

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

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Running title: Cell components of probiotics and immunity

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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 immunoglobulin 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

43 *Rhodococcus*
44 *Vibrio anguillarum*
45 Innate immunity
46 Rainbow trout

47
48 **1. Introduction**
49 Infectious diseases represent major limiting factors for the development of aquaculture
50 production. The administration of probiotics as a control strategy has been shown to provide
51 protection against many bacterial pathogens while reducing the dependency on antibiotics [1–
52 4]. One of the fears surrounding the use of probiotics is that releasing live bacterial cells in large
53 numbers into the vicinity of fish could interfere with the ecosystem [5]. Concerns have been
54 voiced against the development of virulence traits through horizontal gene transfer, the
55 pathogenicity to humans, and antagonism with other beneficial bacteria. In this respect, some
56 probiotics belong to genera containing fish or human pathogens, such as *Aeromonas* [6,7],
57 *Pseudomonas* [2,3], *Roseobacter* [8] and *Vibrio* [1,6]. Therefore, there is a concern about the
58 possible reversion of these bacteria to virulence [9]. However it should be emphasized that, to
59 date, this possibility has never been documented. Certainly, the use of purified cell components
60 from bacteria with beneficial health properties may eliminate any problem associated with
61 virulence. Recently, Abbass et al. [10] demonstrated that cell wall proteins (CWPs), outer
62 membrane proteins (OMPs) and lipopolysaccharides (LPS) of probiotic *Aeromonas sobria* and
63 *Bacillus subtilis* conferred protection against *Yersinia ruckeri*, the causative agent of enteric
64 redmouth (ERM) disease in salmonids. In our previous studies, dietary *Kocuria* SM1 effectively
65 prevented vibriosis in rainbow trout, with protection linked to stimulation of innate immune
66 parameters [11–13]. Moreover, probiotic supplementation was shown to have an adjuvant effect
67 by enhancing immunogenicity of various vaccines in human and animal models [see 14,15].

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

70

71 **2. Materials and methods**

72 *2.1 Fish*

73 Rainbow trout (*Oncorhynchus mykiss*, Walbaum) of 10–15 g average weight were obtained
74 from a commercial fish farm in Scotland. The fish were maintained in continuously aerated
75 free-flowing dechlorinated freshwater at ~12°C, and fed with commercial pelleted diet
76 (Skretting, Glasgow, Great Britain) at ~2% of body weight daily. The fish had neither been
77 vaccinated nor exposed to fish diseases, and the health of the fish (= changes in physical
78 appearance and internal organs followed by swabs from body surface, kidney and liver for
79 bacteriology) was randomly checked initially upon receipt and then at 6–8 weeks intervals
80 [16].

81

82 *2.2 Bacteria*

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

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

94

95 2.3 Sub-cellular proteins of probiotics

96 For upscaling of the probiotics, a loopful of SM1 or SM2 from overnight cultures on TNA
97 plates were inoculated in 10 ml volumes of TNB and incubated overnight at 26°C. Then,
98 cultures were inoculated at a 1:100 dilution in TNB and incubated (18 h, 26°C) on a shaker at
99 4 × g. These cultures were used to prepare the sub-cellular proteins.

100

101 2.3.1 Collection of extracellular proteins (ECPs)

102 ECPs were prepared as described by Barbey *et al.* [18], with slight modifications. Briefly,
103 bacterial cells were removed by centrifuging at 20,000 × g for 30 min at 4°C (Avanti J-26 XP
104 centrifuge; Beckman Coulter, Brea, CA, USA) and the supernatants were filtered through a
105 0.22 µm porosity filter (Millex-GS; Millipore, Cork, Ireland). Then a final concentration of
106 10% (w/v) trichloroacetic acid (TCA; Sigma-Aldrich) was added to the supernatant, mixed
107 well (1 min vortex) and placed in an ice bath for 3 h. The mixture was transferred to a 1.5 ml
108 capacity Eppendorf tube, and the precipitated proteins were harvested by centrifugation
109 (20,000 × g, 30 min) at 4°C using a microcentrifuge (Microfuge 22R centrifuge; Beckman
110 Coulter, High Wycombe, Great Britain). The pellet was washed four times with 1 ml volumes
111 of cold methanol (BDH), and dried in a ~95°C heat block for 5–10 min to drive off the
112 residual methanol. Finally, the pellet was washed twice with [phosphate-buffered saline \(PBS;](#)
113 [Sigma-Aldrich](#)), and redissolved in PBS.

