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Bacterial skin flora variation and *in vitro* inhibitory activity against *Saprolegnia parasitica* in brown and rainbow trout

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ABSTRACT: Variations in the number and diversity of bacteria from the skin of brown trout *Salmo trutta* L. and rainbow trout *Oncorhynchus mykiss* Walbaum were surveyed from different rivers and fish farms in northern Spain. In addition to determining bacterial populations in skin samples of healthy fish, bacterial populations were determined from skin lesions (of brown trout only) infected with *Saprolegnia parasitica*, the causal agent of saprolegniosis. Mean bacterial counts from skin lesions of brown trout suffering from saprolegniosis were nearly 1000 times greater than from the skin of uninfected brown and rainbow trout. More than 20 different genera of bacteria were identified, with isolates of *Aeromonas* and *Iodobacter* being the predominant genera associated with saprolegniosis lesions. The *in vitro* inhibitory activity of 72 of these skin isolates was tested against *S. parasitica* using 3 different assays. These included (1) assessing the inhibition by bacteria of colony growth on agar media, (2) the inhibition of colony growth from colonized hemp seeds in liquid media and (3) the inhibition of cyst germination in liquid media. Finally, the fungicidal effect of the 24 most inhibitory bacterial species, and the inhibitory activity of their culture supernatants, was tested in the same way. Isolates identified as *Aeromonas piscicola, A. sobria, Pantoea agglomerans* and *Pseudomonas fluorescens* achieved the highest inhibition against *S. parasitica*. Many of these inhibitory isolates were obtained primarily from skin lesions of fish with saprolegniosis. It is suggested that some of these isolates might be useful in the biological control of saprolegniosis.

KEY WORDS: *Saprolegnia parasitica* · Inhibition assays · Bacterial skin flora · Trout

INTRODUCTION

Saprolegniosis is a disease of freshwater fish caused by *Saprolegnia* spp., which frequently appears in wild and farmed salmonids. For many years it has been a recurrent problem for brown trout in rivers and hatcheries in north-western Spain, usually emerging in the autumn and winter, coinciding with the spawning period.

Since the banning of malachite green in many countries, control of saprolegniosis has been based on other chemical substances, which, due to their low efficacy, potential toxicity or high cost, are not completely satisfactory. For these reasons, interest in the development of biocontrol measures has increased in the last few years. Bacteria from the skin flora may play a role in development of the disease, and some microorganisms could protect against *Saprolegnia parasitica* infection on fish (Petersen et al. 1994). Therefore, studying the local flora where the infection is established is likely to yield bacterial isolates potentially useful for biocontrol, as has been done with various other fish and amphibian infections (Spanggaard et al. 2001, Silva et al. 2005, Harris et al. 2006, Goldschmidt-Clermont et al. 2008, Aly et al. 2008, Fjellheim et al. 2010).

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However, the selection of bacteria useful for biocontrol is a laborious task based on *in vitro* and *in vivo* tests on a large set of bacteria. *In vitro* tests, which are used as primary screening, usually comprise several independent tests, which means that they are focused on the demonstration of different bacterial properties. In the case of bacteria for control of *Saprolegnia* spp., inhibition of mycelium growth and cyst germination (Hatai & Willoughby 1988, Bly et al. 1997, Hussein & Hatai 2001, Lategan & Gibson 2003, Zhang et al. 2008), the ability to kill the organism and the capacity to attach to the mucus of fish are theoretically advantageous features. Studying production of extracellular substances by inhibitory bacteria would add information about their mechanism of action and might make it possible to isolate the inhibitory substance for management of fish health (Lategan et al. 2006). *In vivo* tests, used as secondary screening, are crucial in determining whether the bacteria are effective in the biocontrol of saprolegniosis (Lategan & Gibson 2003, Lategan et al. 2004).

The aim of the present work was to determine the *in vitro* inhibitory activity of bacteria found on the skin of brown and rainbow trout against *Saprolegnia parasitica*, in order to isolate one or more bacteria potentially useful for the biocontrol of saprolegniosis. We also present information on the inhibitory effects of bacterial supernatants and on variations in the bacterial populations found on the skin of healthy trout and of those infected with *S. parasitica*.

MATERIALS AND METHODS

Fish and isolation of bacteria

From April 2006 to January 2007, adult brown trout *Salmo trutta* L. (n = 96; 78 healthy and 18 with saprolegniosis caused by *Saprolegnia parasitica*) were collected from 3 different sites in the province of León, north-western Spain: (1) the River Porma, which has frequent outbreaks of saprolegniosis caused by *S. parasitica*; (2) the River Omaña, which has no record of the disease; and (3) a hatchery supplied with water from the River Porma, where infected trout are found every year. Healthy specimens were collected at seasonal intervals, and diseased specimens whenever present. Between December 2007 and January 2008, adult healthy rainbow trout *Oncorhynchus mykiss* Walbaum (n = 14) were obtained from 3 commercial fish farms located on the Rivers Duerna, Órbigo and Porma, also in the province of León.

