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# *In vivo* study of phagocytosis, intracellular survival and multiplication of *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum), spleen phagocytes

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## Abstract

The intracellular behaviour of a *Flavobacterium psychrophilum* strain, ingested by spleen phagocytes of rainbow trout, *Oncorhynchus mykiss,* of different ages, was assessed *in vivo.* Three groups of rainbow trout weighing 1 g (aged 10 weeks), 25 g (aged 20 weeks) and 300 g (aged 15 months), respectively, were injected intraperitoneally with $1 \times 10^6$ cfu of a *F. psychrophilum* strain. It was found that only fry, aged 10 weeks, displayed clinical signs and suffered mortality. Bacteriological colony plating of different organs demonstrated that the spleen and to a lesser extent the kidney of only the fry were affected. The number of colony forming units per gram of spleen tissue increased with time. No bacteria were found in the trout aged 5 months and older. Light microscopical examination and epifluorescence microscopy revealed that the fry spleen phagocytes contained an increasing number of viable intracellular *F. psychrophilum* bacteria over time. Again, no bacteria were encountered in the phagocytes collected from older fish.

*Keywords: Flavobacterium psychrophilum,* phagocytes, phagocytosis, rainbow trout

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## Introduction

Rainbow trout fry syndrome (RTFS) is currently recognized as a serious bacterial disease affecting hatchery reared rainbow trout, *Oncorhynchus mykiss* (Walbaum), fry and fingerlings in many parts of Europe (Lorenzen & Olesen 1997; Madsen & Dalsgaard 1999). The aetiological agent is *Flavobacterium psychrophilum* (Bernardet, Segers, Vancanneyt, Berthe, Kersters & Vandamme 1996), a Gram-negative filamentous chromogenic rod (Wood & Yasutake 1956; Holt, Rohovec & Fryer 1993), which was formerly called *Cytophaga psychrophila* or *Flexibacter psychrophilus.* In outbreaks of RTFS, mortalities ranging between 10 and 30% may be involved (Bruno 1992), often rising up to 70% (Chua 1991; Santos, Huntly, Turnbull & Hastings 1992).

Surprisingly, in spite of the increasing significance and severe economic impact of the disease, data relating to its pathogenesis necessary to provide sufficient scientific background for an efficient control strategy are still lacking. Additionally, the reason(s) why only young fish are susceptible to RTFS is (are) still unknown. Rangdale, Richards & Alderman (1999) demonstrated *F. psychrophilum* cells inside spleen phagocytes of rainbow trout fry within phagocytic vesicles. However, the appearance of numerous elongated bacteria and extensive degeneration of the spleen tissue suggested that the response to the pathogen was not effective. The difference in susceptibility between young and old fish may therefore be rooted in a difference in

antibacterial activity of the phagocytes. Lammens, Decostere & Haesebrouck (2000) proved using a chemiluminescence technique that *F. psychrophilum* and their metabolites were able to stimulate phagocytes from mature rainbow trout *in vitro*. To our knowledge, this is the only study concerned with the interaction between *F. psychrophilum* and phagocytes. This interaction is of interest as fish phagocytes play a vital role in both the specific and non-specific immune system against micro-organisms (Secombes & Fletcher 1992).

This study aims to elucidate the age related susceptibility of rainbow trout to RTFS, focusing on the immune system, and specifically the phagocytes.

Different age groups of rainbow trout were injected intraperitoneally with *F. psychrophilum*. At different times post-inoculation, the internal organs were sampled for bacteriology and the interplay between *F. psychrophilum* and spleen phagocytes was assessed using a number of techniques, aiming to assess phagocytosis, intracellular survival and multiplication of *F. psychrophilum*. A comparison was made between the response in different age groups of rainbow trout.

## Materials and methods

### Bacterial strain

*Flavobacterium psychrophilum* strain Dubois was used in this study. This strain was isolated from an outbreak of RTFS in Belgium during which a mortality rate of 70% was noted among the affected rainbow trout fry. A stock suspension of the strain was stored at –70 °C. After thawing, bacteria were grown for 4 days in 4 mL of Shieh broth (Shieh 1980) at 17 °C. Subsequently, 0.5 mL was transferred to 10 mL of Shieh broth and grown for 3 days at 17 °C. The cultured broth was centrifuged (10 000 ***g***, 10 min, 4 °C) and the resulting pellet and supernatant were separated.