114

115 2.3.2 Separation of cell wall proteins (CWPs)

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116 The modified method of Abbass et al. [10] was used. Thus, suitably upscaled bacterial
117 cultures were centrifuged ($2,200 \times g$, 15 min, 4°C) in a Mark IV refrigerated centrifuge (Baird
118 Tatlock, London, Great Britain). The cell pellets were resuspended in 0.5 mM NaCl and
119 washed twice with 0.05 M Tris-HCl buffer (Sigma-Aldrich), pH 7.8. Then, the cells were
120 resuspended to 20 ml with a solution of 0.05 M Tris-HCl (pH 7.8) containing 1 mM
121 phenylmethylsulphonyl fluoride (PMSF; Sigma-Aldrich), and disrupted by sonication (6
122 $\times 5$ min; after each 5 min sonication the sample was incubated for 4 min in ice) on ice with a
123 sonicator (MSE ultrasonic power unit; MSE, London, Great Britain). Cells disruption was
124 microscopically at $\times 1000$ on a Kyowa (Tokyo, Japan) light microscope. The sonicated
125 product was centrifuged ($2,200 \times g$, 5 min, 4°C) to remove cell debris and the cell-walls were
126 separated by centrifugation ($20,000 \times g$, 30 min) of the supernatant at 4°C . The pellet was
127 resuspended in 100 mM NaCl, washed twice in PBS and suspended in the same buffer.

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129 2.3.3 Preparation of whole cell proteins (WCPs)

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

141 added, and vortexed again before addition of 0.3 ml distilled water. The mixture was vortexed
142 and spun for 2 min at $8,950 \times g$ at 4°C . The top aqueous layer was carefully removed and
143 0.3 ml of methanol added, vortexed and centrifuged ($8,950 \times g$, 5 min, 4°C) to pellet the
144 proteins. The protein pellet was air dried and suspended in PBS.

145

146 *2.4 Electrophoresis*

147 One-dimensional denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis
148 (SDS-PAGE) separation of soluble protein fractions in ECPs, CWPs and WCPs extracts was
149 carried out according to Laemmli [20]. Briefly, concentrated proteins ($\sim 1 \text{ mg ml}^{-1}$) were
150 measured (see Section 2.5), mixed 1:1 with $2\times$ Laemmli sample buffer [2.5 ml (w/v) 0.5 M
151 Tris-HCl (pH 6.8), 2 ml (v/v) glycerol, 4 ml (w/v) 10% SDS, 0.31 g (w/v) dithiothreitol
152 (DTT, Sigma-Aldrich), 0.04% (w/v) bromophenol blue; made up to 10 ml with distilled
153 water], boiled at $\sim 100^{\circ}\text{C}$ for 10 min in a heating block, and loaded (10–30 μl protein sample
154 well^{-1}) into Tris-HCl-SDS gels with 4% (w/v) polyacrylamide stacking gel, and 10% (for
155 ECPs and WCPs) or 12% (w/v) polyacrylamide separating (= resolving) gel. Also, 10 μl of
156 prestained molecular-mass standards (Bio-Rad, Hemel Hempstead, Great Britain) were
157 loaded in one lane on all gels. The resolving gel solutions (20 ml) contained 70 μl of 10%
158 ammonium peroxodisulphate (APS; Sigma-Aldrich) and 15 μl of N,N,N',N' -
159 tetramethylethylenediamine (TEMED; Sigma-Aldrich), whereas stacking gels (10 ml) were
160 with 50 μl 10% APS and 10 μl TEMED. Electrophoresis was carried out in a Mini Protean II
161 electrophoresis chamber (Bio-Rad) for $\sim 1.5 \text{ h}$ at 150 V constant voltages, in running buffer
162 [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
163 with distilled water] at room temperature. After the electrophoretic separation, protein bands
164 were visualized by staining the gel for 1 h with Coomassie brilliant blue G solution (Sigma-
165 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, CWP and WCP 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 ($\approx 2 \text{ mg ml}^{-1}$) 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 $2.0 \pm 0.5 \text{ mg ml}^{-1}$ ECPs, CWP and WCP 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 $3 \times 10^5 \text{ cells ml}^{-1}$ [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 $\times 400$] 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 $\sim 18^\circ\text{C}$ with $\sim 50\%$ 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 ($2,000 \times g$ 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

