Subcellular components of probiotics *Kocuria* SM1 and *Rhodococcus* SM2 induce protective immunity in rainbow trout *(Oncorhynchus mykiss*, Walbaum) against *Vibrio anguillarum*

S.M. Sharifuzzaman[1,2], A. Abbass[1,3], J.W. Tinsley[1], B. Austin[1,4*]

[1] *School of Life Sciences, Heriot-Watt University, Edinburgh EH14 4AS, Scotland, UK*

[2] *Institute of Marine Sciences and Fisheries, University of Chittagong, Chittagong 4331, Bangladesh*

[3] *Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Banha University, Egypt*

[4] *Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK*

Running title: Cell components of probiotics and immunity

* Corresponding author at Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK. Tel.: +44 1786 467871; fax: +44 1786 472133

E-mail address: brian.austin@stir.ac.uk (B. Austin).

ABSTRACT

The efficacy of cellular components of probiotics *Kocuria* SM1 and *Rhodococcus* SM2 to protect rainbow trout (*Oncorhynchus mykiss*, Walbaum) against vibriosis was assessed. Groups of fish (average weight = 10–15 g) were immunized intraperitoneally (i.p.) with 0.1 ml of subcellular materials, i.e. 0.2 ± 0.05 mg protein per fish, comprising extracellular proteins (ECPs), cell wall proteins (CWPs) and whole cell proteins (WCPs) of SM1 and SM2, respectively, or with 0.1 ml of phosphate-buffered saline (PBS) to serve as the control. Seven days after administration, fish from each group were challenged i.p. with 0.1 ml of a suspension in PBS of 3×10^5 cells ml^{-1} per fish of *Vibrio anguillarum*. Use of CWPs and WCPs demonstrated significantly ($P < 0.05$) better protection against *V. anguillarum* insofar as mortalities were reduced to 11–17% [relative percent survival (RPS) = 80–87%], although ECPs fared less well (mortalities = 33–38%; RPS = 56–62%; $P > 0.05$), compared to 86% mortalities of the controls. The mode of action reflected activation of innate immune factors by CWPs and WCPs, demonstrating significantly ($P < 0.05$) increased expression of respiratory burst (optical density; OD_{550nm}) from 0.039 to 0.043–0.045, peroxidase (OD_{550nm}) from 0.26 to 0.37–0.55, and bacterial killing activities (i.e. percentage of surviving bacteria reduced from 79% to 56–57% for SM2). Moreover, an elevation of leucocyte number (from 1.93% to 1.98–2.93%; $P > 0.05$) and immunoglubolin level (from 27 mg ml^{-1} to 28.5–33 mg ml^{-1}; $P > 0.05$) were observed with the experimental groups. These results indicate that cell components of the probiotics stimulate an immune response.

Keywords:

Cell components

Probiotics

Kocuria

Rhodococcus

Vibrio anguillarum

Innate immunity

Rainbow trout

1. Introduction

Infectious diseases represent major limiting factors for the development of aquaculture production. The administration of probiotics as a control strategy has been shown to provide protection against many bacterial pathogens while reducing the dependency on antibiotics [1–4]. One of the fears surrounding the use of probiotics is that releasing live bacterial cells in large numbers into the vicinity of fish could interfere with the ecosystem [5]. Concerns have been voiced against the development of virulence traits through horizontal gene transfer, the pathogenicity to humans, and antagonism with other beneficial bacteria. In this respect, some probiotics belong to genera containing fish or human pathogens, such as *Aeromonas* [6,7], *Pseudomonas* [2,3], *Roseobacter* [8] and *Vibrio* [1,6]. Therefore, there is a concern about the possible reversion of these bacteria to virulence [9]. However it should be emphasized that, to date, this possibility has never been documented. Certainly, the use of purified cell components from bacteria with beneficial health properties may eliminate any problem associated with virulence. Recently, Abbass et al. [10] demonstrated that cell wall proteins (CWPs), outer membrane proteins (OMPs) and lipopolysaccharides (LPS) of probiotic *Aeromonas sobria* and *Bacillus subtilis* conferred protection against *Yersinia ruckeri*, the causative agent of enteric redmouth (ERM) disease in salmonids. In our previous studies, dietary *Kocuria* SM1 effectively prevented vibriosis in rainbow trout, with protection linked to stimulation of innate immune parameters [11–13]. Moreover, probiotic supplementation was shown to have an adjuvant effect by enhancing immunogenicity of various vaccines in human and animal models [see 14,15].

This study aimed to determine the protective nature of subcellular components of *Kocuria* SM1 and *Rhodococcus* SM2 in rainbow trout to *Vibrio anguillarum* infection.