The pellet was resuspended in phosphate-buffered saline (PBS). The suspension was checked for purity and the number of colony forming units (cfu) was determined by plating 10-fold serial dilutions on Shieh plates.

### Fish

In total 370 rainbow trout fry, weighing approximately 1 g (10-week-old) (group I), 38 rainbow trout of approximately 25 g (20-week-old) (group II) and 38 rainbow trout of approximately 300 g (15-months-old) (group III) were obtained from a trout farm in Villers le Gambon, Belgium, with no history of RTFS. Fish were acclimatized for at least 1 week in 1000 L aquaria containing recirculated tap water at 12–14 °C. Fish were daily fed dry commercial pellets *ad libitum* (Extruvet, Trouw, The Netherlands). All animals used in the studies appeared to be healthy before experiments were carried out. Ten fish of group I and two from each of groups II and III were killed for parasitological and bacteriological examination using an overdose of a stock solution of benzocaine [1 g of benzocaine (ethyl aminobenzoate) (Federa, Brussels, Belgium) in 10 mL of acetone]. Parasites were not noted upon examination of wet mount preparations made from the skin and gills. *F. psychrophilum* was not isolated on Shieh agar from samples taken from the skin, gills, gut, liver, kidney, spleen and brain.

### *In vivo* experiment

One hundred fish belonging to group I and 10 fish each of groups II and III were injected intraperitoneally with $1 \times 10^6$ cfu of *F. psychrophilum* strain Dubois, suspended in 0.2 mL PBS. Twenty rainbow trout fry of group I together with two fish from each of groups II and III, were injected intraperitoneally with PBS without any bacteria and hence served as negative control fish.

At 0.5, 1, 2, 3, 4, 5 and 6 days post-inoculation, 10 fish from group I and one fish from each of groups II and III were killed using an overdose of benzocaine. At 0.5 and at 6 days post-inoculation, 10 negative control fish from group I together with one negative control fish from each of groups II and III were killed as described above.

All fish were sampled for bacteriological examination and the spleen phagocytes were collected. Both procedures are described below. Disease signs and mortality in rainbow trout of all groups were monitored until 6 days post-inoculation. The experiment was performed three times.

### Quantitative bacteriological examination

At each sampling time post-inoculation, the spleen, kidney, liver, gills and brain of four of the 10 fish sampled from group I were collected for bacteriological examination and pooled. Of the fish belonging to groups II and III, 1 g of various

internal organs (the spleen, anterior kidney, liver, first gill arch and anterior brain) was collected. Each organ sample was suspended in 1 mL of PBS and the number of cfu of *F. psychrophilum* per gram of tissue was determined by plating 10-fold serial dilutions on Shieh plates, which were incubated for at least 3 days at 17 °C.

### Collection of spleen phagocytes and assessment of phagocytosis, intracellular survival and/or multiplication of *F. psychrophilum*

At each sampling time post-inoculation, the spleens of the remaining six fry of group I were collected and pooled. Half of the spleen of the fish from groups II and III was used to obtain phagocytes. The collection of the phagocytes was carried out according to the procedure described by Lammens *et al.* (2000). The phagocytes were resuspended in L-15 medium supplemented with 5% foetal calf serum (Gibco, Merelbeke, Belgium) and 100 U $mL^{-1}$ penicillin/streptomycin (Gibco) and adjusted to $10^3$ phagocytes $mL^{-1}$. Their viability was determined by exclusion of trypan blue. The spleen phagocytes were used within 3 h after collection.

The following techniques were adopted to visualize the possible phagocytosis of *F. psychrophilum* and to evaluate their survival and/or multiplication inside the phagocytes over time.

For assessing phagocytosis, 100 µL of the suspended phagocytes ($10^3$ $mL^{-1}$) were placed onto sterile 13 mm circular glass cover slips (Menzel-Gläser, Braunschweig, Germany). The cover slips were put into sterile plastic conical tubes and incubated for 3 h at 17 °C, allowing adherence of the cells to the cover slips. Subsequently, the cover slips were washed with L-15 medium, stained with the Haemacolor® (Merck, Darmstadt, Germany) and inspected with a light microscope (× 1000). One hundred rounded cells were counted and a morphological differentiation between phagocytes and lymphocytes was made. The number of intracellular *F. psychrophilum* was counted in each phagocytic cell.

