental conditions. Fishes 4 (1), 2. https://doi.org/10.3390/fishes4010002.

Campos-Pérez, J.J., Ellis, A.E., Secombes, C.J., 2000. Toxicity of nitric oxide and peroxynitrite to bacterial pathogens of fish. Dis. Aquat. Organ. 43, 109–115. https://doi.org/10.3354/dao043109.

Cao, L., Pan, L., Gong, L., Yang, Y., He, H., Li, Y., Peng, Y., Li, D., Yan, L., Ding, X., Hu, S., Yu, Z., Sun, Y., Huang, W., Hu, Y., Yi, G., Xia, L., 2019. Interaction of a novel *Bacillus velezensis* (BvL03) against *Aeromonas hydrophila in vitro* and *in vivo* in grass carp. Appl. Microbiol. Biotechnol. 103 (21-22), 8987–8999. https://doi.org/10.1007/s00253-019-10096-7.

Cappellozza, B.I., Segura, A., Milora, N., Galschioet, C., Schjelde, M., Copani, G., 2023. Stability of *Bacillus* and *Enterococcus faecium* 669 probiotic strains when added to different feed matrices used in dairy production. Animals 13 (14), 2350. https://doi.org/10.3390/ani13142350.

Cerezuela, R., Guardiola, F.A., Cuesta, A., Esteban, M.Á., 2016. Enrichment of gilthead seabream (*Sparus aurata* L.) diet with palm fruit extracts and probiotics: effects on skin mucosal immunity. Fish. Shellfish Immunol. 49, 100–109. https://doi.org/10.1016/j.fsi.2015.12.028.

Chang, M.C., Nie, P., Liu, G., Song, Y., Gao, Q., 2005. Identification of immune genes in grass carp *Ctenopharyngodon idella* in response to infection of the parasitic copepod *Sinergasilus major*. Parasitol. Res. 96 (4), 224–229. https://doi.org/10.1007/s00436-005-1340-8.

Chen, Z., Ceballos-Francisco, D., Guardiola, F.A., Esteban, M.A., 2020. Dietary administration of the probiotic *Shewanella putrefaciens* to experimentally wounded gilthead seabream (*Sparus aurata* L.) facilitates the skin wound healing, 3 Sci. Rep. 10 (1), 11029. https://doi.org/10.1038/s41598-020-68024-z.

Das, A., Kakhro, K., Chowdhury, S., Kamilya, D., 2013. Effects of potential probiotics *Bacillus amyloliquifaciens* FPTB16 on systemic and cutaneous mucosal immune responses and disease resistance of catla (*Catla catla*). Fish. Shellfish Immunol. 35, 1547–1553. https://doi.org/10.1016/j.fsi.2013.08.022.

Das, S., Sahoo, P.K., 2018. Ceruloplasmin, a moonlighting protein in fish. Fish. Shellfish Immunol. 82, 460–468. https://doi.org/10.1016/j.fsi.2018.08.043.

Dash, S., Das, S.K., Samal, J., Thatoi, H.N., 2018. Epidermal mucus, a major determinant in fish health: a review. Iran. J. Vet. Res. 19 (2), 72–81. https://doi.org/10.22099/IJVR.2018.48499.

Dautremepuits, C., Betoulle, S., Paris-Palacios, S., Vernet, G., 2004. Immunology-related perturbations induced by copper and chitosan in carp (*Cyprinus carpio* L.). Arch. Environ. Contam. Toxicol. 47, 370–378. https://doi.org/10.1007/s00244-004-3115-0.

Dawood, M.A., Koshio, S., Ishikawa, M., Yokoyama, S., El Basuini, M.F., Hossain, M.S., Nhu, T.H., Dossou, S., Moss, A.S., 2016. Effects of dietary supplementation of *Lactobacillus rhamnosus* or/and *Lactococcus lactis* on the growth, gut microbiota and immune responses of red sea bream, *Pagrus major*. Fish. Shellfish Immunol. 49, 275–285. https://doi.org/10.1016/j.fsi.2015.12.047.

Dhanaraj, M., Haniffa, M.A., Arun Singh, S.V., Muthu Ramakrishnan, C., Manikandaraja, D., James Milton, M., 2009. Antibacterial activity of skin and intestinal mucus of five different freshwater fish species viz., *Channa striatus*, *C. micropeltes*, *C. marulius*, *C. punctatus* and *C. gachua*. Malay. J. Sci. 28 (3), 257–262. https://doi.org/10.22452/mjs.vol28no3.3.

