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Interactions of head-kidney leucocytes from giant grouper, *Epinephelus lanceolatus*, with pathogenic *Streptococcus agalactiae* strains from marine and terrestrial origins

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ABSTRACT

Streptococcus agalactiae (Group B *Streptococcus*, GBS) is emerging as a genetically diverse species infecting farmed and wild fish, including commercially and culturally important groupers. To better understand how *S. agalactiae* are pathogenic in fish, we investigated interactions between isolates from fish and terrestrial hosts and the cellular immune system of Queensland grouper *Epinephelus lanceolatus* using flow cytometry. Adherent head-kidney leucocytes (HKL) from Queensland grouper displayed two main cell populations with distinct forward and side scatter by flow cytometry. The population of smaller and less complex cells (P1) was composed of monocytes, lymphocytes and thrombocytes, while the population of primarily larger and more complex cells (P2) comprised predominantly of macrophages and neutrophils. The cells in P2 had higher phagocytic index and capacity when incubated with fluorescent latex beads. HKL were activated by phorbol myristate acetate (PMA) but were unresponsive to lipopolysaccharide (LPS) and peptidoglycan (PTG), suggesting the absence of specific receptors on the surface of these cells for these ligands or a requirement for intermediates. In *in vitro* phagocytosis assays, all fish isolates of GBS activated a respiratory burst in P2 indicated by significant production of intracellular reactive oxygen species (ROS). Similarly, dog and cat isolates of different serotype and sequence type also induced ROS production in grouper HKL. However, human, crocodile and bovine isolates of GBS did not elicit significant ROS in HKL although they coincided with the highest phagocytic index. This suggests that these strains are capable of quenching ROS production. Terrestrial isolates significantly increased mortality of Queensland grouper leucocytes *in vitro*, aligned with a more diverse repertoire of cellular toxins in these strains. Opsonisation of a marine strain and terrestrial strain of GBS with antiserum raised against the marine strain resulted in an increase in ROS production by HKL in both cases although there was low antigenic cross reactivity between the two strains by flow cytometry, reflecting their diverse serotypes (Ib vs III). However, pre-incubation of either strain with normal serum from grouper also increased ROS production of HKL suggesting other opsonins may be involved. Based on these results it appears that piscine and terrestrial GBS isolates have contrasting strategies when interacting with the cellular immune system of Queensland grouper; the former seemingly evading phagocytosis, whilst the latter are readily phagocytosed but counteract ROS production.

1. Introduction

Teleosts (bony fish) are the earliest vertebrate group that have an immune system comparable to higher vertebrates [1]. They possess a repertoire of non-specific and specific humoral and cell-mediated mechanisms to resist bacterial diseases [2]. In order to infect the host and disseminate in the organs and tissues, bacterial pathogens must defeat or elude these defence mechanisms. The major interface between host and bacterial pathogen is the innate cellular immune system, orchestrated by phagocytic cells, including macrophages and neutrophils that act collaboratively with other leucocyte populations to play a central role in both innate and adaptive immunity [2–4]. The cephalic portion of the kidney (pronephros or head-kidney) is a major haematopoietic tissue in fish where progenitor cells, from which many of these

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leucocyte populations are derived, are present [5]. Macrophages are professional phagocytic cells specialised in killing invading micro-organisms. In fish, they are found in connective and other tissues and organs and are the predominant phagocytic cells [6]. Phagocytosis is mediated by interaction between pattern recognition receptors (PRRs), either directly or via intermediates, and pathogen/microbe associated molecular patterns (PAMPs/MAMPs) such as lipopolysaccharide (LPS), peptidoglycan (PTG) and mannose. Following phagocytosis, macrophages enter a state of heightened respiratory activity termed respiratory burst, during which molecular oxygen is reduced to superoxide anion (O_2^-) via the macrophage membrane enzyme nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase). Although superoxide anion has low redox potential in highly polar environments, it is active in hydrophobic regions such as at the membrane. Moreover, it is a precursor for the production of other reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH^-$), single oxygen, and hypohalites [6,7]. Together, these comprise an important microbicidal component of the cellular innate defence [6,8]. Phagocytosis and downstream respiratory burst activity have been widely used in a number of fish species as a proxy to measure the intensity of the cellular innate immune response in response to various stimuli such as pathogens, immuno-modulators and environmental stress [9–12].

Recently, *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) has been identified as a cause of mortality in wild Queensland grouper (*Epinephelus lanceolatus*) and in other marine teleost and elasmobranch species [13,14]. Moreover, susceptibility of Queensland grouper to infection by GBS via different routes of exposures has been demonstrated, confirming the previous hypothesis that *S. agalactiae* was causative for the wild fish mortality [13,15]. Recent genome sequencing has indicated that GBS isolates causing mortalities in these wild fish cases are substantially different from their terrestrial counterparts [16,17], although strains belonging to clonal complexes associated with human colonisation have been found in diseased fish [18]. Therefore, comparison of how representatives of the fish pathogenic ST-261 and isolates from terrestrial animals counteract phagocytic cells in order to colonise and cause disease may provide vital clues to key processes in pathogenesis in fish. In the present study, we use flow cytometry to investigate phagocyte functions in response to GBS by challenging primary leucocyte cultures from Queensland grouper head-kidney (HKL) with different strains of GBS isolated from marine and terrestrial hosts.

2. Materials and methods

2.1. Bacterial isolates

Strains were chosen based on differences observed from multilocus sequence typing (MLST), capsular polysaccharide (CPS) molecular serotyping and whole genome sequencing [17]. Five marine strains were isolated from wild Queensland groupers and mullet. An additional four terrestrial strains were isolated from mammals including dog, cat, cow and human, and one strain was isolated from saltwater crocodile (Table 1).

2.2. Experimental fish and husbandry

Queensland grouper juveniles (*E. lanceolatus*), weighing approximately 30 g, were obtained from Northern Fisheries Centre (NFC, Cairns, QLD, Australia) and held in seawater at 35 parts per thousand salinity in 100 L plastic circular tanks. Aquaria were organized into two banks of four tanks supplied by a common recirculation system comprising of 2 × 250 L sump, 2 × 50 L bio-filter, and a protein skimmer. The temperature was maintained at 28 °C by a heating and cooling system with a recirculation rate of approximately 120 L per hour. Fish were fed a maintenance diet comprising a commercial 4 mm floating diet twice a day (Pelagic, Ridley Aqua Feed, Narangba, QLD), equivalent to 5% body weight. Water exchange (15%) was performed every week and water quality was checked for ammonium, nitrite, nitrate and pH levels on a daily basis. Fish were graded by size throughout the experiment in order to prevent aggression, social hierarchies and cannibalism.

2.3. Isolation, preparation and culture of head-kidney leucocytess

Head-kidney leucocytes (HKL) were collected from euthanized juvenile Queensland groupers essentially as described by Secombes [6] with some modifications. Briefly, following euthanasia by overdose of anaesthetic (Aqui-S), fish were completely exsanguinated by bleeding from the caudal vein followed by sectioning of the gills. Subsequently, an incision was made ventrally to open the peritoneal cavity. The swim bladder and other organs were removed aseptically in order to expose the head-kidney, which was dissected out and placed in L-15 Leibovitz medium containing phenol red (Invitrogen, Melbourne, Australia), supplemented with heat-inactivated (HI, 55 °C for 30 min) 2% foetal bovine serum (FBS; Bovogen, Victoria, Australia), 100 units mL^{-1} penicillin streptomycin (P/S) and 10 units mL^{-1} heparin. Head-kidney was cut into small pieces and gently pushed through a sterile 100 μm cell strainer with the plunger of a 1 mL tuberculin syringe into a sterile Petri dish to obtain a single cell suspension in supplemented medium. The resultant disaggregated kidney suspension was layered onto a 34% (1.050 g mL^{-1})/51% (1.072 g mL^{-1}) v/v Percoll gradient and centrifuged at 600 × *g* for 30 min at 23 °C. The leucocyte fractions lying at the 34–51% interface were collected, washed in L-15 containing 0.1% FBS (L-15 (0.1%), centrifuged at 400 × *g* for 10 min and resuspended in L-15 (0.1%). Total number and percentage of viable leucocytes was determined by exclusion of propidium iodide (PI) positive cells using epifluorescence microscopy (Texas-red: U-MWIY, excitation filter BP545-580, emission filter 610IF, dichromatic filter 600, Olympus). Viable leucocytes were counted on a haemocytometer then plated, when necessary, at a concentration of 5×10^6 cells mL^{-1} in 24 well plates, 1 mL per well, for a minimum of 4 h in L-15 (0.1% FBS).

Table 1
Streptococcus agalactiae (Group B *Streptococcus*, GBS) isolates from marine, terrestrial animals and humans used in the *in vitro* cellular immune assays.

Strain	Genus	species	Host	Scientific name	Year	Tissue/organ	Geographic origin
QMA0284	*Streptococcus*	*agalactiae*	Grouper 1	*Epinephelus lanceolatus*	2008	Eye	Cairns
QMA0285	*Streptococcus*	*agalactiae*	Grouper 2	*E. lanceolatus*	2008	Head-kidney	Cairns
QMA0281	*Streptococcus*	*agalactiae*	Grouper 5	*E. lanceolatus*	2010	Heart	Townsville
QMA0368	*Streptococcus*	*agalactiae*	Grouper 6	*E. lanceolatus*	2010	Eye	Gulf of Carpentaria
QMA0274	*Streptococcus*	*agalactiae*	Squaretail Mullet	*Ellochelon vaigiensis*	2009	Heart	Cairns
QMA0336	*Streptococcus*	*agalactiae*	Crocodile	*Crocodylus porosus*	2010	Joint	Darwin
QMA0355	*Streptococcus*	*agalactiae*	Human foot	*Homo sapiens*	2011	Foot	Townsville
QMA0300	*Streptococcus*	*agalactiae*	Dog	*Canis lupus*	2008	Canine urine	unknown
QMA0303	*Streptococcus*	*agalactiae*	Cat	*Felis catus*	2009	Feline left fore paw	unknown
QMA0306	*Streptococcus*	*agalactiae*	Cow	*Bos taurus*	2005	Bovine milk	unknown

Leucocytes showed viability of 95% or above for cell samples used in this study. The plates were then washed to remove non-adherent cells and fresh culture medium (L-15 10% FBS) added to the wells. The cells were then incubated at 28 °C overnight before being used for subsequent assays the following day.

2.4. Cell identification and characterisation by light microscopy

The different leucocyte sub-populations isolated from Queensland grouper head-kidney were characterised based on their morphology using microscopy (cytospin preparations). After separation on a Percoll gradient, both top and bottom layers were carefully collected and cells diluted to give a final concentration of 300,000 cells mL^{-1}. Cell cytospins were prepared on glass slides using 100 µL of cell suspension with a Tharmac, Cellspin II cytospin centrifuge (3 min, 140 × *g* for 3 min). Air-dried slides were fixed for 5 s in 100% methanol then stained with Hemacolor (Merck). Slides were rinsed in phosphate buffered saline (PBS, pH 7.4; AMRESCO) and left to air-dry at room temperature before being mounted with Permount mounting medium (Fisher Scientific) for observation by light microscopy.

2.5. Spectrophotometric determination of leucocyte activation

The responses of HKL to the PAMPs LPS and PTG, and the mitogen and specific activator of protein kinase C (PKC), phorbol myristate acetate (PMA), were measured quantitatively via the detection of intracellular ROS with the cell-permeable non-fluorescent probe 2′,7′-dichlorodihydrofluorescin-diacetate (DCFH-DA). Briefly, DCFH-DA diffuses rapidly into the cells and is de-esterified (deacetylated) by cellular esterases to non-fluorescent 2′,7′-dichlorodihydrofluorescin (DCFH), which is oxidised by ROS into the highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF). The fluorescence intensity of DCF is assumed to be directly proportional to the concentration of hydrogen peroxide produced within the cell cytoplasm [19,20].