All specimens were transported live to the laboratory, killed within 2 h of collection and their skin surface gently rinsed with sterile distilled water. Samples were obtained from the skin surface of healthy specimens and from cutaneous lesions in those with saprolegniosis. In hatchery brown trout displaying saprolegniosis, samples were also taken from areas of healthy skin. Skin surface samples were obtained by scraping the 2 cm^2 area inside the outline of a 16 mm punch placed on the skin surface, and containing 1 ml phosphate buffered saline (PBS) added as diluent. The resulting mixture was serially diluted in PBS, and 100 µl of the appropriate dilutions were spread onto tryptose soya agar (TSA, Cultimed) and sheep blood agar (bioMérieux®) plates. After aerobic cultivation for 7 d at 20°C, the plates were analysed for total counts using dilutions, as far as possible, with between 30 and 300 colonies. The number of colony-forming units (CFU) cm^{-2} of skin was estimated from the mean of CFU in blood agar and TSA, as the mean counts on these 2 media were similar (ANOVA, $p > 0.05$).

One colony of the most numerous bacterial types from plates derived from different fish was removed and sub-cultured until pure cultures were obtained. The selected bacterial isolates were stored frozen at −80°C in tryptose soya broth (TSB, Cultimed) with 30% v/v glycerol.

ANOVA or the Kruskal-Wallis test (K-W) was used to determine whether the differences between means or medians of bacterial counts (after log transformation) were significant ($p < 0.05$). All statistical tests were performed using Epi Info™ version 3.2.2 software.

Bacterial identification

Identification of bacterial isolates was carried out first using basic phenotypic methods and then molecular methods. The latter consisted of sequencing the fragment of the 16S rDNA gene amplified by the universal bacterial primers (both from Invitrogen) 27F (5′-AGA GTT TGA TC(A:C) TGG CTC AG-3′) and 1492R (5′-TAC GG(C:T) TAC CTT GTT ACG ACT T-3′). For identification to species level of isolates identified as belonging to the genus *Pseudomonas*, a fragment of the 23S rDNA gene was amplified by the primers (both from Invitrogen) fPs16S (5′-ACT GAC ACT GAG GTG CGA AAG CG-3′) and rPs23S (5′-ACC GTA TGC GCT TCT TCA CTT GAC C-3′) (Locatelli et al. 2002).

The sequences were compared with those available in the GenBank database using the BLASTn

algorithm at http://www.ncbi.nlm.nih.gov/genbank. Representative sequencing outputs are available at http://www.ebi.ac.uk/embl/Access/index.html under the accession numbers FN908434-FN908459.

Inhibition assays with bacteria

Bacterial and *Saprolegnia* isolates. A total of 72 bacterial isolates from trout skin, including 61 representatives of the different species identified in this study as well as 11 others from previous isolations in our laboratory (see Table 1), were tested against the *Saprolegnia parasitica* TRU 8 isolate obtained from a wild brown trout with saprolegniosis (Fregeneda-Grandes et al. 2000). To test the inhibitory activity of the bacterial isolates, 3 assays were carried out. The first tested the inhibition of hyphal growth on solid media (plate assay), the second and third tested the inhibition of hyphal growth and germination of cysts, respectively, in liquid media (broth assays).

Plate assay. This test was performed in Petri dishes with brain-heart infusion agar (BHI, Cultimed) inoculated with 2 parallel streaks of each bacterium, as described by Hussein & Hatai (2001). Two plates were prepared per bacterial isolate for each incubation time tested: 3, 5 or 7 d at 20°C, for a total of 6 plates per isolate. After incubation, a block of agar with *Saprolegnia parasitica* young hyphal tips, 3 mm diameter, was placed between the 2 bacterial streaks. The plates were subsequently incubated for 4 d at 20°C and the diameter of the *S. parasitica* colony measured. A negative control plate, without bacterial streaks, was assayed. The diameter reached by the *S. parasitica* colony was used as an indicator of inhibitory activity.

Broth assays (hemp seed test and cyst test). First, the bacterial isolates were inoculated into TSB and incubated at 20°C overnight. Following this, a 250 µl aliquot of the culture broth was inoculated into 12.5 ml of TSB and incubated until the estimated middle of the exponential growth phase on a rotary orbital shaker (Innova® 44, New Brunswick Scientific) at 20°C and 200 rpm (the estimate being based on previously established growth curves). The bacteria were pelleted by centrifuging at 1000 × *g* for 15 min and resuspended in sterile saline solution. The concentration of bacteria was adjusted (based on the optical density of the cultures) to 2×10^5 bacteria ml^{-1} for the assays with colonized hemp seeds, and to 4×10^5 bacteria ml^{-1} for the assays with zoospores, so that the final concentrations of bacteria in the 2 tests were equal. Taking this as the first dilution, five 10-fold dilutions of bacterial suspension were prepared.

In parallel, *Saprolegnia parasitica* was incubated for 3 d at 20°C on glucose peptone (GP) agar (Willoughby & Pickering 1977). Autoclaved half hemp seeds C*annabis sativa* were then placed on the edges of the colony and incubation was continued for another 24 h at 20°C. To obtain zoospores, the method described by Fregeneda-Grandes et al. (2000) was used. Briefly, half hemp seeds colonised by hyphae were placed in Petri dishes with filtered autoclaved river water, and incubated for 36 h at 20°C. The concentration of zoospores was then estimated using a Hawksley Cristalite BS 748 counting chamber and adjusted to 4×10^4 zoospores ml^{-1}.