Di, J., Chu, Z., Zhang, S., Huang, J., Du, H., Wei, Q., 2019. Evaluation of the potential probiotic *Bacillus subtilis* isolated from two ancient sturgeons on growth performance, serum immunity and disease resistance of *Acipenser dabryanus*. Fish. Shellfish Immunol. 93, 711–719. https://doi.org/10.1016/j.fsi.2019.08.020.

Docando, F., Nuñez-Ortiz, N., Gonçalves, G., Serra, C.R., Gomez-Casado, E., Martín, D., Abós, B., Oliva-Teles, A., Tafalla, C., Díaz-Rosales, P., 2022b. *Bacillus subtilis* expressing the infectious pancreatic necrosis virus VP2 protein retains its immunostimulatory properties and induces a specific antibody response, 1 Front. Immunol. 13, 888311. https://doi.org/10.3389/fimmu.2022.888311.

Docando, F., Nuñez-Ortiz, N., Serra, C.R., Arense, P., Enes, P., Oliva-Teles, A., Díaz-Rosales, P., Tafalla, C., 2022a. Mucosal and systemic immune effects of *Bacillus subtilis* in rainbow trout (*Oncorhynchus mykiss*). Fish. Shellfish Immunol. 124, 142–155. https://doi.org/10.1016/j.fsi.2022.03.040.

Domínguez-Maqueda, M., Cerezo, I.M., Tapia-Paniagua, S.T., De La Banda, I.G., Moreno-Ventas, X., Moriñigo, M.Á., Balebona, M.C., 2021. A tentative study of the effects of heat-inactivation of the probiotic strain *Shewanella putrefaciens* Ppd11 on Senegalese Sole (*Solea senegalensis*) intestinal microbiota and immune response. Microorganisms 9 (4), 808. https://doi.org/10.3390/microorganisms9040808.

Done, H.Y., Venkatesan, A.K., Halden, R.U., 2015. Does the recent growth of aquaculture create antibiotic resistance threats different from those associated with land animal production in agriculture? AAPS J. 17 (3), 513–524. https://doi.org/10.1208/s12248-015-9722-z.

Du, Y., Hu, X., Miao, L., Chen, J., 2022. Current status and development prospects of aquatic vaccines, 10 Front. Immunol. 13, 1040336. https://doi.org/10.3389/fimmu.2022.1040336.

Dunier, M., Siwicki, A.K., 1994. Effects of lindane exposure on rainbow trout (*Oncorhynchus mykiss*) immunity. Ecotoxicol. Environ. Saf. 27 (1), 1–6. https://doi.org/10.1006/eesa.1994.1001.

El-Saadony, M.T., Alagawany, M., Patra, A.K., Kar, I., Tiwari, R., Dawood, M.A., Dhama, K., Abdel-Latif, H.M., 2021. The functionality of probiotics in aquaculture: An overview. Fish. Shellfish Immunol. 117, 36–52. https://doi.org/10.1016/j.fsi.2021.07.007.

Encarnação, P., 2016. Functional feed additives in aquaculture feeds. Aquafeed Formul. 217–237. https://doi.org/10.1016/B978-0-12-800873-7.00005-1.

Fekih-Zaghbib, S., Ksouri, A., Bouhaouala-Zahar, B., 2023. Differences in fish mucus proteomes identify potential antimicrobial peptide biomarkers. Dev. Comp. Immunol. 145, 104730 https://doi.org/10.1016/j.dci.2023.104730.

Floris, G., Medda, R., Padiglia, A., Musci, G., 2000. The physiological significance of ceruloplasmin. Biochem. Pharmacol. 60, 1735–1741. https://doi.org/10.1016/S0006-2952(00)00399-3.

Forlenza, M., Nakao, M., Wibowo, I., Joerink, M., Arts, J.A., Savelkoul, H.F., Wiegertjes, G.F., 2009. Nitric oxide hinders antibody clearance from the surface of *Trypanoplasma borreli* and increases susceptibility to complement-mediated lysis. Mol. Immunol. 46 (16), 3188–3197. https://doi.org/10.1016/j.molimm.2009.08.011.