HKL from Queensland grouper were seeded into 96-well black flat-bottom plates (250,000 cells mL^{-1}) and left to attach in L-15 (0.1% FBS) for 4 h at 28 °C. Subsequently supernatant was removed and leucocytes were pre-incubated with DCFH-DA (Sigma, Castle Hill, Australia) (10 µM) in L-15 without phenol-red or serum, to minimize extracellular hydrolysis of the dye, and for 30 min, to allow penetration of the dye into the cells. After 30 min, HKL were supplemented in triplicate with the following treatments: LPS 1, 10, and 100 µg mL^{-1}, PMA 10, 100, and 1000 ng mL^{-1}, or PTG 1, 10, and 100 µg mL^{-1} (Sigma, Castle Hill, Australia). The negative control consisted of leucocytes left unstimulated, and supplemented with L−15 medium and DCFH-DA. The background noise was obtained with cells incubated without DCFH-DA. When all treatments were added, the plate was incubated at 28 °C for 2 h and relative fluorescence unit (RFU) was measured with a Fluostar Optima plate reader (BMG Labtech, Melbourne, Australia) (excitation: 485 nm, emission: 520 nm).

2.6. Flow cytometry

2.6.1. Analysis and sorting of HKL by flow cytometry

The BD FACSAria™ II cell sorting flow cytometer (BD Biosciences, NSW, Australia) was used to collect and analyse data on cellular functions of specific sub-populations of leucocytes in the presence or absence of different pathogenic isolates of GBS. Samples were prepared as described above, and run on the cytometer collecting 10,000 cells in logarithmic mode from each sample using the FACSDiva™ software version 6.1.3 (BD Biosciences). Data were collected for forward scatter (FSC) and side scatter (SSC) representing the size and granularity of the cells respectively. Due to the presence of very large cells in our samples, a neutral density (ND) filter in front of the forward scatter was used to visualize all the cells in the scatter plot. Gating of the cells was performed to exclude debris and dead cells from subsequent analysis using SyBr green and PI respectively (Life Technologies, VIC, Australia).

Gates around the two main populations observed on the scatter plot (SSC vs. FSC) were created and populations sorted in separate tubes. Cytospin preparations were made with the sorted populations as previously described and stained using Hemacolor stain for morphological analysis.

2.6.2. Flow cytometric assay of phagocytosis

The preliminary assessment of HKL phagocytic rate and capacity was performed using fluorescent latex beads by microscopy and flow cytometry. The beads (Fluoresbrite ® Yellow Green carboxylate microspheres 0.2 µm; ~5.68×10^{12} particles mL^{-1}; 2.65% latex, Polysciences, Inc.) were opsonised using a 10,000-fold dilution in PBS supplemented with 10% heated inactivated (HI) Queensland grouper serum. The leucocyte concentration was adjusted to 2.5×10^6 cells per well (500 µL) and the medium was changed to 10%FBS/L-15 containing opsonised beads at a cell:bead ratio of 1:10 per well in 24-well culture plates (Greiner). The plate was then centrifuged at 400 × *g*, 23 °C for 5 min to bring the beads and cells into contact (acceleration 6, brake 4, Eppendorf RC5810 centrifuge), as modified from Bassity and Clark [21]. Following 2 h incubation with beads at 28 °C, the cell suspensions were removed and the wells were washed twice with PBS. Adherent cells were detached by trypsinisation for 2 min using 200 µL of trypsin-EDTA per well (Invitrogen, Melbourne, Australia). The reaction was stopped by addition of 800 µL of PBS with 10% FBS. The cells were transferred to 5 mL polystyrene falcon tube (BD, NSW, Australia) and put on ice to stop the cell activity until data were acquired on the BD FACSAria™ II for green fluorescence (detected with 530/30 bandpass filter; FITC) from 488 nm blue argon-ion laser excitation. Phagocytic rate was expressed as the percentage of total cells with ingested beads, while the phagocytic index was defined as the average number of fluorescent beads engulfed per cell. A negative control consisting of a cell suspension without beads was used to determine fluorescence threshold. Cytospin preparations were made and mounted using DABCO anti-fading medium (Sigma, Castle Hill, Australia) for microscopy morphological observations.

Further characterisation of HKL phagocytic capability was performed using a selection of marine and terrestrial GBS strains (Table 1) and analysed by flow cytometry. Briefly, bacterial suspensions in late exponential phase were adjusted to an optical density at 600 nm (OD_{600}) of 1 (~10^8 bacteria mL^{-1}). They were then stained with 0.5 µM BacLight Green (BLG, Thermo, Melbourne Australia) for 15 min at room temperature (RT), rinsed five times in PBS before being diluted 100-fold in 10% FBS/L-15 phenol red. Fluorescently labelled bacteria were added to the cells to give a final multiplicity of infection (MOI) of 1. Plates were centrifuged at 400 × *g* to bring bacterial into contact with the cell monolayers. After incubation for 2 h at 28 °C, the cells were washed once, detached from the wells with trypsin-EDTA, and transferred to 5 mL falcon tubes on ice to stop the cell activity until flow cytometry analysis, measuring the fluorescence emission at 530 nm using 488 nm excitation laser.

2.6.3. Flow cytometric assay of ROS production

Cellular oxidative activity of Queensland grouper HKL in response to different isolates of GBS (Table 1) was evaluated by flow cytometry.

To avoid overstimulation of the cells and in order to detect maximum activity of the leucocytes in response to the bacteria, a pilot experiment was designed to establish the effect of different MOI (bacteria to HKL cell ratio) on the production of ROS (using two representative strains of *S. agalactiae*, QMA0285 and QMA0355). The different MOI used were 10, 1 and 0.1 bacteria per cell corresponding respectively to a 1:10, 1:100 and 1:1000 dilution from a bacterial suspension standardised to an optical density (OD_{600}) of 1.

In brief, after HKL isolation and incubation overnight at 28 °C in 24-well plate, the different GBS isolates were added to the cells at MOI = 1 in phenol red- and serum-free L-15 with DCFH-DA (10 µM). Several

controls were included in all experiments for the respiratory burst analyses: leucocytes stimulated with PMA were used as positive controls, unstimulated cells incubated in L-15 with DCFH-DA was used to define the oxidative activity in resting cells, untreated leucocytes without stimulants or DCFH-DA served for detection of any possible auto-fluorescence from the leucocytes. After 2 h incubation, samples were put on ice to stop the reaction until being analysed by flow cytometry measuring the fluorescence emission at 530 nm using a 488 nm excitation laser.

2.6.4. Flow cytometric assay for cell viability and apoptosis

Cell viability was assayed using the Alexa Fluor® 488 annexin V/PI dead cell apoptosis kit for flow cytometry (Invitrogen, Melbourne, Australia), according to the manufacturer instructions. Briefly, HKL were incubated with different GBS isolates (Table 1) for 2 h. Positive controls were prepared for both annexin V and PI including camptothecin (Sigma, Castle Hill, Australia), a potent inducer of apoptosis, for annexin V and fixed leucocytes in 4% formaldehyde for PI. Negative control consisted of cells without bacteria or inducing agents. Stained cells were analysed as soon as possible by flow cytometry, measuring green and red fluorescence emissions at 530 and 610 nm using the 488 and 561 nm excitation lasers respectively. Flow cytometry results were confirmed by viewing the cells under a fluorescence microscope, using filters appropriate for fluorescein (FITC) and PI (Texas Red).

2.7. Vaccine preparation, fish vaccination and analysis of sera for specific and cross-reactive antibody response by enzyme-linked immunosorbent assay (ELISA)

A formalin-killed bacterin vaccine based on strain QMA0285 isolated from wild Queensland grouper was prepared as previously described [22]. Cohorts of fish were anaesthetised with Aqui-S and vaccinated by IP injection using a calibrated self-refilling syringe (Socorex, Switzerland) with 100 µL dose per fish of either the vaccine (adjuvanted by emulsifying in an equal volume of Freund's incomplete adjuvant, FIA), PBS or FIA used as controls. After 900 degree-days (32 days at 28 °C) to allow development of immunity, all fish were euthanized by lethal overdose of Aqui-S and exsanguinated by caudal venipuncture. Blood was allowed to clot at 4 °C overnight, and sera were separated by centrifugation (5000 × *g*, 15 min, 4 °C) and retained at −20 °C for ELISA and flow cytometry assays. Antibody response of vaccinated Queensland groupers (QMA0285) and PBS or FAI injected fish were determined against the vaccine strain (QMA0285) by indirect whole-cell ELISA as described previously [23].

2.8. Evaluation of antigen exposure by FACS

Evaluation of antigen exposure on the bacterial surface of five marine strains and five terrestrial strains of GBS was performed as described previously [24] with some modifications. GBS strains were grown overnight in Todd-Hewitt broth. Optical density (OD_{600}) was fixed to 0.5, and then cells washed twice with PBS. A volume of 200 µL of bacterial suspension was dispensed in 1.5 mL tubes, centrifuged and resuspended in 600 µL FBS for 60 min at room temperature. Bacteria were then incubated overnight at 4 °C with high titre polyclonal sera from fish that had responded positively to the QMA0285 formalin killed vaccine or with PBS immune serum as negative control both diluted 1:50 in dilution buffer (PBS, 20% FBS, 0.1% bovine serum albumin (BSA)). After centrifugation and washing twice in PBS and 0.1% BSA, samples were incubated for 90 min at 4 °C with polyclonal sheep anti-barramundi IgM (kindly provided by Richard Whittington, The University of Sydney), which was previously shown to cross-react with Queensland grouper IgM. The polyclonal antibody was diluted 1:200 (PBS, 5% FBS, 0.1% BSA). After centrifugation and washing in PBS and 0.1% BSA twice, samples were incubated at 4 °C for 30 min with donkey polyclonal tertiary antibody anti-goat IgG (Alexa Fluor 647) (shown to cross-react with sheep anti-barramundi IgM) diluted 1:250 (PBS, 5% FBS, 0.1% BSA). Bacteria were washed twice and suspended in a final volume of 200 µL PBS. Control samples with omission of the primary antibodies and with only the tertiary antibody were included to determine the non-specific binding of the secondary and tertiary immunoglobulins respectively. Bacteria alone without antibodies were used as a negative control. For each strain, the difference between the mean fluorescence intensity (MFI) of the immune and PBS injected fish sera were defined (ΔMFIs). All samples were kept on ice after incubation until analysis with the BD FACSAria™ II flow cytometer. Forward, side scatters and red fluorescence voltages were set respectively at 250 V, 300 V and 670 V.

2.9. Opsonophagocytosis

The effect of pre-opsonisation of bacteria with antibodies on the phagocytosis and respiratory burst activities of HKL was determined as follows: Bacterial suspensions were incubated with heat-inactivated non-immune serum (from PBS injected fish) or antiserum against QMA0285 vaccine (Ab) (10% v/v) for 1 h at room temperature. Then the bacteria were centrifuged at 14,100 × *g* for 10 min and resuspended with HKL in L-15 medium at a MOI of 1 (bacteria/macrophage). Phagocytosis assay was carried out as previously described with Baclight Green opsonised labelled bacteria, whereas the oxidative activity was performed with bacteria opsonised but not fluorescently labelled.

2.10. Microscopy

Cultures were viewed with an Olympus CKX41 inverted microscope and cytospin slides were viewed using an Olympus BX41 microscope. On both microscopes, images were captured with an Olympus DP26/U-CMAD3 camera and optimised with the imaging software CellSens (Olympus Optical Co. Ltd, Japan).

2.11. Statistical analysis

Data analysis was performed using GraphPad Prism version 7.0 for Mac OS X (GraphPad Software, San Diego California, USA). Before analysis, homogeneity of variance was checked using Cochran's test. Phagocytosis of beads was analysed by a two-tailed unpaired *t*-test. Data from the oxidative activity, phagocytosis and viability obtained by flow cytometry and spectrofluorimetry were analysed by one-way analysis of variance (ANOVA), specific differences between treatments were isolated using Tukey's *post-hoc* tests.