For the test with a hemp seed colonized by *Saprolegnia parasitica* (hemp-seed test), 1 ml of each bacterial dilution, 1 ml of TSB and a half hemp seed colonized by *S. parasitica* were dispensed by duplicate into each well of a 24-well tissue culture plate (Falcon). Bacteria and *S. parasitica* negative controls were included on each plate. For the assay with the zoospores/cysts of *S. parasitica* (cyst test), the procedure was basically like the hemp-seed test, the only difference being that 0.5 ml of the zoospore suspension was added in place of a colonized hemp seed, and 0.5 ml of each bacterial dilution was used instead of 1 ml. Bacteria and *S. parasitica* zoospore negative controls were also included on each plate. Plates were incubated for 3 d at 20°C. Presence/absence of macroscopic or microscopic hyphal growth and germination of cysts was observed throughout the incubation period using a Nikon Diaphot inverted microscope and results were recorded on Day 3.

Assay to determine fungicidal effects

For this assay we used the 24 bacterial isolates with the highest inhibitory activity, as determined by the previous 3 tests. The bacterial and *Saprolegnia parasitica* isolates were prepared as for the hemp-seed test in broth assays. Then, 1 ml of each inhibitory bacterial dilution, 1 ml of TSB and a half hemp seed colonized by *S. parasitica* were dispensed by duplicate into each well of a 24-well plate and incubated at 20°C. Incubation times of 1, 2 or 3 d were tested. At the end of each period, the hemp seeds in the wells showing no *S. parasitica* growth were removed, washed 3 times with sterile distilled water, placed in a Petri dish with 20 ml of filtered and autoclaved river water containing 2×10^2 mg streptomycin l^{-1} and 2×10^5 UI penicillin l^{-1}, and incubated for 20 d at 20°C. False positive results due to inhibitory effects of bacteria remaining viable in the mycelium were avoided

by accepting the results only when there was no growth of bacteria from a sample of the water collected after 24 h. Negative control was achieved by incubating a hemp seed colonized by *S. parasitica* in water both with and without antibiotics. Absence of viability was determined when there was no growth of the mycelium.

Inhibition assays with bacterial culture supernatants

For these assays the same 24 bacterial isolates used in the test to study fungicidal effects were selected. The method described by Lategan et al. (2006) was used to prepare supernatants. In brief, after the bacteria had been cultivated in tryptone-neutralized peptone broth (TNPB) on a rotary shaker at 180 rpm for 36 h at 25°C, they were killed by heating in a water bath for 1 h at 70°C. Then the cultures were centrifuged at 15 000 × *g* for 20 min and the supernatant collected. Following this, the supernatants were filtered through a 0.22 µm syringe filter (Acrodisc®) and their pH was measured and adjusted to 7.0. This supernatant and three 2-fold serial dilutions in TNPB were tested.

Two broth assays were carried out. The purpose of the first was to investigate the capacity of the supernatants to inhibit hyphal growth (hemp-seed test). In this assay, 2 ml of each supernatant dilution and a half hemp seed colonized by *S. parasitica* were dispensed by duplicate into each well of a 24-well tissue culture plate. Wells with TNPB instead of the supernatants were included as negative controls. The purpose of the second assay was to investigate the capacity of the supernatants to inhibit cyst germination (cyst test). In this assay, 1 ml of each supernatant dilution and 1 ml of the zoospore suspension (prepared as described under 'Broth assays', above) were added to each well. Wells with TNPB instead of supernatants were included as negative controls. The same incubation times and observation procedures as in 'Broth assays', were completed.

RESULTS

Skin bacterial counts and identification of bacteria

The bacterial counts cm^{-2} of skin after log transformation of each type of sample are shown in Fig. 1. The geometric means of bacterial counts in healthy skin of brown trout with and without saprolegniosis were 57 and 179 CFU cm^{-2} respectively, with no significant differences between bacterial counts (K-W, $p > 0.05$). However, in lesions of saprolegniosis the geometric mean was much higher (57 518 CFU cm^{-2}). It was found that, on the skin of healthy brown trout, the mean bacterial counts in each season (from 23 to 168 CFU cm^{-2}) and at each sampling point (from 22 to 90 CFU cm^{-2}) were significantly different (K-W, $p < 0.001$ and ANOVA, $p = 0.0029$ respectively). A considerable difference (ANOVA, $p < 0.005$) was also found between the mean bacterial counts in fish from the 3 rainbow trout fish farms, which varied from 12 to 834 CFU cm^{-2} (ANOVA, $p < 0.005$).

In total, 137 bacteria were selected (119 from healthy and diseased brown trout and 18 from healthy rainbow trout) and 23 bacterial genera and 28 species were identified (Table 1). The identification to genus level was achieved by sequencing the 16S rRNA gene in all the isolates in which this method was used. However, using the phenotypic tests, only 56.2% of isolates were identified to genus level. Using molecular methods, identification to species level was achieved in 59.4% of the isolates. The results from phenotypic tests and from molecular identification were identical with respect to genera identified.