Galagarza, O.A., Smith, S.A., Drahos, D.J., Eifert, J.D., Williams, R.C., Kuhn, D.D., 2018. Modulation of innate immunity in Nile tilapia (*Oreochromis niloticus*) by dietary supplementation of *Bacillus subtilis* endospores. Fish. Shellfish Immunol. 83, 171–179. https://doi.org/10.1016/j.fsi.2018.08.062.

Gao, Y., Huo, X., Wang, Z., Yuan, G., Liu, X., Ai, T., Su, J., 2021. Oral administration of *Bacillus subtilis* subunit vaccine significantly enhances the immune protection of grass carp against GCRV-II infection. Viruses 14 (1), 30. https://doi.org/10.3390/v14010030.

Garcia, B., Dong, F., Casadei, E., Rességuier, J., Ma, J., Cain, K.D., Castrillo, P.A., Xu, Z., Salinas, I., 2022. A novel organized nasopharynx-associated lymphoid tissue in teleosts that expresses molecular markers characteristic of mammalian germinal centers, 1 J. Immunol. 209 (11), 2215–2226. https://doi.org/10.4049/jimmunol.2200396.

Gatesoupe, F.J., 1999. The use of probiotics in aquaculture. Aquaculture 180, 147–165. https://doi.org/10.1016/S0044-8486(99)00187-8.

Gitlin, J.D., 1988. Transcriptional regulation of ceruloplasmin gene expression during inflammation. J. Biol. Chem. 263, 6281–6287. https://doi.org/10.1016/S0021-9258(18)68783-6.

Gonçalves, G., Santos, R.A., Coutinho, F., Pedrosa, N., Curado, M., Machado, M., Costas, B., Bonneville, L., Serrano, M., Carvalho, A.P., Díaz-Rosales, P., Oliva-Teles, A., Couto, A., Serra, C.R., 2022. Oral vaccination of fish against vibriosis using spore-display technology, 13 Front. Immunol. 13, 1012301. https://doi.org/10.3389/fimmu.2022.1012301.

Grayfer, L., Kerimoglu, B., Yaparla, A., Hodgkinson, J.W., Xie, J., Belosevic, M., 2018. Mechanisms of fish macrophage antimicrobial immunity, 28 Front. Immunol. 9, 1105. https://doi.org/10.3389/fimmu.2018.01105.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S., 1982. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. Anal. Biochem 126, 131–138. https://doi.org/10.1016/0003-2697(82)90118-X.

Guardiola, F.A., Cuesta, A., Abellán, E., Meseguer, J., Esteban, M.A., 2014. Comparative analysis of the humoral immunity of skin mucus from several marine teleost fish. Fish. Shellfish Immunol. 40 (1), 24–31. https://doi.org/10.1016/j.fsi.2014.06.018.

Guardiola, F.A., Bahi, A., Bakhrouf, A., Esteban, M.A., 2017. Effects of dietary supplementation with fenugreek seeds, alone or in combination with probiotics, on gilthead seabream (*Sparus aurata* L.) skin mucosal immunity. Fish. Shellfish Immunol. 65, 169–178. https://doi.org/10.1016/j.fsi.2017.04.014.

Han, S.R., Munang'andu, H.M., Yeo, I.K., Kim, S.H., 2020. *Bacillus subtilis* inhibits viral hemorrhagic septicemia virus infection in olive flounder (*Paralichthys olivaceus*) intestinal epithelial cells, 25 Viruses 13 (1), 28. https://doi.org/10.3390/v13010028.

Hoseinifar, S.H., Mirvaghefi, A., Amoozegar, M.A., Sharifian, M., Esteban, M.Á., 2015. Modulation of innate immune response, mucosal parameters and disease resistance in rainbow trout (*Oncorhynchus mykiss*) upon synbiotic feeding. Fish. Shellfish Immunol. 45 (1), 27–32. https://doi.org/10.1016/j.fsi.2015.03.029.

Hosseini, M., Miandare, H.K., Hoseinifar, S.H., Yarahmadi, P., 2016. Dietary *Lactobacillus acidophilus* modulated skin mucus protein profile, immune and appetite genes expression in gold fish (*Carassius auratus gibelio*). Fish. Shellfish Immunol. 59, 149–154. https://doi.org/10.1016/j.fsi.2016.10.026.