3. Results

3.1. Structural and functional characterisation of HKL from Queensland grouper

When analysed by flow cytometry, HKL from Queensland grouper comprised two main cell populations with distinct forward and side scatter (FSC/SSC) characteristics (Fig. 1A). The population of smaller, less complex cells (low FSC/SSC) was designated P1 and the population of cells with increased FSC/SSC values as P2 (Fig. 1A). Further characterisation of the P1 and P2 sub-populations by microscopy confirmed morphological differences. Following sorting by flow cytometry based on their different forward (Fig. 2A) and side scatter values (Fig. 2B), the sorted sub-populations were prepared by cytospin and stained with Hemacolor (Fig. 2C and D). Population P1 was dominated by small monocytes (Fig. 2C i), it also included a large number of thrombocytes (Fig. 2C ii), and small lymphocytes (Fig. 2C iii). Population P2 contained primarily larger cells including macrophages (Fig. 2D i), a small percentage of small monocytes (Fig. 2D i), large lymphocytes (Fig. 2D ii), granular cells such as polymorphonucleated neutrophils consistent

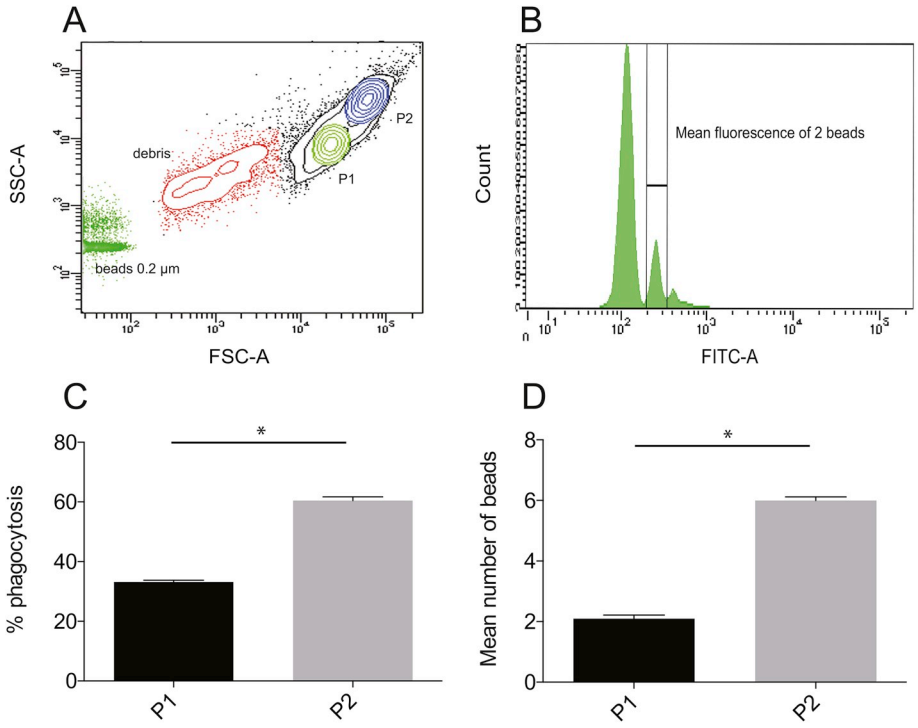

Fig. 1. Flow cytometric analysis of the phagocytic activity of Queensland grouper HKL. A. Representative dot-plot of HKL based on the size (FSC-A value) and internal complexity (SSC-A value) of leucocytes collected from the Percoll density gradient obtained by flow cytometry. Two main populations of cells could be differentiated based on their scatter properties: P1 (green), P2 (blue), debris (red). Note the 0.2 µm beads do not overlap with the leucocytes. B. Representative histogram of cell counts against the green fluorescence (FITC-A) of cells that have phagocytosed 1 (1st peak), 2 (2nd peaks) or more beads. C. Percentage phagocytosis (phagocytic rate) of P1 and P2. C. Phagocytosis index (mean number of beads ingested) per P1 and P2. Values are expressed as the mean (± SEM) of three replicate assays with leucocytes from three fish. Stars indicate a significant difference between cell populations P1 and P2 ($p < 0.05$, two-tailed unpaired *t*-test).

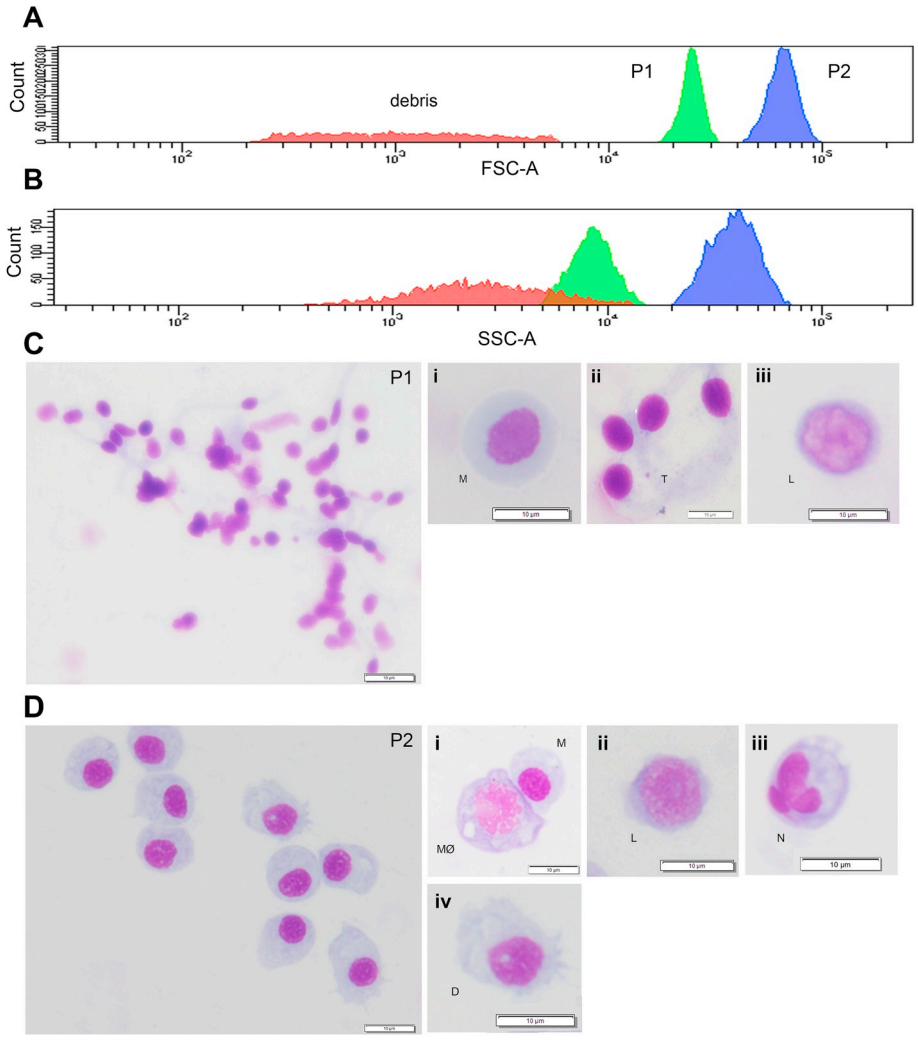

Fig. 2. Morphological analysis of Queensland grouper HKL sub-populations sorted by flow cytometry. A. Representative flow cytometry histograms of cell count against the size (FSC-A) and B. Granularity (SSC-A) of cells present in P1 (green) and P2 (blue). Note the debris gate is designated in red. Gated populations P1 and P2 were sorted and cytospin preparations analysed by light microscopy. C. Cytospins of P1 and D. Cytospins of P2 stained with hemacolor respectively. The overview photos in C and D (left) and representative single cells (right). In each case, MØ = macrophages; M = monocytes; L = lymphocytes; N = neutrophils; T = thrombocytes; D = dendritic-like cells. Scale bar = 10 µm.

with high SSC values (Fig. 2D iii), and some dendritic-like cells (Fig. 2D iv).

When analysed for phagocytic rate (percentage of phagocytic cells) and index (number of particles ingested per phagocytic cell), both populations P1 and P2 were capable of phagocytosis of 0.2 µm fluorescent latex beads (Fig. 1C and D). Phagocytic index was determined with a representative histogram of the cell count against green fluorescence (Fig. 1B). The mean fluorescence of one bead was determined by gating the second peaks on the histogram corresponding to the mean fluorescence of cells that had ingested two beads, and divide this value by two. Total percentage of phagocytic P2 cells (phagocytic rate) was significantly higher than P1 (Fig. 1C) ($p < 0.05$). P2 had a significantly higher phagocytic index than P1 (Fig. 1D) ($p < 0.05$), with an average of six beads being ingested compared to two beads by P1.

To determine the activation status of the HKL, an experiment was conducted to investigate the total redox potential generated by the entire leucocyte population after stimulation with LPS, PTG, or the surface receptor-independent protein kinase C activator, PMA. Head-kidney cells produced significantly more ROS in response to stimulation by PMA at 100 and 1000 ng mL^{-1} compared to control unstimulated cells but were not activated at the lowest concentration 10 ng mL^{-1} (Fig. 3). Whilst there was a slight increase in relative fluorescence with 10 and 100 µg mL^{-1} PTG, neither LPS nor PTG induced ROS production that was significantly different from non-stimulated control at any concentration used (Fig. 3).

3.2. Interactions of GBS isolates with Queensland grouper HKL

A pilot study to establish the optimal MOI for subsequent experiments indicated that at a MOI of 10 the ROS production in P1 and P2 were significantly lower than at a MOI of 1 or 0.1 (Fig. 4A and B). The pattern of ROS production with respect to strain and concentration in P1 and P2 are the same, however P2 produced significantly more ROS than P1, almost 10 times more (Fig. 4). For all subsequent experiments a MOI of 1 was used.

3.3. All piscine and some terrestrial GBS isolates trigger ROS production in HKL sub-populations

The intracellular oxidative metabolism of the P1 population was significantly different from the control in response to all the marine strains (Fig. 5A). Strains from human foot (QMA0355) and cow (QMA0306) did not significantly induce ROS production in P1 compared to control ($p > 0.05$). A small, but significant increase in ROS production compared to control was observed with the crocodile (QMA0336), cat (QMA0303) and dog (QMA0300) isolates. Isolates from mullet (QMA0274) and grouper 6 (QMA0368) induced the highest response in P1. Mean ROS production in HK P1 leucocyte population when stimulated with 1 µg mL^{-1} PMA was two-fold higher than that induced by QMA0368 (Fig. 5A inset graph). The pattern of activity stimulated by the differing isolates and PMA control between P1 and P2 cell populations were similar, but P2 was metabolically more active with values 10 times higher than P1 (see scales Fig. 5A and B). All GBS isolates had a significant effect on the production of ROS in P2 compared to control except the isolates from human foot (QMA0355), crocodile (QMA0336) and cow (QMA0306) (Fig. 5B). The strongest activation was obtained with the isolates from grouper 1 (QMA0284), grouper 6 (QMA0368) and mullet (QMA0274) for the marine strains and with the cat (QMA0303) and the dog (QMA0300) for the terrestrial isolates. Overall the effect in response to the different strains of GBS could be categorized as no stimulation, medium stimulation and high stimulation. However, the response to positive control (PMA 1 µg mL^{-1}) was approximately five times greater than the highest response measured with the bacteria (Fig. 5 inset graphs).

3.4. Phagocytic rate and capacity of grouper HKL is higher for terrestrial GBS isolates

When HKL phagocytosis was analysed in terms of their phagocytic rate and phagocytic capacity, or index for different isolates of *S. agalactiae*, the percentage of positive HK P2 leucocyte for phagocytosis was higher than P1 for all bacteria tested (Table 2). However, the difference between percentage positive phagocytic P1 and P2 within each treatment was quite small except with the isolates from grouper 5 (QMA0281) and grouper 6 (QMA0368) amongst the marine isolates and cat (QMA0303), dog (QMA0300) and cow (QMA0306) amongst the terrestrial isolates, which were phagocytosed by approximately two times more P2 than P1 cells (Table 2). The highest phagocytic rate for P2 and lowest phagocytic rate for P1 were obtained with the isolate from grouper 5 (QMA0281) with respectively 66.6% and 24.4% of cells being phagocytic (Table 2).