2. Materials and methods

2.1 Fish

Rainbow trout (*Oncorhynchus mykiss*, Walbaum) of 10–15 g average weight were obtained from a commercial fish farm in Scotland. The fish were maintained in continuously aerated free-flowing dechlorinated freshwater at ~12°C, and fed with commercial pelleted diet (Skretting, Glasgow, Great Britain) at ~2% of body weight daily. The fish had neither been vaccinated nor exposed to fish diseases, and the health of the fish (= changes in physical appearance and internal organs followed by swabs from body surface, kidney and liver for bacteriology) was randomly checked initially upon receipt and then at 6–8 weeks intervals [16].

2.2 Bacteria

Vibrio anguillarum was originally recovered from diseased salmonids in Tasmania and obtained from the fish pathogen collection of the School of Life Sciences, Heriot-Watt University with authenticity verified after Austin and Austin [17]. Putative probiotic *Rhodococcus* SM2, which was part of the allochthonous microbiota and isolated from the intestine of rainbow trout, was identified as a probiotic after Sharifuzzaman and Austin [11]. Probiotic *Kocuria* SM1 was confirmed previously [11–13]. Bacterial cultures were routinely grown on tryptone soya agar (TSA; Oxoid, Basingstoke, Great Britain) plates and in tryptone soya broth (TSB; Oxoid) supplemented with 1% (w/v) sodium chloride (NaCl; BDH, Poole, Great Britain) referred to as TNA and TNB, respectively, with incubation at 26°C for 18–72

h. Stock cultures were stored in TNB containing sterile (121°C for 15 min) 20% (v/v) glycerol (Sigma-Aldrich, Basingstoke, Great Britain) at −70°C.

2.3 Sub-cellular proteins of probiotics

For upscaling of the probiotics, a loopful of SM1 or SM2 from overnight cultures on TNA plates were inoculated in 10 ml volumes of TNB and incubated overnight at 26°C. Then, cultures were inoculated at a 1:100 dilution in TNB and incubated (18 h, 26°C) on a shaker at 4 × *g*. These cultures were used to prepare the sub-cellular proteins.

2.3.1 Collection of extracellular proteins (ECPs)

ECPs were prepared as described by Barbey *et al.* [18], with slight modifications. Briefly, bacterial cells were removed by centrifuging at 20,000 × *g* for 30 min at 4°C (Avanti J-26 XP centrifuge; Beckman Coulter, Brea, CA, USA) and the supernatants were filtered through a 0.22 μm porosity filter (Millex-GS; Millipore, Cork, Ireland). Then a final concentration of 10% (w/v) trichloroacetic acid (TCA; Sigma-Aldrich) was added to the supernatant, mixed well (1 min vortex) and placed in an ice bath for 3 h. The mixture was transferred to a 1.5 ml capacity Eppendorf tube, and the precipitated proteins were harvested by centrifugation (20,000 × *g*, 30 min) at 4°C using a microcentrifuge (Microfuge 22R centrifuge; Beckman Coulter, High Wycombe, Great Britain). The pellet was washed four times with 1 ml volumes of cold methanol (BDH), and dried in a ~95°C heat block for 5–10 min to drive off the residual methanol. Finally, the pellet was washed twice with phosphate-buffered saline (PBS; Sigma-Aldrich), and redissolved in PBS.

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2.3.2 Separation of cell wall proteins (CWPs)

The modified method of Abbass et al. [10] was used. Thus, suitably upscaled bacterial cultures were centrifuged (2,200 × *g*, 15 min, 4°C) in a Mark IV refrigerated centrifuge (Baird Tatlock, London, Great Britain). The cell pellets were resuspended in 0.5 mM NaCl and washed twice with 0.05 M Tris-HCl buffer (Sigma-Aldrich), pH 7.8. Then, the cells were resuspended to 20 ml with a solution of 0.05 M Tris-HCl (pH 7.8) containing 1 mM phenylmethylsulphonyl fluoride (PMSF; Sigma-Aldrich), and disrupted by sonication (6 × 5 min; after each 5 min sonication the sample was incubated for 4 min in ice) on ice with a sonicator (MSE ultrasonic power unit; MSE, London, Great Britain). Cells disruption was microscopically at ×1000 on a Kyowa (Tokyo, Japan) light microscope. The sonicated product was centrifuged (2,200 × *g*, 5 min, 4°C) to remove cell debris and the cell-walls were separated by centrifugation (20,000 × *g*, 30 min) of the supernatant at 4°C. The pellet was resuspended in 100 mM NaCl, washed twice in PBS and suspended in the same buffer.