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

222

223 2.8 Statistics

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

230

231 3. Results

232 3.1 Protective efficacy

233 Fish injected with ECPs, CWP_s and WCP_s of SM1 and SM2, respectively, followed by
234 challenge on day 8 with *V. anguillarum* experienced 11–38% mortalities (RPS = 56–87%)
235 compared with 86% mortalities in the controls (Fig. 1). In particular, use of CWP_s (SM1 =
236 17%; RSP = 80%, and SM2 = 14%; RPS = 84%) and WCP_s (SM1 = 13%; RPS = 85%, and
237 SM2 = 11%; RPS = 87%) of the probiotics led to significant ($P < 0.05$) decreases in
238 mortalities. However, the total mortalities (33–38%; RPS = 56–62%; $P > 0.05$) for the ECP_s
239 did not differ statistically when compared to controls (Fig. 1). Overall, these results pointed to
240 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 CWP. 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 CWP 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 CWP and WCP, with the highest level 0.045 in
251 fish inoculated with WCP 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 CWP (i.e. percentage of surviving bacteria = 56%) and WCP
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).

266

267 4. Discussion

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

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

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

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

327

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

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

343

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

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

362

363 **Acknowledgements**

364 The authors thank Dr. D.A. Austin and Mrs. M.B.C. Stobie for their assistance in the
 365 maintenance of fish. This study was supported by a Heriot-Watt University James Watt
 366 Scholarship to SMS.

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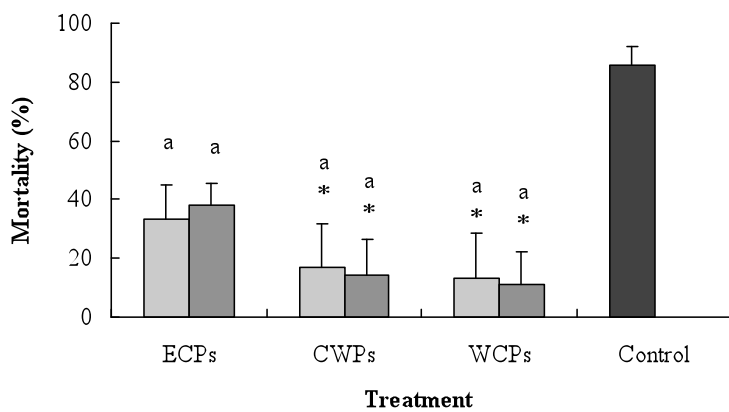
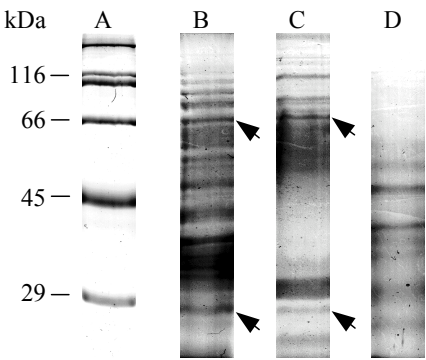


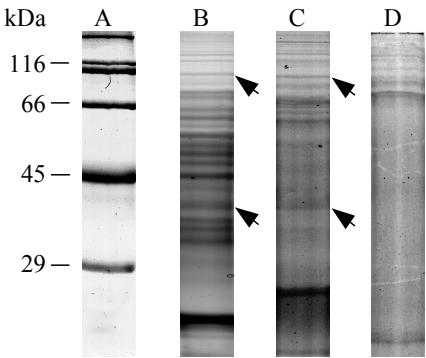
Fig. 1. Percent mortality (%) of rainbow trout following i.p. challenge with *V. anguillarum* after inoculation with cellular components of the probiotics *Kocuria* SM1 (□) and *Rhodococcus* SM2 (■) for 7 days, compared with controls (■). Data represent the average \pm SD from a triplicate set of 10 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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551 **Fig. 2.** Coomassie brilliant blue stained SDS-PAGE analysis of proteins extracted from the
552 probiotic *Kocuria* SM1. Lanes: (A) protein markers, (B) whole cell proteins (WCPs), (C) cell
553 wall proteins (CWPs) and (D) extracellular proteins (ECPs). Arrows [point](#) to likely common
554 proteins.