Species most frequently isolated from the skin of healthy brown trout were *Iodobacter* sp., *Deefgea rivuli* and *Yersinia kristensenii* (with all *Yersinia* isolates being obtained in summer); from the healthy skin of brown trout with saprolegniosis, *Iodobacter* sp. and *D. rivuli*; from saprolegniosis lesions on these individuals, *Iodobacter* sp., *Aeromonas sobria* and *Aeromonas* sp.; and from the skin of healthy rainbow trout, *Chryseobacterium* sp., *Pseudomonas fluorescens* and *Staphylococcus* sp., each species predominating at a different fish farm (Table 1).

Inhibition assays with bacteria

Plate assays. Because the highest difference in the inhibitory action upon mycelium growth among bacteria tested was observed when *Saprolegnia parasitica* was inoculated onto plates with bacterial cultures previously grown for 7 d, it was this incubation period against which degree of inhibition was measured. On the negative control plates the colony grew to 4 cm in diameter (Fig. 2). Presence of inhibitory activity was determined when the *S. parasitica* colony reached a diameter of 2.5 cm or less, and was observed in all 72 isolates. Inhibitory activity was then grouped into 3 inhibition levels depending on

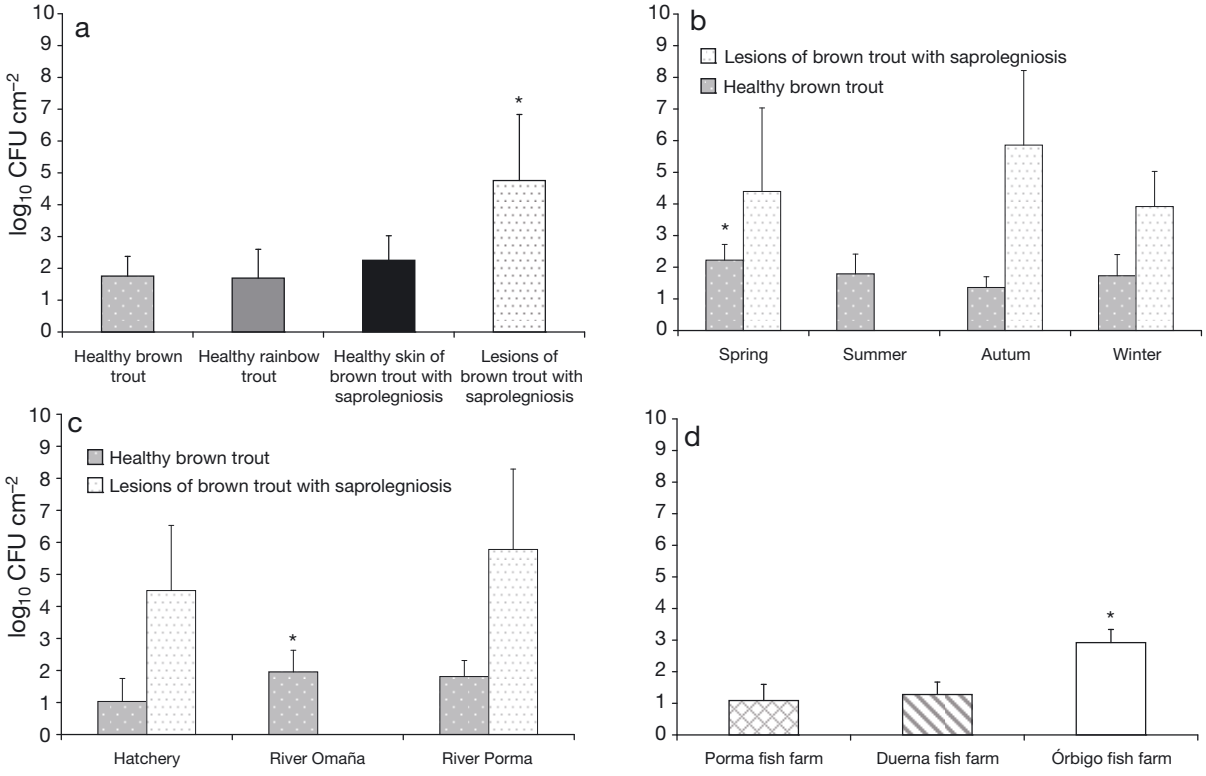

Fig. 1. Bacterial plate counts (mean CFU cm^{-2} + SD) from skin or lesions of brown trout *Salmo trutta* and rainbow trout *Oncorhynchus mykiss*. (a) Total; (b) by season; (c) by sampling location; (d) from 3 rainbow trout fish farms. No trout with saprolegniosis were found either in summer or in the river Omaña. Asterisk: significantly higher mean ($p < 0.05$) than the mean of the other samples; (b) and (c) only in healthy trout

the diameter reached by the *S. parasitica* colony (Tables 2 & 3).

Broth assays. For the hemp-seed test, the absence of macroscopic hyphal growth after 3 d of incubation was used to indicate inhibitory activity (Fig. 2). It was found that 41 out of the 72 bacterial isolates (56.9%) tested exhibited this activity at any dilution. The minimum number of bacteria added to the wells necessary for the inhibition of *S. parasitica* was used to establish 3 levels of inhibitory activity. The results of this assay are shown in Tables 2 & 3.