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2.3.3 Preparation of whole cell proteins (WCPs)

The WCPs were prepared according to Abbass et al. [10], with slight modification. Thus, the cells were collected by centrifugation (2,200 × *g*, 15 min, 4°C), and the cell pellets were collected and resuspended with 0.5 mM NaCl, washed twice with Milli-Q water, then resuspended in Milli-Q water containing 1mM PMSF and frozen at −20°C. Cells in suspension were thawed and disrupted by sonication on ice for 6 × 5 min, mixed with equal volumes (v/v) of lysis buffer [4 g (w/v) SDS, 20 ml (v/v) glycerol, 10 ml (v/v) β-mercaptoethanol (Sigma-Aldrich) and 12.5 ml 0.5 M (w/v) Tris-HCl (pH 6.8) per 100 ml of Milli-Q water] and kept over ice for 30 min. The supernatant was collected following centrifugation and contained the WCPs. Precipitation of proteins was done following the methanol-chloroform method by Wessel and Flugge [19]. Briefly, 0.4 ml (v/v) methanol (BDH) was added to 0.1 ml lysate, vortexed well, then 0.1 ml (v/v) chloroform (BDH) was

added, and vortexed again before addition of 0.3 ml distilled water. The mixture was vortexed and spun for 2 min at 8,950 × *g* at 4°C. The top aqueous layer was carefully removed and 0.3 ml of methanol added, vortexed and centrifuged (8,950 × *g*, 5 min, 4°C) to pellet the proteins. The protein pellet was air dried and suspended in PBS.

2.4 Electrophoresis

One-dimensional denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separation of soluble protein fractions in ECPs, CWPs and WCPs extracts was carried out according to Laemmli [20]. Briefly, concentrated proteins (~1 mg ml^{-1}) were measured (see Section 2.5), mixed 1:1 with 2× Laemmli sample buffer [2.5 ml (w/v) 0.5 M Tris-HCl (pH 6.8), 2 ml (v/v) glycerol, 4 ml (w/v) 10% SDS, 0.31 g (w/v) dithiothreitol (DTT, Sigma-Aldrich), 0.04% (w/v) bromophenol blue; made up to 10 ml with distilled water], boiled at ~100°C for 10 min in a heating block, and loaded (10–30 µl protein sample $well^{-1}$) into Tris-HCl-SDS gels with 4% (w/v) polyacrylamide stacking gel, and 10% (for ECPs and WCPs) or 12% (w/v) polyacrylamide separating (= resolving) gel. Also, 10 µl of prestained molecular-mass standards (Bio-Rad, Hemel Hempstead, Great Britain) were loaded in one lane on all gels. The resolving gel solutions (20 ml) contained 70 µl of 10% ammonium peroxodisulphate (APS; Sigma-Aldrich) and 15 µl of N,N,N',N'-tetramethylethylenediamine (TEMED; Sigma-Aldrich), whereas stacking gels (10 ml) were with 50 µl 10% APS and 10 µl TEMED. Electrophoresis was carried out in a Mini Protean II electrophoresis chamber (Bio-Rad) for ~1.5 h at 150 V constant voltages, in running buffer [12.0 g (w/v) Tris, 57.6 g (w/v) glycine (Sigma-Aldrich), 2.0 g (w/v) SDS; make up to 2.0 l with distilled water] at room temperature. After the electrophoretic separation, protein bands were visualized by staining the gel for 1 h with Coomassie brilliant blue G solution (Sigma-Aldrich) followed by destaining in methanol-acetic acid-water solution (40:10:50) for 3 h.

166 Densitometry of gels was performed with the aim of assigning relative molecular masses to
167 the ECPs, CWPs and WCPs separated bands. Protein bands were digitally imaged using a
168 Canon CanoScan 3000F scanner (Canon, Lake Success, NY, USA).
169
170 *2.5 Total protein*
171 Before inoculation into fish, the concentration of total protein present in the cellular
172 components dissolved in PBS was measured with BioAssay Systems (Hayward, CA, USA)
173 QuantiChrom™ protein assay kit (QCPR-500). Thus, standard [Bovine serum albumin
174 (BSA)] and samples were diluted in PBS according to the manufacturer's instructions. For
175 this, 10 µl volumes of diluted standard and samples were transferred into wells of flat bottom
176 96-well plates (Nalge Nunc, Loughborough, Great Britain). Then, 200 µl of working reagent,
177 which was supplied with the kit, was added to each well and mixed gently. The intensity of
178 colour obtained was measured at 620 nm in a microplate absorbance reader (Sunrise; Tecan,
179 Reading, Great Britain). The optical density (OD) of a blank was deducted from the OD of
180 standard, and plotted against the protein concentrations of standard to produce the standard
181 curve. Then, the OD values of the samples were plotted onto the standard curve to obtain the
182 protein concentration in the sample. If necessary, the proteins were re-diluted in PBS to
183 achieve the required concentration (= ~2 mg ml^{-1}) and stored at −20°C for subsequent use.
184
185 *2.6 Fish experiment*
186 Groups of 10 rainbow trout in triplicate were inoculated intraperitoneally (i.p.) with 0.1 ml
187 volumes of 2.0 ± 0.5 mg ml^{-1} ECPs, CWPs and WCPs derived from SM1 and SM2, or with
188 PBS as controls. The fish were fed with control diet for 7 days before challenge i.p. with
189 0.1 ml volumes of a suspension of *V. anguillarum* in 0.9% (w/v) saline containing
190 3×10^5 cells ml^{-1} [determined by cell counts using a haemocytometer slide (Improved