In order to investigate intracellular survival and/or multiplication, epifluorescence microscopy was used. For this purpose, 500 µL of the suspended phagocytes ($10^3$ $mL^{-1}$) were filtered over a cycloblack coated polyester membrane filter (25 mm diameter, 0.4 µm pore size; Chemunex, Ivry-sur-Seine, France). The filter was transferred to a cellulose pad (Millipore, Bedford, MA, USA) saturated with a 100-fold dilution of ChemChrome V3 (Chemunex) in labelling buffer ChemSol B1 (Chemunex). ChemChrome V3 is a proprietary mixture containing an originally non-fluorescent fluorescein derivative. This substrate is cleaved by intracellular esterases into intensely green fluorescent carboxyfluorescein, which can only be retained in viable cells with intact membranes. The substrate cleavage takes place during a short incubation (45 min, 30 °C) of the membrane filter. Three hundred fluorescently labelled round cells were counted using an Olympus BX40 epifluorescence microscope (Olympus, Tokyo, Japan) (× 500). The number of rounded cells containing fluorescent label and hence viable intracellular *F. psychrophilum* was determined and for each positive cell the number of intracellular fluorescent *F. psychrophilum* was counted. The percentage of phagocytic cells was calculated on the basis of the results obtained by the morphological differentiation of the rounded cells during light microscopical examination.

### Statistical analysis

Student's *t*-test (STATISTIX 4.1, Analytical software) was used to compare the differences in cfu per gram of tissue and the data on phagocytosis, intracellular survival and/or multiplication of *F. psychrophilum* between the age groups tested and within each age group between the different sampling periods. A significance level of 0.05 was accepted.

## Results

### *In vivo* experiments

Fish from group I that served as negative controls (injected with PBS without any bacteria) and all fish belonging to groups II and III did not exhibit any clinical signs or mortality.

In fish from group I that were inoculated intraperitoneally with *F. psychrophilum*, mortalities started to occur from day 2 post-inoculation. Twenty-seven fish died between day 2 and 6. At necropsy, the spleen was severely swollen and anaemia, as reflected by pale gills and internal organs, was observed.

### Quantitative bacteriological examination

*Flavobacterium psychrophilum* was not isolated from the spleen, kidney, liver, gills and brain of the

**Table 1** The mean number of colony forming units (cfu) in the spleen of rainbow trout fry (group I) following intraperitoneal injection of $10^6$ cfu of *Flavobacterium psychrophilum*

| Time post-injection of *F. psychrophilum* (days) | Number of cfu per gram of spleen tissue (mean ± SE) |
|---|---|
| 0.5 | $5.9 \times 10^3 \pm 2.3 \times 10^3$ |
| 1 | $2.3 \times 10^4 \pm 5.9 \times 10^3$ |
| 2 | $1.7 \times 10^5 \pm 9.8 \times 10^4$ |
| 3 | $7.4 \times 10^5 \pm 3.2 \times 10^5$ |
| 4 | $3.3 \times 10^6 \pm 2.2 \times 10^6$ |
| 5 | $4.2 \times 10^6 \pm 2.1 \times 10^6$ |
| 6 | $8.3 \times 10^6 \pm 2.8 \times 10^6$ |

negative control fish from group I and all fish belonging to groups II and III.

In group I, *F. psychrophilum* was not isolated from the liver, gills and brain of any of the fish examined. *F. psychrophilum* was, however, isolated at each sampling time from the pooled spleens of the fry that were inoculated with *F. psychrophilum*. The mean number of cfu of *F. psychrophilum* per gram of spleen tissue increased significantly over time (Table 1).

From day 3 onwards, *F. psychrophilum* was isolated from the kidney (on average $6 \times 10^3$ cfu $gm^{-1}$ of kidney tissue; data not shown). However, no significant increase in the number of cfu per gram of kidney tissue was noted over time.

## Collection of spleen phagocytes and assessment of phagocytosis, intracellular survival and/or multiplication of *F. psychrophilum*

Approximately $10^4$ rounded cells were harvested from the six pooled spleens of the rainbow trout fry from group I. At least $10^5$ rounded cells were collected from half of the spleen of the rainbow trout from groups II and III. The viability of the phagocytes, based on trypan blue exclusion, exceeded 98% in all cases.

On light microscopical examination, it was noted that the rounded cells that adhered onto the cover slips consisted of 80% phagocytes and 20% lymphocytes.

*Flavobacterium psychrophilum* were not observed in any phagocytes collected from the spleen of control fry from group I and from any fish belonging to groups II and III.