For the cyst test, it was observed that none of the 72 bacterial isolates inhibited cyst germination, but that the mycelium growth was inversely proportional to the concentration of bacteria added to the wells. Therefore, absence of macroscopic growth at any dilution after 3 d of incubation was considered as inhibitory activity, and it was found in 52 out of the 72 isolates (72.2%). Depending on the minimal concentration of bacteria necessary for this effect, 3 levels of inhibition were fixed. The results of this assay are shown in Tables 2 & 3.

Fungicidal effect of the bacteria. Fifteen of the 24 bacteria species tested proved to be lethal for *Saprolegnia parasitica* under any of the conditions assayed (Table 3). A small and atrophied brown mycelium was observed after 20 d of incubation in water, in contrast to the control plates and plates with the non-fungicidal bacteria, in which the mycelium grew and produced zoospores. The bacteria caused the lethal effect on *S. parasitica* only up to the second dilution (2×10^4 bacteria per well), and with at least 2 d of incubation together at 20°C.

Most inhibitory isolates. The isolates with high inhibitory activity in the plate assay, capable of inhibiting *Saprolegnia parasitica* with a lower concentration of bacteria in the broth assays and also with fungicidal effect, were considered the best (Table 3). These isolates belonged to the species *Aeromonas piscicola, A. sobria, Pantoea agglomerans* and *Pseudomonas fluorescens*, many of them (66.6%) obtained from saprolegniosis lesions.

Inhibition assays with the bacterial culture supernatants. In the broth assay with colonized hemp seeds, it was observed that 19 of the 24 bacterial supernatants tested showed inhibition of *Saprolegnia parasitica* mycelium growth after 3 d of incubation. Total inhibition was observed at the first dilu-

Table 1. Taxonomic identification of bacteria isolated from skin of brown trout *Salmo trutta* and rainbow trout *Oncorhynchus mykiss*, and of bacterial isolates used in the *in vitro* inhibition assays against *Saprolegnia parasitica*

Bacteria	Healthy brown trout	Brown trout with saprolegniosis		Healthy rainbow trout	Total isolates	Isolates used in the inhibition assays
		Healthy skin	Lesions			
Achromobacter xylosoxidans	2	0	0	0	2	1
Acinetobacter lwoffii	0	0	1	0	1	1
Acinetobacter johnsonii[a]	–	–	–	–	–	2
Acinetobacter sp.	1	0	1	0	2	0
Aeromonas piscicola	0	0	1	0	1	1
Aeromonas popoffii	0	0	3	0	3	2
Aeromonas salmonicida	0	0	3	0	3	1
Aeromonas salmonicida[a]	–	–	–	–	–	1
Aeromonas sobria	2	1	10	0	13	9
Aeromonas sp.	0	1	6	0	7	0
Brevundimonas nasdae	1	0	0	0	1	0
Carnobacterium maltaromaticum	1	1	1	0	3	1
Chryseobacterium sp.	3	0	0	6	9	3
Deefgea rivuli	6	2	0	0	8	3
Flavobacterium succinicans	1	0	0	0	1	0
Flavobacterium sp.	1	1	1	0	3	2
Flavobacterium sp.[a]	–	–	–	–	–	1
Frigoribacterium sp.	0	0	0	1	1	1
Gram-negative rods	0	0	0	1	1	0
Gram-positive rods	0	0	0	1	1	0
Iodobacter fluviatilis	2	0	1	0	3	1
Iodobacter sp.	9	2	11	0	22	6
Iodobacter sp.[a]	–	–	–	–	–	1
Janthinobacterium lividum	2	0	0	0	2	1
Janthinobacterium sp.	1	0	0	0	1	1
Kocuria rhizophila	2	0	0	0	2	1
Kurthia zopfii	0	0	1	0	1	1
Microbacterium foliorum	1	0	0	0	1	1
Microbacterium hydrocarbonoxydans	1	0	0	0	1	1
Microbacterium phyllosphaerae	1	0	0	0	1	0
Microbacterium sp.	1	0	0	0	1	1
Moraxella osloensis	0	0	0	1	1	1
Pantoea agglomerans	3	0	0	0	3	3
Plantibacter agrosticola	0	1	0	0	1	1
Pseudomonas fluorescens	1	1	4	4	10	5
Pseudomonas fluorescens[a]	–	–	–	–	–	1
Rhodococcus fascians	1	0	0	0	1	1
Rhodococcus fascians[a]	–	–	–	–	–	1
Rhodococcus sp.	2	1	0	1	4	1
Serratia fonticola	2	0	0	0	2	1
Staphylococcus aureus[a]	–	–	–	–	–	1
Staphylococcus equorum[a]	–	–	–	–	–	3
Staphylococcus pasteuri	1	1	0	0	2	1
Staphylococcus sp.	3	1	1	3	8	2
Xanthomonas retroflexus	1	0	0	0	1	1
Yersinia enterocolitica	2	0	0	0	2	2
Yersinia intermedia	2	0	0	0	2	1
Yersinia kristensenii	5	0	0	0	5	2
Total	61	13	45	18	137	72

[a]From previous isolations, skin of brown trout *S. trutta*

tion, and was achieved by only 10 bacterial isolates. This was considered high inhibition. If mycelium growth was reduced in comparison to the control, but not inhibited totally, it was considered low inhibition (Table 3).