Overall the phagocytic index was lower in P1 than in P2 regardless of the isolate used. The phagocytic index of the HKL for the marine isolates ranged from 2.3 to 3.7 bacteria per cell from P1 and from 15.9 to 27 bacteria per cell from P2. Queensland grouper HKL were generally capable of phagocytising more of the terrestrial GBS isolates compared

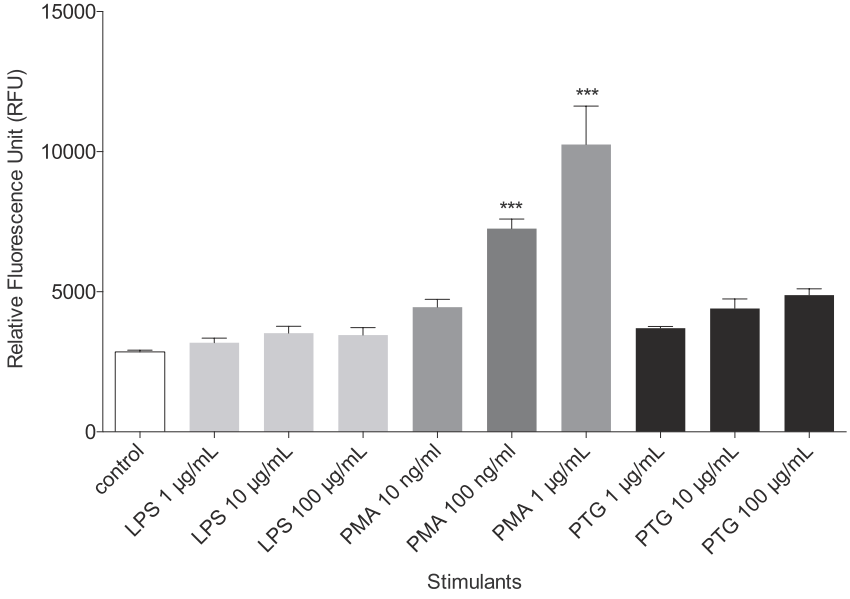

Fig. 3. Direct stimulation of respiratory burst in Queensland grouper HKL assayed by fluorometry. Percoll-separated HKL (1×10^6 mL^{-1}) were pre-incubated in L-15 medium containing 10 µM DCFH-DA without phenol-red and FBS for 30 min. Drugs were added for 2 h and fluorescence (relative fluorescence unit) was measured in a spectrophotometer (excitation 485 nm/emission 520 nm) in plate mode fluorescence. LPS: lipopolysaccharide, PMA: phorbol myristate acetate, PTG: peptidoglycan. Mean values (± SEM) of replicate assays with leucocytes from three fish are expressed in relative fluorescence unit (RFU). Stars indicate a significant difference with the unstimulated control ($p < 0.05$, one-way ANOVA). ***$p < 0.001$.

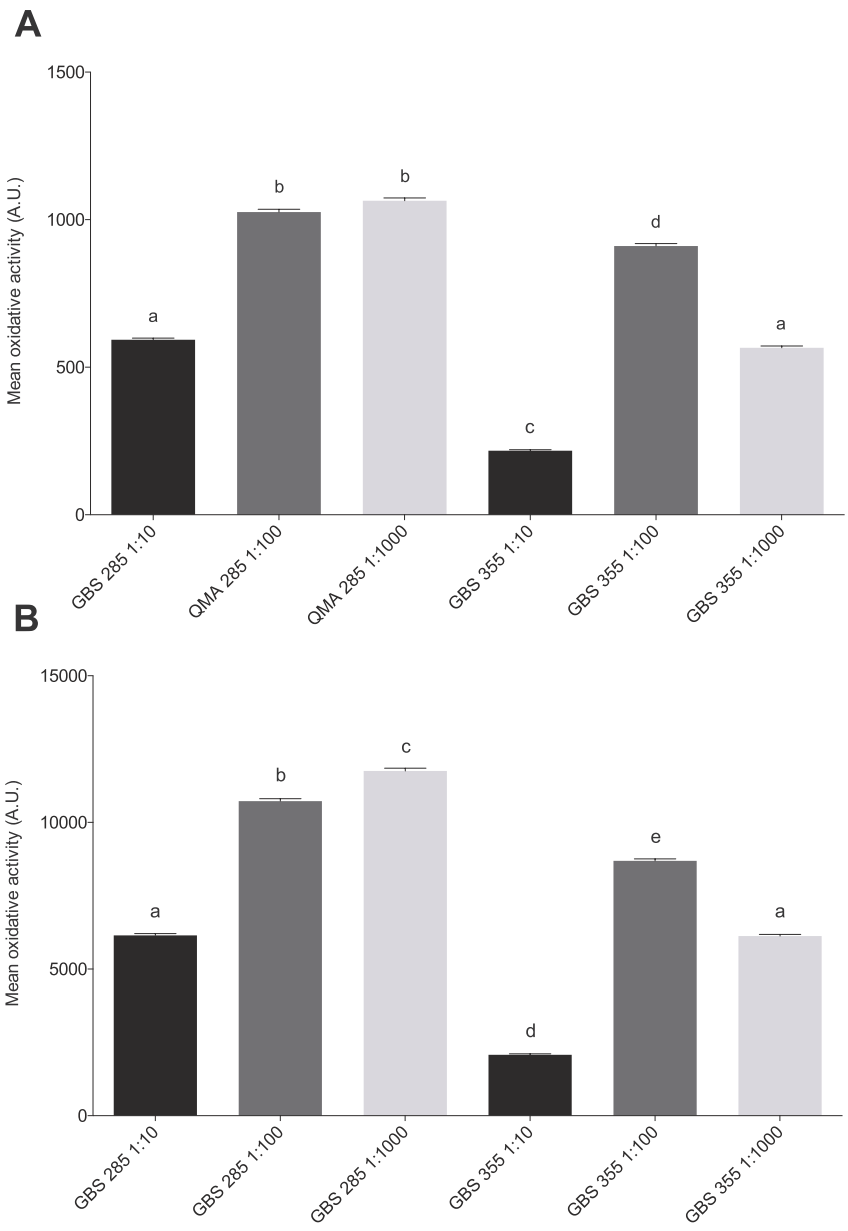

Fig. 4. Respiratory burst activity of Queensland grouper HKL sub-populations infected with GBS at different MOI. A. P1 and B. P2 in response to different MOI using strain QMA0285 (grouper 2) and QMA0355 (human foot). HKL (2.5×10^6 mL^{-1}) were incubated in L-15 medium containing 10 µM DCFH-DA without phenol-red and FBS and the different MOI for 2 h and then analysed by flow cytometry. All bacteria were fixed at an optical density (OD_{600} = 1), a MOI of 10 corresponds to a dilution of 1:10, a MOI of 1 corresponds to a dilution of 1:100 and a MOI of 0.1 corresponds to a dilution of 1:1000. Mean values (± SEM) are expressed in flow cytometry arbitrary units (A.U) of replicate assays with leucocytes from three fish. Different letters indicate a significant difference between the different treatments and strains ($p < 0.05$, one-way ANOVA).

to the marine strains. The range of phagocytic indices for cells from P1 was from 5.7 to 6.6 and from 27 to 49.1 for cell from P2. The marine GBS strain from grouper 2 (QMA0285) resulted in the lowest phagocytic index with 2.3 and 15.9 bacteria ingested per cell from P1 and P2 respectively and the terrestrial crocodile isolate (QMA0336) resulted in the highest phagocytic index with 6.6 and 49.1 bacteria per cell from P1 and P2 respectively (Table 2).

3.5. *Terrestrial isolates cause higher mortality of grouper HKL than marine isolates*

All GBS isolates induced some mortality in the P1 and P2 populations after 2 h incubation when compared to control cells without bacteria (Table 2). Marine strains only induced a maximum of 1.3-fold increased mortality (QMA0274) in P1, whereas three terrestrial strains: human foot (QMA0355), crocodile (QMA0336), and dog (QMA0300) induced 2.0, 2.3, and 3.5 fold increased mortality respectively in P1. For most strains, mortality in P2 was greater than in P1 except with isolates from human foot (QMA0355), crocodile (QMA0336) and dog (QMA0300) (Table 2).

Presence of GBS did not significantly affect apoptosis of either HK cell populations. In the P1 population, fold increase in apoptosis across the marine and terrestrial isolates ranged from 0.9 to 1.4 and from 1.1 to 1.5 in the P2 population.

Phagocytosis, oxidative burst activity and viability of Queensland grouper HKL in response to various GBS were confirmed by microscopy using the BX41 epifluorescent microscope. Different levels of oxidative burst were observed in leucocytes with some cells having more intense fluorescence than others (Fig. 6A). GBS, after being stained with the non-nucleic acid Baclight green dye and washed several times before being used in the phagocytosis assay, appeared as cocci in chains and were brightly fluorescent (Fig. 6B). Large phagocytic cells with green internalised bacteria were observed after 2 h incubation (Fig. 6C), as well as with internalised 0.2 µm fluorescent latex beads (Fig. 6D). After 2 h incubation with different strains of GBS, mortality and apoptosis in HKL could be observed by the fluorescence of PI (red) and annexin V (green) respectively (Fig. 6E and F). The nuclei of the leucocytes were stained blue using DAPI for better observation of internalised bacteria that could be seen inside the phagolysosome (Fig. 6G).

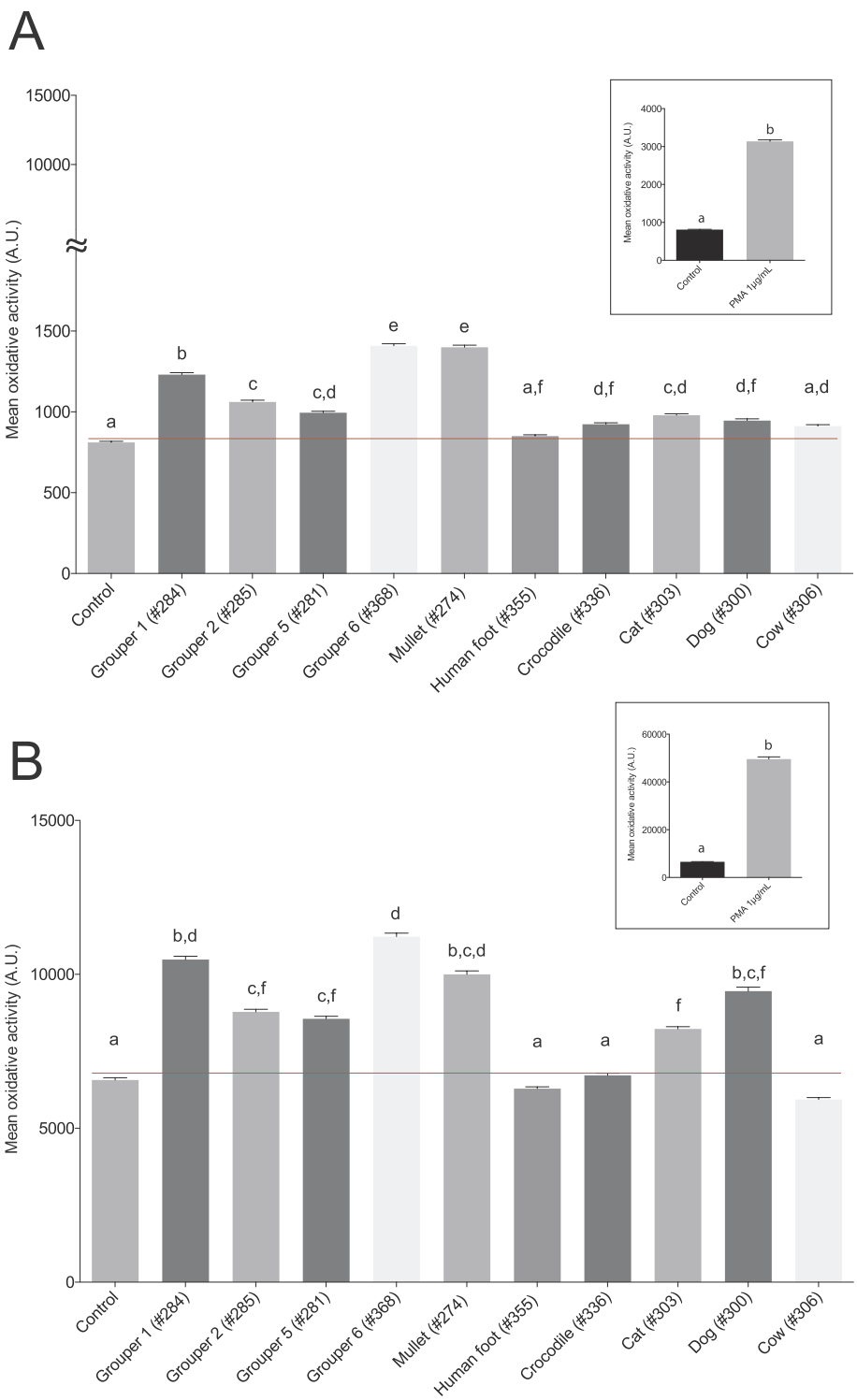

Fig. 5. Respiratory burst activity of Queensland grouper HKL sub-populations in the presence of different GBS isolates. A. Mean oxidative activity of Queensland grouper HKL P1 population in response to incubation with marine and terrestrial GBS isolates for 2 h. B. Mean oxidative activities of Queensland grouper HKL P2 population with the same marine and terrestrial GBS isolates for 2 h after incubation. Mean values (± SEM) are expressed in flow cytometry arbitrary units (A.U) of replicate assays with leucocytes from three fish. Different letters represent a statistically significant difference between strains and the control ($p < 0.05$, one-way ANOVA).