On the other hand, *F. psychrophilum* were clearly visible at each sampling time inside phagocytes from fry of group I inoculated with *F. psychrophilum*. The percentage of phagocytes containing intracellular *F. psychrophilum* increased significantly from 12 h up to 3 days post-inoculation (Table 2). Likewise, the mean number of *F. psychrophilum* per phagocytic cell increased significantly over time (Table 2).

**Table 2** Light microscopical examination of spleen phagocytes collected from fry inoculated intraperitoneally with $10^6$ cfu of *Flavobacterium psychrophilum* (group I). The percentage of phagocytes containing *F. psychrophilum* and the number of *F. psychrophilum* per phagocytic cell are presented

| Time post-injection of *F. psychrophilum* (days) | Percentage of phagocytes containing *F. psychrophilum* (mean ± SE) | Number of *F. psychrophilum* per phagocyte (mean ± SE) |
|---|---|---|
| 0.5 | 1.5 ± 0.5 | 3.6 ± 0.7 |
| 1 | 4.1 ± 1.3 | 5.3 ± 1.9 |
| 2 | 6.1 ± 2.1 | 8.6 ± 3.2 |
| 3 | 9.5 ± 1.7 | 21.3 ± 8.7 |
| 4 | 9.9 ± 1.1 | 36.4 ± 12.4 |
| 5 | 10.0 ± 3.3 | 50.0 ± 11.5 |
| 6 | 10.4 ± 3.0 | >70.0 |

**Table 3** Epifluorescence microscopy of spleen phagocytes collected from rainbow trout fry inoculated with $10^6$ cfu of *Flavobacterium psychrophilum* (group I). The percentage of phagocytes containing *F. psychrophilum* and the number of *F. psychrophilum* per phagocytic cell are presented

| Time post-injection of *F. psychrophilum* (days) | Percentage of phagocytes containing intracellular *F. psychrophilum* (mean ± SE) | Number of *F. psychrophilum* per phagocyte (mean ± SE) |
|---|---|---|
| 0.5 | 2.5 ± 0.6 | 7.2 ± 2.3 |
| 1 | 2.1 ± 1.2 | 5.3 ± 2.0 |
| 2 | 7.1 ± 2.4 | 12.2 ± 3.5 |
| 3 | 11.7 ± 1.8 | 20.2 ± 6.5 |
| 4 | 11.2 ± 2.4 | 40.2 ± 8.3 |
| 5 | 10.4 ± 1.2 | 50.3 ± 4.2 |
| 6 | 12.5 ± 1.8 | >70.0 |

Using epifluorescence microscopy, no *F. psychrophilum* bacteria were observed in spleen phagocytes of control fish from group I or in any fish of groups II and III. In contrast, fluorescent, and thus viable, *F. psychrophilum* bacteria were seen at each sampling time in spleen phagocytes of fry of group I inoculated with *F. psychrophilum*. As seen with light microscopy, the converted percentage of phagocytes exhibiting phagocytosis increased significantly from day 1 to day 3 post-inoculation (Table 3). The mean number of *F. psychrophilum* per phagocytic cell similarly increased significantly over time.

## Discussion

*Flavobacterium psychrophilum* is now recognized as one of the most important constraints to a successful rainbow trout culture industry in Europe. However, data on the pathogenesis of the disease are lacking. In this study, an attempt was made to elucidate the age related susceptibility of rainbow trout to RTFS by focusing on phagocytic activity. To our knowledge, this is the first study in which the age related development of resistance to *F. psychrophilum* has been examined.

This study has shown that spleen phagocytic cells of rainbow trout fry, aged 10 weeks, contained an increasing number of *F. psychrophilum* over time. This growing number of intracellular *F. psychrophilum* may be caused by the presence of an increasing number of extracellularly located bacteria which are subsequently ingested and/or an active division of the bacteria within the phagocytes. Further *in vitro* studies are underway to elucidate the origin of the increasing number of intracellularly located *F. psychrophilum* over time.

The percentage of spleen phagocytic cells of trout fry containing *F. psychrophilum* increased significantly from 12 h until 2 days post-inoculation. From day 3 onwards, no significant increase in the percentage of phagocytic cells containing *F. psychrophilum* was noted. This may reflect the establishment of an equilibrium, in which newly recruited phagocytes ingest *F. psychrophilum* present extracellularly, while at the same time other phagocytic cells that have already engulfed bacterial cells die.