When bacterial supernatants were used in the cyst test, the growth of mycelia from the cysts, but not their germination, was inhibited in 20 of the 24 isolates tested. After 3 d of incubation, 14 of these isolates showed high inhibition at first dilution (Table 3).

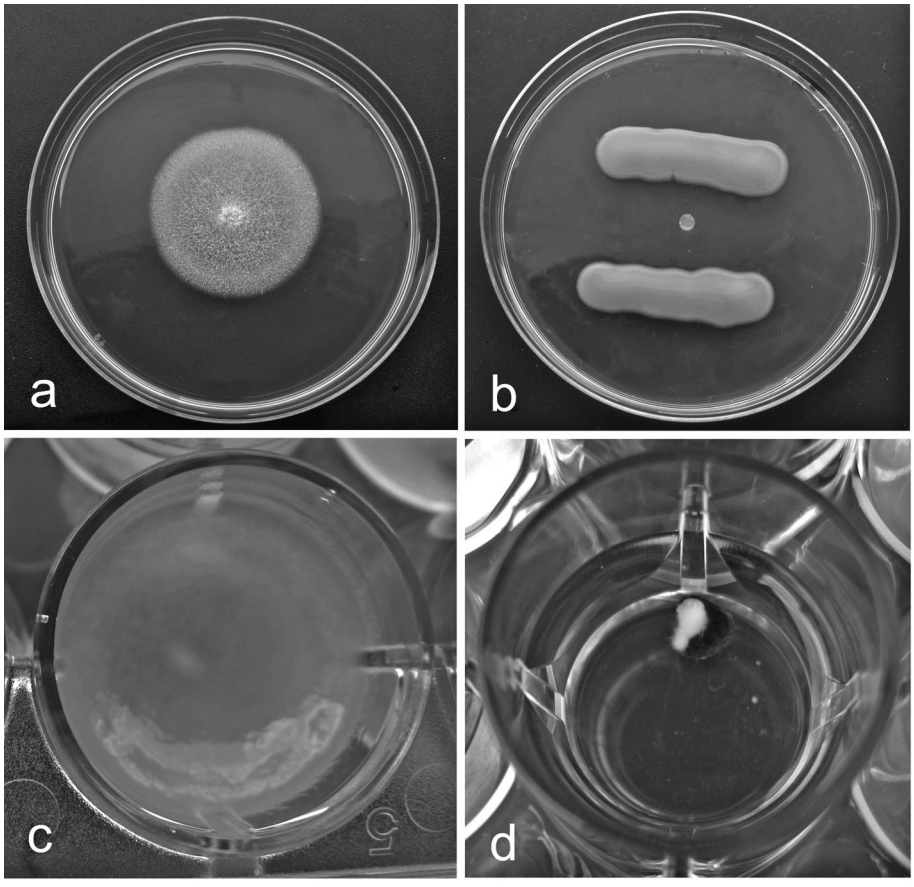

Fig. 2. *In vitro* inhibition assays of *Saprolegnia parasitica* with bacteria on solid media and in 24-well tissue culture plates with colonized hemp seed and liquid media. (a) Control plate (solid medium) with *S. parasitica* after 4 d at 20°C. (b) High inhibition of *S. parasitica* (solid medium) caused by a *Xanthomonas retroflexus* isolate after 4 d at 20°C, with bacteria previously grown for 7 d. (c) Control well (liquid medium) with *S. parasitica* after 3 d at 20°C. (d) High inhibition of *S. parasitica* (liquid medium) caused by an *Aeromonas sobria* isolate after 3 d at 20°C

Table 2. Degree of inhibition of *Saprolegnia parasitica* achieved by 72 bacterial isolates in assays on BHI agar (plate assays), in liquid media with hemp seed colonized by *S. parasitica* (hemp seed test) and in liquid media with cysts of *S. parasitica* (cyst test)

Test	Degree of inhibition	Indicator of degree of inhibition	Bacterial isolates with inhibitory activity No.	Total (% of total tested)
Plate assay		Diameter of *S. parasitica* colony after 4 d at 20°C[a]		72 (100%)
	Low	1.5–2.5 cm	13	
	Medium	0.7–1.5 cm	12	
	High	<0.7 cm	47	
Hemp seed test		Total inhibition of macroscopic mycelium growth after 3 d at 20°C		41 (56.9%)
	Low	with 2×10^5 bacteria	10	
	Medium	with 2×10^4 bacteria	5	
	High	with $\leq 2 \times 10^3$ bacteria	26	
Cyst test		Total inhibition of macroscopic mycelium growth after 3 d at 20°C		52 (72.2%)
	Low	with 2×10^5 bacteria	16	
	Medium	with 2×10^4 to 2×10^3 bacteria	9	
	High	with $\leq 2 \times 10^2$ bacteria	27	

[a]*S. parasitica* added after incubation of bacteria for 7 d at 20°C

Table 3. Results of all *in vitro* tests performed with 24 bacterial isolates with high inhibitory activity against *Saprolegnia parasitica*. HBT: healthy brown trout; BTSH: brown trout with saprolegniosis, healthy skin; BTSL: brown trout with saprolegniosis, lesions; HRT: healthy rainbow trout; bracketed nos.: minimum number of bacteria causing total inhibition of macroscopic mycelium growth. Supernatants inhibition: High (total absence of macroscopic mycelium growth achieved with first dilution after 3 d of incubation at 20°C); Low (partial inhibition of macroscopic mycelium growth achieved with first dilution