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191 Neubauer type; Merck, Lutterworth, Great Britain) on a Kyowa light microscope at a
192 magnification of ×400] per fish. Previous work had determined that these cell numbers led to
193 the death of >80% of the fish populations [11–13]. The groups of control and treated fish
194 were observed daily for two weeks, and all dead fish and the survivors were examined
195 bacteriologically using swabs from the peritoneal cavity, kidney and liver to determine the
196 presence of the pathogen [17]. The relative percent survival (RPS) was calculated after
197 Amend [21]. Challenge experiments were maintained in static, aerated dechlorinated
198 freshwater at ~18 °C with ~50% water exchange daily. The care and use of experimental
199 animals complied with local animal welfare regulations.
200
201 Additional sub-groups of 5 fish were used for immunological assays involving blood
202 and serum. Individual fish were sampled once to avoid multiple bleeding and/or handling
203 stress.
204
205 *2.7 Immunological assay*
206 Blood was collected by venepuncture using syringes coated with heparin (Sigma-Aldrich) and
207 transferred immediately into 9 ml capacity lithium heparin vacuettes (Greiner, Stonehouse,
208 Great Britain) on ice. For serum, the blood was transferred into vacuettes containing Z Serum
209 Clot Activator (Greiner) and allowed to clot at 4°C for 4 h. The sera were separated by
210 centrifugation (2,000 × *g* for 25 min at 4°C) and stored at −70°C until required.
211
212 Unclotted blood was used to determine the respiratory burst activity [11], and serum
213 was used to assess the peroxidase and leukocrit content, and bacterial killing activity [13]
214 according to the reported methods of Sharifuzzaman and Austin [11,13]. The method of
215 Siwicki and Anderson [22] as described by Panigrahi et al. [23] was followed to determine the

total immunoglobulin (Ig) level in serum. For this, 100 µl of serum (100-fold dilutions in PBS) was mixed with an equal volume of 12% (v/v) solution of polyethylene glycol (10,000 MW, PEG; Sigma-Aldrich) and incubated for 2 h at room temperature to deposit the Ig molecules. These were removed by centrifugation (5,000 × *g*, 4°C) and the protein content was determined by the Bradford method [11]. This value was subtracted from the total protein content of serum, which corresponded to the total Ig content (mg ml^{-1}).

2.8 Statistics

Data were analysed statistically by one-way analysis of variance (ANOVA) and Duncan's comparison of means when necessary. Percentage data were transformed to square-root arcsine values to homogenize variance. All statistical tests were conducted using the computerized software Statistical Package for Social Sciences (SPSS; Release 14.0, SPSS, Chicago, IL, USA). Differences were considered statistically significant when $P < 0.05$. The data were plotted using the program Microsoft Excel (Microsoft, Seattle WA, USA).

3. Results

3.1 Protective efficacy

Fish injected with ECPs, CWPs and WCPs of SM1 and SM2, respectively, followed by challenge on day 8 with *V. anguillarum* experienced 11–38% mortalities (RPS = 56–87%) compared with 86% mortalities in the controls (Fig. 1). In particular, use of CWPs (SM1 = 17%; RSP = 80%, and SM2 = 14%; RPS = 84%) and WCPs (SM1 = 13%; RPS = 85%, and SM2 = 11%; RPS = 87%) of the probiotics led to significant ($P < 0.05$) decreases in mortalities. However, the total mortalities (33–38%; RPS = 56–62%; $P > 0.05$) for the ECPs did not differ statistically when compared to controls (Fig. 1). Overall, these results pointed to the potential of using cellular components of probiotics in controlling bacterial fish diseases

241 and may well explain the parts of the cells involved in protection. Moreover, SDS-PAGE
242 profiles of WCPs of probiotics revealed 23–26 protein bands (range: 17.3 to 209 kDa) in
243 comparison to 11–12 well stained bands between 13.1 kDa and 209 kDa in the CWPs. This
244 compares with 6–8 bands (range: 22 to 182 kDa) for the ECPs (Figs. 2 and 3). Some likely
245 common proteins (arrows on the gel image) were also evident between WCPs and CWPs for
246 both probiotics (Figs. 2 and 3).
247
248 *3.2 Mode of action*
249 A significant ($P < 0.05$) increase in the respiratory burst activity, i.e. production of superoxide
250 anion, was observed in groups receiving CWPs and WCPs, with the highest level 0.045 in
251 fish inoculated with WCPs of SM2 as compared to the controls, 0.039. Although non-
252 significant ($P > 0.05$), the level of induction maintained at elevated levels with ECPs, 0.041–
253 0.042 (Fig. 4). The serum peroxidase activity was significantly different from the controls (=
254 0.26) in experimental (= 0.37–0.55; $P < 0.05$) groups except that inoculated with ECPs ranged
255 from 0.25–0.28 (Fig. 5). Moreover, all groups of fish inoculated with cellular proteins of
256 probiotics had an increased ($P > 0.05$) number of white blood cells between 1.98% and
257 2.93%, as examined by measurement of the leukocrit value, compared with the controls,
258 1.93% (Fig. 6). Significantly ($P < 0.05$) enhanced bacteriocidal activity was recorded
259 following inoculation with CWPs (i.e. percentage of surviving bacteria = 56%) and WCPs
260 (i.e. percentage of surviving bacteria = 57%) of SM2 compared with the controls (i.e.,
261 percentage of surviving bacteria = 79%). Improved bacterial killing was also noted in the rest
262 of the treated groups than those of the controls, the differences were not significant (Fig. 7).
263 The data for total immunoglobulin levels were recorded with a non-significant increase, and
264 were ranged from 28.5–33 mg ml^{-1} ($P > 0.05$) for experimental fish and 27 mg ml^{-1} for
265 control fish (Fig. 8).