3.6. Effect of antibody on phagocytosis rates and ROS production by HKL

3.6.1. Specific antibody response of Queensland grouper to S. agalactiae and cross reactivity between marine and terrestrial strains

To determine whether fish had responded to the vaccination, serum antibody titres from a cohort of vaccinated and controls fish were determined by enzyme-linked immunosorbent assay (ELISA) (Fig. 7A). After 900 degree-days (32 days at 28 °C), fish vaccinated by intraperitoneal (IP) injection with a formalin-killed *S. agalactiae* (QMA0285) vaccine produced significantly more serum antibody against QMA0285 than fish injected with PBS or FIA ($p < 0.05$) (Fig. 7A). Only one vaccinated fish with QMA0285 did not respond and had the lowest level of specific antibody produced, and thus was removed from the study as an outlier. Fish injected with either PBS or FIA had low antibody titres when tested against the vaccine antigen (QMA0285) and were not statistically different from each other ($p > 0.05$).

In order to determine whether high titre antisera from fish that had responded to the GBS vaccine (QMA0285) could cross-react with other marine and terrestrial GBS isolates, a flow cytometry assay was conducted using antisera against the QMA0285 vaccine and serum from PBS injected fish with five marine and five terrestrial GBS isolates

Table 2
Phagocytosis, apoptosis and mortality of Queensland grouper HKL in the presence of different GBS isolates. Phagocytic rate and phagocytic index of P1 and P2 subpopulations of Queensland grouper head-kidney leucocytes were determined by flow cytometry after incubation for 2 h with various GBS isolates (five marine and five terrestrial). Apoptosis and mortality are expressed as fold increase compared to control leucocytes (without bacteria). Phagocytic index was defined as the average number of fluorescent bacteria engulfed per cell and phagocytic rate as the percentage of phagocytic cells in the analysed population.

P1	Treatments									
	GBS0284	GBS0285	GBS0281	GBS0368	GBS0274	GBS0355	GBS0336	GBS0303	GBS0300	GBS0306
	Grouper 1	Grouper 2	Grouper 5	Grouper 6	Mullet	Human foot	Crocodile	Cat	Dog	Cow
Phagocytic rate (%)	44.8	42.7	24.4	32.8	45.1	41.6	43.6	29.3	36.6	29.9
Phagocytic index	2.6	2.3	3.7	3.1	3.6	5.7	6.6	3.8	6.4	6.0
Fold increase mortality	1.1	1.1	1.1	1.0	1.3	2.0	2.3	1.2	3.5	1.2
Fold increase apoptosis	1.3	1.2	1.4	1.3	1.3	1.0	1.1	1.2	0.9	1.1
P2										
Phagocytic rate (%)	45.5	47.2	66.6	57.7	46.4	49.7	47.1	62.0	52.4	61.6
Phagocytic index	19.2	15.9	17.5	19.9	27.0	38.6	49.1	27.0	45.1	28.1
Fold increase mortality	1.7	1.7	1.7	1.9	1.7	1.7	2.0	1.7	3.0	2.4
Fold increase apoptosis	1.2	1.1	1.3	1.5	1.1	1.4	1.4	1.4	1.3	1.5

(Fig. 7B). The highest reactivity with the antibodies was recorded for the strain used for the vaccine (QMA0285) (Fig. 7B). Antibody cross-reacted with all the marine strains. However, there was low cross-reactivity with the terrestrial isolates. Negative ΔMFI values were recorded with the human foot (QMA0355) and the cow isolate (QMA0306) (Fig. 7B).

3.6.2. Serum and antiserum increase phagocytosis and respiratory burst of Queensland grouper head-kidney leucocytes

In order to evaluate the effect of antibodies on the phagocytosis and respiratory burst activity of Queensland grouper HKL in response to GBS bacteria, two strains were chosen: the marine isolate from grouper 2 (QMA0285) due to its high binding affinity with its homologous sera antibodies (Fig. 7B) and the terrestrial isolate from human foot (QMA0355) because it had the lowest binding affinity with the same serum antibodies produced against QMA0285 (Fig. 7B). When bacteria QMA0285 and QMA0355 were incubated with antiserum from fish vaccinated (QMA0285) and normal serum from fish injected with PBS, there were no significant differences in terms of leucocyte phagocytic rate and capacity (data not shown) between any of the sera analysed compared to control (no serum).

A significant increase in oxidative activity of Queensland grouper HKL, as determined via measurement of ROS production, was observed when both QMA0285 and QMA0355 were incubated with antiserum from fish vaccinated with QMA0285 or with serum from fish injected with PBS, when compared to the control leucocytes (no bacteria) and with leucocytes incubated with the bacteria without antiserum (Fig. 8). There was significantly more ROS produced in HKL when QMA0285 was incubated with anti-285 antiserum compared to QMA0285 incubated with control serum from fish injected with PBS (Fig. 8). Oxidative burst activity was lower in HKL in response to QMA355 incubated with antiserum (QMA0285) compared to QMA0355 incubated with control serum (Fig. 8).

4. Discussion

Virulence of GBS for *E. lanceolatus* has been demonstrated in *in vivo* infectivity challenges [15]. However, although various clonal complex groups have been isolated from diseased fish, little is known about the ability of this pathogen to cross the interspecies barrier allowing human

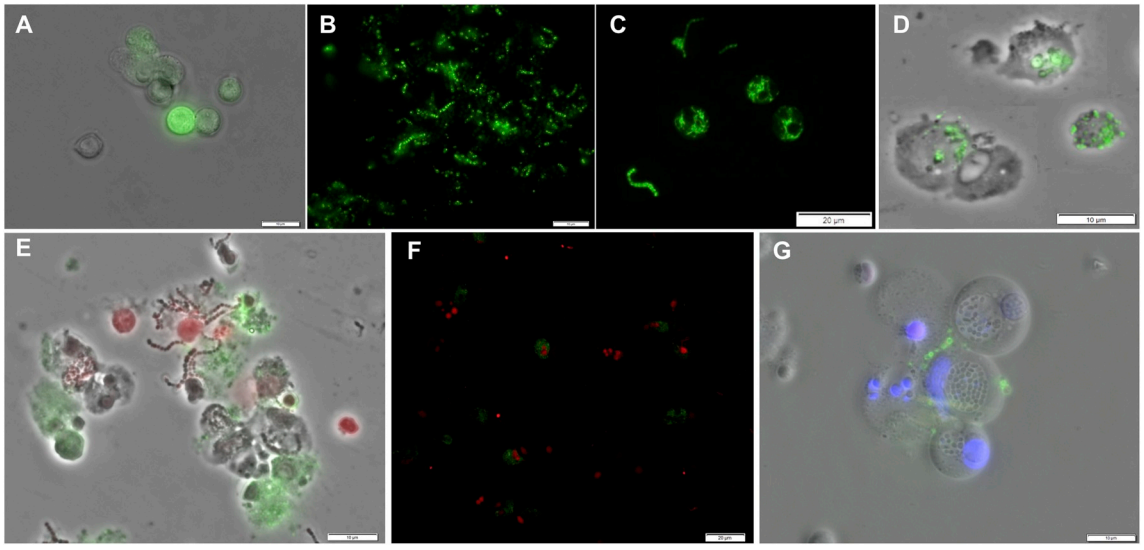

Fig. 6. Confirmation of oxidative activity, phagocytosis and cellular viability of Queensland grouper HKL by fluorescent microscopy. Queensland grouper HKL used in the different assays were prepared by cytospin for fluorescent microscopy. A. Cells that have produced reactive oxygen species are detected with the green fluorescence (DCF). Note the difference in fluorescence intensity and the negative cell on the left. B. Group B *Streptococcus* (QMA0285) stained with Baclight green stain showing chains of typical cocci. C. Macrophages from HKL with ingested fluorescent bacteria. D. HKL with internalised 0.2 μm beads. E. Cell viability where apoptotic cells are green (annexin V) and dead cells red (propidium iodide). F. Overlays of apoptotic and dead cells. G. Internalised bacteria QMA0300 in the phagolysosome with nuclei stained with DAPI (blue) and some apoptotic cells are stained with annexin-V (green).

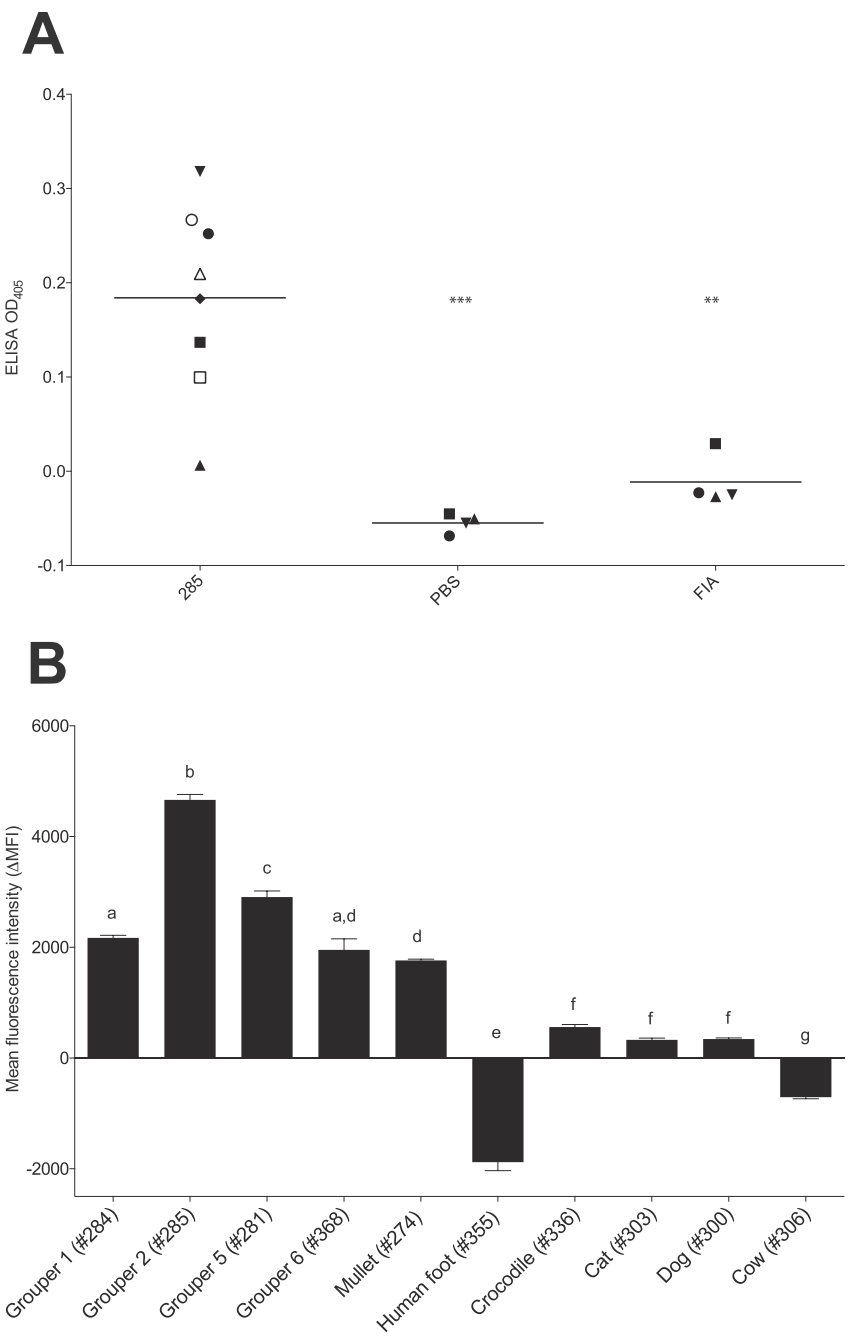

Fig. 7. Specific antibody response by whole cell ELISA of Queensland grouper following vaccination with QMA0285 (serotype Ib, ST261). A. Serum antibody response by ELISA from vaccinated (n = 8) and control Queensland groupers (PBS injected (n = 4) or Freund's incomplete adjuvant (FIA) injected (n = 4)) tested against the vaccine antigen (QMA0285) **p < 0.01, ***p < 0.001 (one-way ANOVA). B. Surface antigen exposure of five marine and five terrestrial strains evaluated by flow cytometry using a high titre sera antibody produced against the marine isolate QMA0285 Different letters represent statistically significant differences between mean ELISA OD values (p < 0.05, one-way ANOVA, *n* = 3).

and other terrestrial isolates to infect fish, or *vice versa*. The objective of this study was to better understand how these strains of GBS are pathogenic in fish, using *in vitro* cellular immune assays with juvenile Queensland grouper HKL in the presence of different aquatic and terrestrial isolates as an *ex vivo* model. However, as this study represents the first assessment of the immune function of *E. lanceolatus,* some preliminary characterisation of cellular immune composition and function was required.