We have demonstrated that intraperitoneal injection of rainbow trout aged 5 months and older with *F. psychrophilum* does not induce phagocytosis. It is tempting to speculate that the ability of *F. psychrophilum* to enter the phagocytes and disseminate throughout the body of rainbow trout fry only may be at least a partial explanation for the age related susceptibility of rainbow trout to RTFS. Bacterial cells located inside the phagocytes are protected against complement and lysozyme activity and other humoral defence mechanisms of the host, which is undoubtedly a major advantage for the invader and hence may constitute an important virulence trait. In fish aged 5 months and older, it is likely that *F. psychrophilum*, which are not phagocytosed and therefore located extracellularly, are inactivated by these components of the immune system.

It is of interest to note that epifluorescence microscopy demonstrated that the intracellularly located *F. psychrophilum* were still viable. For *F. psychrophilum* to be able to survive within phagocytes, the bacteria must be able to escape from the bactericidal mechanisms of these immune cells. In general terms, intracellularly located bacteria may subvert the killing mechanisms of phagocytes in three ways. Firstly, bacteria such as *Mycobacterium tuberculosis* (Armstrong & Hart 1971) and *Rhodococcus equi* (Zink, Yager, Prescott & Fernando 1987) inhibit fusion of the lysosomes with the phagocytic vacuole containing the ingested bacteria, thereby preventing exposure to toxic lysosomal contents. A second group of intracellular bacteria resists oxidative and lysosomal attack; representatives of this group include *Mycobacterium lepraemurium* (Lowrie, Aber & Jackett 1979) and *Salmonella typhimurium* (Kagaya, Miyakawa, Watanabe & Fukazawa 1992). Finally, organisms such as *Listeria monocytogenes* (Tilney & Portnoy 1989) and *Shigella flexneri* (Clerc, Ryter, Mounier & Sansonetti 1987) use a special lysin to escape from the phagosome. Transmission electron microscopical examination of spleen tissue from naturally infected rainbow trout fry with *F. psychrophilum* revealed the presence of numerous phagosomes and residual bodies indicative of extensive lysosomal activity within the granulocytes (Rangdale *et al.* 1999). Further studies are needed in order to elucidate the mechanisms whereby *F. psychrophilum* counteracts the microbiocidal processes of rainbow trout phagocytes.

The initial stage of interaction of *F. psychrophilum* with phagocytes is probably caused by the recognition of bacterial surface sugars by the phagocytic cell. Indeed, for several bacterial pathogens the capsular polysaccharides have been shown to mediate adherence to phagocytes (Pruimboom,

Rimler, Ackermann & Brogden 1996; Keisari, Kabha, Nissimov, Schepper-Schafer & Ofek 1997; Maganti, Pierce, Hoffmaster & Rodgers 1998). Rangdale *et al.* (1999) suggested that bacterial cells of *F. psychrophilum* possess a surface extracellular polysaccharide layer which is presented as a capsule. These investigators speculated that this capsule aids in motility and adhesion to host cells. It would be worthwhile to compare *F. psychrophilum* with *F. columnare*, its warmwater counterpart, in this respect. *F. columnare* possesses a polysaccharide capsule which probably mediates adhesion to fish cells (Decostere, Haesebrouck, Charlier & Ducatelle 1999).

Splenomegaly and anaemia were the most important disease signs found in the present study in the fish that died following the intraperitoneal inoculation of *F. psychrophilum*. This is largely consistent with descriptions of clinical signs of RTFS reported by other authors (Chua 1991; Santos *et al.* 1992; Rangdale *et al.* 1999).

In conclusion, this study clearly demonstrates the age related phagocytosis of *F. psychrophilum* by spleen phagocytes and the capacity of *F. psychrophilum* to survive in increasing numbers in rainbow trout fry phagocytes over time. Further studies will be carried out in the future to pinpoint the mechanisms involved in intracellular survival and/or multiplication and to fully describe the role of this phenomenon in the pathogenesis of RTFS.

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## References

Armstrong J.A. & Hart P.D. (1971) Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. *Journal of Experimental Medicine* **134,** 713–740.

Bernardet J.F., Segers P., Vancanneyt M., Berthe F., Kersters K. & Vandamme P. (1996) Cutting a Gordian Knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *International Journal of Systematic Bacteriology* **46,** 128–148.

Bruno D.W. (1992) *Cytophaga psychrophila* (='*Flexibacter psychrophilus*') (Borg), histopathology associated with mortalities among farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum) in the UK. *Bulletin of the European Association of Fish Pathologists* **12,** 215–216.