4. Discussion

The use of cell wall proteins (CWPs) and whole cell proteins (WCPs) of SM1 and SM2 led to significant resistance to *V. anguillarum* infection in rainbow trout. In contrast, extracellular proteins (ECPs) of both probiotic fared less well. From previous work, cell-free supernatants of probiotics *A. sobria* and *Clostridium butyricum* contributed less protection compared to viable cells [4,7]. Moreover, immunization with membrane vesicle (MVs) rich supernatant of *Flavobacterium psychrophilum* cells did not protect rainbow trout against infection [24]. Further support of the ineffectiveness of ECPs of *A. salmonicida* subsp. *salmonicida* and *A. hydrophila*, respectively, was reported in Atlantic salmon (*Salmon salar*) against classical furunculosis [25] and in Indian major carp (*Catla catla*) against *A. hydrophila* disease [26] when compared to whole cell or cell-associated antigen preparations of vaccine. These results were in contrast with the observations of Evenberg et al. [27] and Gudmundsdóttir and Magnadóttir [28], who noted relatively better protection against atypical *A. salmonicida* in carp (*Cyprinus carpio*) and salmon by vaccination with ECPs. It is should be noted that the protection rate by extracellular components of bacteria can vary with the growth phase, nutrient level (= growth medium), pH, temperature and owing to differences of other unknown *in vivo* vs. *in vitro* growth conditions, and due to the quantity of inocula. Pasnik et al. [29] highlighted the decreasing efficacy of ECP vaccine prepared from *Streptococcus agalactiae* when stored for one year at 4°C, and noted apparent loss of high molecular weight antigens (i.e. the stored ECP showed bands of <55 kDa compared to 47–75 kDa bands with freshly prepared ECP). Thus, considerations of these factors deserve further attention while evaluating the usefulness of ECPs of probiotics.

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The most comparable study to the present one was that of Abbass et al. [10] in which CWPs, WCPs, OMPs (outer membrane proteins) and LPS (lipopolysaccharides) of probiotics *A. sobria* and *B. subtilis* when administered to rainbow trout led to complete protection compared with 90% mortality in the controls against a new biogroup of *Y. ruckeri* that had been resistant to conventional vaccines. Likewise, enhanced resistance against vibriosis (= *V. anguillarum*) in rainbow trout and Japanese flounder (*Paralichthys olivaceus*), and *Enterococcus seriolicida* infection of yellowtail (*Seriola quinqueradiata*) resulted after dietary supplementation of peptidoglycan (PGN) derived from *Bifidobacterium* sp. [30–32]. Moreover, vaccination with outer membrane fraction of *F. psychrophilum* induced significantly higher protection against coldwater disease, achieving RPS values of 93–95% in rainbow trout and 64–71% in ayu (*Plecoglossus altivelis*), respectively [33].

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The cell components of micro-organisms have been reported to activate the immune system of many animals, including fish. For example, LPS, which is a component of the outer cell wall membrane of Gram-negative bacteria, possesses immunogenic properties, and a small dose (i.e., μg ml^{-1}; Iliev et al. [34]) can induce the production/activation of antibody, lysozyme, alternative complement pathway, B and T lymphocytes, cytokines like interleukin (IL)-2 and -6, pro-inflammatory cytokines like IL-1β, tumour necrosis factor (TNF)-α and several other factors from macrophages, including phagocytic activity in fish [35,36]. OMPs of Gram-negative bacteria are also known to be immunodominant antigens, and may provoke strong humoral and cellular immune responses in fish [37,38]. Moreover, PGN, present in Gram-positive and -negative bacterial cell walls and lipoteichoic acids (LTA) from Gram-positive bacteria demonstrated to be to an excellent immunostimulant in fish [32,39]. After inoculation, ECPs may often lead to adequate immunity against piscine pathogens, and some authors have suggested the importance of including inactivated ECPs in the design of