Flow cytometry analysis of Queensland grouper head-kidney derived leucocytes purified on Percoll gradients showed two clusters of cells. Cytospin preparations observed by microscopy confirmed the differences in morphology of these two populations. P1 had a lower SSC/FSC profiles and comprised of small lymphocytes, monocytes, thrombocytes and some macrophages. The cells in P2 population, on the other hand, were more homogeneous, comprising larger and more complex cells and consisted of macrophages and neutrophils. Similarly, two populations comprising similar cell types were found in the European sea bass (*Dicentrarchus labrax*) and common carp (*Cyprinus carpio*) peripheral blood leucocytes [25,26]. However, three sub-populations were found in leucocytes derived from the head-kidney of barramundi, *Lates calcarifer* [7], dab, *Limanda limanda* [27] and Atlantic cod, *Gadus morhua* [28].

In terms of HKL activation by different microbial stimulants, only PMA induced significant ROS production in HKL compared to non-stimulated controls, while no effects were detected in cells stimulated with LPS or PTG. Similar observations have been reported in barramundi (*L. calcarifer*), Atlantic salmon (*Salmo salar*) and Atlantic cod (*G. morhua*) head-kidney cells [7,29]. PMA, which bypasses cell surface receptors, triggers leucocytes respiratory burst activity directly at the intracellular side of the membrane through MAP kinase/protein kinase C activated signal transduction [10]. In this study, PMA was used as a positive control to confirm that these cells could indeed activate a

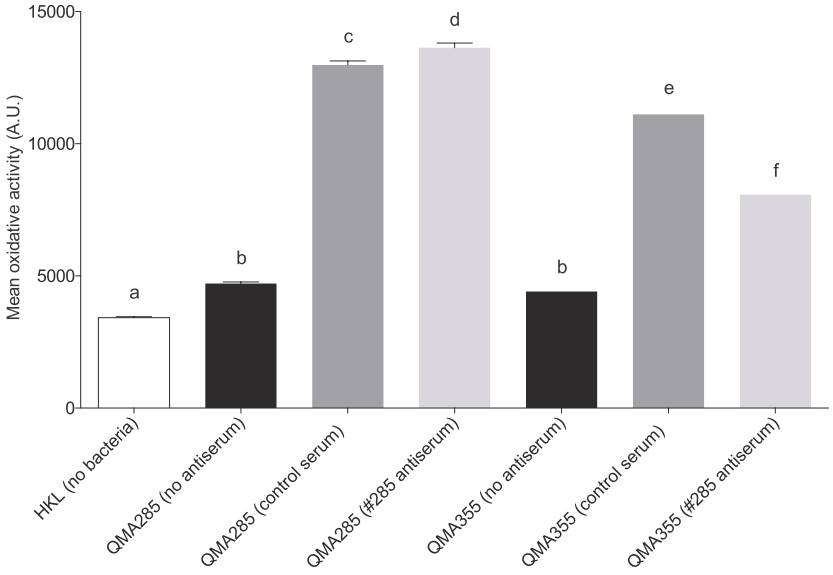

Fig. 8. GBS opsonised by antibodies increased respiratory burst activity by Queensland grouper HKL. Effect of pre-incubation of live QMA0285 (grouper 2) and QMA0355 (human foot) with antiserum from vaccinated fish with a formalin-killed vaccine based on strain QMA0285 and normal serum from fish injected with PBS on the mean oxidative activity (A.U.) of Queensland grouper HKL. Mean values (± SEM) are expressed in flow cytometry arbitrary units (A.U) of replicate assays with leucocytes from two individual fish. Different letters represent a statistically significant difference between strains and the control ($p < 0.05$, one-way ANOVA).

respiratory burst, but it is not a relevant route of activation in terms of determining how these cells interact with putative pathogens, as it bypasses critical membrane receptor/signal transduction complexes. Therefore, microbial PAMPs were also investigated. LPS is an endotoxin forming part of the outer membrane of Gram-negative bacteria, and the hydrophobic lipid component can induce a strong immune response in normal teleostean macrophages [30–32]. The molecular mechanism by which LPS stimulates an immune response in fish has been reviewed by Forlenza et al. [33]. In mammals, two essential accessory proteins mediate the transfer of LPS to its toll-like receptor TLR4 on the macrophage surface: CD14 and LPS-binding protein (LBP) [34–36]. Genome analyses of many fish species, including Japanese pufferfish (*Takifugu rubripes*), Spotted green pufferfish (*Tetraodon nigroviridis*) and Australian barramundi (*Lates calcarifer*) indicate absence of a TLR4 homologue and several receptor molecules (LBP, CD14) that are essential for recognition of LPS by mammals [37–40]. Queensland grouper HKL were insensitive to LPS at high concentration up to 100 μg mL^{-1}, which suggests the absence of membrane receptors specific for LPS and/or the lack of potential essential co-stimulatory factors from the medium. On the other hand, HKL of goldfish, *Carassius auratus* [32], Atlantic salmon, *S. salar* [31], and European sea bass, *D. labrax* [30], reportedly up-regulate ROS production upon priming or stimulation with LPS. It is therefore evident that not all fish respond the in the same way to LPS and that ligand recognition by TLRs and other PRRs needs to be deciphered for each fish species. It is important to note that a highly purified LPS (Sigma L3034), experimentally determined to be free from stimulatory contaminants, was employed in the present study. Contamination with, for example, nucleic acids or peptidoglycan in less purified preparations can result in pro-inflammatory stimulation via alternative receptors such as TLR9 or TLR2.

The cell wall of Gram-positive bacteria is largely made up of peptidoglycan, a complex carbohydrate polymer that confers rigidity. In mammalian vertebrates, recognition of PTG is mediated by TLR2 [41–43]. PTG purified from *Staphylococcus aureus* has been shown to elicit the activation of macrophages through carp TLR2 [44]. TLR 1 and 2 have been recently cloned and expressed from Orange-spotted grouper, *Epinephelus coioides* [45]. In this study, the lack of detectable response of *E. lanceolatus* HKL to PTG suggests that sequence homology of TLR2 does not necessarily mean functional conservation. In *in vivo* experiments, where PTG was administrated orally or by injection to Olive flounder, *Paralichthys olivaceus*, it was demonstrated that immune functions such as phagocytic activity, production of ROS, and resistance to injection challenge with *Vibrio anguillarum* were enhanced [11,46,47]. This might suggest that a combination of factors is necessary for PTG to activate macrophages and granulocytes in *E. lanceolatus*. Other PAMPs such as poly(I:C) and zymosan failed to increase phagocytosis and microbicidal activity in acidophilic granulocytes from *S. aurata* [48]. This further emphasises the likely diversity of PAMPs recognition and activation of phagocytic cells in different species of teleost fish.

Both P1 and P2 subpopulations of HKL were capable of phagocytosis and ROS production, but 90% of this activity was in the P2 population. Mixed sub-populations of *C. auratus* primary kidney macrophages were differentially phagocytic depending on their stage of maturation [49–51]. Flow cytometry and electron microscopy showed that large macrophages and acidophilic granulocytes from *S. aurata* head-kidney could actively ingest *V. anguillarum* [52]. In Queensland grouper, P2 was mainly composed of mature macrophages and neutrophils, whereas cells in P1 were smaller monocytes, lymphocytes and thrombocytes. Monocytes are precursor cells with moderate phagocytic capability until they mature [3]. Thrombocytes are nucleated cells in fish and are the morphofunctional equivalents to mammalian platelets, originating from a multipotent myelo-erythroid progenitor [5]. Beyond their primary function in homeostasis, it is uncertain if thrombocytes play an immunological role. However, both phagocytic and bactericidal activity have been attributed to *C. carpio* thrombocytes [53], while others suggest an interaction of thrombocytes with peripheral macrophages, erythrocytes, polymorphonuclear cells and lymphocytes in the case of trout infected with *Candida albicans* [54]. These reports in other fish species support the different oxidative activity observed in P1 and P2 in response to marine and terrestrial GBS isolates.

The Group B streptococci represent a diverse species comprising multiple clonal complexes [16,55–57]. To date, almost all GBS primary pathogens of fish belong to, or are closely related to, the MLST sequence type ST-261 [16,55], although there have been occasional reports of isolates normally associated with human colonisation found in aquatic animals [56,58,59]. All isolates from different fish hosts in this study belonged to ST-261, serotype Ib, and were capable of triggering an oxidative burst in both P1 and P2 populations, which support that these bacteria are recognised in a strain-specific manner. Recognition of pathogens is mediated through a number of receptors in addition to TLRs, including macrophage mannose receptor and other scavenger receptors and involvement of these receptors in phagocytosis of Gram-positive and Gram-negative bacteria by teleost leucocytes has been reported [60,61]. Lack of activation of Queensland grouper HKL by PTG, but activation by ST261 serotype 1b GBS implicates such alternative

receptors in this process.

The highest phagocytic rate was observed in P2 when leucocytes were incubated with ST261 sertotype 1b GBS isolate from grouper with a large deletion in its capsular operon resulting in the loss of the capsule, as evidenced by a buoyant density assay [17]. Previous studies have shown that the presence of a capsule can greatly impair phagocytic clearance by fish leucocytes and CPS is the major virulence factor in fish-pathogenic streptococci [62–64].

While, the GBS isolates in this study were genetically diverse and from different host origins as evidenced by different CPS sequences and MLST sequence type [17], two of the terrestrial isolates, cat (QMA0303) and dog (QMA0300), both serotype V, significantly activated Queensland grouper macrophages in a similar way to some of the marine isolates. These marine and terrestrial isolates probably share common antigens on their surfaces that interact with Queensland grouper leucocytes in a similar fashion, although there was low cross-reactivity with antiserum raised in grouper against the marine isolate QMA0285.

The highest mortality increase in both P1 and P2 leucocyte subpopulations was observed when they were incubated with the GBS isolate from a dog. This terrestrial strain expresses virulence factors that promote host-cell death, including toxin members of the *cyl* locus (β-hemolysin/cytolysin) [17,65]. These toxins are absent from the genomes of our piscine isolates and they are non-hemolytic on sheep blood agar [17]. The underlying reasons for the general tendency for the terrestrial isolates to cause higher mortality in grouper leucocytes compared to the piscine isolates are still unclear. However, most of the major virulence factors found in human and bovine isolates are absent from the ST260-261 GBS piscine lineages [16,56,66], including those used in the present study [17].

Surprisingly the low production of ROS by P2 in the presence of human, crocodile and bovine GBS strains coincided with high phagocytic index, suggesting that these bacteria possess virulence factors specialised detoxifying singlet oxygen and superoxide, such as superoxide dismutase (SodA) and other protein members of the *cyl* locus [67,68]. In fish, similar inhibition of the leucocyte respiratory burst has been described by *V. anguillarum* in *D. labrax* [69], which is probably used as a strategy for the pathogen to survive inside the cells and disseminate to other organs and tissues. In view of these findings, the role of grouper immunoglobulin in the phagocytosis and respiratory activity of Queensland grouper HKL was evaluated in an opsonophagocytosis assay.