Chua F.H.C. (1991) *A study on the rainbow trout fry syndrome.* MSc Thesis, Institute of Aquaculture, University of Stirling, Stirling, UK.

Clerc P.L., Ryter A., Mounier J. & Sansonetti P. (1987) Plasmid-mediated early killing of eucaryotic cells by *Shigella flexneri* as studied by infection of J774 macrophages. *Infection and Immunity* **55,** 521–527.

Decostere A., Haesebrouck F., Charlier G. & Ducatelle R. (1999) The association of *Flavobacterium columnare* strains of high and low virulence with gill tissue of black mollies (*Poecilia sphenops*). *Veterinary Microbiology* **67,** 287–298.

Holt R.A., Rohovec J.S. & Fryer J.L. (1993) Bacterial cold-water disease. In: *Bacterial Diseases of Fish* (ed. by V. Inglis., R.J. Roberts & N.R. Bromage), pp. 3–23. Blackwell Scientific Publications, Oxford, UK.

Kagaya K., Miyakawa Y., Watanabe K. & Fukazawa Y. (1992) Antigenic role of stress-induced catalase of *Salmonella typhimurium* in cell-mediated immunity. *Infection and Immunity* **60,** 1820–1825.

Keisari Y., Kabha K., Nissimov L., Schlepper-Schafer J. & Ofek I. (1997) Phagocyte–bacteria interactions. *Advances in Dental Research* **11,** 43–49.

Lammens M., Decostere A. & Haesebrouck F. (2000) Effect of *Flavobacterium psychrophilum* strains and their metabolites on the oxidative activity of rainbow trout *Oncorhynchus mykiss* phagocytes. *Diseases of Aquatic Organisms* **41,** 173–179.

Lorenzen E. & Olesen N.J. (1997) Characterization of isolates of *Flavobacterium psychrophilum* associated with cold-water disease or rainbow trout fry syndrome II: serological studies. *Diseases of Aquatic Organisms* **31,** 209–220.

Lowrie D.B., Aber V.R. & Jackett P.S. (1979) Phagosome-lysosome fusion and cyclic adenosine 3′:5′-monophosphate in macrophages infected with *Mycobacterium microti, Mycobacterium bovis BCG* or *Mycobacterium lepraemurium. Journal of Genetic Microbiology* **110,** 431–441.

Madsen L. & Dalsgaard I. (1999) Reproducible methods for experimental infection with *Flavobacterium psychrophilum* in rainbow trout *Oncorhynchus mykiss. Diseases of Aquatic Organisms* **36,** 169–176.

Maganti S., Pierce M.M., Hoffmaster A. & Rodgers F.G. (1998) The role of sialic acid in opsonin-dependent and opsonin-independent adhesion of *Listeria monocytogenes* to murine peritoneal macrophages. *Infection and Immunity* **66,** 620–626.

Pruimboom I.M., Rimler R.B., Ackermann M.R. & Brogden K.A. (1996) Capsular hyaluronic acid-mediated adhesion of *Pasteurella multocida* to turkey air sac macrophages. *Avian Diseases* **40,** 887–893.

Rangdale R.E., Richards R.H. & Alderman D.J. (1999) Histopathological and electron microscopical observations on rainbow trout fry syndrome. *Veterinary Record* **144,** 251–254.

Santos Y., Huntly P.J., Turnbull A. & Hastings T.S. (1992) Isolation of *Cytophaga psychrophila (Flexibacter psychrophilus)* in association with rainbow trout mortality in the United Kingdom. *Bulletin of the European Association of Fish Pathologists* **12,** 209–210.

Secombes C.J. & Fletcher T.C. (1992) The role of phagocytes in the protective mechanisms of fish. *Annual Review of Fish Diseases* **2,** 53–71.

Shieh H.S. (1980) Studies on the nutrition of a fish pathogen, *Flexibacter columnaris. Microbios Letters* **13,** 129–133.

Tilney L.G. & Portnoy D.A. (1989) Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes. Journal of Cellular Biology* **109,** 1597–1608.

Wood E.M. & Yasutake W.T. (1956) Histopathology of fish-peduncle (cold water) disease. *Progressive Fish Culturist* **18,** 58–61.

Zink M.C., Yager J.A., Prescott J.F. & Fernando M.A. (1987) Electron microscopic investigation of *Rhodococcus equi* by foal alveolar macrophages. *Veterinary Microbiology* **14,** 295–305.

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