Species	Bacterial isolate	Origin	Plate assays	Broth assays		Fungicidal effect	Inhibitory activity of supernatants	
				Hemp seed test	Cyst test		Hemp seed test	Cyst test
Aeromonas piscicola[a]	LE 21	BTSL	High	High (200)	High (20)	Yes	High	High
A. popoffii	LE 65	BTSL	High	High (20)	High (20)	No	High	High
A. popoffii	LE 69	BTSL	High	High (200)	High (200)	Yes	High	High
A. salmonicida	LE 9	BTSL	High	High (20)	High (20)	No	High	High
A. salmonicida	LE 99	BTSL	High	High (20)	High (20)	No	High	High
A. sobria	LE 17	BTSL	High	High (20)	High (20)	No	High	Low
A. sobria	LE 51	HBT	High	High (2000)	High (20)	Yes	High	High
A. sobria	LE 68	BTSL	High	High (20)	High (20)	No	High	High
A. sobria[a]	LE 74	BTSL	High	High (200)	High (20)	Yes	Low	High
A. sobria	LE 76	BTSL	High	High (20)	High (20)	No	High	High
A. sobria	LE 100	BTSL	High	High (20)	High (20)	No	Low	High
A. sobria	LE 104	BTSH	High	High (20)	High (20)	Yes	High	High
Pantoea agglomerans	LE 35	HBT	High	High (2000)	High (200)	Yes	No	No
P. agglomerans	LE 36	HBT	High	High (2000)	High (200)	Yes	No	No
P. agglomerans[a]	LE 37	HBT	High	High (200)	High (20)	Yes	Low	Low
Pseudomonas fluorescens	LE 89	HBT	High	High (2000)	High (20)	Yes	Low	High
P. fluorescens[a]	LE 98	BTSL	High	High (200)	High (20)	Yes	Low	Low
P. fluorescens[a]	LE 122	BTSL	High	High (200)	High (20)	Yes	Low	High
P. fluorescens	LE 141	HRT	High	High (200)	High (20)	No	Low	Low
P. fluorescens[a]	LE 143	HRT	High	High (200)	High (20)	Yes	Low	Low
Serratia fonticola	LE 52	HBT	High	High (2000)	High (200)	Yes	No	No
Xanthomonas retroflexus	LE 38	HBT	High	High (2000)	High (200)	No	Low	High
Yersinia kristensenii	LE 54	HBT	High	High (2000)	High (200)	Yes	No	No
Y. kristensenii	LE 58	HBT	High	High (2000)	High (200)	Yes	No	Low

[a]These isolates considered the best, taking into account minimum number of bacteria necessary for inhibition of *S. parasitica* in broth assays, and fungicidal effect

DISCUSSION

In the present study, it was observed that the bacterial skin flora of brown trout is dependent on factors such as season and habitat. Furthermore, the predominant bacterial genera and species of this flora were identified and some of them shown to have *in vitro* inhibitory capability against *Saprolegnia parasitica*. Thus, these are candidates for further study in the biocontrol of saprolegniosis.

Making comparisons among studies involving bacterial counts is complex, as several factors are involved. Among these are the method of taking the sample from the fish and the culture media used (Spanggaard et al. 2000, Nedoluha et al. 2001, Huber et al. 2004, Romero & Navarrete 2006). Austin (2006) reported that, generally, bacterial populations on the skin of fish range from 10^2 to 10^4 bacteria cm^{-2}. In the present study, the aerobic-bacteria counts on the healthy skin of brown trout and rainbow trout were markedly below those found by González et al. (1999) in the same species of fish from the province of León using the skin scraping method. This disagreement of findings may be caused by differences in sampling method, given that these investigators transported the fish dead in plastic boxes to the laboratory within 6 h of collection, and in the culture media used. In the current study, high variability was found in bacterial counts, as well as significant differences among bacterial mean counts on the skin of healthy brown trout during different seasons. Al-Harbi & Uddin (2004) found seasonal differences in the intestinal flora of tilapia and related them to oscillations in the water temperature, higher bacterial concentrations being more likely at increased water temperature. However, the flora of fish can also be influenced by other environmental factors, which may explain why, in the present study, bacterial counts were highest in spring and lowest in autumn, when

the first trout with saprolegniosis usually appear in the province of León. This fact, in addition to the fact that the very highest counts were found in specimens from the river Omaña, which has no record of outbreaks of the disease, might indicate that microflora on the skin are related to the presence of saprolegniosis.

In fish farms, disinfectants are often added to the water to control a range of organisms. This may have an adverse effect on bacterial flora of the skin, and may well explain the fact that the lowest bacterial counts were observed in hatchery brown trout. Moreover, the highest counts in rainbow trout were obtained from the Órbigo fish farm, where chemical treatments were not used.