Antibodies produced against vaccine (QMA0285, serotype Ib) cross-reacted with all the marine isolates, suggesting that these strains have common antigens on their surfaces consistent with molecular serotype (Ib) determined by WGS [17]. In contrast, Queensland grouper antibodies against QMA0285 did not cross-react strongly with the terrestrial isolates of serotypes Ia, III and V. Indeed, a negative ΔMFI was detected for two isolates, QMA0355 (crocodile, serotype Ia) and QMA0306 (cow, serotype III). Streptococci are capable of binding immunoglobulin by a non-immune mechanism. Many strains of group A and B streptococci express surface proteins such as M and M-like proteins that can bind human IgA and IgG antibodies via their Fc region [70–73]. Although IgM, the predominant serum antibody in teleost fish, is polymeric, the fish pathogen *S. iniae* is capable of binding trout and barramundi IgM in a non-immune manner, with Fc-domain binding demonstrated experimentally with trout IgM [74,75].

Opsonisation of pathogens by specific antibodies is key to a coordinated response between the cellular innate and adaptive immune systems. However, while opsonisation of QMA0285 and QMA0355 with antibodies raised against QMA0285 (vaccine) significantly increased the ROS productions, there was a decrease in the number of bacteria being phagocytosed with strain QMA0355, which supports a potential binding of IgM by the Fc region, making the antigen-antibody complex invisible for phagocytosis as with other pathogenic streptococci [75,76]. Indeed, isolates belonging to ST-7, which infect terrestrial animals including humans, have been reported to also cause disease in aquatic animals and fish [56,59].

The relatively high level of ROS detected in unstimulated control HKL may be a result of stress applied to the cells during handling and culture post-isolation. These results, however, are in accordance with previous findings with European sea bass (*D. labrax*) and barramundi (*L. calcarifer*) macrophages [7,12], which together indictate that there is room for improvement (optimum culture medium, better cell sorting techniques, faster processing time and analysis etc) when conducting immune assays using primary cell cultures.

5. Conclusions

In conclusion, the results of these *in vitro* experiments clearly demonstrate the potential for terrestrial animal or human isolates of GBS to invade Queensland grouper leucocytes, quench their radical oxygen production and cause a significant increase in cell mortality. There is an increasing body of evidence that describes the presence and virulence of human and bovine GBS isolates in teleosts [18,56,77–80], which supports interspecies infection by GBS. Future controlled experiments should investigate further the susceptibility of *E. lanceolatus* to non-piscine GBS isolates to confirm the premise that, regardless of its genetic diversity, GBS can cross interspecies barriers and establish itself within fish populations. As some variants of this pathogen could be transmitted to humans via handling or consumption of infected fish products, this could have major impacts on local seafood industries and consumer health.

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References

[1] C. Uribe, H. Folch, R. Enriquez, G. Moran, Innate and adaptive immunity in teleost fish: a review, Vet. Med. 56 (10) (2011) 486–503.
[2] A.E. Ellis, Immunity to bacteria in fish, Fish Shellfish Immunol. 9 (4) (1999) 291–308.
[3] A.E. Ellis, The leucocytes of fish: a review, J. Fish Biol. 11 (5) (1977) 453–491.
[4] S.K. Whyte, The innate immune response of finfish – a review of current knowledge, Fish Shellfish Immunol. 23 (6) (2007) 1127–1151.
[5] J.H.W.M. Rombout, H.B.T. Huttenhuis, S. Picchietti, G. Scapigliati, Phylogeny and ontogeny of fish leucocytes, Fish Shellfish Immunol. 19 (5 SPEC. ISS.) (2005) 441–455.
[6] C.J. Secombes, Isolation of salmonid macrophages and analysis of their killing activity, in: J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Roberson, W.B. van Muiswinkel (Eds.), Techniques in Fish Immunology, SOS Publications, Fair Haven NJ, 1990, pp. 137–154.
[7] R.A. Tumbol, J.C.F. Baiano, A.C. Barnes, Differing cell population structure reflects differing activity of Percoll-separated pronephros and peritoneal leucocytes from barramundi (Lates calcarifer), Aquaculture 292 (3–4) (2009) 180–188.
[8] J.L. Stafford, F. Galvez, G.G. Goss, M. Belosevic, Induction of nitric oxide and respiratory burst response in activated goldfish macrophages requires potassium channel activity, Dev. Comp. Immunol. 26 (5) (2002) 445–459.
[9] H.T. Boesen, M.H. Larsen, J.L. Larsen, A.E. Ellis, In vitro interactions between rainbow trout (Oncorhynchus mykiss) macrophages and Vibrio anguillarum serogroup O2a, Fish Shellfish Immunol. 11 (5) (2001) 415–431.
[10] B. Kudrenko, N. Snape, A.C. Barnes, Linear and branched ß(1-3) d-glucans activate but do not prime teleost macrophages in vitro and are inactivated by dilute acid: implications for dietary immunostimulation, Fish Shellfish Immunol. 26 (3) (2009) 443–450.

[11] J. Zhou, X.L. Song, J. Huang, X.H. Wang, Effects of dietary supplementation of A3α-peptidoglycan on innate immune responses and defense activity of Japanese flounder (Paralichthys olivaceus), Aquaculture 251 (2–4) (2006) 172–181.
[12] A. Sarmento, L. Guilhermino, A. Afonso, Mercury chloride effects on the function and cellular integrity of sea bass (Dicentrarchus labrax) head kidney macrophages, Fish Shellfish Immunol. 17 (5) (2004) 489–498.
[13] R.O. Bowater, J. Forbes-Faulkner, I.G. Anderson, K. Condon, B. Robinson, F. Kong, G.L. Gilbert, A. Reynolds, S. Hyland, G. McPherson, J.O. Brien, D. Blyde, Natural outbreak of Streptococcus agalactiae (GBS) infection in wild giant Queensland grouper, Epinephelus lanceolatus (Bloch), and other wild fish in northern Queensland, Australia, J. Fish Dis. 35 (3) (2012) 173–186.
[14] R.O. Bowater, M.M. Dennis, D. Blyde, B. Stone, A.C. Barnes, J. Delamare-Deboutteville, M.A. Horton, M. White, K. Condon, R. Jones, Epizootics of Streptococcus agalactiae infection in captive rays from Queensland, Australia, J. Fish Dis. 41 (2) (2018) 223–232.
[15] J. Delamare-Deboutteville, R. Bowater, K. Condon, A. Reynolds, A. Fisk, F. Aviles, A.C. Barnes, Infection and pathology in Queensland grouper, Epinephelus lanceolatus, (Bloch), caused by exposure to Streptococcus agalactiae via different routes, J. Fish Dis. 38 (12) (2015) 1021–1035.
[16] I. Rosinski-Chupin, E. Sauvage, B. Mairey, S. Mangenot, L. Ma, V. Da Cunha, C. Rusniok, C. Bouchier, V. Barbe, P. Glaser, Reductive evolution in *Streptococcus agalactiae* and the emergence of a host adapted lineage, BMC Genomics 14 (1) (2013) 252.
[17] M. Kawasaki, J. Delamare-Deboutteville, R.O. Bowater, M.J. Walker, S. Beatson, N.L. Ben Zakour, A.C. Barnes, Microevolution of Streptococcus agalactiae ST-261 from Australia indicates dissemination via imported Tilapia and ongoing adaptation to marine hosts or environment, Appl. Environ. Microbiol. 84 (16) (2018).
[18] J.J. Evans, J.F. Bohnsack, P.H. Klesius, A.A. Whiting, J.C. Garcia, C.A. Shoemaker, S. Takahashi, Phylogenetic relationships among Streptococcus agalactiae isolated from piscine, dolphin, bovine and human sources: a dolphin and piscine lineage associated with a fish epidemic in Kuwait is also associated with human neonatal infections in Japan, J. Med. Microbiol. 57 (11) (2008) 1369–1376.
[19] D.A. Bass, J.W. Parce, L.R. Dechatelet, Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation, J. Immunol. 130 (4) (1983) 1910–1917.
[20] J.A. Royall, H. Ischiropoulos, Evaluation of 2',7'-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H2O2 in cultured endothelial cells, Arch. Biochem. Biophys. 302 (2) (1993) 348–355.
[21] E. Bassity, T.G. Clark, Functional identification of dendritic cells in the teleost model, rainbow trout (Oncorhynchus mykiss), PLoS One 7 (3) (2012) e33196.
[22] F. Aviles, M.M. Zhang, J. Chan, J. Delamare-Deboutteville, T.J. Green, C. Dang, A.C. Barnes, The conserved surface M-protein SiMA of Streptococcus iniae is not effective as a cross-protective vaccine against differing capsular serotypes in farmed fish, Vet. Microbiol. 162 (1) (2013) 151–159.
[23] J. Delamare-Deboutteville, D. Wood, A.C. Barnes, Response and function of cutaneous mucosal and serum antibodies in barramundi (Lates calcarifer) acclimated in seawater and freshwater, Fish Shellfish Immunol. 21 (1) (2006) 92–101.
[24] G. Bensi, M. Mora, G. Tuscano, M. Biagini, E. Chiarot, M. Bombaci, S. Capo, F. Falugi, A.G. Manetti, P. Donato, E. Swennen, M. Gallotta, M. Garibaldi, V. Pinto, N. Chiappini, J.M. Musser, R. Janulczyk, M. Mariani, M. Scarselli, J.L. Telford, R. Grifantini, N. Norais, I. Margarit, G. Grandi, Multi high-throughput approach for highly selective identification of vaccine candidates: the Group A Streptococcus case, Mol. Cell. Proteomics 11 (6) (2012) M111 015693.
[25] M.A. Esteban, J. Munoz, J. Meseguer, Blood cells of sea bass (Dicentrarchus labrax L.). Flow cytometric and microscopic studies, Anat. Rec. 258 (1) (2000) 80–89.
[26] C. Nakayasu, M. Omori, S. Hasegawa, O. Kurata, N. Okamoto, Production of a monoclonal antibody for carp (Cyprinus carpio L.) phagocytic cells and separation of the cells, Fish Shellfish Immunol. 8 (2) (1998) 91–100.
[27] A. Skouras, T. Lang, M. Vobach, D. Danischewski, W. Wosniok, J.P. Scharsack, D. Steinhagen, Assessment of some innate immune responses in dab (Limanda limanda L.) from the North Sea as part of an integrated biological effects monitoring, Helgol. Mar. Res. 57 (3–4) (2003) 181–189.
[28] A. Ronneseth, H.I. Wergeland, E.F. Pettersen, Neutrophils and B-cells in atlantic cod (Gadus morhua L.), Fish Shellfish Immunol. 23 (3) (2007) 493–503.
[29] C.A.K. Kalgraff, H.I. Wergeland, E.F. Pettersen, Flow cytometry assays of respiratory burst in Atlantic salmon (Salmo salar L.) and in Atlantic cod (Gadus morhua L.) leucocytes, Fish Shellfish Immunol. 31 (3) (2011) 381–388.
[30] A. Sarmento, F. Marques, A.E. Ellis, A. Afonso, Modulation of the activity of sea bass (Dicentrarchus labrax) head-kidney macrophages by macrophage activating factor (s) and lipopolysaccharide, Fish Shellfish Immunol. 16 (2) (2004) 79–92.
[31] S.T. Solem, J.B. Jørgensen, B. Robertsen, Stimulation of respiratory burst and phagocytic activity in Atlantic salmon (Salmo salar L.) macrophages by lipopolysaccharide, Fish Shellfish Immunol. 5 (7) (1995) 475–491.
[32] N.F. Neumann, D. Barreda, M. Belosevic, Production of a macrophage growth factor (s) by a goldfish macrophage cell line and macrophages derived from goldfish kidney leukocytes, Dev. Comp. Immunol. 22 (4) (1998) 417–432.
[33] M. Forlenza, I.R. Fink, G. Raes, G.F. Wiegertjes, Heterogeneity of macrophage activation in fish, Dev. Comp. Immunol. 35 (12) (2011) 1246–1255.
[34] P.S. Tobias, K. Soldau, J.A. Gegner, D. Mintz, R.J. Ulevitch, Lipopolysaccharide binding protein-mediated complexation of lipopolysaccharide with soluble CD14, J. Biol. Chem. 270 (18) (1995) 10482–10488.
[35] J. Pugin, R.J. Ulevitch, P.S. Tobias, Activation of endothelial cells by endotoxin: direct versus indirect pathways and the role of CD14, Prog. Clin. Biol. Res. 392 (1995) 369–373.
[36] J.C. Mathison, P.S. Tobias, E. Wolfson, R.J. Ulevitch, Plasma lipopolysaccharide (LPS)-binding protein: a key component in macrophage recognition of Gram-negative LPS, J. Immunol. 149 (1) (1992) 200–206.
[37] A. Rebl, T. Goldammer, H.M. Seyfert, Toll-like receptor signaling in bony fish, Vet. Immunol. Immunopathol. 134 (3–4) (2010) 139–150.
[38] Y. Palti, Toll-like receptors in bony fish: from genomics to function, Dev. Comp. Immunol. 35 (12) (2011) 1263–1272.
[39] D.B. Iliev, J.C. Roach, S. Mackenzie, J.V. Planas, F.W. Goetz, Endotoxin recognition: in fish or not in fish? FEBS (Fed. Eur. Biochem. Soc.) Lett. 579 (29) (2005) 6519–6528.
[40] E. Zoccola, S. Kellie, A.C. Barnes, Immune transcriptome reveals the mincle C-type lectin receptor acts as a partial replacement for TLR4 in lipopolysaccharide-mediated inflammatory response in barramundi (Lates calcarifer), Mol. Immunol. 83 (2017) 33–45.
[41] A.O. Aliprantis, R.B. Yang, D.S. Weiss, P. Godowski, A. Zychlinsky, The apoptotic signaling pathway activated by Toll-like receptor-2, EMBO J. 19 (13) (2000) 3325–3336.
[42] R. Schwandner, R. Dziarski, H. Wesche, M. Rothe, C.J. Kirschning, Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2, J. Biol. Chem. 274 (25) (1999) 17406–17409.
[43] K. Takeda, O. Takeuchi, S. Akira, Recognition of lipopeptides by Toll-like receptors, J. Endotoxin Res. 8 (6) (2002) 459–463.
[44] C.M.S. Ribeiro, T. Hermsen, A.J. Taverne-Thiele, H.F.J. Savelkoul, G.F. Wiegertjes, Evolution of recognition of ligands from gram-positive bacteria: similarities and differences in the TLR2-mediated response between mammalian vertebrates and teleost fish, J. Immunol. 184 (5) (2010) 2355–2368.
[45] Y.C. Wei, T.S. Pan, M.X. Chang, B. Huang, Z. Xu, T.R. Luo, P. Nie, Cloning and expression of Toll-like receptors 1 and 2 from a teleost fish, the orange-spotted grouper Epinephelus coioides, Vet. Immunol. Immunopathol. 141 (3–4) (2011) 173–182.
[46] T. Kono, M. Sakai, The analysis of expressed genes in the kidney of Japanese flounder, Paralichthys olivaceus, injected with the immunostimulant peptidoglycan, Fish Shellfish Immunol. 11 (4) (2001) 357–366.
[47] T. Kono, R. Kusuda, E. Kawahara, M. Sakai, The analysis of immune responses of a novel CC-chemokine gene from Japanese flounder Paralichthys olivaceus, Vaccine 21 (5–6) (2003) 446–457.
[48] M.P. Sepulcre, G. López-Castejón, J. Meseguer, V. Mulero, The activation of gilthead seabream professional phagocytes by different PAMPs underlines the behavioural diversity of the main innate immune cells of bony fish, Mol. Immunol. 44 (8) (2007) 2019–2026.
[49] A.M. Rieger, B.E. Hall, D.R. Barreda, Macrophage activation differentially modulates particle binding, phagocytosis and downstream antimicrobial mechanisms, Dev. Comp. Immunol. 34 (11) (2010) 1144–1159.
[50] L. Grayfer, J.G. Walsh, M. Belosevic, Characterization and functional analysis of goldfish (Carassius auratus L.) tumor necrosis factor-alpha, Dev. Comp. Immunol. 32 (5) (2008) 532–543.
[51] N.F. Neumann, D.R. Barreda, M. Belosevic, Generation and functional analysis of distinct macrophage sub-populations from goldfish (Carassius auratus L.) kidney leukocyte cultures, Fish Shellfish Immunol. 10 (1) (2000) 1–20.
[52] M.A. Esteban, V. Mulero, J. Muñoz, J. Meseguer, Methodological aspects of assessing phagocytosis of Vibrio anguillarum by leucocytes of gilthead seabream (Sparus aurata L.) by flow cytometry and electron microscopy, Cell Tissue Res. 293 (1) (1998) 133–141.
[53] M. Stosik, W. Deptuła, M. Trávniček, K. Baldy-Chudzik, Phagocytic and bactericidal activity of blood thrombocytes in carps (Cyprinus carpio), Vet. Med. 47 (1) (2002) 21–25.
[54] L. Passantino, A. Cianciotta, R. Patruno, M.R. Ribaud, E. Jirillo, G.F. Passantino, Do fish thrombocytes play an immunological role? Their cytoenzymatic profiles and function during an accidental piscine candidiasis in aquarium, Immunopharmacol. Immunotoxicol. 27 (2) (2005) 345–356.
[55] J.J. Evans, J.F. Bohnsack, P.H. Klesius, A.A. Whiting, J.C. Garcia, C.A. Shoemaker, S. Takahashi, Phylogenetic relationships among Streptococcus agalactiae isolated from piscine, dolphin, bovine and human sources: a dolphin and piscine lineage associated with a fish epidemic in Kuwait is also associated with human neonatal infections in Japan, J. Med. Microbiol. 57 (Pt 11) (2008) 1369–1376.
[56] C.M.J. Delannoy, M. Crumlish, M.C. Fontaine, J. Pollock, G. Foster, M.P. Dagleish, J.F. Turnbull, R.N. Zadoks, Human Streptococcus agalactiae strains in aquatic mammals and fish, BMC Microbiol. 13 (1) (2013) 41.
[57] D.T. Godoy, G.A. Carvalho-Castro, C.A.G. Leal, U.P. Pereira, R.C. Leite, H.C.P. Figueiredo, Genetic diversity and new genotyping scheme for fish pathogenic Streptococcus agalactiae, Lett. Appl. Microbiol. 57 (6) (2013) 476–483.
[58] D. Zhang, A. Li, Y. Guo, Q. Zhang, X. Chen, X. Gong, Molecular characterization of Streptococcus agalactiae in diseased farmed tilapia in China, Aquaculture 412–413 (2013) 64–69.
[59] J.J. Evans, P.H. Klesius, D.J. Pasnik, J.F. Bohnsack, Human Streptococcus agalactiae isolate in nile Tilapia (Oreochromis niloticus), Emerg. Infect. Dis. 15 (5) (2009) 774–776.
[60] M.K. Frøystad, M. Rode, T. Berg, T. Gjøen, A role for scavenger receptors in phagocytosis of protein-coated particles in rainbow trout head kidney macrophages, Dev. Comp. Immunol. 22 (5–6) (1998) 533–549.
[61] Z. Meng, X.Y. Zhang, J. Guo, L.X. Xiang, J.Z. Shao, Scavenger receptor in fish is a lipopolysaccharide recognition molecule involved in negative regulation of NF-κB activation by competing with TNF receptor-associated factor 2 recruitment into the TNF-α signaling pathway, J. Immunol. 189 (8) (2012) 4024–4039.
[62] J.B. Locke, K.M. Colvin, A.K. Datta, S.K. Patel, N.N. Naidu, M.N. Neely, V. Nizet, J.T. Buchanan, Streptococcus iniae capsule impairs phagocytic clearance and contributes to virulence in fish, J. Bacteriol. 189 (4) (2007) 1279–1287.
[63] T.R. Martin, J.T. Ruzinski, C.E. Rubens, E.Y. Chi, C.B. Wilson, The effect of type-