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effective vaccines [40–42]. β-glucans, which are polysaccharides from the cell walls of yeast and fungi, are also found in plants, algae and some bacteria, and have been determined to enhance specific and non-specific immune responses in several fish species, i.e. yellow croaker, Asian catfish, carp and zebra fish [43,44]. Furthermore, nucleotides and RNA shown to have immunostimulatory effects, and thus enhance fish resistance to pathogens [45]. Similarly, bacterial DNA is reported to activate antigen-presenting cells (APCs), in mice and fish models [see 46]. These data suggest that the non-specific defence of vertebrates has evolved towards recognition of conserved microbial structures, i.e. yeast/fungal cell wall β-glucan, bacterial LPS and peptidoglycan, and oligonucleotides – all of which have been reported to enhance the host resistance against microbial diseases [47]. Therefore, in this study, a protective immune response in rainbow trout after inoculation with subcellular components of probiotics was not surprising.

This study demonstrated an enhanced respiratory burst, peroxidase and bacteriocidal activity, and white blood cell numbers and total Ig levels following inoculation with CWPs and WCPs of SM1 and SM2 compared with the controls. Similarly, immune factors such as complement, lysozyme and phagocytic activity [32], and IL-1β, IL-6 and interferon gamma (IFN-γ) expression [39,48] were upregulated in fish by administration of PGN. Recently, MacKenzie et al. [39] observed that both LTA (from *B. subtilis*) and PGN (from *S. aureus* and *B. subtilis*) hold an equal potency to induce cytokine gene expression in rainbow trout macrophages. Moreover, they proposed that the induction of cytokines in trout by crude LPS was primarily due to the contaminating PGN and nucleic acids, since ultrapure LPS found inactive. It is mentionable that the use of whole probiotics cells, i.e. dietary supplementation of live or inactivated probiotic bacteria cells [12,49] or intraperitoneal injection of yeast (*Saccharomyces cerevisiae*) cells [50] were also induced innate immunity in fish. Based on

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these data, it is entirely possible that an improved innate defense mechanisms non-specifically inhibited the growth of or directly destroyed microbial pathogens, and contributed to the host protection from pathogen invasion [11–13].

Exactly why the preparations of cellular protein induced higher protection than the ECPs is unclear. However, it is likely that CWPs and WCPs – i) are very immunogenic (i.e. interact efficiently with the immune system of the host) and hold higher quantity of protective antigen, ii) have multiple immunodominant protein antigens with higher molecular masses, and iii) composed of more complex chemical structure and different particle form, influencing immunogenicity. Of relevance, the 57 kDa protein of the Gram-positive fish pathogen *Renibacterium salmoninarum* has been the target for vaccine studies [51]. In particular, low molecular weight antigens, i.e. < ~30 kDa, from *V. ordalii* and *F. psychrophilum* led to minimal protection in salmonids [42,52]. Nevertheless, PGN (<40% of bacterial cell mass; Hessle et al. [53]), LTA (~2% of the dry cell and ~6 mol% of the cytoplasmic membrane; Gutberlet et al. [54]) and lipoproteins/peptides have been recognized as prominent representatives of Gram-positive cell components modulating the innate immune system in various animal species [39,48,53,55-58].

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Taken together, these data highlight the potential of using cellular components of probiotics in controlling bacterial fish diseases and may well explain the parts of the cells involved in protection. Thus, the cell components of probiotics may consider being a good candidate as adjuvant or vaccine.

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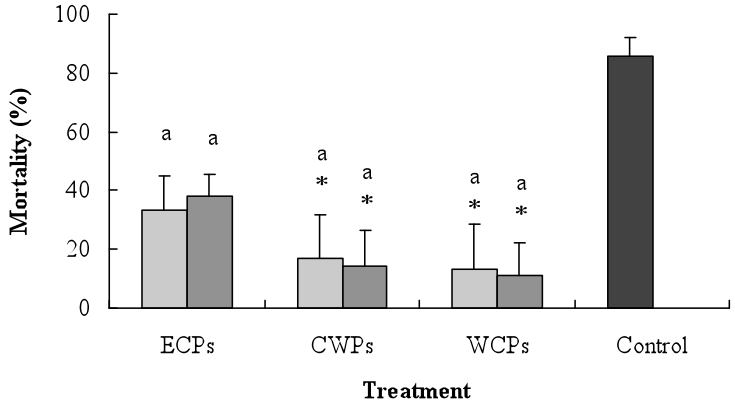

Fig. 1. Percent mortality (%) of rainbow trout following i.p. challenge with *V. anguillarum* after inoculation with cellular components of the probiotics *Kocuria* SM1 (□) and *Rhodococcus* SM2 (■) for 7 days, compared with controls (■). Data represent the average ± SD from a triplicate set of 10 fish. *Significantly different ($P < 0.05$) from the control group. Means without a common letter differ significantly ($P < 0.05$) among different treatments with cellular proteins of the probiotics.