Jiang, Y., Zhou, S., Chu, W., 2019. The effects of dietary *Bacillus cereus* QSI-1 on skin mucus proteins profile and immune response in Crucian Carp (*Carassius auratus gibelio*). Fish. Shellfish Immunol. 89, 319–325. https://doi.org/10.1016/j.fsi.2019.04.014.

Kelly, C., Takizawa, F., Sunyer, J.O., Salinas, I., 2017. Rainbow trout (*Oncorhynchus mykiss*) secretory component binds to commensal bacteria and pathogens, 2 Sci. Rep. 7, 41753. https://doi.org/10.1038/srep41753.

Kuebutornye, F.K.A., Abarike, E.D., Lu, Y., Hlordzi, V., Sakyi, M.E., Afriyie, G., Wang, Z., Li, Y., Xie, C.X., 2020b. Mechanisms and the role of probiotic *Bacillus* in mitigating fish pathogens in aquaculture. Fish. Physiol. Biochem. 46 (3), 819–841. https://doi.org/10.1007/s10695-019-00754-y.

Kuebutornye, F.K.A., Wang, Z., Lu, Y., Abarike, E.D., Sakyi, M.E., Li, Y., Xie, C.X., Hlordzi, V., 2020a. Effects of three host-associated *Bacillus* species on mucosal immunity and gut health of Nile tilapia, *Oreochromis niloticus* and its resistance against *Aeromonas hydrophila* infection. Fish. Shellfish Immunol. 97, 83–95. https://doi.org/10.1016/j.fsi.2019.12.046.

Lazado, C.C., Caipang, C.M.A., 2014. Mucosal immunity and probiotics in fish. Fish. Shellfish Immunol. 39 (1), 78–89. https://doi.org/10.1016/j.fsi.2014.04.015.

Lu, A., Hu, X., Xue, J., Zhu, J., Wang, Y., Zhou, G., 2012. Gene expression profiling in the skin of zebrafish infected with *Citrobacter freundii*. Fish. Shellfish Immunol. 32 (2), 273–283. https://doi.org/10.1016/j.fsi.2011.11.016.

Mantis, N.J., 2019. Role of B cells and antibodies in controlling bacterial pathogens. Encycl. Microbiol. 194–200. https://doi.org/10.1101/cshperspect.a018432.

Martín-Martín, A., Simón, R., Abós, B., Díaz-Rosales, P., Tafalla, C., 2020. Rainbow trout mount a robust specific immune response upon anal administration of thymus-independent antigens. Dev. Comp. Immunol. 109, 103715 https://doi.org/10.1016/j.dci.2020.103715.

McCord, J.M., Fridovich, I., 1969. Superoxide dismutase. J. Biol. Chem. 244 (22), 6049–6055. https://doi.org/10.1016/S0021-9258(18)63504-5.

Modanloo, M., Soltanian, S., Akhlaghi, M., Hoseinifar, S.H., 2017. The effects of single or combined administration of galactooligosaccharide and *Pediococcus acidilactici* on cutaneous mucus immune parameters, humoral immune responses and immune related genes expression in common carp (*Cyprinus carpio*) fingerlings. Fish. Shellfish Immunol. 70, 391–397. https://doi.org/10.1016/j.fsi.2017.09.032.

Monzón-Atienza, L., Bravo, J., Fernández-Montero, L., Charlie-Silva, I., Montero, D., Ramos-Vivas, J., Galindo-Villegas, J., Acosta, F., 2022. Dietary supplementation of *Bacillus velezensis* improves *Vibrio anguillarum* clearance in European sea bass by activating essential innate immune mechanisms. Fish. Shellfish Immunol. 124, 244–253. https://doi.org/10.1016/j.fsi.2022.03.032.

Nayak, S.K., 2010. Probiotics and immunity: a fish perspective. Fish. Shellfish Immunol. 29 (1), 2–14. https://doi.org/10.1016/j.fsi.2010.02.017.

Nayak, S.K., 2021. Multifaceted applications of probiotic *Bacillus* species in aquaculture with special reference to *Bacillus subtilis*. Rev. Aquac. 13 (2), 862–906. https://doi.org/10.1111/raq.12503.

Newaj-Fyzul, A., Al-Harbi, A.H., Austin, B., 2014. Review: developments in the use of probiotics for disease control in aquaculture. Aquaculture (431), 1–11. https://doi.org/10.1002/9781119152125.ch8.