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

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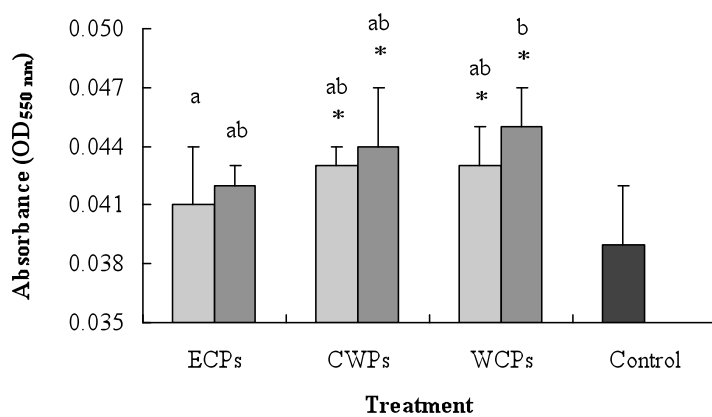
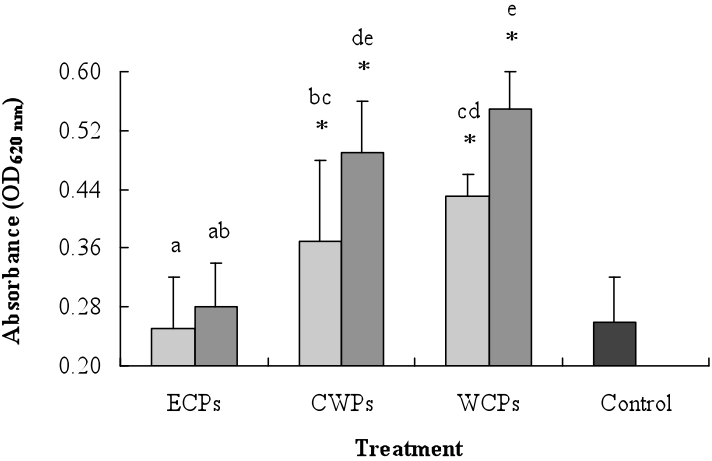


Fig. 4. Blood respiratory burst activity in rainbow trout inoculated with cellular components of the probiotics *Kocuria* SM1 (□) and *Rhodococcus* SM2 (■), compared with controls (■). Data represent the average \pm SD from a triplicate set of 5 fish. *Significantly different ($P < 0.05$) from the control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