With regard to the bacterial species found on the skin of our trout specimens, a greater diversity of species was observed on healthy skin than in lesions. *Iodobacter* was the genus most frequently isolated from the skin of healthy brown trout, and *Aeromonas* from saprolegniosis lesions. In contrast to the report of González et al. (1999), we found *Aeromonas* sp. to be sparse on healthy trout, probably because of the differences in sampling methods used.

Among the 4 inhibition assays performed in the present study, the assays on solid media seemed to be less useful than the others in judging the extent of the inhibitory activity of the bacterial isolates, because on BHI agar all of them inhibited growth of *Saprolegnia parasitica* to different degrees, with about two-thirds of the isolates inhibiting growth completely. Moreover, bacteria that were inhibitory in broth assays also showed considerable inhibition on solid media, but the opposite was not true. Hussein & Hatai (2001) observed inhibition with only 5 out of 47 strains using HI agar, but afterwards they demonstrated that the inhibition was increased by using BHI. However, Zhang et al. (2008) observed a low inhibitory activity of bacteria against pathogenic members of *Saprolegnia* on BHI. These authors demonstrated that the composition of the culture media could affect the inhibitory activity of *Serratia marcescens* against *Saprolegnia* spp. and that the highest inhibition was obtained on potato dextrose agar followed by BHI.

In liquid media, it was observed that none of the isolates tested inhibited the germination of cysts, but they did reduce the growth of the resulting mycelium. The inhibition of cyst germination has already been studied by other investigators with contradictory results (Bly et al. 1997, Lategan & Gibson 2003, Zhang et al. 2008). In the present study *Saprolegnia parasitica* mycelium growth from cysts was inhibited at lower concentrations of the bacteria than were hyphae from colonized hemp seed. This may be due to the longer time necessary for hyphae to grow from the cysts than for the hemp seed, where the mycelium was already present.

The bacterial isolates with the strongest inhibitory activity against *Saprolegnia parasitica* in the assays with bacterial cells belonged to the genera *Aeromonas*, *Pantoea* and *Pseudomonas*, many of them being isolated from lesions of saprolegniosis. Hatai & Willoughby (1988) and Hussein & Hatai (2001) likewise isolated *Aeromonas* and *Pseudomonas* with inhibitory properties from saprolegniosis lesions. The latter investigators stated that the presence of inhibitory bacteria in saprolegniosis lesions may be relevant, given the fact that microbial interactions in the lesions are a possible reason for the spontaneous recovery of some trout with saprolegniosis. In contrast, Petersen et al. (1994) found unidentified bacteria antagonistic to *S. parasitica* in the skin mucus of healthy rainbow trout, but not in those with saprolegniosis.

The *in vitro* inhibitory activity of *Pseudomonas fluorescens* and *Serratia* spp. against *Saprolegnia* spp. has been described by several authors (Hatai & Willoughby 1988, Bly et al. 1997, Zhang et al. 2008). In the present study, most of the isolates identified as *P. fluorescens* and one belonging to the genus *Serratia* showed a fairly strong antagonistic activity.

It was also observed that, in agreement with Lategan et al. (2006), some bacteria were able to kill *Saprolegnia parasitica*. This is an interesting finding. Not only did these bacteria not cause infection, but they could also cure incipient lesions.

The mechanism involved in anti-*Saprolegnia* bacterial activity is unknown, but the fact that both the vegetative and cyst stages were harmed could have significant implications for *in vivo* control of *Saprolegnia* spp. infections in fish farms and hatcheries (Lategan & Gibson 2003). In the present study most of the bacterial supernatants inhibited the *in vitro* growth of *Saprolegnia parasitica,* which suggests that a substance secreted into the medium causes or participates in the inhibitory effect of the bacteria. Furthermore, higher sensitivity to the bacterial supernatants of mycelia grown from cysts than of those grown from hemp seed was demonstrated, as was also observed by Zhang et al. (2008). The strongest inhibitory activity was shown by the supernatants of bacteria belonging to the genus *Aeromonas*, whereas those produced by *Enterobacteriaceae* did not show this inhibitory effect — although, because these bacteria are fungicidal, other mecha-

nisms might be implicated for this property. Lategan & Gibson (2003) found a bacteriocin-like substance secreted by a strain of *Aeromonas media*, which had inhibitory activity against *Saprolegnia* sp. and other pathogens; its main component was identified to be indole (Lategan et al. 2006). Zhang et al. (2008) also found that the supernatant of a *Serratia marcescens* strain was inhibitory against *Saprolegnia* sp. However, other investigators found no inhibition to be caused by bacterial supernatants (Bly et al. 1997, Hussein & Hatai 2001), and speculated that other mechanisms might be implicated, such as competition for iron, or capacity to degrade cellulose or to liquefy gelatine.

In the present work, bacteria from both healthy skin and saprolegniosis lesions in trout were isolated. Some of them, mostly *Aeromonas* and *Pseudomonas* isolates, showed strong inhibitory activity against *Saprolegnia parasitica* in the various *in vitro* assays performed. These bacteria are good candidates for use in the biological control of saprolegniosis (by oral route or added to water), but further work is necessary to determine the degree of danger of these bacteria to fish (given the fact that some inhibitory isolates belong to species that cause diseases in fish) and other characteristics, such as their capacity to adhere to the skin mucus of fish.

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