Nigam, A.K., Kumari, U., Mittal, S., Mittal, A.K., 2012. Comparative analysis of innate immune parameters of the skin mucous secretions from certain freshwater teleosts, inhabiting different ecological niches. Fish. Physiol. Biochem. 38 (5), 1245–1256. https://doi.org/10.1007/s10695-012-9613-5.

Olmos, J., Acosta, M., Mendoza, G., Pitones, V., 2020. *Bacillus subtilis*, an ideal probiotic bacterium to shrimp and fish aquaculture that increase feed digestibility, prevent microbial diseases, and avoid water pollution. Arch. Microbiol. 202 (3), 427–435. https://doi.org/10.1007/s00203-019-01757-2.

Ordás, M.C., Ordás, A., Beloso, C., Figueras, A., 2000. Immune parameters in carpet shell clams naturally infected with. Fish. Shellfish Immunol. 10 (7), 597–609. https://doi.org/10.1006/fsim.2000.0274.

Paul, S.I., Rahman, M.M., 2022. Draft genome sequence of *Bacillus subtilis* YBS29, a potential fish probiotic that prevents motile *Aeromonas* septicemia in *Labeo rohita*, 20 Microbiol. Resour. Announc. 11 (10), e0091522. https://doi.org/10.1128/mra.00915-22.

Perdiguero, P., Martín-Martín, A., Benedicenti, O., Díaz-Rosales, P., Morel, E., Muñoz-Atienza, E., García-Flores, M., Simón, R., Soleto, I., Cerutti, A., Tafalla, C., 2019. Teleost IgD^{+}IgM^{-} B cells mount clonally expanded and mildly mutated intestinal IgD responses in the absence of lymphoid follicles, 24 Cell Rep. 29 (13), 4223–4235. https://doi.org/10.1016/j.celrep.2019.11.101.

Peskin, A.V., Winterbourn, C.C., 2000. A microtiter plate assay for superoxide dismutase using a water-soluble tetrazolium salt (WST-1). Clin. Chim. Acta 293 (1-2), 157–166. https://doi.org/10.1016/S0009-8981(99)00246-6.

Quade, M.J., Roth, J.A., 1997. A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Vet. Immunol. Immunopathol. 58 (3-4), 239–248. https://doi.org/10.1016/S0165-2427(97)00048-2.

Rangel, F., Monteiro, M., Santos, R.A., Ferreira-Martins, D., Cortinhas, R., Gasco, L., Gai, F., Pousão-Ferreira, P., Couto, A., Oliva-Teles, A., Serra, C.R., Enes, P., 2024. Novel chitinolytic *Bacillus* spp. increase feed efficiency, feed digestibility, and survivability to *Vibrio anguillarum* in European seabass fed with diets containing *Hermetia illucens* larvae meal. Aquaculture 579, 740258. https://doi.org/10.1016/j.aquaculture.2023.740258.

Reyes-Becerril, M., Alamillo, E., Angulo, C., 2021. Probiotic and immunomodulatory activity of marine yeast *Yarrowia lipolytica* strains and response against *Vibrio parahaemolyticus* in fish. Probiotics Antimicrob. Proteins 13, 1292–1305. https://doi.org/10.1007/s12602-021-09769-5.

Rohani, M.F., Islam, S.M., Hossain, M.K., Ferdous, Z., Siddik, M.A., Nuruzzaman, M., Padeniya, U., Brown, C., Shahjahan, M., 2022. Probiotics, prebiotics and synbiotics improved the functionality of aquafeed: upgrading growth, reproduction, immunity and disease resistance in fish. Fish. Shellfish Immunol. 120, 569–589. https://doi.org/10.1016/j.fsi.2021.12.037.

Salinas, I., 2015. The mucosal immune system of teleost fish. Biol. (Basel) 4 (3), 525–539. https://doi.org/10.3390/biology4030525.

Salinas, I., Abelli, L., Bertoni, F., Picchietti, S., Roque, A., Furones, D., Cuesta, A., Meseguer, J., Esteban, M.A., 2008. Monospecies and multispecies probiotic formulations produce different systemic and local immunostimulatory effects in the gilthead seabream (*Sparus aurata* L.). Fish. Shellfish Immunol. 25 (1-2), 114–123. https://doi.org/10.1016/j.fsi.2008.03.011.

Salinas, I., Fernández-Montero, A., Ding, Y., Sunyer, J.O., 2021. Mucosal immunoglobulins of teleost fish: a decade of advances. Dev. Comp. Immunol. 121, 104079 https://doi.org/10.1016/j.dci.2021.104079.