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

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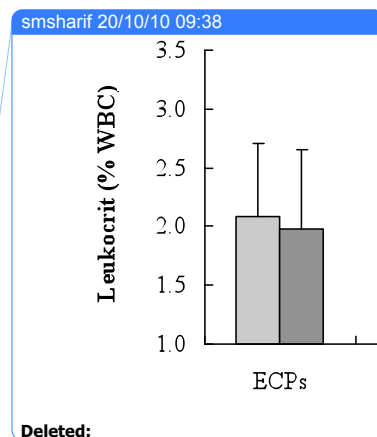
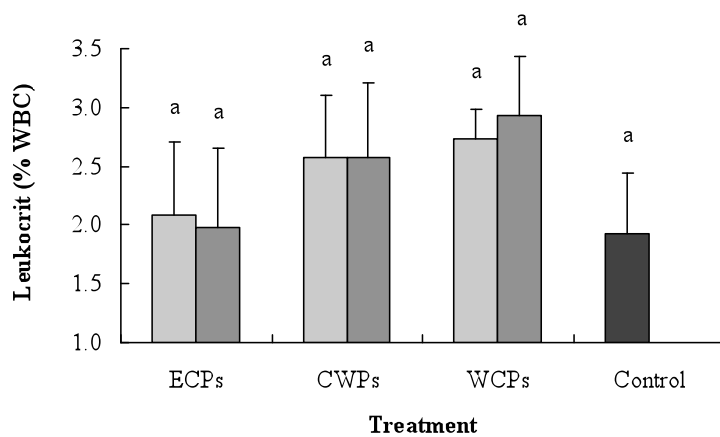
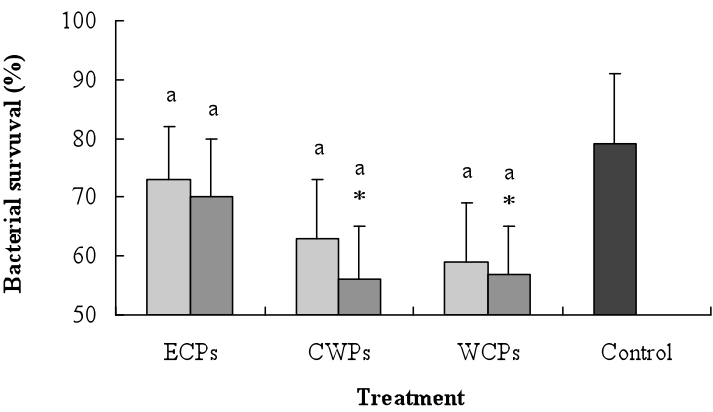


Fig. 6. The leukocrit content, i.e. white blood cell numbers (% WBC) in rainbow trout inoculated with cellular components of the probiotics *Kocuria* SM1 (□) and *Rhodococcus* SM2 (■), compared with controls (■). Data represent the average \pm SD from a triplicate set of 5 fish. Means without a common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

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581 **Fig. 7.** Survival percentage (%) of *V. anguillarum* incubated with serum of rainbow trout
582 inoculated with cellular components of the probiotics *Kocuria* SM1 (□) and *Rhodococcus*
583 SM2 (■), compared with controls (■). Data represent the average \pm SD from a triplicate set of
584 5 fish. *Significantly different ($P < 0.05$) from the control group. Means without a common
585 letter differ significantly ($P < 0.05$) among different treatments with cellular proteins of
586 probiotics.

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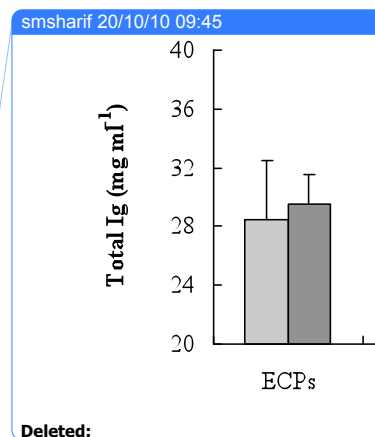
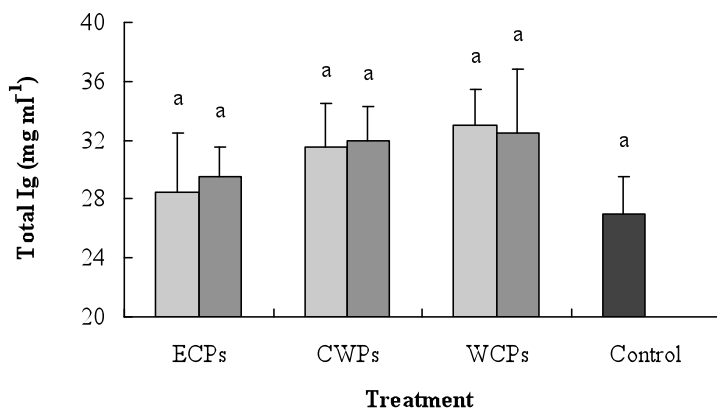


Fig. 8. Total immunoglobulin (Ig) content in the blood of rainbow trout inoculated with cellular components of the probiotics *Kocuria* SM1 (□) and *Rhodococcus* SM2 (■), compared with controls (■). Data represent the average \pm SD from a triplicate set of 5 fish. Means without a common letter differ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

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