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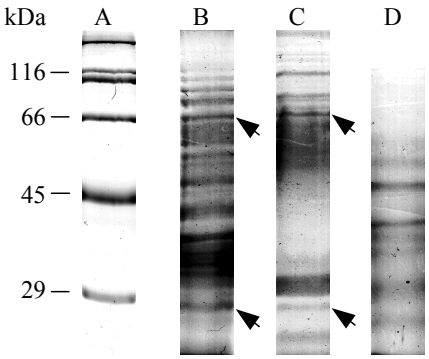

Fig. 2. Coomassie brilliant blue stained SDS-PAGE analysis of proteins extracted from the probiotic *Kocuria* SM1. Lanes: (A) protein markers, (B) whole cell proteins (WCPs), (C) cell wall proteins (CWPs) and (D) extracellular proteins (ECPs). Arrows point to likely common proteins.

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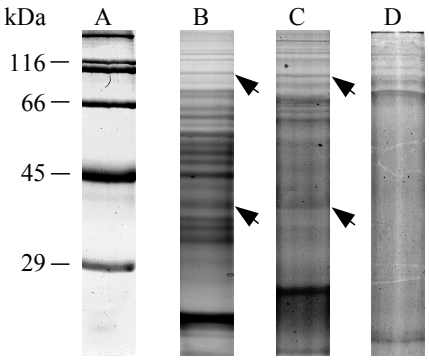

Fig. 3. Coomassie brilliant blue stained SDS-PAGE analysis of proteins extracted from the probiotic *Rhodococcus* SM2. Lanes: (A) protein markers, (B) whole cell proteins (WCPs), (C) cell wall proteins (CWPs) and (D) extracellular proteins (ECPs). Arrows point to likely common proteins.

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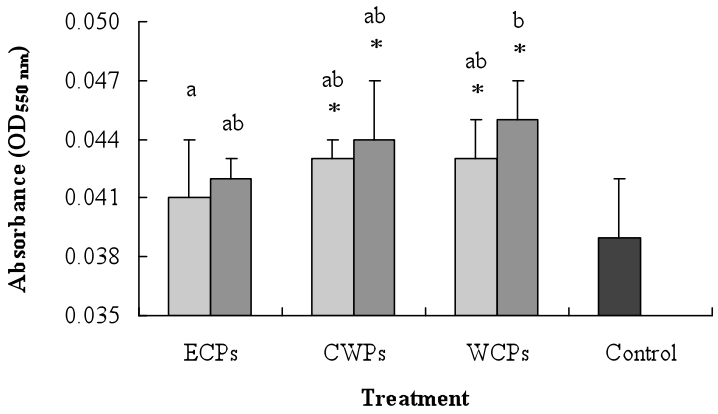

Fig. 4. Blood respiratory burst activity in rainbow trout inoculated with cellular components of the probiotics *Kocuria* SM1 (▫) and *Rhodococcus* SM2 (▪), compared with controls (■). Data represent the average ± SD from a triplicate set of 5 fish. *Significantly different ($P < 0.05$) from the control group. Means without a common letter differ significantly ($P < 0.05$) among different treatments with cellular proteins of probiotics.

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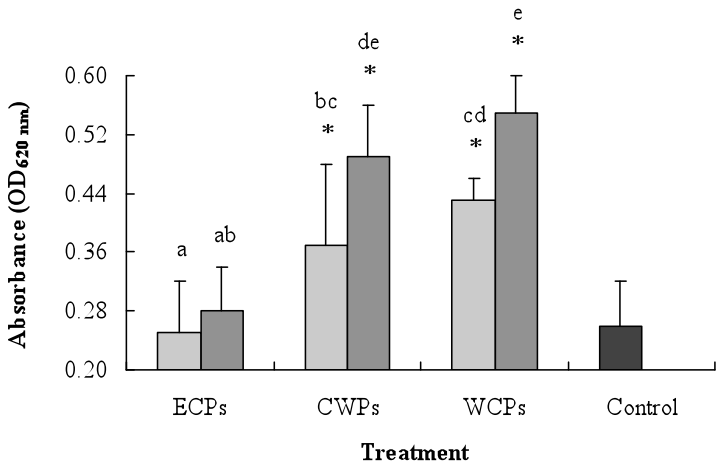

Fig. 5. Serum peroxidase activity of rainbow trout inoculated with cellular components of the probiotics *Kocuria* SM1 (□) and *Rhodococcus* SM2 (▩), compared with controls (■). Data represent the average ± SD from a triplicate set of 5 fish. *Significantly different ($P < 0.05$) from the control group. Means without a common letter differ significantly ($P < 0.05$) among different treatments with cellular proteins of probiotics.