Sanahuja, I., Fernández-Alacid, L., Ordóñez-Grande, B., Sánchez-Nuño, S., Ramos, A., Araujo, R.M., Ibarz, A., 2019. Comparison of several non-specific skin mucus immune defences in three piscine species of aquaculture interest. Fish. Shellfish Immunol. 89, 428–436. https://doi.org/10.1016/j.fsi.2019.04.008.

Santos, L., Ramos, F., 2018. Antimicrobial resistance in aquaculture: current knowledge and alternatives to tackle the problem. Int. J. Antimicrob. Agents 52 (2), 135–143. https://doi.org/10.1016/j.ijantimicag.2018.03.010.

Santos, R.A., Monteiro, M., Rangel, F., Jerusik, R., Saavedra, M.J., Carvalho, A.P., Oliva-Teles, A., Serra, C.R., 2021. *Bacillus* spp. inhibit *Edwardsiella tarda* quorum-sensing and fish infection, 23 Mar. Drugs 19 (11), 602. https://doi.org/10.3390/md19110602.

Serra, C.R., Earl, A.M., Barbosa, T.M., Kolter, R., Henriques, A.O., 2014. Sporulation during growth in a gut isolate of *Bacillus subtilis*. J. Bacteriol. 196 (23), 4184–4196. https://doi.org/10.1128/jb.01993-14.

Setlow, P., 2014. Spore resistance properties. Microbiol. Spectr. 2 (5) https://doi.org/10.1128/microbiolspec.tbs-0003-2012.

Simón, R., Docando, F., Nuñez-Ortiz, N., Tafalla, C., Díaz-Rosales, P., 2021. Mechanisms used by probiotics to confer pathogen resistance to teleost fish. Front. Immunol. 12, 653025 https://doi.org/10.3389/fimmu.2021.653025.

Soltani, M., Pakzad, K., Taheri-Mirghaed, A., Mirzargar, S., Shekarabi, S.P.H., Yosefi, P., Soleymani, N., 2019. Dietary application of the probiotic *Lactobacillus plantarum* 426951 enhances immune status and growth of rainbow trout (*Oncorhynchus mykiss*) vaccinated against *Yersinia ruckeri*. Probiotics Antimicrob. Proteins 11, 207–219. https://doi.org/10.1007/s12602-017-9376-5.

Soltis, R.D., Hasz, D.E., 1983. The effect of serum immunoglobulin concentration on immune complex detection by polyethylene glycol, 25 J. Immunol. Methods 57 (1-3), 275–282. https://doi.org/10.1016/0022-1759(83)90087-X.

de Souza, R.D., Batista, M.T., Luiz, W.B., Cavalcante, R.C.M., Amorim, J.H., Bizerra, R.S. P., Martins, E.G., de Souza Ferreira, L.C., 2014. *Bacillus subtilis* spores as vaccine adjuvants: further insights into the mechanisms of action. PLoS ONE 9 (1), e87454. https://doi.org/10.1371/journal.pone.0087454.

Subramanian, S., MacKinnon, S.L., Ross, N.W., 2007. A comparative study on innate immune parameters in the epidermal mucus of various fish species. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 148 (3), 256–263. https://doi.org/10.1016/j.cbpb.2007.06.003.

Subramanian, S., Ross, N.W., MacKinnon, S.L., 2008. Comparison of antimicrobial activity in the epidermal mucus extracts of fish. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 150 (1), 85–92. https://doi.org/10.1016/j.cbpb.2008.01.011.

Sun, R., Zhang, M., Chen, H., Wei, Y., Ning, D., 2020. Germination-arrest *Bacillus subtilis* spores as an oral delivery vehicle of grass carp reovirus (GCRV) Vp7 antigen augment protective immunity in grass carp (*Ctenopharyngodon idella*), 14 Genes (Basel) 11 (11), 1351. https://doi.org/10.3390/genes11111351.

Sunyer, J.O., 2012. Evolutionary and functional relationships of B cells from fish and mammals: insights into their novel roles in phagocytosis and presentation of particulate antigen, 1 Infect. Disord. Drug Targets (Former. Curr. Drug Targets Infect. Disord.) 12 (3), 200–212. https://doi.org/10.2174/187152612800564419.