specific polysaccharide capsule on the clearance of group B streptococci from the lungs of infant and adult rats, J. Infect. Dis. 165 (2) (1992) 306–314.
[64] J. Källman, J. Schollin, C. Schalèn, A. Erlandsson, E. Kihlström, Impaired phagocytosis and opsonisation towards group B streptococci in preterm neonates, Arch. Dis. Child. Fetal Neonatal Ed. 78 (1) (1998) F46–F50.
[65] F.C.O. Los, T.M. Randis, R.V. Aroian, A.J. Ratner, Role of pore-forming toxins in bacterial infectious diseases, Microbiol. Mol. Biol. Rev. 77 (2) (2013) 173–207.
[66] U.P. Pereira, S.C. Soares, J. Blom, C.A.G. Leal, R.T.J. Ramos, L.C. Guimaraes, L.C. Oliveira, S.S. Almeida, S.S. Hassan, A.R. Santos, A. Miyoshi, A. Silva, A. Tauch, D. Barh, V. Azevedo, H.C.P. Figueiredo, In silico prediction of conserved vaccine targets in Streptococcus agalactiae strains isolated from fish, cattle, and human samples, Genet. Mol. Res. 12 (3) (2013) 2902–2912.
[67] C. Poyart, E. Pellegrini, O. Gaillot, C. Boumaila, M. Baptista, P. Trieu-Cuot, Contribution of Mn-cofactored superoxide dismutase (SodA) to the virulence of Streptococcus agalactiae, Infect. Immun. 69 (8) (2001) 5098–5106.
[68] L. Rajagopal, Understanding the regulation of Group B Streptococcal virulence factors, Future Microbiol. 4 (2) (2009) 201–221.
[69] M.P. Sepulcre, E. Sarropoulou, G. Kotoulas, J. Meseguer, V. Mulero, Vibrio anguillarum evades the immune response of the bony fish sea bass (Dicentrarchus labrax L.) through the inhibition of leukocyte respiratory burst and down-regulation of apoptotic caspases, Mol. Immunol. 44 (15) (2007) 3751–3757.
[70] P. Nordenfelt, S. Waldemarson, A. Linder, M. Mörgelin, C. Karlsson, J. Malmström, L. Björck, Antibody orientation at bacterial surfaces is related to invasive infection, J. Exp. Med. 209 (13) (2012) 2367–2381.
[71] D.E. Bessen, Localization of immunoglobulin A-binding sites within M or M-like proteins of group A streptococci, Infect. Immun. 62 (5) (1994) 1968–1974.
[72] G.J. Russell-Jones, E.C. Gotschlich, M.S. Blake, A surface receptor specific for human IgA on group B streptococci possessing the Ibc protein antigen, J. Exp. Med. 160 (5) (1984) 1467–1475.
[73] G. Lindahl, B. Akerstrom, J.P. Vaerman, L. Stenberg, Characterization of an IgA receptor from group B streptococci: specificity for serum IgA, Eur. J. Immunol. 20 (10) (1990) 2241–2247.
[74] J.C. Baiano, R.A. Tumbol, A. Umapathy, A.C. Barnes, Identification and molecular characterisation of a fibrinogen binding protein from Streptococcus iniae, BMC Microbiol. 8 (2008) 67.
[75] A.C. Barnes, M.T. Horne, A.E. Ellis, Streptococcus iniae expresses a cell surface non-immune trout immunoglobulin-binding factor when grown in normal trout serum, Fish Shellfish Immunol. 15 (5) (2003) 425–431.
[76] J.C. Baiano, R.A. Tumbol, A. Umapathy, A.C. Barnes, Identification and molecular characterisation of a fibrinogen binding protein from Streptococcus iniae, BMC Microbiol. 8 (2008) 67.
[77] J.J. Evans, P.H. Klesius, D.J. Pasnik, J.F. Bohnsack, Human Streptococcus agalactiae isolate in Nile tilapia (Oreochromis niloticus), Emerg. Infect. Dis. 15 (5) (2009) 774–776.
[78] U.P. Pereira, G.F. Mian, I.C.M. Oliveira, L.C. Benchetrit, G.M. Costa, H.C.P. Figueiredo, Genotyping of Streptococcus agalactiae strains isolated from fish, human and cattle and their virulence potential in Nile tilapia, Vet. Microbiol. 140 (1–2) (2010) 186–192.
[79] G. Liu, W. Zhang, C. Lu, Comparative genomics analysis of Streptococcus agalactiae reveals that isolates from cultured tilapia in China are closely related to the human strain A909, BMC Genomics 14 (1) (2013) 775.
[80] N. Suanyuk, F. Kong, D. Ko, G.L. Gilbert, K. Supamattaya, Occurrence of rare genotypes of Streptococcus agalactiae in cultured red tilapia Oreochromis sp. and Nile tilapia O. niloticus in Thailand-Relationship to human isolates? Aquaculture 284 (1–4) (2008) 35–40.