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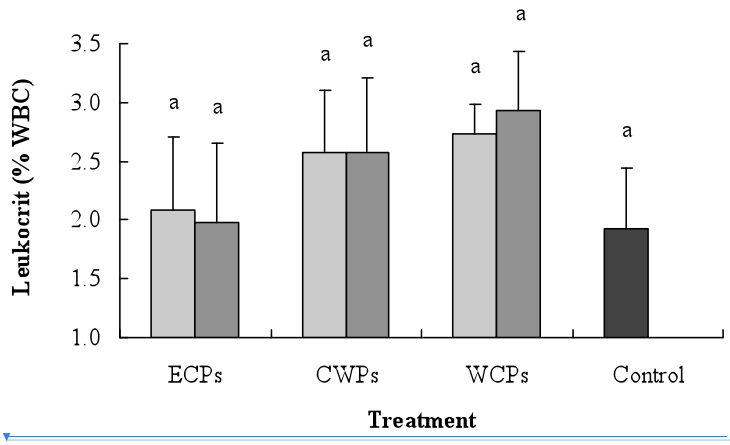

Fig. 6. The leukocrit content, i.e. white blood cell numbers (% WBC) in rainbow trout inoculated with cellular components of the probiotics *Kocuria* SM1 (□) and *Rhodococcus* SM2 (■), compared with controls (■). Data represent the average ± SD from a triplicate set of 5 fish. Means without a common letter differ significantly ($P < 0.05$) between treatments.

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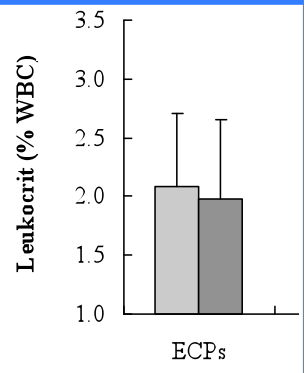

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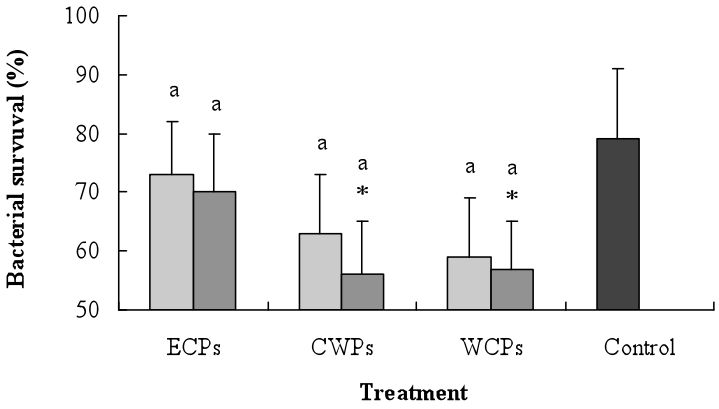

Fig. 7. Survival percentage (%) of *V. anguillarum* incubated with serum of rainbow trout inoculated with cellular components of the probiotics *Kocuria* SM1 (▫) and *Rhodococcus* SM2 (▪), compared with controls (■). Data represent the average ± SD from a triplicate set of 5 fish. *Significantly different ($P < 0.05$) from the control group. Means without a common letter differ significantly ($P < 0.05$) among different treatments with cellular proteins of probiotics.

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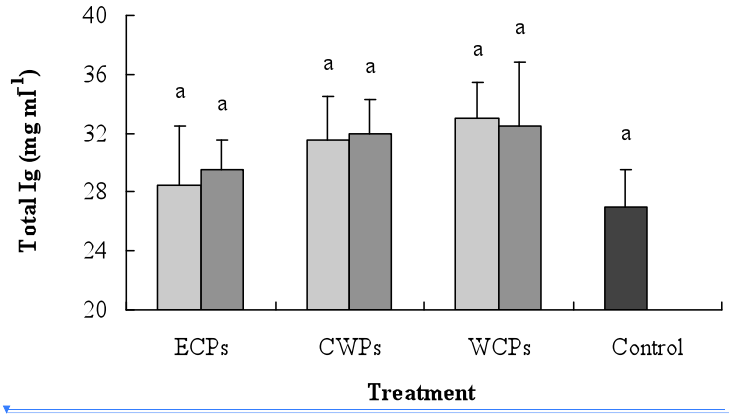

Fig. 8. Total immunoglobulin (Ig) content in the blood of rainbow trout inoculated with cellular components of the probiotics *Kocuria* SM1 (□) and *Rhodococcus* SM2 (■), compared with controls (■). Data represent the average ± SD from a triplicate set of 5 fish. Means without a common letter differ significantly ($P < 0.05$) between treatments.

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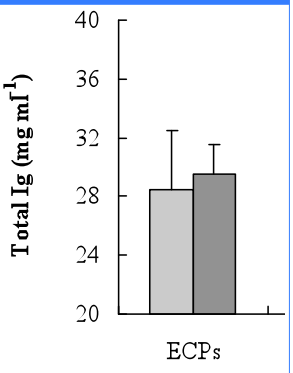

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