Tafalla, C., Figueras, A., Novoa, B., 1999. Role of nitric oxide on the replication of viral haemorrhagic septicemia virus (VHSV), a fish rhabdovirus. Vet. Immunol. Immunopathol. 72, 249–256. https://doi.org/10.1016/S0165-2427(99)00109-9.

Tarkhani, R., Imani, A., Hoseinifar, S.H., Ashayerizadeh, O., Moghanlou, K.S., Manaffar, R., Doan, H.V., Reverter, M., 2020. Comparative study of host-associated and commercial probiotic effects on serum and mucosal immune parameters, intestinal microbiota, digestive enzymes activity and growth performance of roach (*Rutilus caspicus*) fingerlings. Fish. Shellfish Immunol. (98), 661–669. https://doi.org/10.2478/aoas-2021-0026.

Vallejos-Vidal, E., Reyes-López, F., Teles, M., MacKenzie, S., 2016. The response of fish to immunostimulant diets. Fish. Shellfish Immunol. 56, 34–69. https://doi.org/10.1016/j.fsi.2016.06.028.

Van Doan, H., Hoseinifar, S.H., Khanongnuch, C., Kanpiengjai, A., Unban, K., Kim, V.V., Srichaiyo, S., 2018. Host-associated probiotics boosted mucosal and serum immunity, disease resistance and growth performance of Nile tilapia (*Oreochromis niloticus*). Aquaculture (491), 94–100. https://doi.org/10.1007/s12602-019-09554-5.

Van Doan, H., Hoseinifar, S.H., Naraballobh, W., Paolucci, M., Wongmaneeprateep, S., Charoenwattanasak, S., Dawood, M.A.O., Abdel-Tawwab, M., 2021. Dietary inclusion of watermelon rind powder and *Lactobacillus plantarum*: effects on Nile tilapia's growth, skin mucus and serum immunities, and disease resistance. Fish. Shellfish Immunol. 116, 107–114. https://doi.org/10.1016/j.aqrep.2022.101271.

Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W., 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. Rev. 64 (4), 655–671. https://doi.org/10.1128/mmbr.64.4.655-671.2000.

Wang, M., Lv, C., Chen, Y., Bi, X., Yang, D., Zhao, J., 2022. Effects of the potential probiotic *Bacillus subtilis* D1-2 on growth, digestion, immunity and intestinal flora in juvenile sea cucumber, *Apostichopus japonicus*. Fish. Shellfish Immunol. 124, 12–20. https://doi.org/10.1016/j.fsi.2022.03.043.

Wiegertjes, G., Forlenza, M., 2010. Nitrosative stress during infection-induced inflammation in fish: lessons from a host-parasite infection model. Curr. Pharm. Des. 16 (38), 4194–4202. https://doi.org/10.2174/138161210794519138.

Won, S., Hamidoghli, A., Choi, W., Park, Y., Jang, W.J., Kong, I.S., Bai, S.C., 2020. Effects of *Bacillus subtilis* WB60 and *Lactococcus lactis* on growth, immune responses, histology and gene expression in Nile tilapia, *Oreochromis niloticus*, 1 Microorganisms 8 (1), 67. https://doi.org/10.3390/microorganisms8010067.

Xu, Z., Parra, D., Gómez, D., Salinas, I., Zhang, Y.A., von Gersdorff Jørgensen, L., Heinecke, R.D., Buchmann, K., LaPatra, S.E., Sunyer, J.O., 2013. Teleost skin, an ancient mucosal surface that elicits gut-like immune responses, 6 Proc. Natl. Acad. Sci. USA 110 (32). https://doi.org/10.1073/pnas.1304319110.

Xu, Z., Takizawa, F., Parra, D., Gómez, D., von Gersdorff Jørgensen, L., LaPatra, S.E., Sunyer, J.O., 2016. Mucosal immunoglobulins at respiratory surfaces mark an ancient association that predates the emergence of tetrapods, 12 Nat. Commun. 7, 10728. https://doi.org/10.1038/ncomms10728.

Xu, Z., Takizawa, F., Casadei, E., Shibasaki, Y., Ding, Y., Sauters, T.J.C., Yu, Y., Salinas, I., Sunyer, J.O., 2020. Specialization of mucosal immunoglobulins in pathogen control and microbiota homeostasis occurred early in vertebrate evolution, 7 Sci. Immunol. 5 (44), eaay3254. https://doi.org/10.1126/sciimmunol.aay3254.

Yi, Y., Zhang, Z., Zhao, F., Liu, H., Yu, L., Zha, J., Wang, G., 2018. Probiotic potential of *Bacillus velezensis* JW: antimicrobial activity against fish pathogenic bacteria and immune enhancement effects on *Carassius auratus*. Fish. Shellfish Immunol. 78, 322–330. https://doi.org/10.1016/j.fsi.2018.04.055.

Yin, Z., Lam, T., Sin, Y., 1997. Cytokine-mediated antimicrobial immune response of catfish, *Clarias gariepinus*, as a defence against *Aeromonas hydrophila*. Fish. Shellfish Immunol. 7 (2), 93–104. https://doi.org/10.1006/fsim.1996.0066.

Yu, Y., Wang, Q., Huang, Z., Ding, L., Xu, Z., 2020. Immunoglobulins, mucosal immunity and vaccination in teleost fish. Front. Immunol. 11, 567941 https://doi.org/10.3389/fimmu.2020.567941.

Yu, Y.Y., Kong, W.G., Xu, H.Y., Huang, Z.Y., Zhang, X.T., Ding, L.G., Dong, S., Yin, G.M., Dong, F., Yu, W., Cao, J.F., Meng, K.F., Liu, X., Fu, L., Zhang, X.Z., Zhang, Y.A., Sunyer, J.O., Xu, Z., 2019. Convergent evolution of mucosal immune responses at the buccal cavity of teleost fish, 27 iScience 19, 821–835. https://doi.org/10.1016/j.isci.2019.08.034.

Zahl, I.H., Samuelsen, O., Kiessling, A., 2012. Anaesthesia of farmed fish: implications for welfare. Fish. Physiol. Biochem. 38, 201–218. https://doi.org/10.1007/s10695-011-9565-1.

Zaineldin, A.I., Hegazi, S., Koshio, S., Ishikawa, M., Bakr, A., El-Keredy, A.M., Dawood, M.A., Dossou, S., Wang, W., Yukun, Z., 2018. *Bacillus subtilis* as probiotic candidate for red sea bream: Growth performance, oxidative status, and immune response traits. Fish. Shellfish Immunol. 79, 303–312. https://doi.org/10.1016/j.fsi.2018.05.035.

Zhang, N., Zhang, X.J., Chen, D.D., Sunyer, J.O., Zhang, Y.A., 2017. Molecular characterization and expression analysis of three subclasses of IgT in rainbow trout (*Oncorhynchus mykiss*). Dev. Comp. Immunol. 70, 94–105. https://doi.org/10.1016/j.dci.2017.01.001.

Zhang, Q., Yu, H., Tong, T., Tong, W., Dong, L., Xu, M., Wang, Z., 2014. Dietary supplementation of *Bacillus subtilis* and fructooligosaccharide enhance the growth, non-specific immunity of juvenile ovate pompano, *Trachinotus ovatus* and its disease resistance against *Vibrio vulnificus*. Fish. Shellfish Immunol. 38 (1), 7–14. https://doi.org/10.1016/j.fsi.2014.02.008.

Zhang, X.T., Yu, Y.Y., Xu, H.Y., Huang, Z.Y., Liu, X., Cao, J.F., Meng, K.F., Wu, Z.B., Han, G.K., Zhan, M.T., Ding, L.G., Kong, W.G., Li, N., Takizawa, F., Sunyer, J.O., Xu, Z., 2021. Prevailing role of mucosal Igs and B cells in teleost skin immune responses to bacterial infection, 1 J. Immunol. 206 (5), 1088–1101. https://doi.org/10.4049/jimmunol.2001097.

Zhang, Y.A., Salinas, I., Li, J., Parra, D., Bjork, S., Xu, Z., LaPatra, S.E., Bartholomew, J., Sunyer, J.O., 2010. IgT, a primitive immunoglobulin class specialized in mucosal immunity. Nat. Immunol. 11 (9), 827–835. https://doi.org/10.1038/ni.1913.

Zhou, X., Tian, Z., Wang, Y., Li, W., 2010. Effect of treatment with probiotics as water additives on tilapia (*Oreochromis niloticus*) growth performance and immune response. Fish. Physiol. Biochem. 36, 501–509. https://doi.org/10.1007/s10695-009-9320-z.